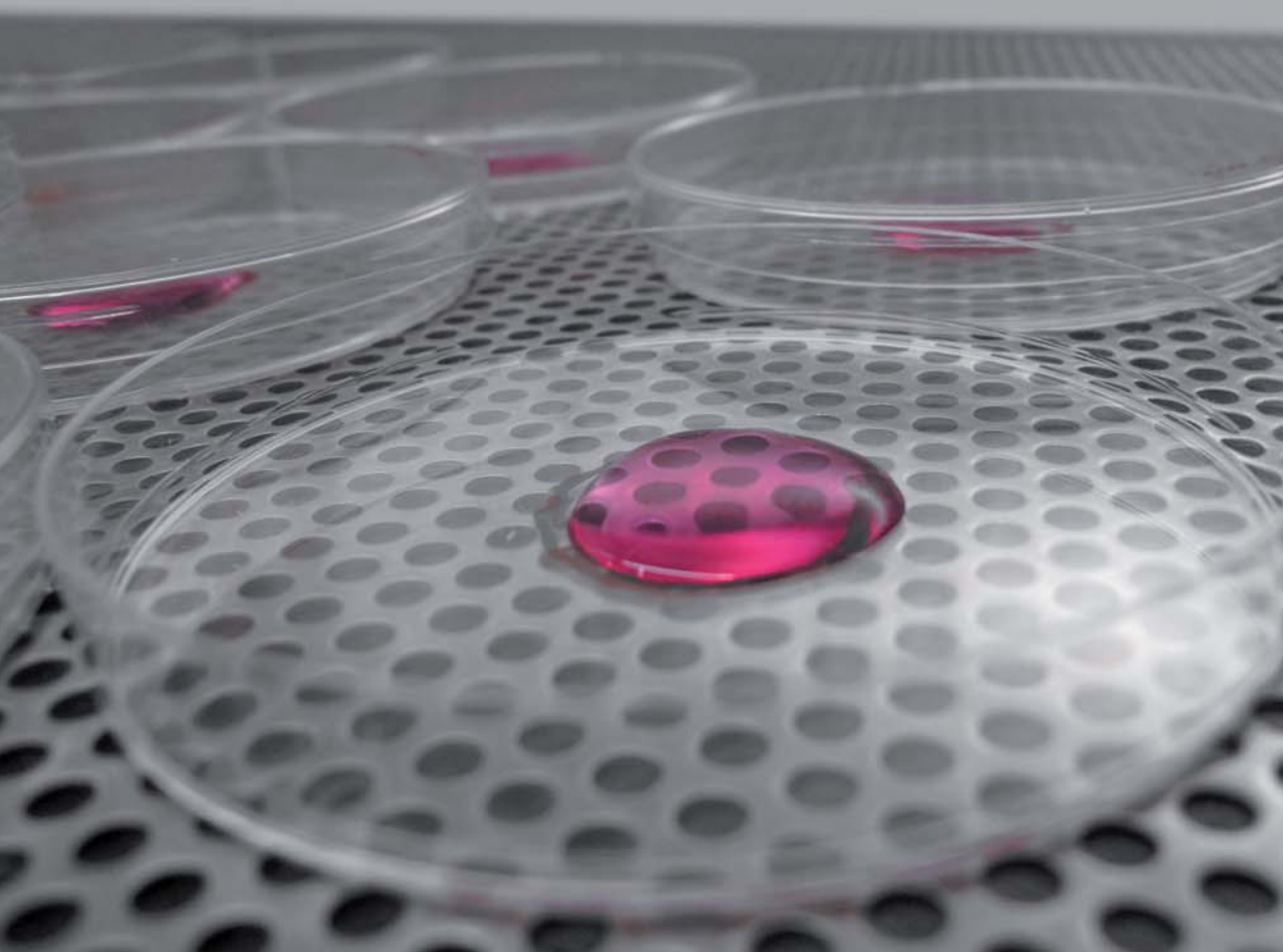
Monitoring, Identification, Genotyping and Inactivation of Enteric Viruses in the Food Chain

Marta Diez Valcarce



TESIS DOCTORAL

MONITORING, IDENTIFICATION, GENOTYPING AND INACTIVATION OF ENTERIC VIRUSES IN THE FOOD CHAIN

Presentada por Marta Diez Valcarce para optar

al grado de doctor por la Universidad de León

Dirigida por:

Marta Hernández Pérez y David Rodríguez-Lázaro

León, Septiembre 2012

A mis padres

Sinopsis

Para reducir el riesgo asociado a la contaminación vírica de los alimentos y conseguir así productos seguros y saludables para los consumidores son esenciales dos elementos. Por un lado la disponibilidad de métodos fiables para la detección de virus y por otro es necesario explorar, y explotar, el uso de tecnologías eficaces para la inactivación de los virus presentes en las muestras de alimentos. Con este doble objetivo, y dentro del marco del proyecto europeo FP7 KBBE "*Integrated Monitoring and Control of Foodborne Viruses in European Food Supply Chains"* (VITAL, www.eurovital.org), en esta Tesis se ha trabajado en la estandarización de la metodología de análisis, desde la toma de muestras de alimento, hasta la concentración, extracción y detección de los virus de origen alimentario y se ha evaluado la eficacia de tecnologías emergentes de inactivación vírica que pueden ser aplicadas en la industria alimentaria.

Para ello, se han diseñado, o bien optimizado, tres controles analíticos para su implantación en los protocolos estandarizados de detección de virus de origen alimentario mediante métodos moleculares. El uso de un control de procesado de la muestra (SPCV) y de controles internos de amplificación (IAC) han demostrado ser una estrategia fiable y sólida que permite evaluar el funcionamiento correcto de los métodos de análisis de alimentos. En el caso del SPCV, no sólo se demostró su aplicación experimentalmente, sino que su uso se caracterizó más en profundidad mediante un estudio que permitió dilucidar las diferencias entre dos posibles virus candidatos (el norovirus murino- MNV-1, y el mengovirus-vMC$_0$). No se encontraron diferencias significativas en el rendimiento de extracción de ambos virus incluso hasta 24 horas después de ser añadidos a la muestra. Sin embargo, se observaron diferencias significativas dependiendo de la etapa en la que se incorporaron al proceso analítico; los rendimientos de extracción fueron mayores cuanto más tarde se añadió el SPCV a la muestra, lo que indica que durante el proceso hay una pérdida sustancial de virus. Por ello es recomendable la incorporación del SPCV al comienzo del procesado de la muestra, favoreciendo de esta manera un seguimiento más preciso del análisis. La identificación del virus no es suficiente a la hora de tomar decisiones sobre la salubridad de los alimentos, es necesario cuantificar el grado de contaminación vírica. Para llevar a cabo una cuantificación precisa de los virus se utilizaron ácidos nucleicos sintéticos. Se diseñaron dos moléculas que contenían 3 y 4 dianas específicas para varias especies de virus de origen alimentario con genomas ADN y ARN, respectivamente. Además de para su uso cómo estándares de cuantificación, estas moléculas sirven como controles positivos en los sistemas de PCR, puesto que suponen una alternativa al uso de ácido nucleicos extraídos de los virus, que tan frecuentemente son escasos de manera natural.

La incorporación de estos controles en los métodos de detección de virus fue evaluada mediante un estudio de validación inter-laboratorio en el que participaron once laboratorios de nueve países europeos diferentes. Se determinó la presencia del adenovirus humano - HAdV en frambuesas y se utilizó el MNV-1 como SPCV. Se trata del primer estudio de validación de un método de detección de virus en alimentos a nivel internacional. Los resultados en su conjunto se consideraron suficientemente robustos, con una sensibilidad y una especificidad del ensayo del 98,5% y 69,7%, respectivamente.

La detección de virus mediante PCR a tiempo real, pese a ser el método de elección por su sensibilidad y especificidad, presenta un inconveniente importante, la imposibilidad de distinguir entre partículas infecciosas y no infecciosas. Mediante la aplicación de un tratamiento enzimático a las muestras antes de la etapa de amplificación de ácidos nucleicos, se pretendió superar este problema. A pesar de tratarse de una aproximación teóricamente correcta, el tratamiento enzimático no resultó útil para cuantificar la capacidad infecciosa del virus, puesto que no se encontró una correlación clara entre la pérdida de capacidad infecciosa del virus medida mediante los tradicionales métodos celulares ($TCDI_{50}$) y los métodos moleculares.

Se llevaron a cabo tres estudios de muestreo a nivel europeo: dos estudios de prevalencia en dos cadenas distintas de suministro de alimentos (producción de carne de cerdo y producción de moluscos bivalvos) y un estudio de prevalencia en granjas porcinas. En la cadena de producción de carne de cerdo, los virus estudiados fueron el virus de la hepatitis E - HEV, por tratarse de un agente zoonótico emergente y el adenovirus porcino-PAdV, como un indicador de contaminación fecal de origen porcino. También se llevó a cabo un estudio sobre la presencia de HEV en granjas porcinas y se estimó la tasa de transmisión de la infección entre animales infectados y

susceptibles. En el caso de los moluscos, los virus estudiados fueron los norovirus humanos- NoV (genogrupo I y II), HEV y el virus de la hepatitis A- HAV. HAdV fue también objeto de estudio por su posible uso como indicador de la presencia de virus patógenos. Nuestros resultados muestran la presencia de ARN de HEV en toda la cadena de producción de carne de cerdo en Europa (desde la granja a la mesa), lo que representa un riesgo potencial para la salud de los consumidores. La detección frecuente de adenovirus porcino en las heces de cerdo, junto con su escasa presencia en los productos del cerdo (carne y salchichas), y su total ausencia en las muestras hígado, indica que el riesgo de contaminación con heces de cerdo durante el sacrificio y manipulación de los alimentos parece ser bajo pero no completamente inexistente. En las granjas europeas, se estimó que la tasa de transmisión de la infección de HEV de un animal infectado a uno susceptible es de 10 a 27 días, en base a la presencia de HEV en las heces recogidas en granjas de seis países europeos. Asimismo, se detectaron virus patógenos (NoVGI, NoVGII y HEV) en mejillones muestreados en el punto de venta. Teniendo en cuenta que este producto se puede consumir crudo o poco cocido, se utilizaron modelos de dosis-respuesta para evaluar el riesgo asociado a su consumo y se observó que tan sólo en el caso de NoV el consumo de mejillones representaba un riesgo. Las muestras de mejillones mostraron también una alta prevalencia de HAdV, aunque no se encontró ninguna correlación entre su presencia y la de los virus patógenos. Podemos concluir tan solo que las muestras estuvieron en contacto con aguas contaminadas con heces humanas, pero este hecho por sí solo no apoya el uso del adenovirus humano como indicador de la presencia de virus patógenos.

Finalmente, con el fin de reducir el riesgo asociado a la contaminación vírica de los alimentos se llevaron a cabo ensayos con dos tecnologías emergentes para la inactivación de virus: las altas presiones hidrostáticas (HHP) y la utilización de

compuestos naturales presentes en los aceites esenciales (EO) de las plantas. Se trata de dos tecnologías no térmicas, por lo tanto de potencial uso sobre alimentos termolábiles como los vegetales y los frutos rojos. En los ensayos se utilizaron dos virus, MNV 1-y HAdV-2, un virus subrogado del norovirus humano y un virus patógeno humano, con genomas de ARN y ADN, respectivamente. Los resultados obtenidos fueron dispares, mientras que las HHP demostraron ser una opción eficaz ya que tratamientos de 400 MPa o superiores alcanzaron un objetivo de seguridad alimentario (FSO) de al menos 4 $\log_{10}$ de reducción en la capacidad infecciosa del virus en 2,5 min o ~1,5 min, en el caso de MNV-1 y HAdV -2, respectivamente. En el caso de los EO, la aplicación de los extractos de mejorana e hisopo no alcanzaron los niveles de inactivación vírica esperados para ser considerados un procedimiento adecuado para la descontaminación de alimentos.

Abstract

To effectively reduce the risk associated to virus contamination in food and to render foodstuffs safe and healthy for consumers, two elements are essential. First, reliable methodology for virus detection is necessary and second, the effectiveness of inactivation technologies must be assayed. With this double aim and within the framework of the European FP7 KBBE project "*Integrated Monitoring and Control of Foodborne Viruses in European Food Supply Chains" (VITAL,* www.eurovital.org), in the present Thesis much work has been performed towards the standardization of methodology for the sampling, concentration, extraction and detection of relevant foodborne viruses in foods. Furthermore, the effectiveness of emerging inactivation technologies to be applied in the Food Industry has been explored.

Three analytical controls have been designed or optimized for their successful implementation in standardised protocols for the detection of foodborne viruses by

molecular methods. The use of sample process control viruses (SPCV) and internal amplification controls (IAC) has been demonstrated to be a reliable and robust approach for assessing the correct performance of the analysis of samples from different food supply chains. The use of SPCV has been further characterized in a study to clarify the differences between two SPCV candidates (murine norovirus-MNV-1, and mengovirus- vMC_0). No significant differences were found in the performance of the control up to 24 hours after their addition to the sample. However, significant differences were observed, depending on the stage in which the SPCV was incorporated to the process. The efficiencies of extraction were higher the later the SPCV was added to the sample, indicating that during the process there is a substantial loss of virus, thus, the early addition of SPCV is recommended to allow a more complete monitoring of the analysis. The sole identification of the virus is not sufficient to decide whether food is healthy for consumers or not, accurate virus quantification is required. A strategy consisting in the use of synthetic nucleic acids for virus quantification was also implemented. These synthetic nucleic acids were designed containing a multi-target sequence, which permit us to use the same molecule for up to four different virus species. These standards can be also used as positive controls in molecular detection methods; they constitute an alternative to the use of natural nucleic acids extracted from the virus, which are often scarce in the nature.

The suitability of the methodology was evaluated by an international interlaboratory exercise in which eleven laboratories from nine different countries were involved. The performance of a PCR-based method to detect human adenovirus-HAdV in raspberries using MNV-1 as SPCV was determined. To our knowledge, this is the first validation study on a virus detection method in food. The overall results were

considered acceptably robust (trial sensitivity and specificity of 98.5% and 69.7%, respectively).

Virus detection using real-time PCR (RTi-PCR), despite being a sensitive and specific method, has an important drawback, the impossibility to distinguish infective from non infective particles. Aiming to overcome this issue, a preenzymatic treatment of the samples prior to deliver them to the amplification step was assessed. Despite its theoretically correct principle, the preenzymatic treatment did not seem to be a feasible approach to quantify virus infectivity since no correlation between the decrease in virus infectivity measured by cell culture ($TCID_{50}$) and the molecular method (ET-RTi-PCR) was found.

Three sampling studies were performed; two major prevalence studies in two different European food supply chains (pork and shellfish production) and in the European swine populations in farms. Hepatitis E virus- HEV and porcine adenovirus- PAdV were the viruses tested in the pork production chain; HEV as an emergent zoonotic agent and PAdV as an indicator of faecal contamination of porcine origin. To gather also information on farm prevalence, one study of HEV prevalence in European farms as well as its transmission rate from infected to susceptible animals was also performed. In the case of shellfish, target viruses were human noroviruses- NoV (genogroup I and II), HEV and hepatitis A virus- HAV, and HAdV as a potential pathogenic virus indicator. Our results show that HEV RNA is present throughout the pork production chain in Europe (from farm to fork) and this presents a potential health risk for consumers. The frequent detection of PAdV in pig faeces, along with its low presence in the pork products (i.e. meat and sausages), and complete absence in liver, indicates that risks for contamination with swine faeces during slaughtering and food manipulation appear to be low but not absent. In the European farm setting, the

HEV estimation of the transmission from an infected to a susceptible animal was calculated to range from 10 to 27 days, based on HEV presence in faeces taken from farms of six different European countries. Pathogenic viruses (NoVGI, NoVGII and HEV) were found in shellfish at point of sale. Considering that shellfish can be consumed raw or slightly cooked, dose-response models were used and only NoV was found to represent a health risk. Shellfish samples purchased at retail level showed high prevalence of HAdV, although no correlation with pathogenic viruses was found. This finding indicates that samples were in contact with waters polluted with human faeces but does not support the use of adenovirus as pathogenic virus indicator.

To manage this risk associated with viral contamination of food, emergent technologies for virus inactivation were assayed. Two different non-thermal inactivation technologies (High hydrostatic pressure processing- HHP and natural compounds present in the essential oil- EO fraction of plants) were used with two viruses, MNV-1 and HAdV-2, a surrogate of human norovirus and a pathogenic human virus, with RNA and DNA genomes, respectively. The results obtained were disparate, whereas HHP proved to be an efficient option; the application of EOs did not meet the expected levels of virus inactivation. HHP, using treatments of 400 MPa or higher, showed an efficient non-thermal inactivation performance achieving a food safety objective of, at least, 4 $\log_{10}$ reduction in virus infectivity in 2.5 min or ~1.5 min, in case of MNV-1 and HAdV-2, respectively. On the contrary, the application of the EO of marjoram and hyssop did not meet the expected levels of virus inactivation to be considered an appropriate procedure for food decontamination.

TABLE OF CONTENTS

ÍNDICE

Chapter 1

Introduction

1.1 Foodborne viruses: a general overview

There are 31 known major foodborne pathogens causing 19.7% of the total foodborne illness in the USA. Among these pathogens, viruses are the leading causative agents (58.7%), followed by bacteria (38.8%) and parasites (2.5%); important foodborne bacteria such as non-typhoidal *Salmonella* spp., *Clostridium perfringens* and *Campylobacter* spp., all together only represent the 30% of cases due to known microorganisms in comparison to the 58% of cases represented solely by norovirus (NoV). Taking into consideration only the viral foodborne illnesses (caused by astrovirus, hepatitis A virus, norovirus, rotavirus and sapovirus), norovirus represents the 99.1% (Scallan *et al.*, 2011) and cost annually about $2 billion for healthcare and lost in productivity (www.cdc.gov). The importance of viruses as causative agent of foodborne illness in Europe shows an increasing trend in terms of number of viral foodborne outbreaks. In recent years, figures have raised from 38 outbreaks in 2008 through 70 in 2009, to 87 in 2010 (European Centre for Disease Prevention and Control – European Food Safety Authority, ECDC – EFSA, 2012). NoV and hepatitis A virus (HAV) have been listed as priority foodborne virus hazards and shellfish, soft fruit and salad vegetables are considered as the food products most at risk of contamination with these agents (World Health Organisation, WHO, 2008). Therefore, much research effort is devoted to reducing the impact of foodborne viruses by developing and establishing effective methods for their monitoring and control.

Foodborne viruses can be classified into three main groups based on the associated symptoms of the illness they produce (Table 1).

Table 1. Likelihood of foodborne or waterborne transmission of enterically transmittable viruses, according to the type of illness associated with infection (Koopmans and Duizer, 2004).

Likelihood	*Illness*		
	Gastroenteritis	Hepatitis	Other [a]
Common	NoV	HAV	
Less common	hAdV F40-F41 HRV (A-C) SpV hAstV AiV	HEV	EV

NoV: norovirus, HRV: human rotavirus groups A to C, hAstV: human astrovirus, hAdV F40-F41: human adenovirus species F serotype 40 and 41, SpV: sapovirus, AiV: aichi virus, HAV: hepatitis A virus, HEV: hepatitis E virus, EV: enterovirus.

[a] Group of viruses which replicate in the human intestine, but only cause illness after they migrate to other organs such as the central nervous system (e.g. poliovirus).

Pathogenic enteric viruses can enter into the food chain from two different sources: humans and animals. HAV and the strains of NoV which infect humans, both originate from humans themselves. In the case of hepatitis E virus (HEV), genotypes 1 and 2 primary circulate in humans and are less frequently isolated in animals, whereas genotypes 3 and 4 show a high diversity apparently related to their zoonotic origin from different animals (Lu *et al.*, 2006). Genotype 3 appears endemic in pig herds, and it has been identified as a significant emerging hazard for human infection due to consumption of contaminated pork products (WHO, 2008). As part of this Thesis, a study to evaluate the prevalence of HEV in the pork production chain was performed (chapter 3, section 3.2.1).

Fresh produce such as soft fruit and salad vegetables can become contaminated in the farm during growing (use of contaminated fertilizers or sewage or the use of inadequate irrigation water) or harvesting. Consumption of raw or undercooked fruit

or vegetables can subsequently be the cause of a foodborne viral episode. Bivalve shellfish, due to their filter feeding nature, can concentrate and retain human pathogens derived from sewage contamination in the shellfish-growing water, which can also lead to a foodborne viral disease (Formiga-Cruz *et al.*, 2002). To further contribute in the gathering of information on viral prevalence in the shellfish supply chain, one study was performed and it is included in chapter 3, section 3.2.3 of this Thesis.

Contamination can also occur after the production phase, e.g. it can take place during food preparation or handling by infected food handlers. Bidawid and collaborators (2000) showed that 9.2% of infectious virus particles on contaminated hands can be transferred to lettuce during its manipulation. Figure 1 shows a scheme of the spectrum of transmission routes of foodborne viruses within the food supply chain.

The concentration of pathogenic viruses in the food supply chains may not be high enough to detect them, so the detection of other enteric viruses which may be found in higher concentrations may be necessary to show that a food supply chain is at risk of contamination. These latter viruses are known as "index" viruses, as their presence in a sample would indicate that a route of contamination exists from source to sampling point. Viruses of the Adenoviridae family infect both humans and a wide variety of animal species and are shed in large numbers in the faeces of infected individuals making them an appropriate tool for tracing the source of faecal viral contamination (Hundesa *et al.*, 2006; Maluquer de Motes *et al.*, 2004; Wyn-Jones *et al.*, 2011).

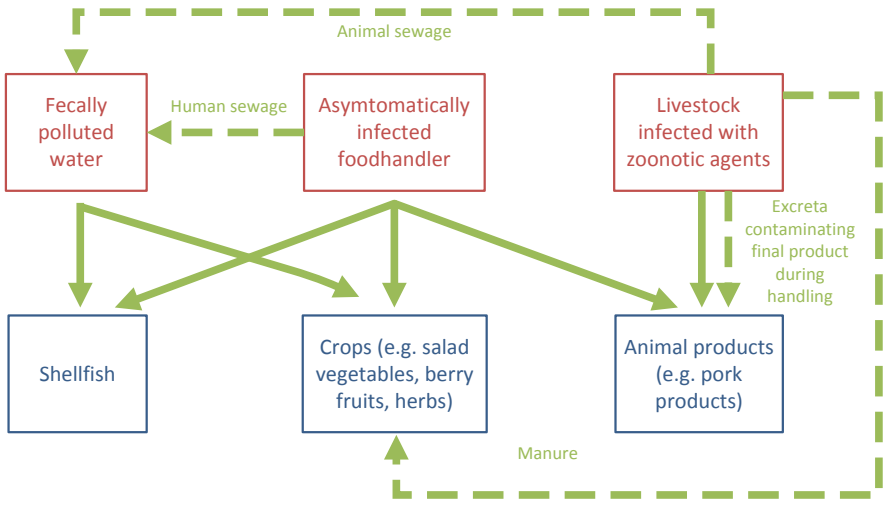


Figure 1. Spectrum of transmission routes of foodborne viruses within the food chain. Continuous green arrows indicate direct transmission routes and discontinuous green arrows indicate indirect transmission routes

1.2 Characteristics of the main foodborne viruses

Norovirus

Noroviruses belong to the family *Caliciviridae* and constitute the leading cause of foodborne outbreaks of acute gastroenteritis and the most common etiologic agent of sporadic infectious gastroenteritis affecting people of all age groups (Green, 2007; Patel *et al.*, 2009). NoVs are icosahedric non-enveloped viruses with a positive-sense, single-stranded RNA genome of between 7.3 and 8.3 kb. They are subdivided into five genogroups (Karst *et al.*, 2003) and several serotypes. Humans can be infected by three genogroups (GI, GII, and GIV) containing more than 20 genotypes. The intra-genotype nucleotide diversity can be as high as 15% (Zheng *et al.*, 2006), hindering their detection; furthermore, currently available methods are not sufficiently powerful and indeed, the prevalence of uncommon variants such as NoV GIV is probably

underestimated (La Rosa *et al.*, 2008). NoVs are highly resistant to disinfectants (Duizer *et al.,* 2004), with long-term stability in the environment (D'Souza *et al.*, 2006), and have an extremely low infectious dose; less than 100 particles are sufficient to produce infection (Teunis *et al.*, 2008), and can be spread by asymptomatic-excreting carriers. All these characteristics make NoV an easily spreadable threat with consequences such as gastroenteritis outbreaks after consumption of shellfish (Le Guyader *et al.*, 2008) or outbreaks in institutions such as healthcare facilities (Schmid *et al.*, 2011).

Hepatitis A virus

Hepatitis A virus (HAV) infects approximately 1.4 million people worldwide each year (Issa and Mourad, 2001), although its incidence varies globally. Developing countries have the highest rates of HAV due to limited hygiene standards and sewage treatment facilities. On the contrary HAV figures have been drastically reduced in countries where effective programmes of immunization have been implemented; as an example, in the USA the number of cases has been reduced by 92% to an infection rate as low as 1 case per 100,000 persons per year (Daniels *et al.*, 2009); i.e. only 2500 cases of hepatitis A were identified in 2008 (www.cdc.gov). HAV is an icosahedric non-enveloped virus species with a positive-sense, single-stranded RNA genome of approximately 7.5 kb, classified in the family of the *Picornaviridae*, genus *Hepatovirus*. HAV is able to survive in several environments, particularly in water and food (Rzeżutka and Cook, 2004). HAV can retain its infectivity for several days on fruits and vegetables which are often consumed raw (Sattar *et al.*, 2000). Water is considered to be the most important source of infectious HAV because it can survive for long periods in this environment; up to 60 days in tap water (Enriquez *et al.*, 1995).

Shellfishborne outbreaks due to HAV have also been reported, one of them lasting seven months and affecting one hundred people in Spain in 2008 (Pintó *et al* 2009).

Hepatitis E virus

Hepatitis E virus (HEV) is a small spherical non-enveloped positive-sense, single-stranded RNA virus with a genome of approximately 7.2 kb. It is the sole species of the genus *Hepevirus* within the *Hepeviridae* family. It can be classified into four major genotypes (1-4) (Lu *et al.*, 2006). Although four genotypes of HEV exist, there only seems to be one serotype present (Herremans *et al.*, 2007; Mushahwar, 2008; Zhou *et al.*, 2004). Genotypes 1 and 2 circulate primarily in humans causing the majority of HEV infections including all epidemics in Asian and African countries, and also in Mexico. By contrast, for genotypes 3 and 4 only isolated cases of human infection have been described and only in more industrialized countries including the USA, Japan, China, and countries in Europe. These latter cases were initially believed to be travel related but recently indigenous cases have been reported (Borgen *et al.*, 2008; Wang *et al.*, 2001; Mansuy *et al.*, 2004; Widdowson *et al.*, 2003). The presence of HEV has been reported in food (Di Bartolo *et al*, 2012), linked to large waterborne outbreaks (Sailaja *et al.*, 2009), and in animals such as pigs (Rutjes *et al.*, 2009), wild boar (Martelli *et al.*, 2008), Sika deer (Tei *et al.*, 2003) and red deer (Rutjes *et al.*, 2010). Swine strains of HEV genotypes 3 and 4, that are closely related to human strains, have been isolated worldwide (Clemente-Casares *et al.*, 2003; Huang *et al.*, 2002; Reuter *et al.*, 2009; Rutjes *et al.*, 2007; Van der Poel *et al.*, 2001) suggesting an emerging zoonotic transmission of HEV where pigs may be the reservoir. Supporting the potential foodborne zoonotic transmission of HEV genotype 3, five cases of hepatitis E have been linked directly to eating raw deer or wild boar meat; identical HEV strains were found in the meat consumed and in the patients (Li *et al.*, 2005; Tei *et al.*, 2003).

Although the case-fatality of Hepatitis E in epidemics is <0.1%; HEV infection is more severe in pregnant women, particularly from certain geographic areas in India and especially during the third trimester, leading to fatal hepatic failure and death in 10-25% of cases (Navaneethan *et al.*, 2008).

Adenovirus

Adenovirus (AdV) is an icosahedric non-enveloped virus with a double-stranded DNA genome of 28 to 45 kb. AdV are classified as members of the *Adenoviridae* family, genus *Mastadenovirus,* including several species infecting humans and other species infecting a wide range of animal species (bovine, swine, rodents...). The fifty-one serotypes of human adenovirus (hAdV) are classified in six sub-groups (A-F), depending on their physicochemical, immunological and biological characteristics. Among the fifty-one serotypes, serotypes 40 and 41, included in sub-group F, are major causes of gastroenteritis in young children and are readily spread by the faecal-oral route through contaminated food or faecally polluted water. Human AdV prevalence in bivalve molluscs grown in contaminated water can be as high as 89% (Formiga-Cruz *et al.*, 2002), although only occasionally has hAdV been proved to be the sole etiologic agent of foodborne outbreaks (Koopmans and Duizer 2004; Domínguez *et al.*, 2008). Human AdV have been proposed as viral indicators of faecal contamination as they are excreted in faeces on a long-term basis and their presence is nearly ubiquitous in sewage, effluent wastewater, sludge, and biosolid samples (Bofill-Mas *et al.*, 2006).

Rotavirus and astrovirus

Rotavirus (RV) is a genus of the *Reoviridae* family; the virions are icosahedral and non-enveloped. Their structure is a triple-layered capsid packaging a segmented double-

stranded RNA genome of approximately 18.5 kb. Estes and Kapikian (2007) classified rotavirus into five major groups named A-E. The majority of RV infections in human beings are associated with group A rotavirus (GARV) and they are of fundamental importance because they are the main causative agent of child mortality due to diarrhoea worldwide. In 2006, Parashar and collaborators estimated that 611,000 cases of rotavirus have a fatal ending, and that more than 80% of all rotavirus-related deaths occurred in low income countries (Parashar *et al.*, 2006). GARV is also widespread in wild and domestic animal species, and the introduction of novel strains of animal origin into the human population via zoonotic transmission has been suggested (Bányai *et al.*, 2009; Cook *et al.*, 2004). Depending on sequence diversity of the genes encoding the two outer capsid proteins (VP7 and VP4), at least 19 G- and 27 P-types can be distinguished (Matthijnssens *et al.*, 2008; van Doorn *et al.*, 2009) and they can combine producing many different combinations of G and P types. In the last few years rotavirus vaccination against the most common circulating types has been introduced and their generalized use may lead to the emergence of novel RV genotypes or the re-emergence of older strains, particularly from animal reservoirs (Cook *et al.*, 2004; Iturriza-Gómara *et al.*, 2004; Kang *et al.*, 2005; Steyer *et al.*, 2008), which could lead to a change in the pattern of the most common circulating types. In the genus *Mamastrovirus,* family *Astroviridae,* there is another important foodborne virus; astrovirus (AstV). AstV are spherical non-enveloped viruses with a positive-sense, single-stranded RNA genome of between 6.8 and 7 kb; six species have been described affecting bovines, felines, mink, ovines, porcines and humans. Human astrovirus (hAstV) is a common cause of gastroenteritis in children, and also in the elderly and in immunocompromised individuals (Guix *et al.*, 2002; Mendez and Arias, 2007). Eight genotypes of hAstVs have been described to date, and are classified into genogroup A (hAstV-1 to 5 and hAstV-8) and genogroup B (hAstV-6 and 7) (Gabbay *et al.*, 2007).

Ocasionally, hAstVs have been associated with gastroenteritis outbreaks involving possible waterborne or foodborne transmission (Domínguez *et al.*, 2008; Leclerc *et al.*, 2002; Maunula *et al.*, 2004; Scarcella *et al.*, 2009; Smith *et al.*, 2006). Depending on rainfall conditions hAstV can be found in seafood (Le Cann *et al.*, 2004; Riou *et al.*, 2007) increasing the risk of human infection due to consumption of these products. Also the possibility of AstV zoonotic transmission from cows has been proposed (Kapoor *et al.*, 2009).

1.3 Detection of foodborne viruses: diagnostic methodology

Due to the high impact of foodborne viral infections in terms of population affected and economic implications the development of rapid and robust diagnostic tools to detect them are necessary (Croci *et al.* 2008; Cook and Rzeżutka 2006; Rodríguez-Lázaro *et al.*, 2012). A key step is the separation and concentration of viruses from the surface of the food matrix into a so-called viral eluate to be assayed. This viral eluate must have a minimal volume before performing any test for detection. But as food items may be contaminated by only few virus particles and still be a hazard, a large sample size needs to be tested, implying a substantial concentration of several orders of magnitude (i.e. from the range of millilitres or even litres in case of water samples to the range of microlitres used in molecular assays) (Sair *et al.*, 2002; Cliver *et al.*, 2008). The concentration step has to be optimized individually for each specific matrix such as fresh produce, soft fruits, pork products or shellfish (Dubois *et al.*, 2002; Bouwknegt *et al.*, 2007; Henshilwood *et al.*, 2003) to take account of the differences in the morphology and hydrophobic interactions of foodstuff surfaces and viral particles, differences in tissue compositions (e.g. containing different amounts of PCR-inhibitory substances), differences in the pH of samples and other qualities. During the concentration step any co-concentration of inhibitory substances such as

polysaccharides and glycogen that can negatively affect the subsequent steps of the detection procedure must be avoided (Atmar *et al.*, 2006; Schwab *et al.*, 1998).

The sample treatment procedure consists of several steps:

(i) Elution of viruses from the food matrix, by washing in case of fresh produce (Dubois *et al.*, 2002; Kurdziel *et al.*, 2001a; Kurdziel *et al.*, 2001b) or homogenization in case of shellfish (Coelho *et al.*, 2003; Croci *et al.*, 1999; Cromeans *et al.*, 1997; Kingsley and Richards, 2001) or pork products (Martínez-Martínez *et al.*, 2011).

(ii) Removal of food solids from the extract using filtration or differential centrifugation.

(iii) A subsequent virus concentration by sedimentation, flocculation, ultracentrifugation or precipitation.

All these steps have to be performed before viruses can be delivered to the next stage in the detection procedure.

After concentration of viruses, the viral eluate must be assayed to identify the viral particles in it. This can be done using direct or indirect methods. Among the direct methods, observation by electron microscopy or the observation of the cytopathic effect in specific cell lines ($TCID_{50}$, median tissue culture infective dose assay, or plaque assay) are the most common. From the indirect methods, immunological or molecular methods are the most used. Figure 2 shows a schematic diagram summarising the above-mentioned methods. However, there is no "gold standard" and all have advantages and limitations.

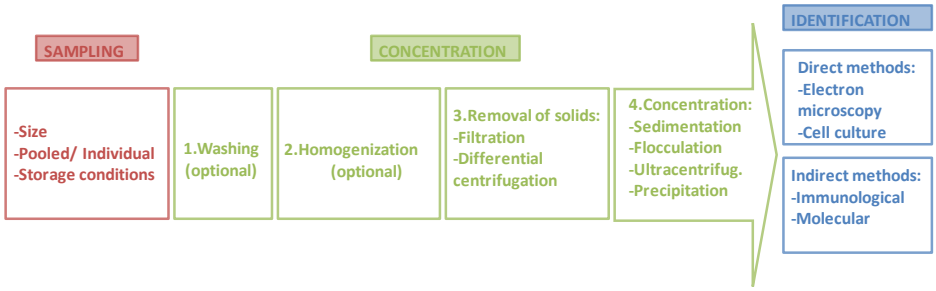


Figure 2. Schematic diagram of the analytical process for the detection of foodborne viruses. Washing and homogenization steps are optional depending on the nature of the matrix (e.g. fresh produce is washed and shellfish is homogenized).

Electron microscopy is a laborious, time-consuming and quite subjective technique as it depends on the expertise of the person performing the observation, and has a limited sensitivity since a minimum concentration of 10^6 viral particles per millilitre is necessary to be observed (Atmar and Estes, 2001). On the other hand, electron microscopy gives undeniably accurate results since they are based on the direct observation of the actual virus particles in the sample.

The use of *specific cell lines* to observe the cytopathic effect of the viral particles is another direct method but at present is not always possible as some of the most important enteric viruses, like NoV and HEV, cannot be consistently propagated in cell lines; or even when possible, is not a simple or cost-effective technique. HEV, for example has been grown on cell lines (Tanaka *et al.*, 2007; Takahashi *et al.*, 2012) but the implementation of this technique is not yet feasible due to the difficulties to reproduce consistently the procedure, which hampers the spread of the technique. Another approach based on cell culture is the integrated cell culture real-time PCR method (ICC-RTi-PCR) (Jiang *et al.*, 2004; Li *et al.*, 2009) but again this option does

not comply with quickness in results, one of the most important requirements of the food industry and this limits its implementation.

Among the indirect methods, *immunological tests* such as enzymatic immunoassay (EIA), radioimmunoassay (RIA) or enzyme linked immunosorbent assay (ELISA) which target the presence of specific antigens in the viral particle are commercially available, which would facilitate their generalized application as detection methods. However the analytical sensitivity of these methods is not high enough for effective testing on samples with such low levels of contamination as some food matrices have. So they are currently used for clinical sample analysis rather than for food sample analysis (Moe *et al.*, 2004; Geginat *et al.*, 2012).

The other indirect methods for the detection of viruses are based on *molecular detection* of the target nucleic acid after an amplification process such as real-time PCR, conventional PCR or NASBA. Real-time PCR, for example can overcome most of the previous limitations; it possess a high analytical sensitivity and specificity as well as promptness in the results. There is a low risk of carry-over contamination due to the closed-tube format and it has a wide dynamic range of quantification as well as the possibility of automation (Rodríguez-Lázaro *et al.*, 2007). Molecular methods have been recommended by international committees such as the ISO/CEN committee CEN/TC275/WG6/TAG4 with the aim that real-time PCR should serve as the basis for the forthcoming international standards for detection of NoV and HAV (Lees and CEN WG6 TAG4, 2010). In fact, a considerable number of molecular methods for detection of different foodborne viruses have already been published and the gradual incorporation of more of these molecular-based foodborne virus detection methods as standards in the analytical laboratory must be aimed. There are, however, still some limitations. The volume of sample used in the amplification is very small (i.e. in the

microlitre range) making the use of concentration protocols that can deliver such small volumes mandatory (Rodríguez-Lázaro *et al.*, 2012). The quality of the nucleic acids used in the assay has a direct effect on the analytical sensitivity of the assay, as incomplete or damaged sequences can prevent primers annealing to the target. Another limiting factor when using molecular methods in food samples is the presence of inhibitory compounds that can hinder the amplification reaction (McMullen, 2003; Goyal, 2006). Notwithstanding all these limitations that render the detection of foodborne viruses quite a challenging matter, the present Thesis is based on the usefulness of molecular methods, particularly real-time PCR and conventional PCR, for the detection, identification and genotyping of viruses in foodstuffs.

1.4 Analytical controls used in the detection process for foodborne viruses

For a successful public health intervention in case of foodborne viral outbreak the early identification of the viral agent(s) causing the outbreak is needed, because the quick identification of the causative viral pathogen markedly increases the probabilities of success of any counter measures applied. However, speed in the identification is not sufficient; reliability of results is a major requirement and any false negative or false positive interpretation of results must be avoided. In response to this need of reliability a suite of controls that serves to verify the correct performance of the analytical method has to be used. These controls can be classified into three different groups: 1) controls used at the beginning of the sample detection process, 2) controls for the nucleic acid extraction step and 3) controls that are used specifically in the amplification step. International standards exist to define the minimum requirements to obtain comparable and reproducible results within individual and between different laboratories when detecting foodborne pathogens using PCR (ISO 22174:2005) or real-time PCR (ISO 22119:2011) methods. Figure 3 shows a schematic representation of the

analytical controls that must be used in the different steps for the detection of foodborne viruses.

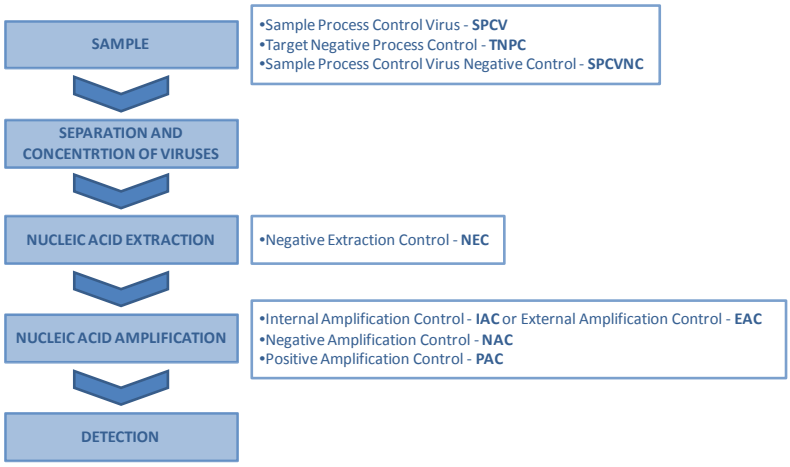


Figure 3. Detection process and associated controls, adapted from D'Agostino *et al.*, 2011.

Controls used at the beginning of the sample detection process.

These controls enable us to monitor the process from the beginning of the detection, including any step for concentration of viruses, to the final amplification step. Within this group there is the sample process control virus (SPCV), the target-negative process control (TNPC) and the sample process control virus-negative control (SPCVNC). The aim and description of these controls is explained below.

SPCV: this control is used to verify that the sample treatment has functioned correctly and consists of a non-target virus added to every sample and to the TNPC at the beginning of the analysis; but it is not added to the SPCVNC. If the detection and amplification steps are performed correctly the SPCV must be detected in every sample into which it was added. A good SPCV must comply with some requirements: it should

be a representative surrogate of the target virus, able to predict as closely as possible the behaviour of the pathogenic virus throughout the analytical procedure, it must be non-pathogenic and it should not be naturally present in the samples to be tested. Murine norovirus (MNV-1) has been proposed as such a surrogate (Deboosere *et al.*, 2012; Cannon *et al.*, 2006) and was used in this Thesis. In chapter 3, sections 3.1.2 and 3.1.3 described two studies on the application of MNV-1 as SPCV for the detection of foodborne viruses.

TNPC: is used to check for any contamination with the target virus or its amplicon in the reagents or the equipment used throughout the procedure. It is a sample which does not contain any food matrix (which has been replaced by the same amount or volume of nuclease-free water) and it is processed exactly as a real sample. This control must be included in every batch of samples analysed. In this control only the SPCV signal should be present, but no target signal.

SPCVNC: the purpose of this control is similar to that of the TNPC, but specifically to check for contamination with the SPCV virus or its amplicon in the reagents and in the equipment. It consists of a sample of nuclease-free water that undergoes the whole procedure as a real sample but with no SPCV added at the beginning. Hence, no SPCV signal should be present, and no target signal should be present.

Controls for the nucleic acid extraction step.

NEC: this control is target free, it consists of nuclease-free water that is treated as a sample and undergoes all the steps of the nucleic acid extraction procedure. Its purpose is to ensure that the extraction reagents and equipment used in the nucleic acid extraction step are free of contamination with the target or its amplicon. If a target signal is found in this control all reagents used in the nucleic acid extraction step

should be replaced and all equipment should be decontaminated. Despite having a similar purpose to the TNPC, the information provided by the NEC is different since this latter control gives information on possible contamination of the nucleic acid extraction reagents and the TNPC gives this same information about the concentration reagents if both controls are used.

Controls used specifically in the amplification step.

A specific suite of controls has to be used in the amplification step as several situations can occur and they all must be monitored. These controls can be included in the (Reverse transcription)-PCR reaction mixtures (i.e. internal amplification control, IAC) or in additional reactions (i.e. external amplification control, EAC; negative and positive amplification controls, NAC and PAC, respectively).

IAC: this control is used to verify whether the amplification reaction has functioned correctly or has failed. It is a non target nucleic acid sequence present in every reaction which can be co-amplified simultaneously with the target sequence by using the same set of primers, but can be distinguishable from the target due to detection using an IAC-specific probe, in case of real-time PCR or can be distinguishable by amplicon size (band position), in case of conventional PCR. In case of real-time PCR, the IAC and the target sequences should be preferably identical except for the probe-binding site (Hoorfar *et al.*, 2004). In a reaction without an IAC, a negative response can have two possible explanations, absence of the target or failure of the amplification reaction. On the contrary, if an IAC is included the absence of target signal if the IAC signal is present can only mean that the sample we are analysing is truly negative, if the rest of control signals are correct.

EAC: this control uses an alternative approach to the IAC but with the same purpose: the EAC will aid in the identification of possible inhibited reactions. In this case two separate reactions must be run for each sample, in one (the test) only the sample nucleic acid is added whereas in the other (the control) the sample nucleic acid plus the EAC are included (Costafreda *et al.*, 2006). For the interpretation of results we have to assume that both amplification reactions (the test and the control) performed with similar efficiency and in that case the EAC-positive signal in the control with a non-production of a target signal in the test is considered a true negative, if the rest of control signals are correct.

The choice of IAC or EAC is completely up to the user, although there is currently a controversy over the use of one approach or the other. In this Thesis project the approach of choice was the use of IAC since the control of the inhibition can be performed in the same reaction as the detection, allowing the monitoring of some situations such as individual pipetting errors. Besides, the EAC approach implies increasing the number of reactions required for each sample up to almost double them. Regarding the main concern of EAC defenders, that is, the possible competition between amplification of target sequence and IAC this can be easily overcome if the IAC concentration in the reaction is properly optimised. Chapter 3, section 3.1.1 describes the construction, optimisation and application of IAC for the detection of diverse relevant foodborne viruses.

NAC: this negative control is used to verify that there is no contamination with the target, the SPCV or their amplicons in the reagent mixture used in the amplification reaction. It consists of a sample in which water takes the place of the nucleic acid extract. This control must include an IAC in the same reaction or an EAC in a separate

reaction. The expected result is to obtain neither signal from target virus nor a signal from the SPCV. However, target virus and SPCV IAC signals are expected.

PAC: the positive amplification control is used to verify the performance of the amplification reaction. The use of an IAC or EAC may also fulfil this purpose, but in the case of a molecular method using probes for the detection, a PAC would not only verify the amplification performance of the target sequence but also the performance of the probe. In a PAC reaction a known quantity of nucleic acid from the target virus is included, so a target virus signal should always be observed. The acquisition of known quantities of nucleic acid is not always easy, especially in the case of enteric viruses, as some of them such as HEV and NoV are non-culturable in the laboratory. For this reason in some cases synthetic nucleic acid sequences can be used as a PAC. The use of this synthetic nucleic acid will also serve a second purpose: they can be used as standards for quantification of viral presence in the samples. In chapter 3, section 3.1.4 the design and application of synthetic nucleic acid standards for detection (PAC) and quantification purposes is explained.

1.5 Technological processes to inactivate foodborne viruses

Historically, thermal treatment has been the most widely used procedure for microbial inactivation in foods. Thermally-processed food is subjected to temperatures ranging from 60ºC to 100ºC for a duration that varies from a few seconds to a minute. A large amount of energy is transferred to the food during this treatment, and this energy can trigger reactions leading to undesired organoleptic and / or nutritional effects in the food. Moreover, some foods most prone to becoming the primary source of foodborne viral outbreaks, such as shellfish, soft fruits or salad vegetables are often consumed raw or slightly cooked, so thermal treatments to inactivate viruses are not considered an

option for these foods. Consequently, non-thermal processing technologies to inactivate foodborne microorganisms have drawn considerable attention for their potential ability to overcome those limitations. They can also assist in the development of new products with improved quality attributes for a marketplace in continuous evolution to live up to consumers needs.

A wide variety of non-thermal processes have been in use recently: radiation, ultrasound, chemical disinfectants, pressure treatments and chemical compounds present in natural sources (e.g. essential oils from plants), among others. Table 2 summarizes the main processes used for viral inactivation that are reviewed in this Introduction and the mechanisms these processes use to inactivate viruses.

It must be pointed out however that some of these non-thermal processes alone are not sufficient to inactivate viruses in food. Hence, hurdle technologies that combine several of the above-mentioned systems should also be explored for virus inactivation.

Ultraviolet (UV) electromagnetic radiation has been proposed as an alternative to thermal methods to sanitize foods. The UV mechanism of virus inactivation is predominantly an attack on the viral nucleic acid: UV light causes the adjacent thymine or uracil residues to dimerize and produce photoproducts which have toxic and lethal effects on the virus. UV inactivation is, however, also dose-dependent and at high doses (≥1,000 mWs/cm^2) UV light can also affect the capsid proteins (Nuanualsuwan and Cliver, 2003). Fino and Kniel (2008) studied the UV inactivation of three viruses - feline calicivirus (FCV, a surrogate for NoV), and two picornaviruses, HAV and AiV - on three produce types that have been involved in foodborne viral outbreaks: green onions, lettuce, and strawberries. The inactivation rates differed in the three different produce types, possibly due to differences in their surfaces; spiked

lettuce was the most easily inactivated matrix, followed by green onions and strawberries.

Table 2. Inactivation technologies and the mechanisms of these technologies to inactivate viruses, affecting mainly the genome, the protein capsid or both.

Inactivation technology	*Inactivation mechanism*	
	Genome	Protein capsid
Temperature	-	Changes in the native antigenicity of the capsid
Electromagnetic radiation (UV)	Damage to nucleic acids	At high doses damage to capsid
Ionizing radiation (e-beam and gamma radiation)	Random breaks in nucleic acids and production of radicals by radiolysis of water	-
Ultrasound	Nucleic acid damage due to free radical production	-
Chemical disinfectants	Oxidation reactions	Oxidation reactions
Pressure	-	Effects on weak hydrophobic interactions and capsid disassembly and reassembly to a non-infectious particle
Natural antimicrobials	-	Components of the oil interfere with structures necessary for adsorption and entry into host cell.

Ionizing radiations with electron beam (e-beam) or gamma radiation have also been proposed. When ionizing radiation strikes bacteria and other microorganisms, its high energy breaks chemical bonds in molecules that are vital for growth and integrity. As a result, the microorganisms die, or can no longer multiply. After the Food and Drug Administration (FDA) approved the use of ionizing irradiation up to 4kGy (kilo Greys) as a pathogen kill step in fresh produce, it has been used in different studies. In one study e-beam was used to inactivate MNV-1 on fresh foods (shredded cabbage and diced strawberries) and model systems (phosphate-buffered saline, PBS and Dulbecco's modified Eagle's medium, DMEM) and the results suggest that the food matrix might provide increased survival for viruses (Sanglay *et al.*, 2011). In fact at the authorized

doses, one log or less reduction was observed in cabbage and strawberries; lower than the reduction observed in PBS and DMEM. In a similar study on rotavirus but using spinach as the food matrix the theoretical reduction in infection risks after a dose of e-beam of 3kGy drops from >3 in 10 persons to approximately 5 in 100 persons (Espinosa *et al.*, 2012). These results highlight the important role that the food matrix plays in the efficacy of e-beam radiation treatments. Another type of ionizing radiation, gamma radiation, has also been used to eliminate virus contaminated fresh produce. However, application of gamma radiation at the FDA-approved radiation dose limits proved impractical, as for MNV-1 only a 1.7 to 2.4 log reduction was observed in fresh produce and only when doses higher than the 4kGy authorised (i.e. 5.6kGy) were used (Feng *et al.*, 2011).

Using another principle, the *ultrasound* has also been proposed as a method for viral inactivation in liquid foods. High-intensity ultrasound (HIUS) has a frequency in the 20kHz–2MHz range and power of 100–500W/cm^2, which is destructive for microorganisms as it causes cavitation, which is the extremely fast creation and collapse of bubbles in a liquid medium, causing DNA damage via the production of free radicals. HIUS was applied on three foodborne virus surrogates MNV-1, FCV and MS2 bacteriophage, in two different matrices. Results from PBS and orange juice showed that HIUS effectiveness depended on the virus type, the initial titre of the viruses, and the matrix in which the virus were suspended. In fact, inactivation by HIUS in orange juice was much lower than in PBS (Su *et al.*, 2010).

Chemical disinfectants have also been the object of viral inactivation studies. Ozone has been suggested for produce disinfection as an alternative to the classic washing or immersion in water containing chlorine. Also with this inactivating agent the role of the food matrix appears to be crucial. In a study comparing the effect of ozone

treatment (6.25 parts per million, ppm) on FCV and MNV-1 in green onions, lettuce and sterile water, results showed that to achieve a 2 log MNV-1 inactivation in lettuce and green onions, treatment with ozone should be two times longer than for sterile water (Hirneisen *et al.*, 2012)

Another area of high interest for viral inactivation studies is the application of *pressure*. The use of high pressure to inactivate microorganisms such as *Escherichia coli* and *Staphylococcus aureus* was first described in 1895 by Royer, and in 1914 Hite developed hydrostatic pressure for the inactivation of some microorganisms in order to preserve fruits and vegetables (reviewed in Rivalain *et al.*, 2010). Since then, the application of high hydrostatic pressure (HHP) processing (also called high pressure processing or high pressure pasteurization) has been increased and developed to adapt to an industrial scale. In 1993 the first food product stabilized under high pressure reached the Japanese market and nowadays a wide range of high pressure treated products are available. For food safety industrial purposes HHP is applied to food at pressures of up to 1,000MPa (Kovac *et al.*, 2010). The pressure applied is transmitted instantaneously and isostatically, that is, the pressurization process is volume independent and affects the item to be treated with the same intensity and at the same time regardless its size or shape. HHP does not have effect on the covalent bonds of the primary structure of proteins; it however affects the conformation of proteins at its secondary and tertiary structures causing irreversible denaturation of the proteins which leads to a non-infectious form of the microorganism (Murchie *et al.*, 2005). Sections 3.3.1 and 3.3.2 describe two studies on the effect of HHP processing on infectivity and genome stability of NoV and AdV in different food and beverage matrices (i.e. strawberry puree and mineral water).

The application of *natural antimicrobials* for food preservation has also been proposed as a way of satisfying consumer demand for safer food, preserving as much as possible quality attributes (flavour, odour, colour and texture) and nutritional value. Whereas maintaining the natural character of minimally processed foods, with no artificial additives. In this way, natural preservatives derived from plants, animals or microflora are being largely studied (Tiwari *et al.*, 2009). Among the plant origin antimicrobials, chemical compounds present in the essential oil (EO) fraction of leaves, flowers, bulbs, seeds or other parts of the plant are under investigation.. The effectiveness of EOs with antiviral properties has been demonstrated for enveloped viruses such as dengue virus (DENV). In one study the inhibitory effects of EO on DENV seemed to be the direct cause of virus inactivation if the EO was applied before viral adsorption on the host cells (Ocazionez *et al.*, 2010). Regarding the antiviral effects of EO on non-enveloped viruses, such as the enteric viruses contaminating foods, some studies have been performed using grape seed extract (GSE).Used at concentrations of 0.2 to 2 mg/ml on enteric viruses and their surrogates, GSE has showed promising applications as a natural alternative to reduce viral contamination (Su and D'Souza, 2011). It has been also noted that the EO effect on the reduction of virus infectivity was modulated by the addition of interfering substances (i.e. dried milk and lettuce extract) that can be present in the real food items (Li *et al.*, 2012 in press). It is beyond doubt the potential use of EO on inactivation of enteric viruses, EO in foods can be used with a double scope: as an additive to change or improve taste as well as a preservative and antimicrobial agent, but more studies are needed. To further explore the potential usage of herbs EO to enhance food safety, a study of the antiviral effect of EO in foodborne viruses was performed as part of this Thesis (chapter 3, section 3.3.3

Chapter 2

Scope of the Thesis

2.1 Objectives

The gathering of information on viral prevalence in the food chain seems to be timely since foodborne viruses have become increasingly significant pathogens but they are still relatively understudied. To overcome this lack of information on viral prevalence correct methodology is a key factor and this implies the development of appropriate procedures for the sampling, concentration, extraction and detection of relevant foodborne viruses in foods. Similarly, the acquisition of data on viral prevalence in the food supply chain together with the utilisation of effective inactivation processes are essential to render foodstuffs safe and healthy for consumers.

Hence, the main objective of this Thesis has been *the detection, identification and monitoring of enteric viruses in the food supply chain and the assessment of the effectiveness of diverse inactivation technologies on enteric viruses*.

For the achievement of this main objective, (*i*) the implementation of standardised protocols including a complete suite of controls specifically designed for the detection of foodborne viruses by molecular methods will developed and the feasibility of the standardised protocols will be assessed by means of an international collaborative ring trial, (*ii*) sampling studies for virus surveillance in two different food supply chains (pork production and shellfish), as well as in a pig farm setting, will be performed and (*iii*) the effect on viral particles of high hydrostatic pressure processing and the chemical compounds of plant essential oils as inactivating procedures for foodborne viruses will be studied.

2.2 Thesis outline

The present Thesis work has been developed within the framework of the European FP7 project VITAL (*Integrated Monitoring and Control of Foodborne Viruses in European Food Supply Chains*- www.eurovital.org) under grant agreement No. KBBE 213178, which aimed to span viral contamination at various stages of production, processing and retail in the European food supply chains. As a participant laboratory, The Instituto Tecnológico Agrario de Castilla y León (ITACyL) took part in the development of standard operating procedures (SOPs) for the detection of foodborne viruses as well as in the sampling studies in two food supply chains (i.e. pork and shellfish production) and sampling in pig farms.

The dissertation book is organised in 8 chapters and contains the results obtained during the accomplishment of the VITAL project specific tasks as well as additional studies of viral inactivation using two different food processing technologies.

Chapter 1 contains a general introduction highlighting the relevance of foodborne viruses, and the methods for their detection and inactivation. In Chapter 2 the objectives and the outline are presented.

Chapter 3 contains the results divided in three main sections: detection methodology (section 3.1), sampling studies (section 3.2) and inactivation studies (section 3.3) (Figure 4).

In section 3.1 several studies aiming to improve detection procedures by means of the development of a suite of analytical controls to be used during the detection process, or tools to assess viral infectivity, were performed. The construction and analytical application of internal amplification controls (IACs) for detection of foodborne viruses

by molecular methods is described in section 3.1.1. Sections 3.1.2 and 3.1.3 describe the analytical application of a process control virus in the detection of foodborne viruses. The use of synthetic nucleic acid as standards for quantitative detection of enteric viruses by real-time PCR is described in section 3.1.4. The practical application of the previously developed method is described in section 3.1.5 as a multicenter collaborative trial to evaluate the detection of human adenovirus in berry fruits. Section 3.1.6 describes a study on the potential of enzymatic treatment to assess viral infectivity by molecular methods.

Section 3.2 consists of three studies aiming to gather information on viral prevalence in the pork production chain (section 3.2.1),the viral prevalence and the transmission rate of hepatitis E virus in farmed swine populations (section 3.2.2), and the viral prevalence in the shellfish production chain (section 3.2.3).

Section 3.3 describes three inactivation studies performed using high hydrostatic pressure on norovirus (section 3.3.1) and adenovirus (section 3.3.2) and the effects of essential oils on non-enveloped viruses (section 3.3.3).

Chapter 4 consists of a general discussion, and in chapter 5 the conclusions drawn are described. Chapter 6 and Chapter 7 are the summary and conclusions, respectively, in Spanish. Chapter 8 contains the references cited in the introduction, in section 3.1.3 (unpublished data) and in the general discussion.

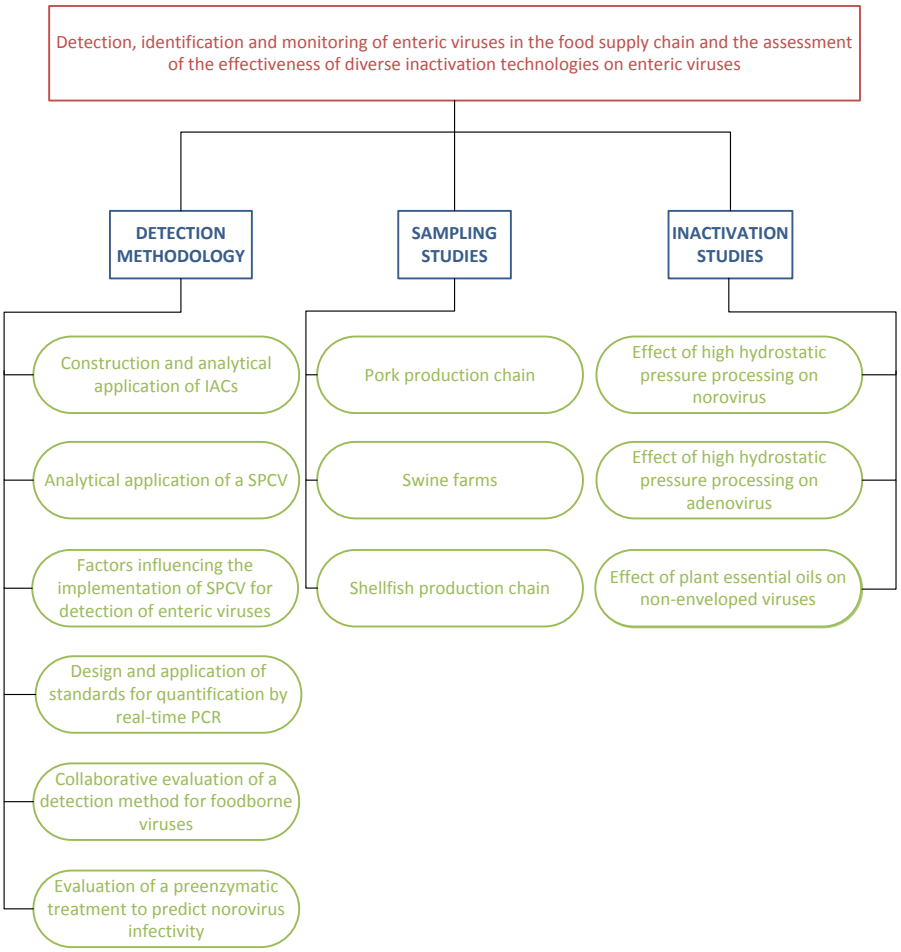


Figure 4. Schematic representation of the contents of this Thesis. Results are organized in three main groups: detection methodology (section 3.1), sampling studies (section 3.2) and inactivation studies (section 3.3).

Chapter 3

Results

Detection Methodology

Section 3.1

3.1.1

Construction and Analytical Application of Internal Amplification Controls (IAC) for Detection of Food Supply Chain-Relevant Viruses by Real-Time PCR-Based Assays

Marta Diez-Valcarce[1], Katarina Kovač[1], Nigel Cook[2], David Rodríguez-Lázaro[1] and Marta Hernández[1]

[1] Instituto Tecnológico Agrario de Castilla y León (ITACyL), Valladolid, Spain [2] Food and Environment Research Agency (FERA), Sand Hutton, York, United Kingdom

Published in Food Analytical Methods, Vol. 4, 437-445

Food Anal. Methods (2011) 4:437–445
DOI 10.1007/s12161-011-9224-2

Construction and Analytical Application of Internal Amplification Controls (IAC) for Detection of Food Supply Chain-Relevant Viruses by Real-Time PCR-Based Assays

Marta Diez-Valcarce · Katarina Kovač · Nigel Cook · David Rodríguez-Lázaro · Marta Hernández

Received: 15 December 2010 / Accepted: 11 February 2011 / Published online: 5 April 2011
© The Author(s) 2011. This article is published with open access at Springerlink.com

Abstract Internal amplification controls (IACs) were constructed for incorporation into real-time nucleic acid amplification assays for bovine polyomavirus, hepatitis A virus, hepatitis E virus, human adenovirus, human norovirus genogroup I, human norovirus genogroup II, murine norovirus and porcine adenovirus. The addition of optimised amounts of IAC into the assays did not affect the limits of detection for each specific target virus. A poorly performed extraction of viral nucleic acids was simulated, and the effectiveness of IACs in identifying failed assays was demonstrated. The IACs constructed in this study can be reliably used in their specific assays to provide a robust control that can be routinely applied in the analysis of foods for viruses.

Keywords Internal amplification control · False negatives · Real-time PCR · Food · Enteric virus

Introduction

Gastroenteritis produced by ingestion of food contaminated with enteric viruses is an important concern for public health. Thus, detection of the presence of enteric viruses—particularly norovirus, hepatitis A and E and adenovirus—in foods is an important issue in food safety, and a rapid and robust diagnostic methodology is needed (Croci et al. 2008; Greening and Hewitt 2008; Cook and Rzezutka 2006). Because at present most of these viruses cannot, or with difficulty, be cultured and integrated cell culture real-time PCR methods are useful but too time-consuming for the quick results required by the food industry, a detection approach based on nucleic acid amplification is necessary (Rodríguez-Lázaro et al. 2007; Bosch et al. 2011). However, the application of nucleic acid amplification to foodstuffs can be complicated by the presence of inhibitory substances (Rodríguez-Lázaro et al. 2004, 2006; Rodríguez-Lázaro and Hernandez 2006), which can cause false negative interpretations of the results. It is imperative therefore for the effective implementation of nucleic acid amplification in food analysis that appropriate controls are used to verify there has been no interference caused by the presence of inhibitory substances (Rodríguez-Lázaro et al. 2007; Bosch et al. 2011). The incorporation of an internal amplification control (IAC) will identify failed reactions (Bosch et al. 2011; Rodríguez-Lázaro et al. 2004, 2007). An IAC is a non-target nucleic acid sequence present in every reaction which can be co-amplified simultaneously with the target sequence (Cone et al. 1992). In a reaction without an IAC, a negative response can mean either that there is no target sequence present in the reaction or that the amplification has been inhibited. However, with the use of an IAC in each reaction, the absence of response both from the target and the IAC indicates that the reaction has failed, and the sample must be retested to avoid any false negative interpretation of its analysis.

The aim of this study was to construct IACs for nucleic acid amplification assays for viruses relevant to the analysis of foods and to define their analytical application. The viruses were human norovirus genogroups I (NoVGI) and II (NoVGII), hepatitis A virus (HAV) and hepatitis E virus (HEV), murine norovirus (MNV; which could be used to

M. Diez-Valcarce · K. Kovač · D. Rodríguez-Lázaro · M. Hernández (✉)
Instituto Tecnológico Agrario de Castilla y León (ITACyL),
Ctra. Burgos, km.119,
47071 Valladolid, Spain
e-mail: ita-herperma@itacyl.es

N. Cook
Food and Environment Research Agency (FERA),
Sand Hutton,
York, UK

assess the efficiency of a pre-nucleic acid amplification sample treatment), human adenovirus (HAdV; which could be used to indicate that routes of virus contamination exist from human sources), porcine adenovirus (PAdV; which could be used to indicate that routes of virus contamination exist from porcine sources) and bovine polyomavirus (BPyV; which could be used to indicate that routes of virus contamination exist from bovine sources).

Materials and Methods

Viruses and Viral Nucleic Acids

Bovine polyomavirus DNA and porcine adenovirus DNA were kindly provided by Professor Rosina Gironés of the University of Barcelona, Spain. Hepatitis A virus suspension was kindly provided by Dr. Dario de Medici of the Istituto Superiore de Sanità, Rome, Italy. Hepatitis E virus RNA was kindly provided by Dr. Malcolm Banks of the Veterinary Laboratories Agency, Weybridge, UK. Murine norovirus (MNV1) was supplied by Herbert W. Virgin IV, Washington University School of Medicine (US) and human adenovirus type 2 (HAdV2) was provided by Dr. Rosina Girones at the University of Barcelona (Spain), both viruses were replicated in the Dr. Franco Ruggeri's laboratory at the Istituto Superiore de Sanità (Rome, Italy). MNV1 was propagated in RAW264.7 cells and titrated by end-point dilution (final stock concentration, 10^7 $TCID_{50}$/ml). HAdV-2 was propagated in A549 cells and titrated by the same technique (final stock concentration, 10^7 $TCID_{50}$/ml). Human norovirus genogroup I RNA and human norovirus genogroup II RNA were kindly provided by Dr. Ana Maria de Roda Husman of RIVM, Bilthoven, The Netherlands.

Primers and Probes

The oligonucleotides used in this study are shown in Tables 1 and 2. IAC primers were designed using the software Primer Express™ version 2.0 (Applied Biosystems, Foster City, CA, USA). All oligonucleotides were purchased from MWG Biotech AG (Ebersberg, Germany), except the minor groove binder (MGB) TaqMan probes HAV150(−), MGB-ORF1/ORF2 and PrfAP that were acquired from Applied Biosystems (Warrington, UK) and NV1LCpr that was from Sigma-Aldrich (St. Louis, MO, USA).

Bovine Polyomavirus Real-Time PCR

This assay was a duplex real-time PCR using the primers and conditions described by Hundesa et al. (2010), with the inclusion of an IAC and a carryover contamination prevention system utilising uracil *N*-glycosylase (UNG). The reaction contained 1× TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.4 μM each primer, 0.120 μM bovine polyomavirus TaqMan probe (labelled with FAM), 50 nM IAC probe (labelled with VIC) and varying copies of bovine polyomavirus IAC. A 10-μl sample of nucleic acid extract was added to make a final reaction volume of 25 μl. The thermocycling conditions were 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C.

Hepatitis A Virus Reverse Transcription Real-Time PCR

This assay was a one-step duplex reverse transcription real-time PCR using the primers and conditions described by Costafreda et al. (2006), with the inclusion of an IAC. The reaction contained 1× RNA Ultrasense reaction mix (Invitrogen, Carlsbad, CA, USA), 0.5 μM primer HAV68, 0.9 μM primer HAV240, 0.25 μM probe HAV150(−) (labelled with FAM), 50 nM IAC probe (labelled with VIC), 1× ROX reference dye (Invitrogen), 1 μl RNA Ultrasense enzyme mix (Invitrogen) and varying copies of HAV IAC. A 10-μl sample of nucleic acid extract was added to make a final reaction volume of 20 μl. The thermocycling conditions were 15 min at 50 °C, 2 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C.

Hepatitis E Virus Reverse Transcription Real-Time PCR

This assay was a one-step duplex reverse transcription real-time PCR using the primers and conditions described by Jothikumar et al. (2006), with the inclusion of an IAC. The reaction contained 1× RNA Ultrasense reaction mix (Invitrogen), 0.25 μM each primer, 0.1 μM probe HEV-P (labelled with FAM), 50 nM IAC probe (labelled with VIC), 1× ROX reference dye (Invitrogen), 1 μl RNA Ultrasense enzyme mix (Invitrogen) and varying copies of HEV IAC. A 10-μl sample of nucleic acid extract was added to make a final reaction volume of 20 μl. The thermocycling conditions were 15 min at 50 °C, 2 min at 95 °C, followed by 45 cycles of 10 s at 95 °C, 20 s at 55 °C and 15 s at 72 °C.

Human Adenovirus Real-Time PCR

This assay was a duplex real-time PCR using the primers and conditions described by Hernroth et al. (2002), with the inclusion of an IAC and a carryover contamination prevention system utilising UNG. The reaction contained 1× TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.9 μM each primer, 0.225 μM adenovirus TaqMan probe (labelled with FAM), 50 nM IAC probe (labelled with VIC) and varying copies of adenovirus IAC. A 10-μl sample of nucleic acid extract was added to

Table 1 Hybrid oligonucleotides designed in this study for the construction of the IACs and probe used for IAC detection

Target	Name	Type	Sequence (5′–3′)	IAC size (bp)
BPyV	QB-F1-1IAC	Forward primer	CTAGATCCTACCCTCAAGGGAAT**GGCTCTATTTGCGGTC**	115
	QB-R1-1IAC	Reverse primer	TTACTTGGATCTGGACACCAAC**TCTTGATGCCATCAGGA**	
HAV	HAVIACF	Forward primer	TCACCGCCGTTTGCCTAG**GGCTCTATTTGCGGTC**	107
	HAVIACR	Reverse primer	GGAGAGCCCTGGAAGAAAG**TCTTGATGCCATCAGGA**	
HEV	HEVIACF	Forward primer	GGTGGTTTCTGGGGTGAC**GGCTCTATTTGCGGTC**	106
	HEVIACR	Reverse primer	AGGGGTTGGTTGGATGAATCTTGATGCCATCAGGA	
HAdV	IACAdF	Forward primer	CWTACATGCACATCKCSGG**GGCTCTATTTGCGGTCAACTT**	107
	IACAdR	Reverse primer	CRCGGGCRAAYTGCACCAG**TCTTGATGCCAT**	
NoVGI	NOR1IACF	Forward primer	CGCTGGATGCGNTTCCAT**GGCTCTATTTGCGGTC**	111
	NOR1IACR	Reverse primer	CCTTAGACGCCATCATCATTTAC**TCTTGATGCCATCAGGA**	
NoVGII	NOR2IACF	Forward primer	ATGTTCAGRTGGATGAGRTTCTCWGA**GGCTCTATTTGCGGTC**	117
	NOR2IACR	Reverse primer	TCGACGCCATCTTCATTCACA**TCTTGATGCCATCAGGA**	
MNV1	IACMuNvF	Forward primer	CACGCCACCGATCTG**TTCTGGGCTCTATTTGCGGT**	107
	IACMuNvR	Reverse primer	GCGCTGCGCCATCACTC**TCTTGATGCCATCAG**	
PAdV	IACPAdF	Forward primer	AACGGCCGCTACTGCAAG**GGCTCTATTTGCGGTC**	105
	IACPAdR	Reverse primer	AGCAGCAGGCTCTTGAGG**TCTTGATGCCATCAGGAG**	
L. monocytogenes	PrfAP	Probe	VIC-CCATACACATAGGTCAGG-MGBNFQ	

The sequences shown in bold are identical in each forward or reverse primer and correspond to a fragment of the *L. monocytogenes prfA* gene. Sequences underlined are identical to the specific primers for each target virus. Bases within the degenerated primers correspond to W=A or T; Y=C or T; K=G or T; R=A or G; S=C or G and N=A, T, C or G

make a final reaction volume of 25 µl. The thermocycling conditions were 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C.

Human Norovirus ggI Reverse Transcription Real-Time PCR

This assay was a one-step duplex reverse transcription real-time PCR using the primers and conditions described by Svraka et al. (2007), with the inclusion of an IAC. The reaction contained 1× RNA Ultrasense reaction mix (Invitrogen), 0.5 µM primer QNIF4, 0.9 µM primer NV1LCR, 0.25 µM probe NV1LCpr (labelled with FAM), 50 nM IAC probe (labelled with VIC), 1× ROX reference dye (Invitrogen), 1 µl RNA Ultrasense enzyme mix (Invitrogen) and varying copies of norovirus ggI IAC. A 10-µl sample of nucleic acid extract was added to make a final reaction volume of 20 µl. The thermocycling conditions were 15 min at 50 °C, 2 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C.

Human Norovirus ggII Reverse Transcription Real-Time PCR

This assay was a one-step duplex reverse transcription real-time PCR using the primers and conditions described by da Silva et al. (2007), with the inclusion of an IAC. The reaction contained 1× RNA Ultrasense reaction mix (Invitrogen), 0.5 µM primer QNIF2, 0.9 µM primer COG2R, 0.25 µM probe QNIFS (labelled with FAM), 50 nM IAC probe (labelled with VIC), 1× ROX reference dye (Invitrogen), 1 µl RNA Ultrasense enzyme mix (Invitrogen) and varying copies of norovirus ggII IAC. A 10-µl sample of nucleic acid extract was added to make a final reaction volume of 20 µl. The thermocycling conditions were 15 min at 50 °C, 2 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C.

Murine Norovirus Reverse Transcription Real-Time PCR

This assay was a one-step duplex reverse transcription real-time PCR using the primers and conditions described by Baert et al. (2008), with the inclusion of an IAC. The reaction contained 1× RNA Ultrasense reaction mix (Invitrogen), 0.2 µM each primer, 0.2 µM probe MGB-ORF1/ORF2 (labelled with FAM), 50 nM IAC probe (labelled with VIC), 1× ROX reference dye (Invitrogen), 1 µl RNA Ultrasense enzyme mix (Invitrogen) and varying copies of murine norovirus IAC. A 10-µl sample of nucleic acid extract was added to make a final reaction volume of 20 µl. The thermocycling conditions were 15 min at 50 °C, 2 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

Porcine Adenovirus Real-Time PCR

This assay was a duplex real-time PCR using the primers and conditions described by Hundesa et al. (2009), with the inclusion of an IAC and a carryover contamination

Table 2 Oligonucleotides used in this study to detect target viruses by real-time or RT real-time-PCR

Target	Name	Type	Sequence (5′–3′)	Amplicon size (bp)	Reference
BPyV	QB-F1-1	Forward primer	CTAGATCCTACCCTCAAGGGAAT	77	Hundesa et al. (2010)
	QB-R1-1	Reverse primer	TTACTTGGATCTGGACACCAAC		
	QB-P1-2	Probe	6FAM-GACAAAGATGGTGTGTATCCTGTTGA -BHQ		
HAV	HAV68	Forward primer	TCACCGCCGTTTGCCTAG	173	Costafreda et al. (2006)
	HAV240	Reverse primer	GGAGAGCCCTGGAAGAAAG		
	HAV150(−)	Probe	6FAM-CCTGAACCTGCAGGAATTAA-MGBNFQ		
HEV	JVHEVF	Forward primer	GGTGGTTTCTGGGGTGAC	70	Jothikumar et al. (2006)
	JVHEVR	Reverse primer	AGGGGTTGGTTGGATGAA		
	JVHEVP	Probe	6FAM-TGATTCTCAGCCCTTCGC-BHQ		
HAdV	AdF	Forward primer	CWTACATGCACATCKCSGG	69	Hernroth et al. (2002)
	AdR	Reverse primer	CRCGGGCRAAYTGCACCAG		
	AdP1	Probe	6FAM-CCGGGCTCAGGTACTCCGAGGCGTCCT-BHQ		
NoVGI	QNIF4	Forward primer	CGCTGGATGCGNTTCCAT	86	Svraka et al. (2007)
	NV1LCR	Reverse primer	CCTTAGACGCCATCATCATTTAC		
	NV1LCpr	Probe	6FAM-TGGACAGGAGAYCGCRATCT-BHQ		
NoVGII	QNIF2d	Forward primer	ATGTTCAGRTGGATGAGRTTCTCWGA	89	da Silva et al. (2007)
	COG2R	Reverse primer	TCGACGCCATCTTCATTCACA		
	QNIFS	Probe	6FAM-AGCACGTGGGAGGGCGATCG-BHQ		
MNV1	Fw-ORF1/ORF2	Forward primer	CACGCCACCGATCTGTTCTG	109	Baert et al. (2008)
	Rv-ORF1/ORF2	Reverse primer	GCGCTGCGCCATCACTC		
	MGB-ORF1/ORF2	Probe	6FAM-CGCTTTGGAACAATG-MGB-NFQ		
PAdV	Q-PAdV-F	Forward primer	AACGGCCGCTACTGCAAG	68	Hundesa et al. (2009)
	Q-PAdV-R	Reverse primer	AGCAGCAGGCTCTTGAGG		
	Q-PAdV-P	Probe	6FAM-CACATCCAGGTGCCGC-BHQ		

Probes were labelled with 6FAM at the 5′ end and MGB-NFQ or BHQ at the 3′ end

6FAM 6-carboxyfluorescein, *MGB-NFQ* minor groove binder non-fluorescent quencher, *BHQ* black hole quencher

prevention system utilising UNG. The reaction contained 1× TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.9 μM each primer, 0.225 μM porcine adenovirus TaqMan probe (labelled with FAM), 50 nM IAC probe (labelled with VIC) and varying copies of porcine adenovirus IAC. A 10-μl sample of nucleic acid extract was added to make a final reaction volume of 25 μl. The thermocycling conditions were 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C, 20 s at 55 °C and 20 s at 60 °C.

IAC Construction

The principles for the construction of IACs can be explained in two different phases: First, PCR amplification of non-target DNA is performed using hybrid oligonucleotide primers. This produces a chimeric DNA molecule containing non-target sequences flanked by target sequences complementary to the virus-specific primers. This molecule is then cloned into a plasmid. If the IAC is for RNA virus detection, the plasmid should contain a T7 RNA polymerase promoter, and IAC RNA transcripts are subsequently produced by T7 RNA polymerase. The plasmid or the RNA transcript is the chimeric IAC which is co-amplified with the virus primers and detected using a fluorescent probe complementary to the internal non-target sequence (Fig. 1). When using a real-time PCR-based assay, the virus target amplicons are detected with specific hydrolysis probes, labelled with one fluorophore (e.g. FAM), and the IAC amplicons are detected with the specific IAC probe, labelled with a different fluorophore (e.g. VIC).

Each IAC in this study was designed as a DNA or RNA molecule containing sequences from the *prfA* gene from *Listeria monocytogenes* (nucleotide positions 2281–2348, AN AY512499) flanked by the sequences complementary to the primers used in the specific assays. With the exception of the *prfA* sequence, the IAC sequences did not show significant similarity to any other sequence deposited in public databases, as shown by BLAST-N searches (National Center for Biotechnology Information, Bethesda, MD, USA; www.ncbi.nlm.nih.gov). The chimeric DNA molecules were generated by PCR as previously described (Rodríguez-Lázaro et al. 2004, 2005) using as template 5 ng of *L. monocytogenes* strain CECT 935 DNA and the

specific set of construction hybrid primers for each IAC (Table 1), which contained the corresponding *prfA* target sequence plus a 5′ tail with the virus forward/reverse primer sequences. The PCR products were excised from a 2% 1× TBE agarose gel and purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), then cloned into the pCR 2.1-TOPO Vector (Invitrogen) in the case of IACs for the HAV, HEV and NoVGII assays or into the pGEM-T Easy Vector (Promega, Madison, WI, USA) in the case of IACs for the NoVGI, BPyV, HAdV, PAdV and MNV1 assays. The concentration and quality of the plasmid DNA stock solutions were determined by fluorimetry using Quant-iT PicoGreen dsDNA reagents (Invitrogen) in a NanoDrop 3300 Fluorospectrometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Production of IAC RNA

In vitro transcription was performed to obtain RNA fragments for the HAV, HEV, NoVGI, NoVGII and MNV1 IACs using the Riboprobe System—T7 (Promega). To prevent carryover contamination by DNA, RNA was treated with DNase (RQ1 RNase-free DNase, Promega) at a concentration of 1 U/μg for 15 min at 37 °C, then purified using an RNeasy Mini Kit (Qiagen) according to the manufacturer's recommendations. The concentration and purity of the RNA stock solutions were determined by fluorimetry using Quant-iT RiboGreen RNA and Quant-iT PicoGreen dsDNA reagents (Invitrogen) in a NanoDrop 3300 Fluorospectrometer (Thermo Fisher Scientific).

Copy Number Calculation

The number of IAC copies was calculated by dividing the amount of IAC in each stock solution by the weight of one IAC molecule, as follows:

$$\text{DNA IAC copies} = \text{g in the IAC stock} / \left(\text{bp} \times 660\ \text{DA/bp} \times 1.6 \times 10^{-27}\ \text{kg/DA} \times 1 \times 10^{-3}\ \text{g/kg}\right)$$

$$\text{RNA IAC copies} = \text{g in the IAC stock} / \left(\text{bp} \times 320\ \text{DA/bp} \times 1.6 \times 10^{-27}\ \text{kg/DA} \times 1 \times 10^{-3}\ \text{g/kg}\right)$$

Optimization of IAC-Containing Nucleic Acid Amplification Assays

After construction and copy number calculation of the IACs, the following steps were performed, in this particular order:

1. Verification that the IAC could be amplified and detected in a uniplex assay
2. Verification that the IAC and the template could be simultaneously amplified and detected in the same reaction tube, i.e. a duplex assay
3. Optimization of the IAC probe concentration by performing (reverse transcription) real-time PCRs without virus nucleic acids but containing 3,000 IAC copies (or 2,000 IAC copies for RNA virus methods), 100 nM of virus target probe and increasing amounts of the IAC probe (25, 50 and 100 nM)
4. Determination of the optimal amount of IAC. First, each assay's target consistent limit of detection (LOD) was determined in the absence of an IAC by establishing the lowest number of genome equivalents (GE) that could be detected in every one of five replicate reactions. Then, decreasing numbers of IACs (down to approximately one copy) were added, and the lowest IAC number which could be consistently detected in five replicate reactions without affecting the LOD of the target was established

Demonstration of IAC Applicability in the Detection of Viruses in Food

To demonstrate the effectiveness of the IAC approach, a foodstuff artificially contaminated with two virus types was analysed. Strawberry puree (25 g) was placed into a sterile plastic bag. Approximately 10^6 $TCID_{50}$ of human adenovirus and 10^6 $TCID_{50}$ of murine norovirus were added to the puree. The sample was then processed using the method of Dubois et al. (2006). Approximately 25 g fruit was placed in a sterile beaker. Forty millilitres of Tris-glycine, pH 9.5, buffer containing 1% beef extract and 6,500 U pectinase (Pectinex™ Ultra SPL Solution, Sigma) was added to the sample, which was then agitated at room temperature for 20 min by rocking at 60 rpm. The pH was maintained at 9.0 throughout (if necessary adjusting using 4% (*w*/*v*) sodium hydroxide, extending the period of agitation by 10 min each time an adjustment was made. In strongly coloured berries, a change in colour of the eluate from blue/purple to red was considered indicative of acidification and was used to trigger pH adjustment). The liquid was decanted from the beaker through a strainer (e.g. a tea strainer) into one 50-ml or two smaller centrifuge tubes and centrifuged at 10,000×*g* for 30 min at 4 °C. The supernatant(s) was decanted into a single clean tube or bottle and the pH adjusted to 7.2. Volumes (0.25) of 50% (*w*/*v*) polyethylene glycol 8000/1.5 M NaCl were then added and mixed by shaking for 1 min. The suspension was then incubated with gentle rocking at 4 °C for 60 min before centrifugation at 10,000×*g* for 30 min at 4 °C. The supernatant was discarded and the

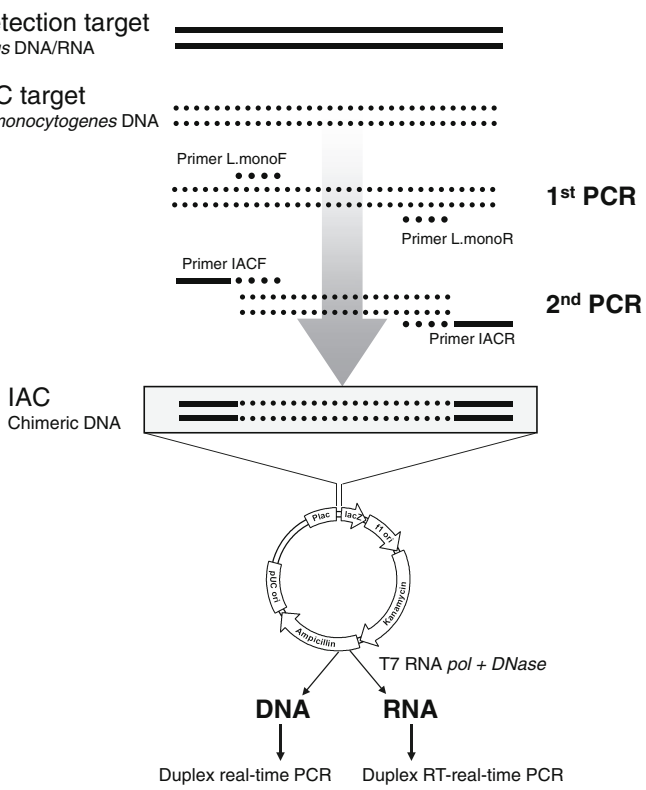


Fig. 1 IAC construction

pellet compacted by centrifugation at 10,000×*g* for 5 min at 4 °C before resuspension in 500 µl phosphate-buffered saline. The suspension was then transferred to a chloroform-resistant tube, and 500 µl chloroform/butanol (1:1) was added and mixed by vortexing. The sample was allowed to stand for 5 min and then centrifuged at 10,000×*g* for 15 min at 4 °C. The aqueous phase was transferred to a clean tube and immediately used for nucleic acid extraction or stored at −20 °C. Nucleic acids were extracted using a NucliSENS miniMAG kit (bio-Mérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. The final elutions were performed with 150 µl elution buffer, resulting in a 300-µl nucleic acid extract. The nucleic acid extract was assayed immediately or stored at −70 °C.

To demonstrate how the IACs would indicate reaction failure, a situation in which nucleic acid purification had been poorly performed was modelled by adding 50 µl of non-extracted strawberry puree to 50 µl nucleic acid extract prior to nucleic acid amplification.

Results

Optimization of the IAC Probe Concentration

For each virus detection method, several experiments were performed as detailed in "Materials and Methods" in duplex (IAC and target probes) and uniplex (IAC probe) formats, and the best performance concentration chosen was that which showed the lowest C_p value with less difference between the duplex and uniplex formats and having the most similar value within the five replicates (Table 3). The results indicated that a probe concentration of 50 nM in all assays exhibits enough fluorescence intensity and that the assay performance in duplex and uniplex was satisfactory.

Determination of the Optimal Amount of IAC for Each Assay

After optimization of IAC amount per reaction as detailed in "Materials and Methods", the target LOD for each virus

was recalculated; norovirus GGI had 100 GE per reaction, and the LOD for all other target viruses was 10 GE (Table 4). These LODs were established in the amount of virus GE when the target signal was present in all five replicates, and if just one target signal was not present in all replicates, the results were not considered robust enough. It is remarkable that the LOD of the HEV system was established at 10 GE when C_p values were as high as 41.50±1.80 whilst at 100 GE were 33.56±0.61 (data not shown), indicating consistency of the results.

Demonstration of IAC Applicability in the Detection of Viruses in Food

The results from the analysis of the purified and inhibitor-containing nucleic acid extracts from the artificially contaminated strawberry puree are shown in Table 5. A signal was obtained for both target viruses and their IACs from the assay of the purified extract. No target virus or IAC signal was obtained from the assay of the inhibitor-containing extract.

Discussion

If monitoring of food supply chains for viruses is to be effectively performed as part of a food safety programme (Rodríguez-Lázaro et al. 2007), then it is vitally necessary that the reliability of the analytical results can be verified. Many matrices from the food supply chains which are most prone to virus contamination—salad vegetable, shellfish and soft fruit—contain substances which can inhibit nucleic acid amplification; therefore, it is essential that this verification includes the recognition of failed assays as these may mask the presence of a virus pathogen by a false negative interpretation of the results (Hoorfar et al. 2004). The use of an amplification control can provide this recognition.

There are two approaches to the use of amplification controls. The first is to run two separate reactions for each sample: One (the test) reaction contains only the sample nucleic acid, but the other (the control reaction) contains the sample nucleic acid plus the amplification control (Costafreda et al. 2006). The latter is thus termed an external amplification control (EAC). If it is successfully amplified to produce a signal, any non-production of a target signal in the test reaction is considered to signify that the sample was uncontaminated. If, however, no signal is produced in both the test and control reactions, it signifies that the nucleic acid extract contains inhibitory substances and the reaction has failed.

In contrast to an EAC, an IAC is a non-target DNA sequence present in the very same reaction as the sample nucleic acid extract (Hoorfar et al. 2004). If it is successfully amplified to produce a signal, any non-

Table 3 Optimization of the IAC probe (PrfAP) by testing three different concentrations (25, 50 and 100 nM) in uniplex and duplex formats for each of the target viruses: BPyV, HAV, HEV, HAdV, NoVGI and NoVGII, MNV1, and PAdV

		BPyV			HAV			HEV			HAdV			NoVGI			NoVGII			MNV1			PAdV		
PrfAP (nM)		25	50	100	25	50	100	25	50	100	25	50	100	25	50	100	25	50	100	25	50	100	25	50	100
Duplex	C_p	26.35	26.50	26.74	27.54	25.00	23.82	24.71	24.12	23.92	41.06	28.65	27.16	27.01	24.41	22.86	28.43	23.31	21.16	31.27	29.05	28.19	27.55	26.95	26.09
	SD	0.14	0.09	0.07	0.05	0.05	0.14	0.07	0.06	0.11	3.11	0.67	0.16	0.07	0.08	0.14	0.70	0.16	0.22	0.15	0.24	0.13	0.19	0.14	0.33
Uniplex	C_p	26.48	26.59	26.79	27.94	25.41	24.16	24.19	23.67	23.48	36.49	30.11	27.46	24.85	24.12	22.92	24.89	23.88	21.65	30.80	28.77	28.00	27.67	27.07	26.43
	SD	0.08	0.04	0.02	0.14	0.09	0.24	0.06	0.38	0.40	1.21	0.18	0.32	0.14	0.08	0.16	0.07	0.29	0.43	0.24	0.16	0.14	0.08	0.07	0.14

BPyV bovine polyomavirus, *HAV* hepatitis A virus, *HEV* hepatitis E virus, *HAdV* human adenovirus, *NoVGI* human norovirus group I, *NoVGII* human norovirus group II, *MNV1* murine norovirus, *PAdV* porcine adenovirus

Table 4 Limits of detection of BPyV, HAV, HEV, HAdV, NoVGI and NoVGII, MNV1 and PAdV assays and optimal number of IAC copies for each assay

	BPyV	HAV	HEV	HAdV	NoVGI	NoVGII	MNV1	PAdV
LOD	10	10	10	10	100	10	10	10
C_p±SD	36.37±0.59	26.28±0.61	41.50±1.80	34.62±0.60	29.78±0.21	27.54±0.09	38.74±0.56	36.54±0.56
IAC	300	300	300	100	300	300	600	100
C_p±SD	28.79±0.06	30.29±0.31	35.48±0.36	33.69±0.25	28.38±0.10	32.65±0.03	36.07±0.44	34.66±0.10

BPyV bovine polyomavirus, *HAV* hepatitis A virus, *HEV* hepatitis E virus, *HAdV* human adenovirus, *NoVGI* human norovirus group I, *NoVGII* human norovirus group II, *MNV1* murine norovirus, *PAdV* porcine adenovirus, *LOD* limit of detection of the specific target virus

production of a target signal in the reaction is considered to signify that the sample was uncontaminated. If, however, the reaction produces neither a signal from the target nor the IAC, it signifies that the reaction has failed.

Optimally, since using different primer sets may cause the amplification control to react to an inhibitory substance differently to the target, it should possess the same primer sequences as the target: This is the "competitive" strategy (Hoorfar et al. 2004). It is so called because the amplification control can compete with the target for the primers. This potential competition issue has led some workers to adopt the EAC approach, but this does contain a degree of ambiguity because one can never be completely certain that the test reaction has not individually failed, for example, through pipetting error or non-homogeneous contamination by inhibitory substances. For example, if an EAC signal is produced in the control reaction but no target signal is produced in the test reaction, one cannot be completely certain that the test reaction has not failed. Using an IAC eliminates this ambiguity since it is present in the mastermix and a signal will always appear when the reaction has not failed or high levels of competing target are not present (if they are, a target signal will be produced anyway).

The concern of the proponents of the EAC approach regards the possibility that a low level of target may be outcompeted by the IAC, leading to a false negative result. However, a thoroughly optimised assay should not present these problems (D'Agostino et al. 2004; Rodríguez-Lázaro et al. 2010). In the current study, the amount of IAC incorporated in each assay was thoroughly optimised to ensure that it did not interfere with the analytical sensitivity of the assay. The limit of detection of the target of each assay remains the same.

Nonetheless, the IAC must be present in the reaction in sufficient quantity to perform its function, and it can only do that reliably if it consistently shows a signal in the absence of a target or in the presence of low target concentrations. The IACs developed in this study all had this capacity.

Finally, it should be demonstrated that an amplification control can identify failed reactions. In this study, using strawberries, which have been implicated in several outbreaks of viral disease (Food and Agriculture Organization of the United Nations/World Health Organization 2008) and which have often been found to contain inhibitory substances (Croci et al. 2008), the IACs showed that the performance of assays for both RNA and DNA viruses could be verified.

In conclusion, this study has produced a suite of IACs for nucleic acid amplification assays suitable for use in the analysis of food supply chain samples for viruses. The IACs constructed in this study can be reliably used in their specific assays and thus provide a robust control that can be routinely applied in the analysis of foods for viruses.

Table 5 Detection of viruses in purified and inhibitor-containing nucleic acid extracts of strawberry puree artificially contaminated with human adenovirus and murine norovirus

	Human adenovirus		Murine norovirus		Interpretation
	Target	IAC	Target	IAC	
Purified extract	20.88±0.15[b] (9/9)[c]	33.52±0.30 (9/9)	35.05±0.35 (9/9)	37.04±0.69 (9/9)	Positive
Inhibitor-containing extract[a]	Undet. (0/9)	Undet. (0/9)	Undet. (0/9)	Undet. (0/9)	False negative

[a] Non-extracted strawberry puree suspension (50 μl) added to 50 μl of nucleic acid extracted from artificially contaminated strawberry puree

[b] Mean and standard deviation of C_p values of three independent nucleic acid amplification reactions using three replicates in each

[c] Positive reactions out of nine reactions

Acknowledgements This work was supported by the EU VITAL project contract no. 213178. M.D.-V. received a Ph.D. studentship from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA). N.C. acknowledges the support of the United Kingdom Food Standards Agency.

Open Access This article is distributed under the terms of the Creative Commons Attribution Noncommercial License which permits any noncommercial use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.

References

Baert L, Wobus CE, Van Coillie E, Thackray LB, Debevere J, Uyttendaele M (2008) Appl Environ Microbiol 74:543

Bosch A, Sánchez G, Abbaszadegan M, Carducci A, Guix S, Le Guyader FS, Netshikweta R, Pinto R, van der Poel WHM, Rutjes S, Sano D, Taylor MB, van Zyl WB, Rodríguez-Lázaro D, Kovač K, Sellwood J (2011) Food Anal Methods 4:4

Cone RW, Hobson AC, Huang ML (1992) J Clin Microbiol 30:3185, Erratum in: J Clin Microbiol 32:2633 (1994)

Cook N, Rzezutka A (2006) Hepatitis viruses. In: Motarjemi Y, Adams M (eds) Emerging foodborne pathogens. Woodhead, Cambridge, pp 282–308

Costafreda MI, Bosch A, Pintó RM (2006) Appl Environ Microbiol 72:3846

Croci L, Dubois E, Cook N, de Medici D, Schultz AC, China B, Rutjes S, Hoorfar J, van der Poel WHM (2008) Food Anal Methods 1:73

da Silva AK, Le Saux JC, Parnaudeau S, Pommepuy M, Elimelech M, Le Guyader FS (2007) Appl Environ Microbiol 73:7891

D'Agostino M, Wagner M, Vazquez-Boland JA, Kuchta T, Karpiskova R, Hoorfar J, Novella S, Scortti M, Ellison J, Murray A, Heuvelink A, Kuhn M, Pazlarova J, Fernandez I, Cook N (2004) J Food Prot 67:1646

Dubois E, Hennechart C, Deboosère N, Merle G, Legeay O, Burger C, Le Calvé M, Lombard B, Ferré V, Traoré O (2006) Int J Food Microbiol 108:164

Food and Agriculture Organization of the United Nations/World Health Organization (2008) Microbiological risk assessment series, no. 13

Greening GE, Hewitt J (2008) Food Anal Methods 1:109

Hernroth BE, Conden-Hansson AC, Rehnstam-Holm AS, Girones R, Allard AK (2002) Appl Environ Microbiol 68:4523

Hoorfar J, Malorny B, Abdulmawjood A, Cook N, Wagner M, Fach P (2004) J Clin Microbiol 42:1863

Hundesa A, Maluquer de Motes C, Albinana-Gimenez N, Rodriguez-Manzano J, Bofill-Mas S, Suñen E, Girones R (2009) J Virol Methods 158:130

Hundesa A, Bofill-Mas S, Maluquer de Motes C, Rodriguez-Manzano J, Bach A, Casas M, Girones R (2010) J Virol Methods 163:385

Jothikumar N, Cromeans TL, Robertson BH, Meng XJ, Hill VR (2006) J Virol Methods 131:65

Rodríguez-Lázaro D, Hernandez M (2006) J Rapid Methods Autom Microbiol 14:395

Rodríguez-Lázaro D, D'Agostino M, Pla M, Cook N (2004) J Clin Microbiol 42:5832

Rodríguez-Lázaro D, Pla M, Scortti M, Monzó HJ, Vázquez-Boland JA (2005) Appl Environ Microbiol 71:9008

Rodríguez-Lázaro D, Lewis DA, Ocampo-Sosa AA, Fogarty U, Makrai L, Navas J, Scortti M, Hernandez M, Vazquez-Boland JA (2006) Appl Environ Microbiol 72:4256

Rodríguez-Lázaro D, Lombard B, Smith H, Rzezutka A, D'Agostino M, Helmuth R, Schroeter A, Malorny B, Miko A, Guerra B, Davison J, Kobilinsky A, Hernández M, Bertheau Y, Cook N (2007) Trends Food Sci Techn 18:306

Rodríguez-Lázaro D, López-Enríquez L, Hernandez M (2010) J Appl Microbiol 109:863

Svraka S, Duizer E, Vennema H, de Bruin E, van der Veer B, Dorresteijn B, Koopmans M (2007) J Clin Microbiol 45:1389

3.1.2

Analytical Application of a Sample Process Control in Detection of Foodborne Viruses

Marta Diez-Valcarce[1], Nigel Cook[2], Marta Hernández[1] and David Rodríguez-Lázaro[1]

[1] Instituto Tecnológico Agrario de Castilla y León (ITACyL), Valladolid, Spain [2] Food and Environment Research Agency (FERA), Sand Hutton, York, United Kingdom

Published in Food Analytical Methods, Vol. 4, 614-618

Food Anal. Methods (2011) 4:614–618
DOI 10.1007/s12161-011-9262-9

Analytical Application of a Sample Process Control in Detection of Foodborne Viruses

Marta Diez-Valcarce · Nigel Cook · Marta Hernández · David Rodríguez-Lázaro

Received: 6 May 2011 / Accepted: 12 June 2011 / Published online: 29 June 2011
© The Author(s) 2011. This article is published with open access at Springerlink.com

Abstract Sample process controls (SPCs) are an essential component of methods to detect viruses in food, as they verify that the sample treatment has operated correctly. Also, the use of an SPC can allow the efficiency of extraction of the target to be estimated for each individual sample analysed. The use of murine norovirus as SPC is here described. Its efficiency of extraction from different food products was 39.47%, 24.79% and 36.29% for strawberry, lettuce and shellfish samples. An incorrectly performed sample treatment was modelled to demonstrate the effectiveness of this control.

Keywords SPCV · False negatives · Real-time PCR · Food · Enteric virus

Introduction

Enteric viruses are recognised as a main cause of outbreaks and sporadic cases of acute gastroenteritis worldwide (Noda et al. 2008; Scallan et al. 2011; Hall et al. 2005). Thus, detection of the presence of enteric viruses in foods is an important issue in food safety, and rapid and robust diagnostic methodology is needed (Greening and Hewitt 2008; Bosch et al. 2011; Croci et al. 2008). Molecular-based methods have become the typical diagnostic approach for the detection of foodborne viruses (Bosch et al. 2011; Rodríguez-Lázaro et al. 2007). If these methods are to be used for monitoring of food supply chains for viruses, then it is vitally necessary that their analytical results can be reliably verified. Many matrices from the food supply chains most prone to virus contamination—soft fruit, salad vegetable and shellfish—are complex and difficult to treat, and can furthermore contain substances which can inhibit nucleic acid amplification. It is essential therefore that verification includes recognition of analyses where the method has failed to perform correctly, as this may mask the presence of a virus in a sample by a false-negative interpretation of the absence of a signal. Incorrect performance can occur during the sample treatment or the assay, and failed methods can be identified by the use of two controls: a sample process control (SPC) and a nucleic acid amplification control. The principles and use of nucleic acid amplification controls are becoming widely recognised (Hoorfar et al. 2004; Diez-Valcarce et al. 2011; Martínez-Martínez et al. 2011; Rodríguez-Lázaro et al. 2004, 2006), but few publications have described the use of an SPC. The incorporation of this control will verify that pre-amplification sample treatment has functioned correctly, and identify those samples in which pre-amplification sample treatment has failed as well as facilitate the determination of the method's efficiency of detection. In a method for detection of viruses, an SPC is a non-target virus added to every test sample including the negative control sample (or blank) at the start of analysis, and must be detected in every sample into which it has been added (D'Agostino et al. 2011). SPC viruses (SPCVs), must comply with some essential characteristics: they must be

M. Diez-Valcarce · M. Hernández (✉) ·
D. Rodríguez-Lázaro (✉)
Instituto Tecnológico Agrario (ITACyL), Junta de Castilla y León,
Ctra. Burgos, km. 119,
47071 Valladolid, Spain
e-mail: ita-herperma@itacyl.es
e-mail: ita-rodlazda@itacyl.es

N. Cook
Food and Environment Research Agency (FERA),
Sand Hutton,
York, UK

structurally similar to foodborne viruses of interest, not found naturally in the samples to be tested and preferably have an identical route of infection. As examples, mengo virus MC_0 (Costafreda et al. 2006) and feline calicivirus (FCV) and murine norovirus 1 (MNV-1) (Cannon et al. 2006) have been proposed as SPCVs for methods for detection of enteric viruses in food products (Bosch et al. 2011).

The aim of this study was to define the analytical application of a SPCV for nucleic acid amplification-based methods for detection of enteric viruses in food, and to assess its application to define the efficiency of a pre-nucleic acid amplification sample treatment. Human adenovirus type 2 (HAdV-2) was selected as a target enteric virus, as it has been suggested as being useful to indicate that routes of contamination from human sources exist (Wyn-Jones et al. 2011); MNV-1 was selected as SPCV since it possesses a similar molecular and biochemical structure and route of infection to human norovirus (Wobus et al. 2006). It is more acid-tolerant than FCV, and therefore it has been proposed as a more suitable human norovirus surrogate (Cannon et al. 2006).

Materials and Methods

Viruses and Cell Cultures MNV-1 was propagated in RAW264.7 cells and titrated by end-point dilution (final stock concentration 4.22×10^6 $TCID_{50}$/ml). HAdV-2 was propagated in A549 cells and titrated by the same technique (final stock concentration 2.1×10^7 $TCID_{50}$/ml). Total viral RNA or DNA was extracted from infected cultures using QIAamp viral RNA mini kit (QIAGEN, GMBH, Inc., Hilden, Germany), following manufacturer's instructions. MNV-1 was supplied by Prof. Herbert W. Virgin IV, Washington University School of Medicine, USA, according to the MTA signed within the EU project VITAL, and HAdV-2 was supplied by Prof. Rosina Girones, University of Barcelona, Spain.

Extraction of Virus Nucleic Acids from Vegetables and Soft Fruits Ready-to-eat lettuce and strawberries were obtained from a local retail outlet. Approximately 25 g of sample was placed in a sterile beaker, and approximately 10^5 $TCID_{50}$ of human adenovirus and 10^4 $TCID_{50}$ of murine norovirus were added. The protocol described by Dubois et al. (2006) was used to concentrate viruses. Briefly, 40 ml of Tris–glycine pH 9.5 buffer containing 1% beef extract (TGBE) were added to the sample [in case of soft fruits, 6,500 U of pectinase (e.g. Pectinex™ Ultra SPL solution, Sigma) were added previously to the TGBE buffer]. Sample was then agitated at room temperature for 20 min by rocking at 60 rpm. In case of soft fruits, it is crucial to maintain the pH at 9.0 throughout. The liquid was decanted from the beaker through a strainer into one 50 ml or two smaller centrifuge tubes and centrifuged at 10,000×*g* for 30 min at 4 °C. The supernatant(s) was decanted into a single clean tube or bottle, and the pH adjusted to 7.2. 0.25 volumes of 50% (*w*/*v*) polyethylene glycol (PEG) 8,000/1.5 M NaCl were then added, and mixed by shaking for 1 min. The suspension was then incubated with gentle rocking at 4 °C for 60 min, before centrifugation at 10,000×*g* for 30 min at 4 °C. The supernatant was discarded, and the pellet compacted by centrifugation at 10,000×*g* for 5 min at 4 °C before resuspension in 500 µl of PBS. The suspension was then transferred to a chloroform-resistant tube, and 500 µl of chloroform: butanol (1:1) was added and mixed by vortexing. The sample was allowed to stand for 5 min and then centrifuged at 10,000×*g* for 15 min at 4 °C. The aqueous phase was transferred to a clean tube and immediately used for nucleic acid extraction or stored at −20 °C. Nucleic acids were extracted using a NucliSENS® miniMAG® kit (bioMérieux) according to the manufacturer's instructions. The final elutions were performed with 150 µl elution buffer (inorganic buffer provided with the kit), resulting in a 300-µl nucleic acid extract. The nucleic acid extract was assayed immediately or stored at −70 °C.

To demonstrate how the SPCV would indicate extraction failure, the above procedure was performed again, but this time replacing the PEG with an equivalent amount of Trizma® HCl, to mimic a situation in which a key reagent had been prepared incorrectly [i.e. the buffer 50% (*w*/*v*) polyethylene glycol (PEG) 8,000/1.5 M NaCl was replaced by 50% (*w*/*v*) Trizma® HCl/1.5 M NaCl]. In addition, the lysis buffer and one of the washing buffers of the nucleic acid extraction kit (NucliSENS® miniMAG® kit—bioMérieux) were replaced by an equivalent volume of PBS.

Extraction of Virus Nucleic Acids from Shellfish Fresh mussels were obtained from a local retail outlet. Approximately 10^5 $TCID_{50}$ of human adenovirus and 10^4 $TCID_{50}$ of murine norovirus were added to the digestive gland of one shellfish (≈1 g). The sample was then processed using the method of Henshilwood et al. (2003). The digestive gland was transferred to a clean Petri dish and chopped finely with a razor blade. The chopped gland was then placed into a sterile plastic bag. The digestive gland was weighed then transferred into a centrifuge tube. One millilitre of 3 U ml^{-1} proteinase K solution was added and mixed well. The sample was incubated at 37 °C in a shaking incubator for 60 min, ensuring that the speed setting for the shaker induced

continual gentle movement of the enzyme/gland mixture. A secondary proteinase K incubation was carried out by placing the tube in a water bath at 65 °C for 15 min. The sample was then centrifuged at 3,000×*g* 5 min, and 500 µl of supernatant was transferred to a clean microcentrifuge tube and immediately used for nucleic acid extraction or stored at −20 °C. Nucleic acids were extracted using a NucliSENS® miniMAG® kit (bioMérieux) according to the manufacturer's instructions. The final elutions were performed with 150 µl elution buffer (inorganic buffer provided with the kit), resulting in a 300-µl nucleic acid extract. The nucleic acid extract was assayed immediately or stored at −70 °C.

To demonstrate how the SPCV would indicate extraction failure, the above procedure was performed again, but this time replacing the proteinase K solution with the equivalent amount of PBS, to mimic a situation in which a key reagent had been prepared incorrectly. In addition, the lysis buffer and one of the washing buffers of the nucleic acid extraction kit (NucliSENS® miniMAG® bioMérieux) were replaced by an equivalent volume of PBS.

Human Adenovirus Real-Time PCR Assay This assay was a duplex real-time PCR using the primers and conditions described by Hernroth et al. (2002), with the inclusion of an internal amplification control (IAC, Diez-Valcarce et al. 2011) and a carry-over contamination prevention system utilising uracil *N*-glycosylase. The reaction contained 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 0.9 µM each primer, 0.225 µM adenovirus TaqMan probe (labelled with FAM), 50 nM IAC probe (labelled with VIC) and 100 copies of adenovirus IAC. Ten microlitres sample of nucleic acid extract was added to make a final reaction volume of 25 µl. The thermocycling conditions were 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C.

Murine Norovirus Reverse Transcription Real-Time PCR Assay This assay was a one-step duplex reverse transcription real-time PCR using the primers and conditions described by Baert et al. (2008), with the inclusion of an IAC (Diez-Valcarce et al. 2011). The reaction contained 1× RNA Ultrasense reaction mix (Invitrogen), 0.2 µM each primer, 0.2 µM probe MGB-ORF1/ORF2 (labelled with FAM), 50 nM IAC probe (labelled with VIC), 1× ROX reference dye (Invitrogen), 1 µl RNA Ultrasense enzyme mix (Invitrogen) and 600 copies of murine norovirus IAC. Ten microlitres sample of nucleic acid extract was added to make a final reaction volume of 20 µl. The thermocycling conditions were 15 min at 50 °C, 2 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

Results

Demonstration of SPC Applicability in Detection of Viruses in Foods The results from analysis of the presence of HAdV-2 and MNV-1 in the artificially contaminated foods in which the concentration and nucleic acid extraction protocols were performed both correctly and incorrectly are shown in Table 1. A signal was obtained for both target (HAdV-2) and sample process control (MNV-1) viruses and their IACs from the assays in which the virus nucleic acid extractions from artificially contaminated food products were performed correctly. The average recoveries of the extractions were 39.47%, 24.79%, and 36.29% for artificially contaminated strawberry, lettuce and shellfish, respectively (Table 1). In contrast, no target (HAdV-2) and sample process control (MNV-1) viruses were obtained from the assays in which the virus nucleic acid extractions from artificially contaminated food products were performed incorrectly.

Table 1 Detection of viruses in different food matrices artificially contaminated with HAdV-2 and MNV-1 in which the analytical process was correctly and incorrectly performed

		Human adenovirus (target virus)			Murine norovirus (sample process control virus)		
		Strawberry	Lettuce	Mussels	Strawberry	Lettuce	Mussels
Correctly performed	Cp value	20.07±0.19[a] (9/9)[b]	20.28±0.21 (9/9)	21.55±0.19 (9/9)	31.08±0.22 (9/9)	31.87±0.30 (9/9)	31.23±0.16 (9/9)
	Efficency[c]	n.a.	n.a.	n.a.	39.47	24.79	36.29
Incorrectly performed[d]	Cp value	Undet. (0/9)	Undet. (0/9)	Undet. (0/9)	Undet. (0/9)	Undet. (0/9)	Undet. (0/9)
	Efficency	n.a.	n.a.	n.a.	0.00	0.00	0.00

Cp crossing point—PCR cycle at which fluorescence intensity rises above background, *n.a.* not applicable, *Undet* undetected

[a] Mean and standard error of Cp values of three independent nucleic acid amplification reactions using three replicates in each

[b] Number of positive reactions out of nine reactions

[c] Percentage of closeness between the results obtained using an artificially contaminated food product and cell culture

[d] Artificially contaminated samples which were subjected to an incorrectly performed concentration and nucleic acids extraction protocols

No significant differences ($p<0.05$) were observed for the IAC signals, which were positive in all the cases (both for the two types of viruses—HAdV-2 and MNV-1—and the two analytical scenarios—correctly and incorrectly performed). This indicates that the amplification step worked correctly and therefore the lack of signal for HAdV-2 and MNV-1 was due to a mistake during the extraction, which indeed was the experimental scenario planned.

Discussion

A mandatory step needed for the effective implementation of molecular diagnostics for the detection of enteric viruses in food supply chains is that the reliability of the analytical results can be verified (Rodríguez-Lázaro et al. 2007). Many matrices from the food supply chains like salad vegetable, shellfish and soft fruit are prone to virus contamination. They contain substances which can affect removal of the virus and its subsequent concentration, extraction of virus nucleic acids, and/or inhibit nucleic acid amplification, and therefore it is essential that this verification includes the recognition of failed methods as these may mask the presence of a virus pathogen by a false-negative interpretation of the results (Hoorfar et al. 2004). The use of a sample process control can provide this recognition.

An important aspect of an SPC is that it must be a virus that shares a very similar biochemical and molecular structure to the virus to be tested. This is a critical issue, as the reliable implementation of this control will rely on it being able to mimic the actions that the target virus will make during the analytical procedure.

Finally, it should be demonstrated that a sample process control can identify any problem during the whole analytical procedure. In this study, using three different types of foods, i.e. strawberries, lettuce and shellfish, which have been implicated in several outbreaks of viral disease (Baker et al. 2010; Maunula et al. 2009; Grotto et al. 2004; Ethelberg et al. 2010) and which have often been found to contain inhibitory substances (Croci et al. 2008), the SPC showed that the performance of the molecular-based analytical procedure for enteric viruses could be verified (concentration, nucleic acid extraction and nucleic acid amplification steps). However the use of an IAC in addition to SPC will allow more precise troubleshooting. The principle of an SPCV is that if it is detected, then the method was performed correctly. If it is not detected, the method has failed and the foodstuff must be reanalysed. In addition to this qualitative interpretation of an analytical result, the SPCV also allow a determination of the recovery efficiency for each individual sample, by comparing the (RT-)PCR results of SPC virus before and after addition to the sample. When the SPC virus is a good surrogate of the target virus, its efficiency of extraction will reflect that of the target, and allows a more precise determination of the target virus load in a sample. Thus, if the result of analysis was that 20 genome copies of a target and 40 genome copies of the SPCV are detected in a sample, and 100 genome copies of the SPCV were initially added, it could be inferred that the original number of target genome copies contaminating the sample was around 50, as the efficiency of the recovery for the SPCV was 40%.

In conclusion, this study has demonstrated the application of the inclusion of an SPC for assessing correct performance of the analytical procedure. The SPCV described in this study can be reliably used, and provide a robust control that can be routinely applied in the analysis of foods for viruses.

Acknowledgements This work was supported by the EU VITAL project Contract No. 213178. MD-V received a Ph.D. studentship from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA). NC acknowledges the support of the United Kingdom Food Standards Agency.

Open Access This article is distributed under the terms of the Creative Commons Attribution Noncommercial License which permits any noncommercial use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.

References

Baert L, Wobus CE, Van Coillie E, Thackray LB, Debevere J, Uyttendaele M (2008) Appl Environ Microbiol 74:543

Baker K, Morris J, McCarthy N et al (2010) An outbreak of norovirus infection linked to oyster consumption at a UK restaurant. J Public Health. doi:10.1093/pubmed/fdq089

Bosch A, Sanchez AG, Abbaszadegan M et al (2011) Food Anal Methods 4:4

Cannon JL, Papafragkou E, Park GW, Osborne J, Jaykus LA, Vinjé J (2006) J Food Prot 69:2761

Costafreda MI, Bosch A, Pintó RM (2006) Appl Environ Microbiol 72:3846

Croci L, Dubois E, Cook N, de Medici D, Charlotte Schultz A, China B, Rutjes SA, Hoorfar J, Van der Poel W-HM (2008) Food Anal Methods 1:73

D'Agostino M, Cook N, Rodríguez-Lázaro D et al (2011) Food Environ Virol 3:92

Diez-Valcarce M, Kovac K, Cook N et al (2011) Food Anal Methods (in press)

Dubois E, Hennechart C, Deboosère N (2006) Int J Food Microbiol 108:164

Ethelberg S, Lisby M, Bottiger B et al. (2010) Euro Surveillance 15(6)

Greening GE, Hewitt J (2008) Food Anal Methods 1:109

Grotto I, Huerta M, Balicer RD et al (2004) Infection 32:339

Hall G, Kirk MD, Becker N, Gregory JE et al (2005) Emerg Inf Dis 11:1257

Henshilwood K, Dore WJ, Anderson S, Lees DN (2003) Investigation of Norwalk like virus elimination during depuration using a real time quantitative PCR. In: Villalba A, Romalde JL, Reura B,

Beiras R (eds) Molluscan shellfish safety. Conselleria de Pesca e Asuntos Maritimos da Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, Galicia, Spain, pp 451–465

Hernroth BE, Conden-Hansson AC, Rehnstam-Holm AS, Girones R, Allard AK (2002) Appl Environ Microbiol 68:4523

Hoorfar J, Malorny B, Abdulmawjood A, Cook N, Wagner M, Fach P (2004) J Clin Microbiol 42:1863

Martínez-Martínez M, Diez-Valcarce M, Hernandez M et al (2011) Food Environ Virol 3:65

Maunula L, Roivainen M, Keränen M et al (2009) Euro Surveillance 14 (49)

Noda M, Fukuda S, Nishio O (2008) Int J Food Microbiol 122:216

Rodríguez-Lázaro D, D'Agostino M, Pla M, Cook N (2004) J Clin Microbiol 42:5832–5836

Rodríguez-Lázaro D, Lewis DA, Ocampo-Sosa AA et al (2006) Appl Environ Microbiol 72:4256

Rodríguez-Lázaro D, Lombard B, Smith H et al (2007) Trends Food Sci Technol 18:306

Scallan E, Hoekstra RM, Angulo FJ et al (2011) Emerg Inf Dis 17:7

Wobus CE, Thackray LB, Virgin HW (2006) J Virol 80:5104

Wyn-Jones A, Carducci A, Cook N, D'Agostino M, Divizia M et al (2011) Water Res 45:1025

3.1.3

Study of Different Factors Influencing the Implementation of Sample Process Control Viruses for Detection of Enteric Viruses in Food

Marta Diez-Valcarce[1], Nigel Cook[2], Marta Hernández[1] and David Rodríguez-Lázaro[1]

[1] Instituto Tecnológico Agrario de Castilla y León (ITACyL), Valladolid, Spain [2] Food and Environment Research Agency (FERA), Sand Hutton, York, United Kingdom

Manuscript in preparation for submission

Study of different factors influencing the implementation of sample process control viruses for detection of enteric viruses in food

ABSTRACT

The aim of this study was to evaluate the impact of two important aspects in the use of sample process control viruses (SPCVs): the moment of addition and the time elapsed until the beginning of the analytical procedure. Two food products (strawberry and iceberg lettuce) were artificially inoculated with two SPCVs (murine norovirus -MNV-1- and mengovirus -vMC$_0$-) at three different stages of the concentration-extraction protocol and the analytical performance was determined. The process efficiencies were also evaluated when the analytical process was performed at different times after SPCV addition. No significant differences were observed regarding the time elapsed from the addition of SPCV. However, a significant difference was observed depending on the step where the SPCV was incorporated, the extraction efficiencies were higher if the SPCV was incorporated later in process, demonstrating that during the process virus lost occurs. Consequenlty, the addition of the SPCV at the beginning of the process is recommended to allow its complete monitoring, while the analysis of the sample can be postpone until, at least, 24 hours without any significant effect on the procedure.

1. Introduction

A realistic risk assessment strategy to assess the risks derived from the contamination of food and water by enteric viruses requires a quantitative focus, and therefore accurate virus quantification is necessary. Molecular-based methods have become the reference for detection of viruses in food and water (Bosch *et al.* 2011; Rodríguez-Lázaro *et al.*; 2012). However, the application of molecular-based methods in foodstuffs can be complicated by the presence of inhibitory substances, which can cause a dramatic decrease in sensitivity and even false negative results, therefore leading to incorrect interpretations of the results (Diez-Valcarce *et al.*, 2011a; Rodríguez-Lázaro *et al.* 2007). If molecular-based methods are to be used for monitoring the presence of viruses in food supply chains, then it is vitally necessary that their analytical results can be reliably verified. For verification, it is essential to recognise those analyses where the method has failed to perform correctly. Incorrect performance of a method can occur during the sample treatment or during the assay, and failed analyses can be identified by the use of a suite of analytical controls, more specifically by using two types of controls: a sample process control (SPC) and a nucleic acid amplification control (Diez-Valcarce *et al.*, 2011a; b).

A SPC is a non-target virus added to every test sample including the target negative process control or blank (TNPC) at the start of analysis, and must be detected in every sample into which it has been added (D'Agostino *et al.*, 2011). As examples, an avirulent genetically-modified mengovirus (vMC_0) (Costafreda *et al.*, 2006), feline calicivirus (FCV) and murine norovirus (MNV-1) (Cannon *et al.*, 2006) have been proposed as SPC viruses (SPCV) in methods for detection of enteric viruses in food products (Bosch *et al.*, 2011; Rodríguez-Lázaro *et al.*, 2012). The incorporation of the SPC will verify that pre-amplification steps of the sample have functioned correctly,

and identify those samples in which those steps have failed as well as facilitate the determination of the method's efficiency. Thus, the inclusion of a SPC within the sample represents an effective quality assurance that validates the entire process from the extraction step to the reverse transcription real-time PCR (RT-RTi-PCR) detection (Jones *et al.*, 2009). The principles and use of SPC (as well as other analytical controls) are becoming widely recognised (D'Agostino *et al.*, 2011; Diez-Valcarce *et al*, 2011b; Rodríguez-Lázaro *et al*, 2012), but few publications have described the effective use of an SPC.

The aim of this study was to evaluate the impact of two important aspects in the use of SPCVs: the moment of addition of the SPCV and the time elapsed since its addition and the beginning of the sample concentration-extraction protocol. For these purposes two model food matrices (strawberry and lettuce) were artificially inoculated with MNV-1 and vMC_0 at three different stages of the concentration-extraction protocol and the analytical performance of the two SPCVs was determined. The process efficiencies were also evaluated when the analytical process was performed at different times after SPCV addition to the sample.

2. Materials and methods

2.1 Viruses and cell cultures.

Murine norovirus 1 (MNV-1) was propagated in RAW 264.7 cells, and titrated by end-point dilution (final stock concentration 6.8×10^5 $TCID_{50}$/ml). Mengo virus (vMC_0) was propagated in HeLa cells and titrated by the same technique (final stock concentration 1.32×10^7 $TCID_{50}$/ml). MNV-1 was supplied by Prof. Herbert W. Virgin IV, Washington University School of Medicine, US according to the MTA signed within the EU project VITAL, and vMC_0 was supplied by the enteric virus group from the University of Barcelona, Spain.

2.2 Extraction of virus nucleic acids from strawberries.

Strawberries were obtained from a local retail outlet. Approximately 25 g of fruit was placed in a sterile beaker, and approximately 10^3-10^4 $TCID_{50}$ of vMC_0 and MNV-1 were added. The samples were processed using the method of Dubois *et al.* (2002). Briefly, 40 ml of Tris Glycine pH 9.5 buffer containing 1% Beef Extract (TGBE), and 6500 U of pectinase (e.g. Pectinex™ Ultra SPL solution, Sigma) were added to the sample, which was then agitated at room temperature for 20 min by rocking at 60 rpm. The pH was maintained at 9.0 throughout the process. The liquid was decanted from the beaker through a strainer into one 50 ml or two smaller centrifuge tubes, and centrifuged at 10,000 × *g* for 30 min at 4°C. The supernatant was decanted into a single clean tube or bottle, and the pH adjusted to 7.2. Then, 0.25 volumes of 50% (w/v) polyethylene glycol (PEG) 8000/ 1.5 M NaCl were added and mixed by shaking for 1 min. The suspension was then incubated with gentle rocking at 4°C for 60 min, before centrifugation at 10,000 × *g* for 30 min at 4°C. The supernatant was discarded, and the pellet compacted by centrifugation at 10,000 × *g* for 5 min at 4°C before resuspension

in 500 µl of PBS. The suspension was then transferred to a chloroform-resistant tube, and 500 µl of chloroform:butanol (1:1) were added and mixed by vortexing. The sample was allowed to stand for 5 min, and then centrifuged at 10,000 × *g* for 15 min at 4°C. The aqueous phase was transferred to a clean tube and immediately used for nucleic acid extraction or stored at –20 °C. Nucleic acids were extracted using a NucliSENS miniMAG kit (bioMérieux) according to the manufacturer's instructions. The final elutions were performed with 150 µl of elution buffer, resulting in a 300 µl nucleic acid extract. The nucleic acid extract was assayed immediately or stored at -70 °C.

2.3 Extraction of virus nucleic acids from lettuce.

Ready-to-eat lettuce was obtained from a local retail outlet. Approximately 25 g of sample was placed in a sterile beaker, and approximately 10^4 $TCID_{50}$ of vMC_0 and 10^3 $TCID_{50}$ of MNV-1 were added. The same protocol as for strawberries was used with the only difference that pectinase was not added to the TGBE buffer.

2.4 Extraction efficiency.

The extraction efficiency value was calculated by comparing the C_p (Crossing point) values for the SPCV in the reagents used for concentration and extraction of the food sample but without any matrix (TNPC) with those obtained for the SPCV in the tested samples (with food matrix); the formula used was*:* $2^{Cp\ TNPC-Cp\ sample} \times 100$. The result was classified as poor (efficiency <1%), acceptable (1-10%), or good (>10%) (da Silva *et al.*, 2007).

2.5 Evaluation of the effect of the step in which the SPCV is added on process efficiency.

To demonstrate which step of the process was the more appropriate to add the SPCV, three different options were assayed for both food matrices, strawberry and lettuce; 10 µl of each SPCV were added (i) onto the matrix surface and let it dry before continuing -option 1-; (ii) into the TGBE buffer –option 2- and (iii) into the supernatant recovered after the first centrifugation of 30 min at 4ºC –option 3-. Three independent experiments were performed for each option.

2.6 Evaluation of the effect of the time elapsed since the addition of the SPCV on process efficiency.

Ten µl of each SPCV were added onto the surface of the lettuce, let it dry and the concentration-extraction protocol was performed immediately, or after 1, 2, 4 or 24 hours.

2.7 Statistical analysis.

For the evaluation of the effect on process efficiencies of the step in which the MNV-1 was added in the analytical process, one way independent ANOVA test was performed using the SPSS Statistics 17.0 software (SPSS Inc., Chicago, IL, USA) and *post hoc* Tukey's and Games-Howell tests were carried out to compare all groups with each other. However, the one way independent ANOVA test was not performed for vMC_0 and for the evaluation of the time elapsed from the addition of the SPCV as the results did not meet some of the assumptions of ANOVA, i.e. homogeneity of variance and/or normal distribution; in those cases a non-parametric test (Kruskal-Wallis) was used, and Mann-Whitney tests and a Bonferroni correction were also applied with a 0.025 level of significance.

3. Results

3.1 Effect of the step in which the SPCV is added.

The C_P values and the efficiencies of extraction for each matrix depending on the step in which the SPCVs were added are summarized in Table 1. In general, the addition of the surrogate virus into the TGBE buffer showed higher process efficiency (Table 1). Process efficiencies in strawberry ranged from 40.54% to 56.75% and from 13.40% to 82.81% for MNV-1 and vMC_0, respectively. Averages efficiencies for MNV-1 were good (i.e. efficiency > 10%) irrespectively of the moment in which the virus surrogate was added (Table 1), and the efficiency results when the SPCV was added onto the strawberry surface were not significantly different (p>0.05) to those when added later in the process (only efficiencies between the addition into the buffer and after the first centrifugation were significantly different – p<0.05). Efficiencies for vMC_0 were significantly different when added onto the strawberry surface to those when added into the buffer (U=0.00, r=-0.84) and significantly different to those when added after the first centrifugation (U=0.00, r=-0.84). Jonckheere's test revealed the median efficiency results increased when the SPCV was added to the matrix later in the process (J=210, z=3.93, r= 0.93).

Process efficiencies in iceberg lettuce ranged from 12.54% to 21.97% and 9.05% to 33.97% for MNV-1 and vMC_0, respectively. Efficiencies for MNV-1 were not affected for the moment when the surrogate was added (p>0.05), and were good (efficiency >10%) irrespectively of the moment in which the virus surrogate was added, except in one experiment when added on the lettuce surface. However, efficiencies for vMC_0 were affected for the moment when the surrogate was added -H(2)=18.317, p<0.05-. In addition, efficiencies for vMC_0 were only acceptable when added onto the surface of

the lettuce (9.05% ± 0.82) (Table 1), and significantly different to those when added into the buffer (U=0.00, r=-0.84) and after the first centrifugation (U=0.00, r=-0.84). Jonckheere's test revealed the median efficiency results increased when the SPCV was added to the matrix later in the process (J=186, z=2.87, r= 0.68).

3.2 Effect of the time elapsed since SPCV addition and further analytical processing.

The C_P values and the efficiencies of extraction are summarized in Table 2. The results in iceberg lettuce gave efficiencies ranged from 8.74% to 13.60% and 9.05% to 20.00% for MNV-1 and vMC_0, respectively. The average efficiencies were good (i.e. >10%) in all the times except for MNV-1 when the lettuce was processed after 2 hours postdrying (with an efficiency acceptable −9.30 ± 0.63−) and after 24 hours postdrying (with an efficiency acceptable −8.74 ± 1.03−) and for vMC_0 when the lettuce was processed immediately after drying (with an efficiency acceptable −9.05 ± 1.50−). Interestingly, there was not a significant effect of the time elapsed since the SPCVs were added in the efficiency (H(4)=7.931, p>0.05 and H(4)=6.816, p>0.05 for MNV-1 and vMC_0, respectively), and the Jonckheere's test also revealed there was not a significant trend in the data (J=330, z=-1.501(<1.65), r= -0.22 and J=441, z=0.72(<1.65), r= 0.11 for for MNV-1 and vMC_0 respectively).

4. Discussion

Many foods prone to virus contamination, such as vegetables and soft fruits, can contain substances that can interfere in the analytical process of the sample; affecting the concentration and extraction of the virus, or inhibiting the nucleic acid amplification. The recognition of any of these possible incidents throughout the process is essential, as otherwise failed methods may mask the presence of a pathogenic virus in the sample (D'Agostino et al., 2011). The inclusion of controls such as IACs and SPCVs and its correct interpretation can disclose these failed methods (Diez-Valcarce *et al.*, 2011a; 2011b), but an optimal methodology must aim to go one step further. When using analytical controls for assessment of molecular methods for virus detection in food, some important questions have to be addressed: how to mimic more realistically a natural contamination in a given food and when (or up to when) the analytical process can be performed after the addition of a sample process control. In this study we tried to answer these two questions by (i) evaluating the differences, if any, in the analytical performance of a SPCV when added in different stages of the process and (ii) checking the time window in which the analysis can be performed after adding the SPCV to the sample without negatively affecting the outcome of the analysis. No significant differences were observed regarding the time elapsed from the addition of SPCV (vMC_0 or MNV-1). Thus, from a practical point of view, in an analytical laboratory, routine analysis of foods can be performed, once the sample is arrived and the SPCV has been added, up to 24 hours after its arrival, without expecting any significant difference in the results. However, a significant difference was observed, as expected, depending on the step where the SPCV was incorporated to the process. Consequently, the addition of the SPCV at the beginning of the process is recommended to allow its complete monitoring.

Furthermore, we compare two of the most commonly proposed virus surrogates for detection of human enteric viruses, vMC_0 and MNV-1 (Costafreda *et al.*, 2006; Cannon *et al.*, 2006). MNV-1 exhibits close similarity in structure and behaviour to human noroviruses; is non-pathogenic to humans; and can be grown in adapted cell lines (RAW 264.7). vMC_0, in turn, is a mutant virus strain of the wild type pMC_0 of mengovirus lacking the poly(C) tract, this latter feature renders it avirulent, but it also makes it a genetically modified organism (GMO), and working with GMOs would require the user laboratories to comply especial legal requirements which could hamper the general implementation of a method. Taking together the results obtained in this study with the previously mentioned facts, we conclude that MNV-1 is the most suitable candidate, especially for the specific purpose of detection of human noroviruses.

Additionally, in this study we evaluate the utilization of the whole viral particle *vs* the extracted nucleic acid as control in the PCR. The purpose of this part of the experiment was to see if any difference was observed when the whole virus particle was added to the PCR to be used as a control *vs* the use of nucleic acids extracted from the virus after it has undergo the whole process of concentration but without the presence of any food matrix (TNPC). Some possible explanations for these differences, if they exist, would be the formation of clumps of viral particles. The viral clumps could prevent the viral solution to be homogeneous, which can give differences in the PCR. A Student *t* test was performed to compare the Cp values observed in both cases and no statistical differences were observed for any of the SPCVs used in this experiment. However, in case of MNV-1 the *t* test value was closer to the significant *p* value of 0.05. It is also important to mentioned that among the values of the whole viral particle the range of values was wider, that is, the intra-group differences observed were bigger, which can

suggest not consistent or repeatable results can be expected when using the whole viral particle as control in the PCR.

5. Acknowledgments

This work was supported by the EU VITAL project Contract No. 213178. M.D.-V. received a Ph.D. studentship from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA).

Table 1. Efficiencies of extraction and Cp values of viruses in different strawberries and iceberg lettuce artificially-contaminated with MNV-1 and vMC_0 at three different steps of the analytical process.

		Murine norovirus		*Mengovirus*	
		Strawberry	*Lettuce*	*Strawberry*	*Lettuce*
Option 1[a]	C_P value[b]	22.47 ± 0.08	24.55 ± 0.25	24.96±0.15	25.50 ± 0.14
	Efficency[c]	47.63 ± 2.53	12.54 ± 2.10	13.40 ±1.50	9.05 ± 0.82
Option 2	Cp value	22.23±0.10	23.74±0.25	22.40±0.07	23.65±0.21
	Efficency	56.75 ± 4.20	21.97 ± 3.63	76.15 ± 3.72	33.97 ± 4.04
Option 3	Cp value	22.72±0.11	24.01±0.14	22.40±0.24	24.19±0.08
	Efficency	40.54 ± 2.71	16.85 ± 1.78	82.81 ± 11.85	22.11 ± 1.37

[a] 10 µl of each SPCV were added onto the matrix surface and let it dry before continuing (option 1); into the TGBE buffer (option 2) and into the supernatant recovered after the first centrifugation of 30 min at 4ºC (option 3). Three independent experiments were performed for each option

[b] C_P: Crossing point- This point is the PCR cycle at which product fluorescence intensity finally rises above background and becomes visible. Mean and standard error of Cp values of 3 independent experiments using 3 replicates in each RT-RTi-PCR.

[c] Closeness between the results obtained comparing the Cp value for the SPCV spiked in the reagents used for concentration and extraction of the sample but without any matrix with that obtained for the SPCV in the tested samples; the formula used was: $2^{Cp\ TNPC - Cp\ sample} \times 100$. Mean and standard error of Cp values of 3 independent experiments using 3 replicates in each RT-RTi-PCR.

Table 2. Efficiencies of extraction and Cp values of viruses in iceberg lettuce artificially-contaminated with MNV-1 and vMC_0 and processed at five different times.

		Time after addition of SPCV and drying (h)				
		0	*1*	*2*	*4*	*24*
MNV-1	Cp^a	24.55 ± 0.25	26.84 ± 0.10	27.35 ± 0.10	26.96 ± 0.27	27.49 ± 0.18
	E^b	12.54 ± 2.10	13.25 ± 0.96	9.30 ± 0.63	13.60 ± 2.16	8.74 ± 1.03
vMC_0	*Cp*	25.50 ± 0.14	25.08 ± 0.04	25.47 ± 0.15	24.64 ± 0.27	25.12 ± 0.16
	E	9.05 ± 1.50	12.95 ± 0.37	10.31 ± 1.13	20.00 ± 3.38	13.14 ± 1.30

[a] Cp: Crossing point- This point is the PCR cycle at which product fluorescence intensity finally rises above background and becomes visible. Mean and standard error of Cp values of 3 independent experiments using 3 replicates in each RT-RTi-PCR.

[b] E: Efficiency- Closeness between the results obtained comparing the Cp value for the SPCV spiked in the reagents used for concentration and extraction of the sample but without any matrix with that obtained for the SPCV in the tested samples; the formula used was: $2^{Cp\ TNPC - Cp\ sample} \times 100$. Mean and standard error of Cp values of 3 independent experiments using 3 replicates in each RT-RTi-PCR.

3.1.4

Design and Application of Nucleic Acid Standards for Quantitative Detection of Enteric Viruses by Real-Time PCR

Mónica Martínez-Martínez, Marta Diez-Valcarce, Marta Hernández and David Rodríguez-Lázaro

Instituto Tecnológico Agrario de Castilla y León (ITACyL), Valladolid, Spain

Published in Food and Environmental Virology, Vol. 3, 92-98

Food Environ Virol (2011) 3:92–98
DOI 10.1007/s12560-011-9062-9

ORIGINAL PAPER

Design and Application of Nucleic Acid Standards for Quantitative Detection of Enteric Viruses by Real-Time PCR

Mónica Martínez-Martínez · Marta Diez-Valcarce · Marta Hernández · David Rodríguez-Lázaro

Received: 5 April 2011 / Accepted: 11 May 2011 / Published online: 21 May 2011
© The Author(s) 2011. This article is published with open access at Springerlink.com

Abstract Synthetic multiple-target RNA and DNA oligonucleotides were constructed for use as quantification standards for nucleic acid amplification assays for human norovirus genogroup I and II, hepatitis E virus, murine norovirus, human adenovirus, porcine adenovirus and bovine polyomavirus. This approach overcomes the problems related to the difficulty of obtaining practical quantities of viral RNA and DNA from these viruses. The quantification capacity of assays using the standards was excellent in each case ($R^2 > 0.998$ and PCR efficiency > 0.89). The copy numbers of the standards were equivalent to the genome equivalents of representative viruses (murine norovirus and human adenovirus), ensuring an accurate determination of virus presence. The availability of these standards should facilitate the implementation of nucleic acid amplification-based methods for quantitative virus detection.

Keywords Foodborne virus · Quantification · Nucleic acid standard · RT real-time PCR

Mónica Martínez-Martínez and Marta Diez-Valcarce have contributed equally this work.

M. Martínez-Martínez · M. Diez-Valcarce · M. Hernández (✉)
Molecular Biology and Microbiology Laboratory, Junta de Castilla y León, Instituto Tecnológico Agrario de Castilla y León (ITACyL), Ctra. Burgos, km, 119, 47071 Valladolid, Spain
e-mail: ita-herperma@itacyl.es

D. Rodríguez-Lázaro (✉)
Food Safety and Technology Research Group, Junta de Castilla y León, Instituto Tecnológico Agrario de Castilla y León (ITACyL), Carretera de Burgos, km. 119, 47071 Valladolid, Spain
e-mail: ita-rodlazda@itacyl.es

Introduction

Molecular-based methods have become the gold standard for routine detection of viruses in food and environmental samples (Bosch et al. 2011; Croci et al. 2008). A realistic risk assessment strategy to assess the risks created by the contamination of food and the environment by enteric viruses will require a quantitative focus, and therefore accurate virus quantification is necessary. When a nucleic acid amplification-based method is applied for quantitative purposes, known concentrations of nucleic acids are used to construct calibration curves for quantification (Rodríguez-Lázaro et al. 2007). According to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al. 2009), nucleic acids used as standards for quantification can be of several types: purified synthetic RNA or DNA oligonucleotides spanning the complete PCR amplicon, plasmid DNA constructs, cDNA cloned into a plasmid, in vitro transcribed RNA, reference DNA pools and RNA or DNA from biological samples. However, important enteric pathogenic viruses such as human norovirus (hNoV) or hepatitis E virus (HEV) are not culturable in the laboratory (Lees and CEN WG6 TAG4 2010) and therefore, a source of viral nucleic acids necessary to make standard solutions can be very restricted, thus being more convenient to use a synthetic nucleic acid.

The application of synthetic nucleic acid molecules as standards for detection and quantification of organisms whose availability is scarce has been already applied successfully to a wide spectrum of organisms such as genetically modified plants (Hernández et al. 2005; Kuribara et al. 2002; Taverniers et al. 2004) and some pathogenic viruses (Kwiatek et al. 2010; Vester et al. 2010; Workenhe et al. 2008). A similar approach has not been followed for

quantitative detection of non-culturable enteric viruses such as hNoV or HEV. However, as the public health implications of their presence in foodstuffs and the environment is becoming increasingly recognised (Anonymous 2008), and methods suitable for their routine monitoring are becoming available (Lees and CEN WG6 TAG4 2010), the availability of specific nucleic acid amplification standards is becoming necessary.

In the present work, we describe the construction of plasmids containing multiple-target sequences, which can be used as standards for quantification of enteric viruses (HEV, hNoV genogroups I and II, bovine polyomavirus—BPyV, human adenovirus—HAdV and porcine adenovirus—PAdV) by (reverse transcription) real-time PCR. The performance in quantitative reactions of these synthetic standards was assessed in comparison to natural nucleic acids extracted from viruses.

Materials and Methods

Viruses and Cell Cultures

Murine norovirus 1 (MNV-1) was propagated in RAW264.7 cells, and titrated by end-point dilution (final stock concentration 4.22×10^6 median tissue culture infective dose ($TCID_{50}$) ml^{-1}). Human adenovirus type-2 (HAdV-2) was propagated in A549 cells and titrated by the same technique (final stock concentration 2.1×10^7 $TCID_{50}$ ml^{-1}). Total viral RNA or DNA was extracted from infected cultures using QIAamp viral RNA mini kit (QIAGEN, GMBH, Inc., Hilden, Germany), following manufacturer's instructions. MNV-1 was supplied by Prof. Herbert W. Virgin IV, Washington University School of Medicine, US according to the MTA signed within the EU project VITAL, and HAdV-2 was supplied by Prof. Rosina Girones, University of Barcelona, Spain.

Construction of a Plasmid for Transcription of Synthetic RNA

A synthetic DNA molecule was designed to contain target sequences for reverse transcription real-time PCR (RT-qPCR) assays for HEV (Jothikumar et al. 2006), hNoV GI (Svraka et al. 2007) and hNoV GII (da Silva et al. 2007). The oligonucleotide was synthesised (Eurofins MWG Operon, Ebersberg, Germany) and cloned into a pCR 2.1-TOPO plasmid (Invitrogen, Breda, The Netherlands). Then, the target sequence for a RT-qPCR assay for murine norovirus (MNV-1) (Baert et al. 2008) was added to the 3′ end of the plasmid. This was done by modifying the primers to add tails containing sites for *Xho*I (primer FwORF1/ORF2) and *Apa*I (primer RvORF1/ORF2). The final sequence was 424 bp (Fig. 1). The recombinant plasmid was designated as pCR2.1TOPO-rSTD.

Production of the Synthetic RNA

Six micrograms of pCR2.1TOPO-rSTD clone were linearised by digestion with *Hin*dIII enzyme (New England Biolabs, Ipswich, MA, USA) prior to in vitro transcription. The linearised plasmid was subsequently purified using the QIAquick PCR purification kit (QIAGEN, GMBH, Inc., Hilden, Germany) and in vitro transcribed using T7 RNA

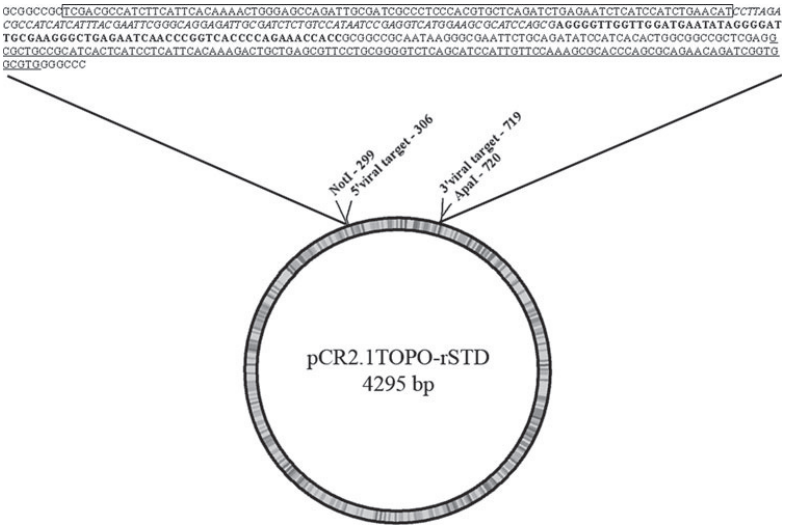


Fig. 1 Graphic representation of pCR2.1TOPO-rSTD containing the sequence of the synthetic rFBV1 RNA. The length of the plasmid pCR2.1TOPO-rSTD is 4295 bp. The viral insert was flanked by *Not*I and *Apa*I sites. The sequences of the RT-qPCR assays are shown (hNoV GII—*within box*, hNoV GI—*italics*, HEV—*bold* and MNV-1—*underlined*. The sequences corresponding to the TOPO vector are in normal type

polymerase (Riboprobe in vitro transcription system, Promega, Madison, WI, USA) following the manufacturer's instructions. As viral target DNA was reverse cloned in pCR-2.1 TOPO vector, transcription was performed in the antisense direction to generate a ssRNA(+). Residual DNA was removed by digestion with 35 U of RNase-free DNase contained in the kit. Subsequently, RNA purification was carried out using RNeasy kit (QIAGEN, GMBH, Inc., Hilden, Germany). Parallel qPCR and RT-qPCR assays for each virus verified that residual DNA had been removed (data not shown). To check the integrity of the RNA, an aliquot was electrophoresed in a native 1.5% agarose gel. To verify that the sequence of the insert was correct, direct sequencing using two pairs of flanking primers M13 Forward/M13 Reverse and FwORF1/ORF2/COG2R was performed using the kits Big Dye v3.1 or v1.1 (Applied Biosystems, Foster City, CA, USA) in an ABI3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) following manufacturer's instructions. Transcription reactions were repeated several times to obtain a higher amount of RNA, and after the integrity and purity was assessed, the RNA solutions were pooled, aliquoted in suitable volumes and stored at −80°C. The synthesised RNA molecule was designated rFBV1.

Construction of the Synthetic DNA

A synthetic DNA molecule was designed to contain target sequences for qPCR assays for BPyV (Hundesa et al. 2010), HAdV (Hernroth et al. 2002) and PAdV (Hundesa et al. 2009). The oligonucleotide was synthesised (Eurofins MWG Operon, Ebersberg, Germany) and cloned into a pCR 2.1-TOPO plasmid (Invitrogen, Breda, The Netherlands). The final sequence was 228 bp (Fig. 2). To verify that the sequence of the insert was correct, direct sequencing using the two flanking primers M13 Forward and M13 Reverse was performed as for synthetic RNA. The plasmid was cloned in *E. coli*, and a purified solution prepared using QIAGEN plasmid Midi kit (QIAGEN, GMBH, Inc., Hilden, Germany). The DNA solutions were pooled, aliquoted in suitable volumes and stored at −80°C. The synthesised DNA was designated as pFBV2.

Quantification of RNA and DNA

RNA and DNA concentrations were determined by UV spectrophotometry in a Nanodrop ND-1000 spectrophotometer (ThermoScientific, Wilmington, NC, USA). The measurements were performed in duplicate and concentration in g was converted to molecule number using the following formulae:

$$\text{RNA molecules } \times \mu l^{-1} = [(g/\mu l)/(\text{transcript length in nucleotides} \times 340)] \times 6.022 \times 10^{23}$$

$$\text{DNA molecules } \times \mu l^{-1} = [(g/\mu l)/(\text{plasmid length in base pairs} \times 660)] \times 6.022 \times 10^{23}$$

RT-qPCRs and qPCRs

All RT-qPCRs and qPCRs were run in an LC 480 II instrument (Roche, Mannheim, Germany). For RT-qPCR assays, 5 µl of the RNA solution were added to 15 µl of master mix consisting of 7.4 µl Light Cycler 480 Master Hydrolysis probes (Roche, Mannheim, Germany), and 1.3 µl activator (50 mM). Primers and probes concentrations

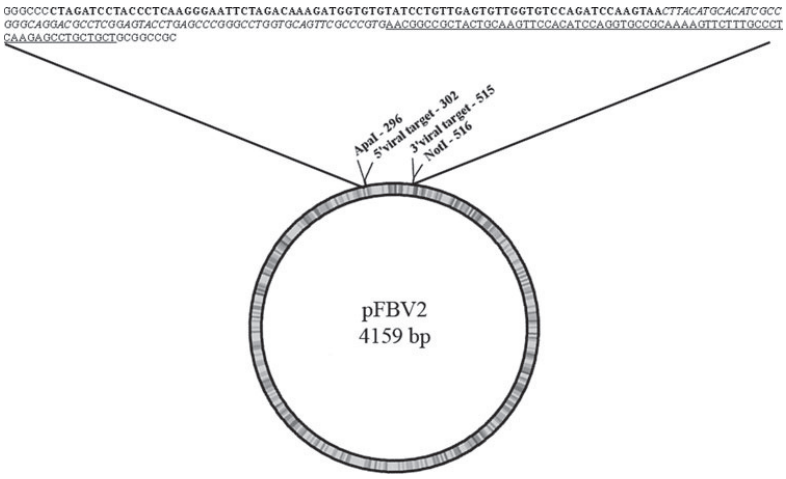


Fig. 2 Graphic representation of pFBV2 containing the sequence of the synthetic DNA. The length of the plasmid is 4,159 bp. The viral insert was flanked by *Apa*I and *Not*I sites. The sequences of the qPCR assays are shown (BPyV—*bold*, HAdV-2—*italics* and PAdV—*underlined*. The sequences corresponding to the TOPO vector are in normal type

were those described before for MNV-1 (Baert et al. 2008) and HEV (Jothikumar et al. 2006). For hNoV GI and GII assays, forward primer was added at a concentration of 500 nM, reverse primer at 900 nM and probe at 250 nM. Cycling conditions consisted of 63°C for 30 min followed by denaturation at 95°C for 5 min, and 45 cycles of denaturation at 95°C for 15 s, and annealing, amplification and detection at 60°C for 1 min. For HEV, cycling conditions were 63°C for 30 min followed by denaturation at 95°C for 5 min 45 cycles of 10 s at 95°C, annealing at 55°C for 20 s and amplification and detection at 72°C for 15 s. For MNV-1, cycling conditions were 63°C for 30 min followed by denaturation at 95°C for 5 min and 40 cycles of denaturation at 95°C for 15 s and annealing, amplification and detection at 60°C for 1 min.

For qPCR assays, 5 µl of the DNA solution were added to 20 µl of master mix consisting of 12.5 µl Light Cycler 480 Probes Master (Roche, Mannheim, Germany). Primers and probes concentrations were those described previously (Hernroth et al. 2002; Hundesa et al. 2010; Hundesa et al. 2009). Cycling conditions for HAdV and PAdV assays consisted of denaturation at 95°C for 10 min, and 45 cycles of denaturation at 95°C for 15 s, and annealing, amplification and detection at 60°C for 1 min. Cycling conditions for BPyV were denaturation at 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 1 min.

Results

Evaluation of the Performance of the RT-qPCR Assays Using Synthesised RNA

The results of the performance of the four RT-qPCR assays (hNoVGI, hNoVGII, HEV and MNV-1) using tenfold dilutions (from 10^6 to 10^1 RNA molecules) of the rFVB1 are shown in Table 1. The capacity for quantification using rFVB1 for each RT-qPCR assay was also calculated based on the linearity and PCR efficiency (E) (Rodríguez-Lazaro et al. 2005). Both parameters were very close to the optimal in all RT-qPCR assays ($R^2 \geq 0.998$ and $E \geq 0.89$; Table 1) demonstrating that the use of the synthetic RNA for constructing standard quantification curves is an excellent approach. The limits of quantification (LOQ) were 1×10^1 rFVB1 copies per reaction in all the assays, with the exception of hNoV GII and HEV where the LOQ was 1×10^2 copies per reaction (Table 1). In addition, reliable quantification was possible over a dynamic range at least of five logs.

The performance of the RT-qPCR assays using tenfold dilutions of rFVB1 were also compared to those assays using tenfold dilutions of native RNA from MNV-1 extracted from infected cells (from 10^6 to 10^1 RNA molecules). The performances were very similar, as the linearity and PCR efficiency values were very similar (R^2 of

Table 1 Quantitative detection of synthetic (hNoVGI, hNoVGII, HEV and MNV-1) and native MNV-1 RNA

Approx. molecules/reaction[a]	Synthetic RNA				Virus RNA
	hNoVGI [b] C_p value[g]	hNoVGII[c] C_p value	HEV[d] C_p value	MNV-1[e] C_p value[g]	MNV-1[f] C_p value[g]
1×10^6	14.75 ± 0.24	12.45 ± 0.07	16.61 ± 0.18	15.57 ± 0.04	15.59 ± 0.02
1×10^5	17.91 ± 0.15	15.89 ± 0.07	19.80 ± 0.15	19.56 ± 0.04	19.32 ± 0.04
1×10^4	21.68 ± 0.30	19.35 ± 0.13	23.35 ± 0.16	23.30 ± 0.04	22.95 ± 0.03
1×10^3	25.01 ± 0.28	23.11 ± 0.15	26.71 ± 0.21	27.07 ± 0.05	26.65 ± 0.02
1×10^2	28.47 ± 0.37	26.92 ± 0.20	29.54 ± 0.30	30.30 ± 0.03	29.85 ± 0.01
1×10^1	32.23 ± 0.45	35.56 ± 0.25	nd[h]	33.74 ± 0.04	33.09 ± 0.03

[a] Estimated number of synthetic rFBV1 or virus RNA molecules in each RT-qPCR run

[b] hNoVGI RT-qPCR results from tenfold serial dilutions of synthetic RNA rFBV1. The standard curve was: $y = -3.497x + 35.58$; and the R^2 and PCR efficiency values were 0.999 and 0.93, respectively

[c] hNoVGII RT-qPCR results from tenfold serial dilutions of synthetic RNA rFBV1. The standard curve was: $y = -3.616x + 34.00$; and the R^2 and PCR efficiency values were 0.999 and 0.89, respectively

[d] HEV RT-qPCR results from tenfold serial dilutions of synthetic RNA rFBV1. The standard curve was: $y = -3.277x + 36.31$; and the R^2 and PCR efficiency values were 0.998 and 1.02, respectively

[e] MNV-1 RT-qPCR results from tenfold serial dilutions of synthetic RNA rFBV1. The standard curve was: $y = -3.624x + 37.60$; and the R^2 and PCR efficiency values were 0.998 and 0.89, respectively

[f] MNV-1 RT-qPCR results from tenfold serial dilutions of RNA purified from MNV-1. The standard curve was: $y = -3.508x + 36.85$; and the R^2 and PCR efficiency values were 0.998 and 0.93, respectively

[g] Cycle number at which fluorescence intensity equals a fixed threshold. Mean value ± standard error of the mean. The experimental results were statistically significant ($P < 0.05$) taking into account unavoidable error associated with serial dilutions

[h] Not detected

0.998 and E of 0.93 and 0.89 for native and synthetic MNV-1 RNA, respectively) (Table 1; Fig. 3a). When the C_p (cycle to positivity) values obtained using each type of RNA were plotted in a graphic (synthetic RNA C_p values vs. MNV-1 RNA C_p values) an excellent correlation (slope of 0.967 and R^2 of 0.999) was found (Fig. 3b).

Evaluation of the Performance of the qPCR Assays Using Synthetic DNA

The results of the performance of the three qPCR assays (BPyV, PAdV and HAdV) using tenfold dilutions (from 10^5 to 10^1 DNA molecules) of the pFBV2 are shown in Table 2. Similarly to the RT-qPCR assays, the capacity for quantification was also calculated based on the linearity and PCR efficiency. Both parameters were very close to the optimal in all qPCR assays ($R^2 \geq 0.96$ and $E \geq 0.997$; Table 2) demonstrating that the use of the synthetic DNA for constructing standard quantification curves is an excellent approach. The limits of quantification (LOQ) were 1×10^1 pFBV2 copies per reaction in all the assays (Table 2). In addition, reliable quantification was possible over a dynamic range at least of five logs.

The performance of the qPCR assays using tenfold dilutions of pFBV2 were also compared to those assays using tenfold dilutions of native DNA from HAdV-2 extracted from infected cells (from 10^5 to 10^1 DNA molecules). The performances were very similar, as the linearity and PCR efficiency values were very similar (R^2 of 0.999 and E of 1.00 and 0.96 for native and synthetic HAdV-2 DNA, respectively) (Table 2; Fig. 4a). When the C_p values obtained using each type of DNA were plotted in a graphic (synthetic DNA C_p values vs. HAdV-2 DNA C_p values) an excellent correlation (slope of the curve of 0.965 and R^2 value of 0.999) was found (Fig. 4b).

Discussion

Accurate quantification of viruses is important to determine not only the level of contamination of food, surfaces, waters, etc., but also to determine any reduction of virus contamination after disinfection treatments. It can also be used to determine a possible linkage of virus levels to risk of infection or outbreaks (Lees and CEN WG6 TAG4 2010). Probably because of the difficulty of obtaining suitable RNA, several previously published methods have used DNA containing virus-complementary sequences as quantification standards. However, this approach is far from optimal, as the reverse transcription step is thus not considered (Boeuf et al. 2005; Terlizzi et al. 2010; Vester et al. 2010; Workenhe et al. 2008).

Absolute quantification will be reliable only if the standard and the unknown samples are retrotranscribed (only for RNA molecules) and amplified with the same efficiency (Boeuf et al. 2005). So standard curves obtained after amplification of tenfold serial dilutions of purified viral RNA or DNA and external RNA or DNA standards were compared (Tables 1, 2; Figs. 1, 2). RNA transcribed from linearised plasmid pCR2.1TOPO-rSTD was found to give more reliable viral RNA copy number estimation than RNA transcribed from circular pCR2.1TOPO-rSTD (data not shown). It has been suggested that an acceptable RT-qPCR standard curve should have a correlation coefficient (R^2) ≥ 0.98 and a slope value (s) between 3.6 and 3.1, corresponding to reaction efficiencies (E) between 0.9

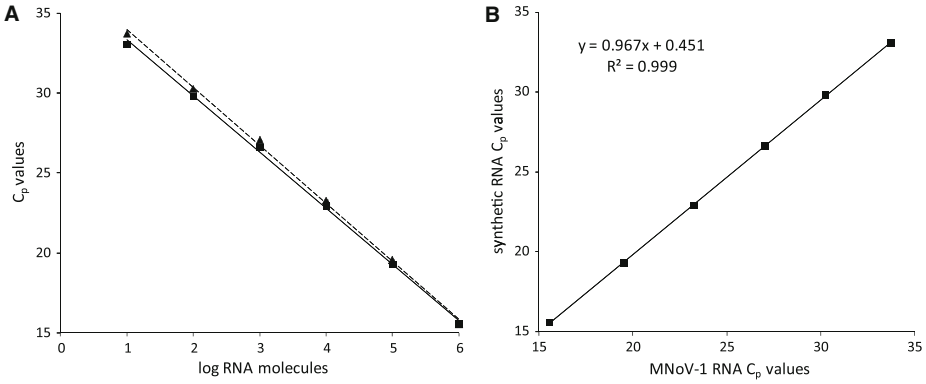


Fig. 3 Comparison of the performance of the RT-qPCR assays using native and synthetic RNA. **a** Standard curve generated by tenfold dilutions (from 10^6 to 10^1 RNA molecules) of rFVB1 (*triangle*) and native RNA from MNV-1 extracted from infected cells (*square*). **b** Representation of the equivalence of the C_p values of tenfold dilutions (from 10^6 to 10^1 RNA molecules) of rFVB1 (y-axis) and MNV-1 (x-axis)

Table 2 Quantitative detection of synthetic (PAdV, BPyV and HAdV) and native HAdV DNA

Approx. molecules/reaction[a]	Synthetic DNA			Virus DNA
	PAdV[b] C_p value[f]	BPyV[c] C_p value[f]	HAdV[d] C_p value[f]	HAdV[e] C_p value[f]
1×10^5	22.46 ± 0.10	23.96 ± 0.07	22.08 ± 0.04	22.15 ± 0.02
1×10^4	25.83 ± 0.13	27.61 ± 0.12	25.49 ± 0.04	25.49 ± 0.04
1×10^3	29.29 ± 0.11	30.98 ± 0.12	28.84 ± 0.05	28.92 ± 0.03
1×10^2	32.32 ± 0.07	34.23 ± 0.04	32.11 ± 0.05	32.06 ± 0.02
1×10^1	35.95 ± 0.09	36.81 ± 0.23	35.92 ± 0.10	35.44 ± 0.06

[a] Estimated number synthetic pFBV2 or virus DNA molecules in each qPCR run

[b] PAdV qPCR results from tenfold serial dilutions of synthetic DNA pFBV2. The standard curve was: $y = -3.347x + 42.55$; and the R^2 and PCR efficiency values were 0.999 and 0.99, respectively

[c] BPyV qPCR results from tenfold serial dilutions of DNA pFBV2. The standard curve was: $y = -3.232x + 43.64$; and the R^2 and PCR efficiency values were 0.999 and 1.04, respectively

[d] HAdV qPCR results from tenfold serial dilutions of DNA pFBV2. The standard curve was: $y = -3.430x + 42.60$; and the R^2 and PCR efficiency values were 0.999 and 1.02, respectively

[e] HAdV qPCR results from tenfold serial dilutions of DNA purified from HAdV-2. The standard curve was: $y = -3.315x + 42.07$; and the R^2 and PCR efficiency values were 0.999 and 1.00, respectively

[f] Cycle number at which fluorescence intensity equals a fixed threshold (Rodríguez-Lázaro et al. 2003). Mean value ± standard error of the mean. The experimental results were statistically significant ($P < 0.05$) taking into account unavoidable error associated with serial dilutions

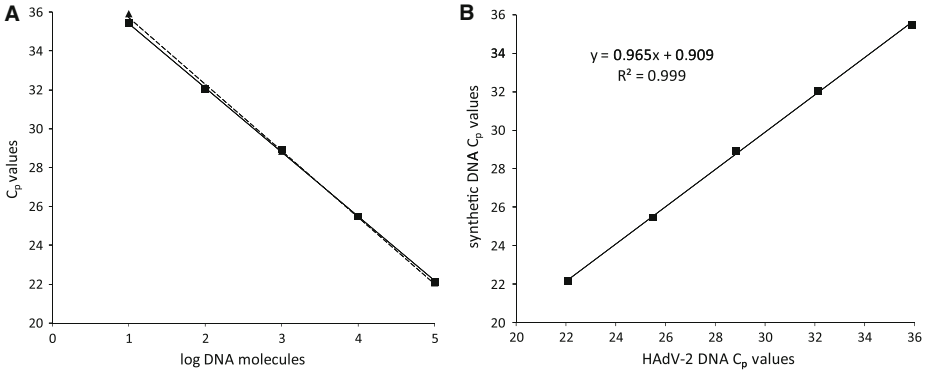


Fig. 4 Comparison of the performance of the qPCR assays using native and synthetic DNA. **a** Standard curve generated by tenfold dilutions (from 10^6 to 10^1 DNA molecules) of pFVB2 (*triangle*) and native DNA from HAdV-2 extracted from infected cells (*square*). **b** Representation of the equivalence of the C_p values of tenfold dilutions (from 10^6 to 10^1 DNA molecules) of pFVB2 (*y*-axis) and HAdV-2 (*x*-axis)

and 1.1 (La Rosa et al. 2010). For rFBV1 and pFBV2 standards, all R^2 and E values conformed to these acceptable limits. Any observed differences in the capacity for quantification of the individual assays using the standards were slight, and can be attributed to the effect of variations in the individual nucleic acid sequences (Boeuf et al. 2005).

The quantification of the DNA viruses was more efficient than the quantification of the RNA viruses (Tables 1, 2). This is probably due to the nature of RNA, and also to the additional RT step, as its efficiency depends on many factors (Levesque-Sergerie et al. 2007). Finally, and most importantly, the copy numbers of rFBV1 are equivalent to the genome equivalents of MNV-1, and the copy numbers of pFBV2 are equivalent to the genome equivalents of HAdV-2. This relationship is confidently expected to pertain also to the other virus species presented in these standards. Thus, when using these standards in monitoring a food or environmental matrix for the viruses, the analyst can be confident that the determination of virus presence he/she obtains is accurate. Thus, the availability of these standards should facilitate the implementation of nucleic acid amplification-based methods for quantitative virus detection.

Acknowledgments The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement no. KBBE-213178 (VITAL project). M.D.-V. received a Ph.D. studentship from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA). The authors thank Dr. Nigel Cook for the critical review of the ms.

Open Access This article is distributed under the terms of the Creative Commons Attribution Noncommercial License which permits any noncommercial use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.

References

Anonymous. (2008). Viruses in food: Scientific advice to support risk management activities. WHO-FAO Microbiological Risk Assessment Series No. 13. Rome. 79 pp.

Baert, L., Wobus, C. E., Van Coillie, E., Thackray, L. B., Debevere, J., & Uyttendaele, M. (2008). Detection of murine norovirus 1 by using plaque assay, transfection assay, and real-time reverse transcription-PCR before and after heat exposure. *Applied and Environmental Microbiology, 74*, 543–546.

Boeuf, P., Vigan-Womas, I., Jublot, D., Loizon, S., Barale, J. C., Akanmori, B. D., et al. (2005). CyProQuant-PCR: A real time RT-PCR technique for profiling human cytokines, based on external RNA standards, readily automatable for clinical use. *BMC Immunology, 6*, 5.

Bosch, A., Sanchez, G., Abbaszadegan, M., Carducci, A., Guix, S., Le Guyader, F. S., et al. (2011). Analytical methods for virus detection in water and food. *Food Analytical Methods, 4*, 4–13.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., et al. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry, 55*, 611–622.

Croci, L., Dubois, E., Cook, N., de Medici, D., Schultz, A. C., China, B., et al. (2008). Current methods for extraction and concentration of enteric viruses from fresh fruit and vegetables: Towards international standards. *Food Analytical Methods, 1*, 73–84.

da Silva, A. K., Le Saux, J. C., Parnaudeau, S., Pommepuy, M., Elimelech, M., & Le Guyader, F. S. (2007). Evaluation of removal of noroviruses during wastewater treatment, using real-time reverse transcription-PCR: Different behaviors of genogroups I and II. *Applied and Environmental Microbiology, 73*, 7891–7897.

Hernández, M., Rodríguez-Lázaro, D., & Ferrando, A. (2005). Current methodology for detection, identification and quantification of genetically modified organisms. *Current Analytical Chemistry, 1*, 203–221.

Hernroth, B. E., Conden-Hansson, A. C., Rehnstam-Holm, A. S., Girones, R., & Allard, A. K. (2002). Environmental factors influencing human viral pathogens and their potential indicator organisms in the blue mussel, *Mytilus edulis*: The first Scandinavian report. *Applied and Environmental Microbiology, 68*, 4523–4533.

Hundesa, A., Bofill-Mas, S., de Maluquer Motes, C., Rodriguez-Manzano, J., Bach, A., Casas, M., et al. (2010). Development of a quantitative PCR assay for the quantitation of bovine polyomavirus as a microbial source-tracking tool. *Journal of Virological Methods, 163*, 385–389.

Hundesa, A., de Maluquer Motes, C., Albinana-Gimenez, N., Rodriguez-Manzano, J., Bofill-Mas, S., Sunen, E., et al. (2009). Development of a qPCR assay for the quantification of porcine adenoviruses as an MST tool for swine fecal contamination in the environment. *Journal of Virological Methods, 158*, 130–135.

Jothikumar, N., Cromeans, T. L., Robertson, B. H., Meng, X. J., & Hill, V. R. (2006). A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. *Journal of Virological Methods, 131*, 65–71.

Kuribara, H., Shindo, Y., Matsuoka, T., Takubo, K., Futo, S., Aoki, N., et al. (2002). Novel reference molecules for quantitation of genetically modified maize and soybean. *Journal of AOAC International, 85*, 1077–1089.

Kwiatek, O., Keita, D., Gil, P., Fernandez-Pinero, J., Jimenez Clavero, M. A., Albina, E., et al. (2010). Quantitative one-step real-time RT-PCR for the fast detection of the four genotypes of PPRV. *Journal of Virological Methods, 165*, 168–177.

La Rosa, G., Pourshaban, M., Iaconelli, M., & Muscillo, M. (2010). Quantitative real-time PCR of enteric viruses in influent and effluent samples from wastewater treatment plants in Italy. *Annali dell'Istituto Superiore di Sanita, 46*, 266–273.

Lees, D., & CEN WG6 TAG4. (2010). International standardisation of a Method for detection of human pathogenic viruses in molluscan shellfish. *Food and Environmental Virology, 2*, 146–155.

Levesque-Sergerie, J. P., Duquette, M., Thibault, C., Delbecchi, L., & Bissonnette, N. (2007). Detection limits of several commercial reverse transcriptase enzymes: impact on the low- and high-abundance transcript levels assessed by quantitative RT-PCR. *BMC Molecular Biology, 8*, 93.

Rodríguez-Lázaro, D., Hernández, M., Esteve, T., Hoorfar, J., & Pla, M. (2003). A rapid and direct real time PCR-based method for identification of *Salmonella* spp. *Journal of Microbiological Methods, 54*, 381–390.

Rodríguez-Lázaro, D., Lombard, B., Smith, H., Rzezutka, A., D' Agostino, M., Helmuth, R., et al. (2007). Trends in analytical methodology in food safety and quality: Monitoring microorganisms and genetically modified organisms. *Trends in Food Sciences and Technology, 18*, 306–319.

Rodríguez-Lazaro, D., Pla, M., Scortti, M., Monzo, H. J., & Vazquez-Boland, J. A. (2005). A novel real-time PCR for *Listeria monocytogenes* that monitors analytical performance via an internal amplification control. *Applied Environmental Microbiology, 71*, 9008–9012.

Svraka, S., Duizer, E., Vennema, H., de Bruin, E., van der Veer, B., Dorresteijn, B., et al. (2007). Etiological role of viruses in outbreaks of acute gastroenteritis in The Netherlands from 1994 through 2005. *Journal of Clinical Microbiology, 45*, 1389–1394.

Taverniers, I., Van Bockstaele, E., & De Loose, M. (2004). Cloned plasmid DNA fragments as calibrators for controlling GMOs: different real-time duplex quantitative PCR methods. *Analytical and Bioanalytical Chemistry, 378*, 1198–1207.

Terlizzi, M. E., Bergallo, M., Astegiano, S., Sidoti, F., Gambarino, S., Solidoro, P., Costa, C., et al. (2010). Improvement of HRV quantification using cRNA-based standards for real time RT-PCR. *Molecular Biotechnology.* doi:10.1007/s12033-010-9343-9.

Vester, D., Lagoda, A., Hoffmann, D., Seitz, C., Heldt, S., Bettenbrock, K., et al. (2010). Real-time RT-qPCR assay for the analysis of human influenza A virus transcription and replication dynamics. *Journal of Virological Methods, 168*, 63–71.

Workenhe, S. T., Kibenge, M. J., Iwamoto, T., & Kibenge, F. S. (2008). Absolute quantitation of infectious salmon anaemia virus using different real-time reverse transcription PCR chemistries. *Journal of Virological Methods, 154*, 128–134.

3.1.5

Multicenter Collaborative Trial Evaluation of a Method for Detection of Human Adenoviruses in Berry Fruit

Martin D'Agostino[1], Nigel Cook[1], Ilaria Di Bartolo[2], Franco M. Ruggeri[2], Alessandra Berto[3], Francesca Martelli[3], Malcolm Banks[3], Petra Vasickova[4], Petr Kralik[4], Ivo Pavlik[4], Petros Kokkinos[5], Apostolos Vantarakis[5], Kirsi Söderberg[6], Leena Maunula[6], Katharina Verhaelen[7], Saskia Rutjes[7], Ana Maria de Roda Husman[7], Renate Hakze[8], Wim Van der Poel[8], Agnieszka Kaupke[9], Iwona Kozyra[9], Artur Rzeżutka[9], Jasna Prodanov[10], Sava Lazic[10], Tamas Petrovic[10], Anna Carratala[11], Rosina Gironés[11], Marta Diez-Valcarce[12], Marta Hernandez[12], David Rodríguez-Lázaro[12]

[1] Food and Environment Research Agency (FERA), Sand Hutton, York, United Kingdom[2] Istituto Superiore di Sanità, Rome, Italy [3] Veterinary Laboratories Agency, Weybridge, New Haw, Surrey, United Kingdom [4]Veterinary Research Institute, Department of Food and Feed Safety, Brno, Czech Republic [5]University of Patras, Greece [6]University of Helsinki, Finland [7]National Institute for Public Health and Environment (RIVM), Bilthoven, Netherlands [8]Central Veterinary Institute, Wageningen University and Research Centre, Lelystad, The Netherlands [9]National Veterinary Research Institute, Pulawy, Poland [10]Scientific Veterinary Institute Novi-Sad, Novi-Sad, Serbia [11]University of Barcelona, Spain [12]Instituto Tecnológico Agrario de Castilla y León (ITACyL), Valladolid, Spain

Published in Food Analytical Methods, Vol. 5, 1-7

Food Anal. Methods (2012) 5:1–7
DOI 10.1007/s12161-011-9287-0

Multicenter Collaborative Trial Evaluation of a Method for Detection of Human Adenoviruses in Berry Fruit

Martin D'Agostino · Nigel Cook · Ilaria Di Bartolo · Franco M. Ruggeri · Alessandra Berto · Francesca Martelli · Malcolm Banks · Petra Vasickova · Petr Kralik · Ivo Pavlik · Petros Kokkinos · Apostolos Vantarakis · Kirsi Söderberg Leena Maunula · Katharina Verhaelen · Saskia Rutjes · Ana Maria de Roda Husman · Renate Hakze · Wim Van der Poel · Agnieszka Kaupke · Iwona Kozyra · Artur Rzeżutka · Jasna Prodanov · Sava Lazic · Tamas Petrovic · Anna Carratala · Rosina Gironés · Marta Diez-Valcarce · Marta Hernandez · David Rodriguez-Lazaro

Received: 24 June 2011 / Accepted: 9 August 2011 / Published online: 9 September 2011
© British Crown Copyright as represented by Fera 2011

Abstract The qualitative performance characteristics of a qPCR-based method to detect human adenoviruses in raspberries were determined through a collaborative trial involving 11 European laboratories. The method incorporated a sample process control (murine norovirus) and an internal amplification control. Trial sensitivity or correct identification of 25-g raspberry samples artificially contaminated with between 5×10^2 and 5×10^4 PFU was 98.5%; the accordance and concordance were 97.0%. The positive predictive value was 94.2%. The trial specificity or percentage correct identification of non-artificially contaminated samples was 69.7%; the accordance was 80.0% and the concordance was 61.7%. The negative predictive value was 100%. Application of a method for the detection of human adenoviruses in food samples could be useful for routine monitoring for food safety management. It would help to determine if a route of contamination exists from human source to food supply chain which pathogenic viruses such as norovirus and hepatitis A virus could follow.

Keywords Human adenovirus · Multicenter ring trial · Detection

M. D'Agostino (✉) · N. Cook
Food and Environment Research Agency (FERA),
Sand Hutton York, UK
e-mail: martin.dagostino@fera.gsi.gov.uk

I. Di Bartolo · F. M. Ruggeri
Istituto Superiore di Sanita,
Rome, Italy

A. Berto · F. Martelli · M. Banks
Animal Health Veterinary Laboratories Agency,
Weybridge, UK

P. Vasickova · P. Kralik · I. Pavlik
Veterinary Research Institute,
Brno, Czech Republic

P. Kokkinos · A. Vantarakis
University of Patras,
Patras, Greece

K. Söderberg · L. Maunula
University of Helsinki,
Helsinki, Finland

K. Verhaelen · S. Rutjes · A. M. de Roda Husman
National Institute for Public Health and the Environment (RIVM),
Bilthoven, the Netherlands

R. Hakze · W. Van der Poel
Wageningen University Research,
Lelystad, the Netherlands

A. Kaupke · I. Kozyra · A. Rzeżutka
National Veterinary Research Institute,
Pulawy, Poland

J. Prodanov · S. Lazic · T. Petrovic
Scientific Veterinary Institute Novi-Sad,
Novi Sad, Serbia

A. Carratala · R. Gironés
University of Barcelona,
Barcelona, Spain

M. Diez-Valcarce · M. Hernandez · D. Rodriguez-Lazaro
Instituto Tecnológico Agrario de Castilla y León (ITACyL),
Junta de Castilla y León,
Valladolid, Spain

Introduction

There have been numerous outbreaks of disease caused by the consumption of berry fruits contaminated with enteric viral pathogens. The World Health Organisation (FAO/WHO 2008) identified norovirus and hepatitis A virus in fresh produce including berry fruits as a priority virus/commodity combination for which control measures should be considered. In the food industry, the major concepts such as HACCP have been directed at bacterial and fungal pathogens only. Equally as importantly, microbiological monitoring methods are used mainly at the end of the production chain. Also, analysing the impact of virus contamination of food has hitherto been based on gathering epidemiological information, which occurs only in response or as a reaction to disease outbreaks, and a coordinated and validated system or network does not yet exist to routinely and proactively monitor actual food samples. It is essential for thorough food safety management that systems are developed whereby viruses can be monitored at critical points throughout food supply chains.

But performing routine monitoring specifically for norovirus and hepatitis A virus may not actually be worthwhile. These viruses may be present as contaminants only very sporadically, or during outbreaks, and might be seldom detected even when food supply chains are vulnerable to contamination. It would be more effective to monitor for agents that would indicate that a route exists from source to points within the food supply chain which norovirus and hepatitis A viruses could follow to cause contamination. Adenoviruses infect both humans and a wide variety of animal species; they are shed in large numbers in the faeces of infected individuals (Granoff and Webster 1999) and are capable of robust survival (Rzeżutka and Cook 2004). Adenoviruses have been shown to be excreted by the populations of all geographical areas and to be the most abundant viruses detected in urban sewage without significant seasonal variation, and for these reasons have been proposed as indicators of human faecal contamination in water and food (Pina et al. 1998; Formiga-Cruz et al. 2002). Specific detection of adenoviruses from human or animal origin should be a useful tool for tracing the source of faecal viral contamination (Maluquer de Motes et al. 2004). Recent studies on the detection of human adenovirus in wastewater (Bofill-Mas et al. 2006), drinking water treatment plants (Albinana-Gimenez et al. 2009) and in recreational waters in Europe (Wyn-Jones et al. 2011) have shown their wide dissemination and support their applicability as indicators of faecal contamination. The European Framework 7 project "Integrated monitoring and control of foodborne viruses in European food supply chains (VITAL)" adopted the use of human adenoviruses as "index viruses" whose presence in a food supply chain such as that for berry fruits will indicate, not specifically the presence of pathogenic virus types, but that a route of contamination exists from source to monitoring point which pathogenic viruses could follow. The study described here was conducted to test the robustness of a polymerase chain reaction (PCR) (qPCR[1])-based method for detecting human adenoviruses in berry fruits, using raspberries as an example. The method incorporates a sample process control and an internal amplification control to verify its correct operation (D'Agostino et al. 2011).

Materials and Methods

Participating Institutes The Food and Environment Research Agency (FERA), UK led the trial. Eleven laboratories from nine EU member states participated in the trials. They comprised the Veterinary Laboratories Agency (UK), Veterinary Research Institute (Czech Republic), University of Patras (Greece), University of Helsinki (Finland), Istituto Superiore di Sanita (ISS) (Italy), National Institute for Public Health and the Environment (the Netherlands), Wageningen University Research (the Netherlands), National Veterinary Research Institute (Poland), Scientific Veterinary Institute Novi-Sad (Serbia), Instituto Tecnológico Agrario de Castilla y León (ITACyL) (Spain) and University of Barcelona (Spain). Each participant was provided with a personalised standard operating procedure (SOP) for performance of this trial.

Viruses Human adenovirus (HAdV) serotype 2, used as the target virus in the trial, was kindly provided by Professor Rosina Girones of the University of Barcelona. It was propagated at ISS for six sequential passages in cultures of A549 cells (European Collection of Cell Culture, UK) and titrated by plaque assay, yielding stock titers of approximately 4×10^7 plaque-forming units (PFU)ml^{-1}. Murine norovirus (MNoV), used as the sample process control (SPCV) in the trial (Diez-Valcarce et al. 2011b), was obtained from Washington University Medical School of St. Louis. It was propagated for six sequential passages in cultures of RAW 267.4 cells (American Type Culture Collection). It was titrated by plaque assays, yielding stock titers of approximately 10^8 PFU ml^{-1} . All virus stock suspensions were prepared by ISS.

Trial Materials Trial materials were prepared at the ISS by FERA staff, who coded each vial and alone knew the identity of the contents. There were nine coded vials, three of which contained 100 µl 1×10^6 PFU ml^{-1} HAdV (HIGH) suspension, three containing 100 µl 1×10^4 PFU ml^{-1}

[1] The term "qPCR" is used for qPCR throughout this article, in accordance with the recommendations of Bustin et al. (2009).

HAdV (LOW) suspension and three containing only cell culture medium (BLANK) were sent to each participant. Each participant was also sent one vial containing 100 µl of 5×10^7 PFU ml^{-1} MNoV (SPCV) suspension.

Preparation of Trial Samples Fresh raspberries were purchased separately by each participant from local sources. Nine 25-g raspberries portions were placed into plastic disposable weighing boats or similar receptacles. Three portions were artificially contaminated with 5×10^4 PFU HAdV by pipetting 5×10 µl of the HIGH suspension onto the surface of the raspberries. Three portions were artificially contaminated with 5×10^2 PFU HAdV by pipetting 5×10 µl of the LOW suspension onto the surface of the raspberries. Three portions were spiked with cell culture medium by pipetting 5×10 µl of the BLANK suspension onto the surface of the raspberries. All samples were left at room temperature for approximately 2 h until the suspending fluid was almost dry, and then processed following the method of Dubois et al. (2002). Immediately prior to commencing the process, all samples were spiked with 1×10^5 PFU murine norovirus by pipetting 10 µl of the SPCV suspension onto the surface of the raspberries.

Extraction of Virus Nucleic Acids from Raspberries The sample was processed using the method of Dubois et al. (2002). Approximately 25 g fruit was placed in a sterile beaker. Forty milliliters of Tris–glycine pH 9.5 buffer containing 1% beef extract and 6,500 U pectinase (e.g. Pectinex™ Ultra SPL solution, Sigma) was added to the sample, which was then agitated at room temperature for 20 min by rocking at 60 rpm. The pH was maintained at 9.0 throughout (if necessary adjusting using 4% *w*/*v* sodium hydroxide, extending the period of agitation by 10 min each time an adjustment was made. In strongly coloured berries, a change in colour of the eluate from blue/purple to red was considered indicative of acidification and was used to trigger pH adjustment). The liquid was decanted from the beaker through a strainer (e.g. a tea strainer) into one 50 ml or two smaller centrifuge tubes and centrifuged at 10,000×*g* for 30 min at 4 °C. The supernatant was decanted into a single clean tube or bottle, and the pH was adjusted to 7.2. Volumes (0.25) of 50% (*w*/*v*) polyethylene glycol 8,000/1.5 M NaCl were then added and mixed by shaking for 1 min. The suspension was then incubated with gentle rocking at 4 °C for 60 min before centrifugation at 10,000×*g* for 30 min at 4°C. The supernatant was discarded, and the pellet was compacted by centrifugation at 10,000×*g* for 5 min at 4°C before resuspension in 500 µl PBS. The suspension was then transferred to a chloroform-resistant tube, and 500 µl 1:1 chloroform:butanol (*v*:*v*) was added and mixed by vortexing. The sample was allowed to stand for 5 min and then centrifuged at 10,000×*g* for 15 min at 4 °C. The aqueous phase was transferred to a clean tube and immediately used for nucleic acid extraction or stored at −20 °C. Nucleic acids were extracted using a NucliSENS® miniMAG® kit (bioMérieux) according to the manufacturer's instructions. The final elutions were performed with 100 µl elution buffer, resulting in a 200-µl nucleic acid extract. The nucleic acid extract was assayed immediately or stored at −70 °C. The extract was diluted to 10^{-1} in nuclease-free water before assaying.

Adenovirus qPCR This assay was a duplex qPCR using the primers and conditions described by Hernroth et al. (2002), with the inclusion of an internal amplification control (IAC) (Diez-Valcarce et al. 2011a) and a carryover contamination prevention system utilising uracil N-glycosylase. The reaction contained 1×TaqMan Universal PCR Master Mix (Applied Biosystems), 0.9 µM each primer, 0.225 µM adenovirus TaqMan probe (labelled with FAM), 50 nM IAC probe (labelled with VIC) and 100 copies of adenovirus IAC (Yorkshire Bioscience Ltd., UK). Ten microliters of the diluted nucleic acid extract was added to make a final reaction volume of 25 µl. The thermocycling conditions were 2 min at 50 °C then 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Two PCR replicates were performed for each sample. In each PCR run, positive and negative amplification controls were included.

Murine Norovirus Reverse Transcription qPCR (RTqPCR) This assay was a one-step duplex reverse transcription qPCR using the primers and conditions described by Baert et al. (2008), with the inclusion of an IAC (Diez-Valcarce et al. 2011a, b). The reaction contained 1×RNA Ultrasense reaction mix (Invitrogen), 0.2 µM each primer, 0.2 µM probe MGB-ORF1/ORF2 (labelled with FAM), 50 nM IAC probe (labelled with VIC), 1×ROX reference dye (Invitrogen), 1 µl RNA Ultrasense enzyme mix (Invitrogen) and 600 copies of murine norovirus IAC (Yorkshire Bioscience Ltd., UK). Ten-microliter sample of the diluted nucleic acid extract was added to make a final reaction volume of 20 µl. The thermocycling conditions were 15 min at 50 °C, 2 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Two RTqPCR replicates were performed for each sample. In each run, positive and negative amplification controls were included.

Definition of Analytical Method In the frame of this collaborative trial, the analytical method is defined as the sample treatment (which includes virus extraction and concentration, and nucleic acid purification) coupled to the nucleic acid amplification assays for the target and the sample process control virus. Equally, a nucleic acid amplification assay is defined as a nucleic acid amplification reaction which contains an IAC.

Reporting and Interpretation of Data Raw data were reported by each participant to the trial leader, who translated the codes and analysed the data in collaboration with ITACyL. When an assay showed a quantification cycle (Cq, previously known as the threshold cycle) value ≤40 or 45 for murine norovirus or adenovirus respectively independently of the corresponding IAC Cq value, the result was interpreted as positive. When an assay showed a Cq value ≥40 or 45 for murine norovirus or adenovirus respectively with the corresponding IAC Cq value ≤40 or 45 for murine norovirus or adenovirus respectively, the result was interpreted as negative. When an assay showed both the target and its corresponding IAC Cq values ≥40 or 45, the reaction was considered to have failed. When a participant reported that at least one of the replicate HAdV assays was positive, they were considered to have identified the sample as being adenovirus contaminated. When a participant reported that both replicate HAdV assays were negative, but at least one replicate MNoV assay was positive, they were considered to have identified the sample as being adenovirus uncontaminated. When a participant reported that both replicate HAdV assays had failed, independently of the results of the MNoV assays, they were considered to have reported that the analysis of that sample had failed. When a participant reported that both replicate HAdV assays were negative and both replicate MNoV assays were negative, they were considered to have reported that the analysis of that sample had failed. Interpretation of the results followed the principles outlined by D'Agostino et al. (2011).

Criteria for Inclusion of Results in the Statistical Analysis The results from each participating laboratory were included unless they fell into one of the following two categories: (1) obvious performance deviation from the SOP and (2) presence of target amplicons in the negative amplification controls, indicating contamination of the reaction.

Qualitative Statistical Analysis The raw data sent by each laboratory were statistically analysed according to the recommendations of Scotter et al. (2001) and by the methods of Langton et al. (2002). The *diagnostic sensitivity* of the analytical method was defined as the percentage of positive samples giving a correct positive signal, i.e. using only the results of the analysis of the artificially contaminated samples. The *diagnostic specificity* of the analytical method was defined as the percentage of negative samples giving a correct negative signal, i.e. using only the results of the analysis of the non-artificially contaminated samples. *Accordance* (repeatability of qualitative data) was defined as the percentage chance of finding the same result, positive or negative, from two identical samples analysed in the same laboratory under predefined repeatability conditions, and *concordance* (reproducibility of qualitative data) was defined as the percentage chance of finding the same result, positive or negative, from two identical samples analysed in different laboratories under predefined repeatability conditions. These calculations take into account different replication in different laboratories by weighting results appropriately. The *concordance odds ratio* (COR) was the degree of inter-laboratory variation in the results and expressed as the ratio between accordance and concordance percentages (Langton et al. 2002). The COR value may be interpreted as the likelihood of getting the same result from two identical samples, whether they are sent to the same laboratory or to two different laboratories. The closer the value is to 1.0, the higher the likelihood is of getting the same result. Confidence intervals for accordance, concordance and COR were calculated by the method of Davison and Hinckley (1997); each laboratory was considered representative of all laboratories in the "population" of laboratories, not just those participating in this analysis.

The positive predictive value of the analytical method is the proportion of the correctly identified contaminated samples. The negative predictive value of the analytical method is the proportion of the correctly identified uncontaminated samples, from all the samples reported as adenovirus uncontaminated. These values were calculated by the ISO 16140 method (Anonymous 2003).

Results

Participants' Results in the Collaborative Trial Table 1 shows the participants' results from the analysis of raspberry samples artificially contaminated with 5×10^4 PFU. All samples were correctly reported as contaminated, except in one case where the analysis of a sample had failed. Table 2 shows the participants' results from the analysis of raspberry samples artificially contaminated with 5×10^2 PFU human adenovirus. Laboratory "4" did not perform analysis of the LOW artificially contaminated test samples. All samples were correctly reported as contaminated. Table 3 shows the participants' results from the analysis of the non-artificially contaminated raspberry samples. Here, four samples were reported as contaminated. Six sample analyses had failed.

Qualitative Statistical Analysis Table 4 gives the diagnostic specificity, diagnostic sensitivity, positive and negative predictive values, accordance and concordance values and the concordance odds ratio for the collaborative trial of the analytical method for the detection of human adenovirus on raspberries. The results of the analysis of the uncontami-

Table 1 Participants' results from the analysis of raspberry samples artificially contaminated with 5×10^4 PFU human adenovirus (HIGH)

Laboratory	Sample A					Sample B					Sample C				
	HAdV		MNoV			HAdV		MNoV			HAdV		MNoV		
	Rep. 1	Rep. 2	Rep. 1	Rep. 2	Int.	Rep. 1	Rep. 2	Rep. 1	Rep. 2	Int.	Rep. 1	Rep. 2	Rep. 1	Rep. 2	Int.
1	+	+	+	+	C	+	+	+	+	C	+	+	+	+	C
2	+	+	+	+	C	+	+	+	+	C	+	+	+	+	C
3	+	+	+	+	C	F	+	−	+	C	F	F	−	−	AF
4	+	+	−	−	C	+	+	−	+	C	+	+	−	+	C
5	+	+	+	+	C	+	+	+	+	C	+	+	+	+	C
6	+	+	+	+	C	+	+	+	+	C	+	+	+	+	C
7	+	+	+	+	C	+	+	+	+	C	+	+	+	+	C
8	+	+	−	−	C	+	+	−	−	C	+	+	−	−	C
9	+	+	+	+	C	+	+	+	+	C	+	+	+	+	C
10	+	+	+	+	C	+	+	+	+	C	+	+	+	+	C
11	+	+	+	+	C	+	+	+	+	C	+	+	+	+	C

HAdV human adenovirus, *MNoV* murine norovirus , *Rep.* replicate qPCR, *Int* interpretation, + target signal present, IAC signal present or absent, − target signal absent, IAC signal present, *F* target signal absent, IAC signal absent, *C* sample contaminated, *AF* analysis failed

nated samples by laboratory "8" were excluded because all their analyses failed.

Discussion

The method under trial proved capable of detecting adenoviruses in berry fruit at a level of at least 10^2 PFU per 25 g in artificially contaminated samples. Out of 66 samples analysed, only 1 had failed. This was due to the failure of the sample process as judged by the absence of a signal from the SPCV in conjunction with the failure of the HAdV qPCR in both replicates. The statistical procedure used to analyse the trial results does not discriminate between negative results and failed analyses; it has been used several times to analyse the results of collaborative trials of PCR-based methods (Abdulmawjood et al. 2004; D'Agostino et al. 2004; Josefsen et al. 2004; Malorny et al. 2004; Wyn-Jones et al. 2011), but it would be advantageous to modify it for future similar studies. In some samples, other controls had failed, but overall, the samples could be legitimately reported as positive for HAdV. And the trial sensitivity was still very high, at 98.5%, which indicates

Table 2 Participants' results from the analysis of raspberry samples artificially contaminated with 5×10^2 PFU human adenovirus (LOW)

Laboratory	Sample A					Sample B					Sample C				
	HAdV		MNoV			HAdV		MNoV			HAdV		MNoV		
	Rep. 1	Rep. 2	Rep. 1	Rep. 2	Int.	Rep. 1	Rep. 2	Rep. 1	Rep. 2	Int.	Rep. 1	Rep. 2	Rep. 1	Rep. 2	Int.
1	+	+	+	+	C	+	+	+	+	C	+	+	+	−	C
2	+	+	+	+	C	+	+	+	+	C	+	−	+	+	C
3	+	+	−	−	C	+	+	−	−	C	+	+	F	−	C
5	+	+	+	+	C	+	+	+	+	C	+	+	+	+	C
6	+	+	+	+	C	+	+	+	+	C	+	+	F	+	C
7	+	+	+	+	C	+	+	+	+	C	+	+	+	+	C
8	+	+	−	−	C	+	+	−	−	C	+	+	−	−	C
9	+	+	+	+	C	+	+	+	+	C	+	+	+	+	C
10	+	+	+	+	C	+	+	+	+	C	+	+	+	+	C
11	+	+	+	+	C	+	+	+	+	C	+	+	+	+	C

HAdV human adenovirus, *MNoV* murine norovirus , *Rep.* replicate qPCR, *Int* interpretation, + target signal present, IAC signal present or absent, − target signal absent, IAC signal present, *F* target signal absent, IAC signal absent, *C* sample contaminated, *AF* analysis failed

Table 3 Participants' results of the analysis of the non-artificially contaminated raspberry samples

Laboratory	Sample A					Sample B					Sample C				
	HAdV		MNoV			HAdV		MNoV			HAdV		MNoV		
	Rep.1	Rep. 2	Rep. 1	Rep. 2	Int.	Rep. 1	Rep. 2	Rep. 1	Rep. 2	Int.	Rep. 1	Rep. 2	Rep. 1	Rep. 2	Int.
1	−	−	+	+	UC	−	−	+	+	UC	−	−	+	+	UC
2	−	−	+	+	UC	−	−	+	+	UC	−	−	+	+	UC
3	+	+	+	+	C	−	−	+	+	UC	F	F	−	−	AF
4	+	−	F	−	C	−	−	−	−	AF	−	−	−	−	AF
5	−	−	+	+	UC	−	−	+	+	UC	−	+	+	+	C
6	−	−	+	+	UC	−	−	+	+	UC	−	−	+	+	UC
7	−	−	+	+	UC	−	−	+	+	UC	−	−	+	+	UC
8	F	F	−	−	AF	F	F	−	−	AF	F	F	−	−	AF
9	−	−	+	+	UC	−	−	+	+	UC	+	+	+	+	C
10	−	F	+	+	UC	−	F	+	+	UC	F	−	+	+	UC
11	−	−	+	+	UC	−	−	+	+	UC	−	−	+	+	UC

HAdV human adenovirus, *MNoV* murine norovirus , *Rep.* replicate qPCR, *Int* interpretation, + target signal present, IAC signal present or absent, − target signal absent, IAC signal present, *F* target signal absent, IAC signal absent, *C* sample contaminated, *AF* analysis failed, *UC* uncontaminated

that the method can be used confidently to detect the presence of human adenovirus in berry fruits.

With the non-artificially contaminated samples, six analyses were reported to have failed. This highlights the value of an interlocking suite of controls when performing routine nucleic acid-based analysis for detection of viruses in foods, as they allow appropriate actions to be identified which should result in accurate reanalysis of failed tests (Bosch et al. 2011; D'Agostino et al. 2011; Rodríguez-Lázaro et al. 2007). It is unclear why the failed tests occurred in the trial, but they left 23 out of 33 samples being reported as uncontaminated, and this skewed the trial specificity to a lower value than that which has been observed in other trials (Abdulmawjood et al. 2004; D'Agostino et al. 2004; Josefsen et al. 2004; Malorny et al. 2004; Wyn-Jones et al. 2011). This proportion may not accurately reflect the actual number of false positives which might be expected in routine application of the current method, where analyses should not be expected to fail so often. The variability of results between laboratories here also affected the accordance and concordance and the concordance odds ratios; however, the confidence intervals of each indicate that if the method was adopted by a wider selection of laboratories there would be a possibility of more uniform results. The negative predictive value of the method is excellent, as none of the artificially contaminated samples were reported as uncontaminated.

Four of the non-artificially contaminated samples were reported as contaminated with adenovirus. As a result, the trial specificity and the positive predictive value indicate that a proportion of false-positive results can be expected when using this method. However, a possible explanation is that the fruit used for these samples had in fact been contaminated with human adenovirus prior to purchase, and the positive results were not actually false. The method described in this study has been subsequently used to analyse berry fruit at point-of-sale in several European countries, and some of these samples have been positive for human adenovirus. It is recommended that any positive target amplicons are sequenced to confirm target identity when performing actual analysis of produce.

Table 4 Statistical evaluation of the data obtained from the collaborative trial

Contamination level	Sensitivity (%)	Specificity (%)	Positive predictive value	Negative predictive value	Accordance (%)	Concordance (%)	Concordance odds ratio (COR)
LOW + HIGH	98.5 (91.9, 99.7)	–	94.2 (86.0, 97.7)	–	97.0 (90.9, 100)	97.0 (91.2, 100)	1.0 (0.96, 1.0)
None	–	69.7 (52.7, 82.6)	–	100 (100, 100)	80.0 (60, 100)	61.7 (46.9, 93.3)	2.48 (0.85, 16.48)

Values in parentheses are the lower and upper 95% confidence intervals

The qPCR HAdV assay used in this study could be applied for quantitation of the target virus by estimating the number of HAdV genome copies based on an external standard. However, when the partners' results were converted into genome copies detected per sample (not shown), the level of between-laboratory variation was too great to be able to describe the performance characteristics of the method in quantitative terms. This is despite the fact that nucleic acid standard solutions were supplied along with the trial materials. The high between-laboratory variation may be caused by several factors, such as the condition in which the standard solutions have reached the partner institutes or operational differences between thermocyclers used in the various laboratories. These possibilities highlight a requirement for reliable reference materials and external quality control systems to be available, if routine monitoring of food supply chains for viruses is to be adopted efficiently.

Notwithstanding the above issues, the overall results of the collaborative trial were considered to show that the qPCR-based method for the detection of human adenoviruses in soft fruits was acceptably robust. The method was then employed within the VITAL project on gathering data on virus presence in various food supply chains. Forthcoming results (manuscripts in preparation) of this data gathering will reveal the usefulness of the index virus approach, and the information gained should assist consideration of measures which can be applied to block routes of virus contamination. The method described and tested in this study is a building block in the foundation of future systems for integrated monitoring and control of viruses in food supply chains.

Acknowledgements This work was supported by the EU VITAL project Contract No. 213178. M.D.-V. received a Ph.D. studentship from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA). M.D. and N.C. acknowledge the support of the United Kingdom Food Standards Agency.

References

Abdulmawjood A, Bülte M, Roth S, Schönenbrücher H, Cook N, D'Agostino M, Malorny B, Jordan K, Pelkonen S, Hoorfar J (2004) J AOAC Int 87:856

Albinana-Gimenez N, Miagostovich M, Calgua B, Huguet JM, Matia L, Girones R (2009) Water Res 43:2011–2019

Anonymous (2003) ISO 16140:2003. International Standards Organisation, Geneva

Baert L, Wobus CE, Van Coillie E, Thackray LB, Debevere J, Uyttendaele M (2008) Appl Environ Microbiol 74:543

Bofill-Mas S, Albiñana-Gimenez N, Clemente-Casares P, Hundesa A, Rodriguez-Manzano J, Allard A, Calvo M, Girones R (2006) Appl Environ Microbiol 72:7894–7896

Bosch A, Sanchez G, Abbaszadegan M, Carducci A, Guix S, Le Guyader FS, Netshikweta R, Pintó RM, van der Poel W, Rutjes S, Sano D, Rodríguez-Lázaro D, Kovac K, Taylor MB, van Zyl W, Sellwood J (2011) Food Anal Methods 4:4

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009) Clin Chem 55:611–622

D'Agostino M, Cook N, Rodriguez-Lazaro D, Rutjes S (2011) Food Environ Virol 3:55

D'Agostino M, Wagner M, Vazquez-Boland JA, Kuchta T, Karpiskova R, Hoorfar J, Novella S, Scortti M, Ellison J, Murray A, Heuvelink A, Kuhn M, Pazlarova J, Fernandez I, Cook N (2004) J Food Protect 67:1646

Davison AC, Hinckley DV (1997) Bootstrap methods and their application. Cambridge University Press, Cambridge

Diez-Valcarce M, Kovac K, Cook N, Rodríguez-Lázaro D, Hernández M (2011a) Food Anal Methods 4:437–445

Diez-Valcarce M, Cook N, Hernández M, Rodríguez-Lázaro D (2011b) Food Anal Methods. doi:10.1007/s12161-011-9262-9

Dubois E, Agier C, Traore O, Hennechart C, Merle G, Cruciere C, Laveran H (2002) J Food Protect 65:1962

FAO/WHO (2008) Viruses in food: scientific advice to support risk management activities. Microbiological risk assessment series. WHO, Geneva

Formiga-Cruz M, Tofiño-Quesada G, Bofill-Mas S, Lees DN, Henshilwood K, Allard AK, Condin-Hansson A-C, Hernroth BE, Vantarakis A, Tsibouxi A, Papapetropoulou M, Furones D, Girones R (2002) Appl Environ Microbiol 68:5990–5998

Granoff A, Webster RG (1999) Encyclopedia of virology, vol. 1, 2nd edn. Academic, London

Hernroth BE, Conden-Hansson AC, Rehnstam-Holm AS, Girones R, Allard AK (2002) Appl Environ Microbiol 68:4523

Josefsen MH, Cook N, D'Agostino M, Hansen F, Wagner M, Demnerova K, Heuvelink AE, Tassios PT, Lindmark H, Kmet V, Barbanera M, Fach P, Loncarevic S, Hoorfar J (2004) Appl Environ Microbiol 70:4379

Langton SD, Chevennement R, Nagelkerke N, Lombard B (2002) Int J Food Microbiol 79:175

Malorny B, Cook N, D'Agostino M, De Medici D, Croci L, Abdulmawjood A, Fach P, Karpiskova R, Aymerich T, Kwaitek K, Kuchta T, Hoorfar J (2004) J AOAC Int 87:86

Maluquer de Motes C, Clemente-Casares P, Hundesa A, Martín M, Girones R (2004) Appl Environ Microbiol 70:1448–1454

Pina S, Puig M, Lucena F, Jofre J, Girones R (1998) Appl Environ Microbiol 64:3376–3382

Rodríguez-Lázaro D, Lombard B, Smith H, Rzezutka A, D'Agostino M, Helmuth R, Schroeter A, Malorny B, Miko A, Guerra B, Davison J, Kobilinsky A, Hernández M, Bertheau Y, Cook N (2007) Trends Food Sci Technol 18:306

Rzeżutka A, Cook N (2004) FEMS Microbiol Revs 28:441

Scotter SL, Langton S, Lombard B, Schulten N, Nagelkerke N, In't Veld PH, Rollier P, Lahellec C (2001) Int J Food Microbiol 64:295

Wyn-Jones A, Carducci A, Cook N, D'Agostino M, Divizia M, Fleischer J, Gantzer C, Gawler A, Girones R, Höller C, de Roda Husman AM, Kay D, Kozyra I, López-Pila J, Muscillo M, São José Nascimento M, Papageorgiou G, Rutjes S, Sellwood J, Szewzyk R, Wyer M (2011) Water Res 45:1025

3.1.6

Virus Genome Quantification Does not Predict Norovirus Infectivity after Application of Food Inactivation Processing Technologies

Marta Diez-Valcarce[1], Katarina Kovač[2], Peter Raspor[2], David Rodríguez-Lázaro[1] and Marta Hernández[1]

[1] Instituto Tecnológico Agrario de Castilla y León (ITACyL), Valladolid, Spain [2] Biotechnology, Microbiology and Food Safety, Biotechnical Faculty, University of Ljubljana, Slovenia

Published in Food and Environmental Virology, Vol. 3, 141-146

Food Environ Virol (2011) 3:141–146
DOI 10.1007/s12560-011-9070-9

ORIGINAL PAPER

Virus Genome Quantification Does not Predict Norovirus Infectivity After Application of Food Inactivation Processing Technologies

Marta Diez-Valcarce · Katarina Kovač · Peter Raspor · David Rodríguez-Lázaro · Marta Hernández

Received: 12 September 2011 / Accepted: 14 October 2011 / Published online: 1 November 2011
© Springer Science+Business Media, LLC 2011

Abstract When determining the effect of food processing on the infectivity of any contaminating virus, it is necessary to distinguish unambiguously between infectious and non-infectious viruses present. However, this can be difficult in the particular case of noroviruses (NoVs) because no reliable cell culture model is available. The aim of this study was to assess the use of molecular methods—RT real-time PCR (RT-qPCR) and enzymatic treatment (ET) coupled to RT-qPCR—to quantify the infectivity of NoV after application of various inactivating food-processing technologies. RT-qPCR and ET-RT-qPCR gave significantly different ($P < 0.01$) results concerning the reduction in viral genome counts by all inactivation procedures and conditions used, except for HHP treatment at 600 MPa for 5 min. These findings indicate that the ET prior to RT-qPCR has an effect on the estimation of the reduction of virus genome counts, and may eliminate genomes of affected virus particles. However, no correlation was found between the results obtained by ET-RT-qPCR and those obtained by cell culture. Therefore, the effect is presumably only partial, and not adequate to allow accurate estimation of virus inactivation. Consequently, our results indicate that the quantification of virus genomes by PCR, regardless of prior ET, is not adequate for establishing virus inactivation and/or infectivity. In addition, our results also illustrate that the general effect of virus inactivation is not directly correlated to effects on the integrity of virus genome and protein capsid. Presumably, inactivation by food processing is the consequence of effects on proteins involved in adhesion and invasion stages.

Keywords Viral infectivity · Inactivation technologies · Enzymatic treatment · RT real-time PCR · Norovirus

Introduction

To establish the effect of food processing on virus infectivity, methods for measuring virus inactivation are required. Molecular methods such as real-time PCR (qPCR) can provide rapid, sensitive and specific quantitative results but have limitations for distinguishing between infectious and inactive virus. The persistence of intact sequences of the genome in the inactivated viruses makes amplification possible and leads to confusing false-positive PCR results (Bhattacharya et al. 2004; Duizer et al. 2004; Sobsey et al. 1998); consequently, it is difficult by this approach to obtain accurate quantitative information about the inactivation and survival of norovirus (NoVs).

Several strategies have been used to adapt PCR to quantify infective virus particles, but so far there are no PCR methods available for accurate quantification of virus infectivity (Rodríguez et al. 2009). One methodological approach that has shown promising results is integrated cell

Marta Diez-Valcarce and Katarina Kovač have contributed equally to this work.

M. Diez-Valcarce · K. Kovač · D. Rodríguez-Lázaro (✉) · M. Hernández
Junta de Castilla y León, Instituto Tecnológico Agrario de Castilla y León (ITACyL), Ctra. Burgos, km, 119, 47071 Valladolid, Spain
e-mail: ita-rodlazda@itacyl.es

K. Kovač · P. Raspor
Biotechnology, Microbiology and Food Safety, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia

M. Hernández (✉)
Molecular Biology and Microbiology Laboratory, Junta de Castilla y León, Instituto Tecnológico Agrario de Castilla y León (ITACyL), Ctra. Burgos, km, 119, 47071 Valladolid, Spain
e-mail: ita-herperma@itacyl.es

culture strand-specific reverse transcription-PCR (ICC-RT-PCR) (Chapron et al. 2000; Jiang et al. 2004). However, this technique includes a culture-based step, and consequently it cannot be applied to NoV. Nuanualsuwan and Cliver (2002) reported that false-positive PCR signals could be eliminated by enzymatic treatment (ET) with proteinase K and RNase prior to nucleic acid extraction and RT-PCR. The use of ET may thus help to differentiate infective from inactivated viruses based on the integrity of their protein capsids: if protein capsid integrity is affected, it is more susceptible to degradation by proteinases. Consequently, the virus genome would be released and be more susceptible to RNase degradation than capsid-enclosed RNA (Nuanualsuwan and Cliver 2003).

The objective of this study was to assess the value of RT-qPCR and of ET coupled to RT-qPCR for quantifying the infectivity of NoV. We particularly focused on (1) trying to establish a quantitative relationship between the loss of the RT-qPCR signal and the loss of infectivity after inactivation treatments and (2) determining if ET improved the correspondence between RT-qPCR and cell culture results. The inactivation treatments used in this study were high-pressure processing (HHP), ultraviolet irradiation (UV) and thermal treatment, and were applied to a NoV surrogate, murine norovirus (MNV-1). Thermal treatment is the most widely used procedure for microbial inactivation in foods; furthermore NoVs are inactivated by cooking and therefore appear to be heat-sensitive (Scientific Committee on Veterinary Measures Relating to Public Health 2002). HHP is an emerging food treatment technique that makes food safer and extends its shelf life while allowing the food to retain many of its original quality and nutritive attributes (Kovač et al. 2010). UV is an attractive alternative to the use of chlorine or ozone for the disinfection of water because it produces minimal disinfectant by-products and is very effective for inactivating highly chlorine-resistant protozoan pathogens such as *Cryptosporidium parvum* and *Giardia lamblia* (Linden et al. 2002; Hijnen et al. 2006). However, little data is available concerning NoV inactivation by UV. These treatments were chosen to represent different strategies of inactivation: thermal treatment principally attacks the viral coat proteins (Breindl 1971), UV predominantly targets the viral nucleic acids depending on the dose (Eischeid et al. 2009; Shin et al. 2009) and HHP seems to not affect viral nucleic acids, but rather denature capsid proteins essential for host cell attachment (Kovač et al. 2010).

Materials and Methods

Viruses and Cell Lines

MNV-1 was kindly provided by Herbert W. Virgin IV (Washington University School of Medicine, USA). It was propagated on confluent monolayers of RAW 264.7 cells, a mouse macrophage cell line. RAW 264.7 cells were grown in Dulbecco's modified Eagle medium (DMEM) (Gibco-Invitrogen, NY, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Gibco-Invitrogen, NY, USA), 1× antibiotic/antimycotic suspension (Gibco-Invitrogen, NY, USA) and 2 mM L-glutamine (Gibco-Invitrogen, NY, USA) at 37°C under 5% CO_2. After the appearance of cytopathic effect, cell lysates were freeze–thawed three times and centrifuged at 1,300×*g* for 25 min at 4°C, to remove the cell debris. Aliquots of the supernatant, containing the virus, were stored at −80°C. Infectivity assays ($TCID_{50}$) were performed to determine the titre of the resulting MNV-1 stock. The titre of the MNV-1 stock used in this study was $\sim 1 \times 10^7$ $TCID_{50}$ ml^{-1}.

Inactivation Treatments

High-Hydrostatic Pressure Treatment

Two-millilitre aliquots of MNV-1 were packed in pressure resistant bags. The bags were heat-sealed using an EU-7 vacuum packaging apparatus (Tecnotrip, Barcelona, Spain). Samples were placed in another bag containing an ice-water mixture, heat-sealed, and then introduced into the HHP device. The samples were subjected to 200 MPa for 5 min and 600 MPa for 5 min in a WAVE 6000/135 High Pressure Processing Equipment (NC Hyperbaric, Burgos, Spain).

Thermal Treatment

Five hundred microlitre aliquots of MNV-1 were placed into 1.5 ml tubes which were heated in a thermoblock (Thermomixer Confort, Eppendorf AG, Hamburg, Germany) at 80°C for 150 or 600 s.

UV Irradiation Treatment

Five hundred microlitre aliquots of MNV-1 were placed into Petri dishes and subjected to UV treatment (100 and 250 J m^{-2}) using a UV Stratalinker® UV Crosslinker 1800 (Stratagene, La Jolla, CA, USA).

Enzymatic Treatment

The MNV-1 samples subjected to the three inactivation protocols were then treated with a combination of Proteinase K (Sigma-Aldrich, St.Louis, MO, USA) and RNase A (Qiagen, Hilden, Germany). Briefly, 20 U of Proteinase K were added per 100 µl of sample, and the sample was incubated at 37°C for 30 min. Then 0.07 U RNase A was added per 100 µl of sample and the sample was incubated

Food Environ Virol (2011) 3:141–146

at 37°C for 1 h. The reaction was stopped by adding 40 U of the RNase inhibitor solution (Qiagen, Hilden, Germany). RNA was extracted from samples immediately thereafter.

RNA Extraction and Reverse Transcription Real-Time PCR

Samples of 100 µl were adjusted to 140 µl by adding PBS, and QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions to extract RNA. In each set of samples extracted at the same time, one negative extraction control was included. Viral RNA was stored at −80°C until further processing. One-step RT real-time PCR (RT-qPCR) assays were performed and analysed essentially as described by Diez-Valcarce et al. (2011) using the Light Cycler 480 RNA Master Hydrolysis Probes Kit (Roche Diagnostics, Mannheim, Germany) in a 20 µl reaction volume containing 1× Light Cycler 480 RNA Master Hydrolysis Probes kit buffer, 3.25 mM Activator, 200 nM ORF1/ORF2 primers, 200 nM of MGB-ORF1/ORF2 probe (Baert et al. 2008) and 10 µl of the RNA solution. Reactions were run on a Light Cycler 480 II apparatus (Roche Diagnostics, Mannheim, Germany) using the following programme: 30 min at 63°C, 30 s at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. The results of one-step RT-qPCR assays were analysed using the SW 1.5 software (Roche Diagnostics, Mannheim, Germany). Virus genome equivalents (GE) were quantified by interpolation with a standard regression curve of Cp values generated from tenfold serial dilutions of RNA from virus samples of known titre (10^7 $TCID_{50}$ ml^{-1} to 10 $TCID_{50}$ ml^{-1}). Reactions with *Cp* value ≥40 or showing an absence of amplification were considered to be negative values. All reactions were performed in triplicate. The reduction of GE associated with treatments was determined by calculating the $\log(N_0/N)$, where N_0 and N are the number of GE as assessed by RT-qPCR in untreated controls and treated samples, respectively.

Infectivity Assay

Freshly prepared RAW 264.7 cells were diluted in complete DMEM to a concentration of 2×10^5 cells ml^{-1} and 100 µl were seeded in each well in a 96-well plate. After 4 h incubation at 37°C under 5% CO_2, 100 µl of tenfold serial dilutions of the MNV-1 stock or samples after treatment, prepared in DMEM (Gibco-Invitrogen, NY, USA) supplemented with 2% FBS (Gibco-Invitrogen, NY, USA), 1× antibiotic/antimycotic suspension (Gibco-Invitrogen, NY, USA) and 2 mM L-glutamine (Gibco-Invitrogen, NY, USA), were added to eight wells per dilution in the 96-well plate. Plates were incubated at 37°C under 5% CO_2 and checked every 2 days for cytopathic effect. After one week, the tissue culture infectious dose ($TCID_{50}$ ml^{-1}) was calculated with the Spearman–Karber equation (Kärber 1931). The decrease of MNV-1 infectivity was determined by calculating the $\log(N_0/N)$, where N_0 and N are the number of viral particles as determined by $TCID_{50}$ assay in untreated controls and treated samples, respectively.

Statistical Analysis

The significance of differences between means for control and treated samples for each processing treatment as measured by RT-qPCR, ET-RT-qPCR or $TCID_{50}$ assay were evaluated using the Student's *t* test with a level of significance $P < 0.01$. SPSS 16.0 Statistical Analysis software (SPSS Inc., Chicago, IL, USA) was used.

Results

ET-RT-qPCR is not an Adequate Methodological Approach for Measuring Reduction of MNV-1 Infectivity

The reduction of virus infectivity was measured by cell culture assay ($TCID_{50}$) and by ET-RT-qPCR (Table 1) (Fig. 1). The results concerning the reduction of infectivity associated with the treatments obtained by $TCID_{50}$ assay and by ET-RT-qPCR were significantly different ($P < 0.01$) for all inactivation procedures and conditions used (Table 2). Whereas the mean results of the $TCID_{50}$ assays showed a reduction of virus infectivity of more than 3.54 $\log_{10}$ after inactivation treatments (except for the HHP treatment of 200 MPa during 5 min), the reductions scored by ET-RT-qPCR were significantly lower and below 0.56 $\log_{10}$ (except for the HHP treatment of 600 MPa during 5 min) (Table 1). To assess the accuracy of the results obtained by ET-RT-qPCR, we calculated the relative accuracy, i.e., the agreement between the results obtained by an accepted method (i.e. $TCID_{50}$) and those obtained by an alternative method (i.e., ET-RT-qPCR) (Rodríguez-Lázaro et al. 2004, 2005). The mean relative accuracy values ranged from 6.86 to 37.85 (Table 2). Consequently, the divergence between results obtained by ET-RT-qPCR and cell culture method after the inactivation treatments (Tables 1, 2) indicates that ET-RT-qPCR is unsatisfactory for evaluating the reduction of virus infectivity and significantly lower.

The apparent absence of correlation between the results of cell culture ($TCID_{50}$) and ET coupled to RT-qPCR (ET-RT-qPCR) assays may have been due to non-optimisation of the enzyme concentrations and conditions used

Table 1 Efficacy against MNV-1 of the different treatments used

Treatment	Intensity[a]	MNV-1 reduction[b]		
		RT-qPCR[c]	ET-RT-qPCR	Infectivity assay
HHP	Mild	0.14 ± 0.02	<0.00	0.46 ± 0.01
	Severe	2.09 ± 0.06	2.15 ± 0.06	>6.13
UV	Mild	0.16 ± 0.06	0.28 ± 0.04	4.08 ± 0.19
	Severe	0.50 ± 0.06	0.37 ± 0.02	5.34 ± 0.18
Heating	Mild	0.12 ± 0.03	0.27 ± 0.04	3.54 ± 0.14
	Severe	0.18 ± 0.02	0.56 ± 0.05	4.67 ± 0.08

[a] Mild treatments involved 200 MPa for 5 min, 100 J m^{-2}, or 80°C for 150 s for HHP, UV and heat treatment, respectively. Severe treatments involved 600 MPa for 5 min, 250 J m^{-2}, or 80°C for 10 min for HHP, UV and heat treatment, respectively

[b] MNV reduction was calculated as the $\log(N_0/N)$, where N and N_0 are the number of viral particles as measured by $TCID_{50}$ assay or GE as measured by RT-qPCR or ET-RT-qPCR in untreated controls and treated samples, respectively, and expressed in log GE ml^{-1} for RT-qPCR and ET-RT-qPCR assays and log $TCID_{50}$ ml^{-1}, for infectivity assays. The values for RT-qPCR and ET-RT-qPCR are means ± standard deviations of nine replicates, and values for the infectivity data ($TCID_{50}$) are means ± standard deviations of three replicates. ">" indicates that the virus titre was below the detection limits for MNV-1 $TCID_{50}$, which were 6.13, 5.79 and 5.88 log-CID_{50} ml^{-1} for HHP, UV and heating treatment, respectively

[c] RT-qPCR indicates reverse transcription real-time PCR; ET-RT-qPCR indicates enzymatic treatment coupled to reverse transcription real-time PCR

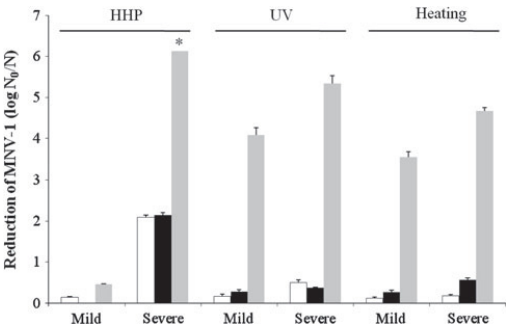


Fig. 1 Reduction of MNV-1 after different food inactivation processing treatments (HHP, UV, and thermal treatments). Two levels of intensity were tested for each processing treatment: mild and severe. Mild treatment involved 200 MPa for 5 min, 100 J m^{-2}, or 80°C for 150 s for HHP, UV and heat treatment, respectively. Severe treatment involved 600 MPa for 5 min, 250 J m^{-2}, or 80°C for 10 min for HHP, UV and heat treatment, respectively. The results for each type of treatment (mean of three independent experiments) are represented by bars. *White bars* represent the inactivation measured by RT-qPCR; *black bars* represent the inactivation measured by ET-RT-qPCR; *grey bars* represent the inactivation measured by $TCID_{50}$ assay. *Error bars* depict the SD of the mean for each treatment. Asterisk signifies a value below the detection limit (6.13 log$TCID_{50}$ ml^{-1} for HHP)

Table 2 Correlation of virus inactivation results as assessed by the different evaluation methods

Treatment	Intensity[a]	Correlation of inactivation results		Relative accuracy
		RT-qPCR vs. ET-RT-qPCR	ET-RT-qPCR vs. $TCID_{50}$	ET-RT-qPCR vs. $TCID_{50}$
HHP	Mild	0.00003[b]	0.00783[b]	30.43
	Severe	0.06250[c]	0.00079[b]	37.85
UV	Mild	0.00039[b]	0.00280[b]	6.86
	Severe	0.00002[b]	0.00097[b]	6.93
Heating	Mild	4.97×10^{-8}[b]	0.00006[b]	7.63
	Severe	2.99×10^{-10}[b]	0.00148[b]	11.99

[a] Mild treatments involved 200 MPa for 5 min, 100 J m^{-2}, or 80°C for 150 s for HHP, UV and heat treatment, respectively. Severe treatments involved 600 MPa for 5 min, 250 J m^{-2}, or 80°C for 10 min for HHP, UV and heat treatment, respectively

[b] The differences observed were significant using Student's t test ($P < 0.01$)

[c] The differences observed were not significant using Student's t test ($P > 0.01$)

for the ET. We therefore evaluated the performance of the ET using a series of concentrations of RNase (0.07, 0.7, 1.4, and 2.1 U) for 1 and 2 h with dilutions of the virus samples from 1 to 1:100. As assessed by using Student's t test, there were no significant differences ($P > 0.01$) between the results obtained using different concentrations of RNase (30-fold range), times (1 or 2 h) and dilutions of the virus (100-fold range) (data not shown). Therefore, the discrepancies observed between the results obtained by $TCID_{50}$ and by ET-RT-qPCR assays concerning virus inactivation were not due to suboptimal design of the ET, but due to the nature of the effect of the inactivation processes.

The Inclusion of an Enzymatic Treatment Coupled to RT-qPCR Shows a Significant Reduction of Genomes Associated with Inactivation Treatments

We also evaluated the effect of the inclusion of ET prior to the PCR on the measurement of the reduction of virus genomes after inactivation treatments (Table 1). The results obtained by RT-qPCR and by ET-RT-qPCR were significantly different ($P < 0.01$) irrespective of the inactivation procedure and conditions used, except for the HHP treatment at 600 MPa for 5 min ($P = 0.06250$) (Table 2). Mean values for reduction obtained by ET-RT-qPCR were significantly higher than those obtained by RT-qPCR except for the UV treatment at 250 J m^{-2} (0.50 vs. 0.37) and the HHP treatment at 200 MPa for 5 min (0.14 vs. <0.00) (Table 1) (Fig. 1). Therefore, the ET prior to RT-qPCR affected the estimation of the reduction of the virus

genome count, and may eliminate genomes of affected virus particles. However, comparison of these findings (RT-qPCR vs. ET-RT-qPCR) with those reported above (ET-RT-qPCR vs. $TCID_{50}$) clearly indicates that this effect is only partial and small, such that even with ET, RT-qPCR is unsatisfactory for estimating virus inactivation and/or virus infectivity.

Discussion

The elimination of emergent pathogens from foods is an important issue in food safety. Enteric viruses, such as NoVs or hepatitis A, have been involved in gastroenteritis outbreaks associated with different food sources (Baker et al. 2010; Maunula et al. 2009; Grotto et al. 2004; Ethelberg et al. 2010), and consequently, the effect of standard and novel inactivation processes on food borne viruses needs to be assessed. The use of a surrogate virus is currently the most common approach. However, the surrogate must be selected sensibly and the results obtained carefully extrapolated to real-life situations, as discrepancies have been observed between different surrogates (Kovač et al. 2010). Other experimental approach is to use ET with endonucleases to eliminate the genomes of affected virus particles and then assay the intact virus particles by quantitative molecular methods such as (RT-) qPCR (Rodríguez et al. 2009). In this study, we evaluated the suitability of this experimental approach using different inactivation processes with different effects on the integrity of virus particles and/or genomes: HHP, UV, and thermal treatments. We compared the results obtained with those obtained by a standard cell culture technique ($TCID_{50}$) and by RT-qPCR without ET. We found that there was no correlation between the results obtained by RT-qPCR coupled to a prior ET (ET-RT-qPCR) and those obtained by $TCID_{50}$. There are at least three different explanations for these observations: (1) the inactivation processes may affect virus infectivity without affecting the virus structure in a way that would make the virus genome sensitive to the ET, (2) the ET may have little or no effect on the virus genomes in the affected virus particles, and/or (3) the ET conditions were not optimised and therefore inappropriate. However, we evaluated a series of enzyme (RNase) concentrations, different incubation times and a range of virus concentrations, and we did not observe any significant differences ($P > 0.01$); therefore, the last of these three explanations, that the treatment conditions were inappropriate, seems highly unlikely. There were also significant differences ($P < 0.01$) between the results obtained with and without ET before the PCR, for all inactivation procedures and conditions used, and higher inactivation ratios were scored by RT-qPCR with than without ET. This makes the second explanation unlikely (that the ET did not affect the virus genomes of the affected virus particles). The inactivation methodologies (HHP, UV and heating) were selected because they involve different inactivating factors (pressure, irradiation, and temperature), and are therefore expected to have different effects on virions and/or virus genomes. Consequently, our results seem to indicate that the general effect of virus inactivation, at least for these three different treatments, is not directly associated with inactivation effects on the virus genome and protein capsid, but rather with effects on proteins associated with adhesion to and invasion of eukaryotic cells. This conclusion agrees with previous findings concerning the virus inactivation effect of HHP (Kovač et al. 2011; Tang et al. 2010).

Nevertheless, the significant differences between RT-qPCR and ET-RT-qPCR findings, and ET-RT-qPCR giving higher reduction scores than RT-qPCR following inactivation treatment, indicate that the ET with proteinase K and RNase improves the measurement of the reduction of virus genomes. However, there was no direct correlation between the reduction of the virus genome count as assessed by PCR and infective virus particle titre. Therefore, quantification of the virus genome count is not an adequate experimental approach for establishing virus inactivation and/or infectivity in contrast with conclusions arisen in a previous work (Topping et al. 2009). However, they used a different norovirus surrogate (FCV F-9), and only evaluated the effect of one inactivating treatment (temperature). In addition, they used two different models for interpreting the molecular method of predicting virus infectivity and FCV-9 best fit with one model and the real norovirus GII.4 isolates best fit to the other predictive model, showing that FCV-9 can not be appropriate as norovirus surrogate. In addition, our findings for MNV-1 inactivation after thermal treatment were done at 80°C, a temperature close to the 76.6°C of predicted maximal exposure of RNA in norovirus GII.4 isolates calculated in Topping's work (2009). Furthermore, as we used three different inactivation procedures with different inactivation principles, our results can probably be extrapolated to other inactivation treatments. We conclude that quantification of virus genomes, even if using an ET for eliminating the genomes of structurally affected virus particles, is not suitable for estimations of virus infectivity.

Acknowledgments We thank Dr. Herbert W. Virgin IV (Washington University School of Medicine, USA) for providing the murine norovirus 1 strain. This study was supported by a national grant INIA RTA 11-79 from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Spanish Ministry of Science and Innovation. M.D.-V. received a Ph.D. studentship from the *Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria* (INIA) (Spain). Katarina Kovač was supported by a FEMS Research Fellowship 2009-2, a short-term scientific mission from COST Action Number 929 (COST-STSM-929-05122) and a PhD student fellowship from the University of Leon (Spain).

References

Baert, L., Wobus, C. E., Van Coillie, E., Thackray, L. B., Debevere, J., & Uyttendaele, M. (2008). Detection of murine norovirus 1 by using plaque assay, transfection assay, and real-time reverse transcription-PCR before and after heat exposure. *Applied Environmental Microbiology, 74*(2), 543–546.

Baker, K., Morris, J., McCarthy, N., Lowther, J., Collinson, A., & Young, M. (2010). An outbreak of norovirus infection linked to oyster consumption at a UK restaurant, February 2010. *Journal of Public Health (Oxf), 33*(2), 205–211.

Bhattacharya, S. S., Kulka, M., Lampel, K. A., Cebula, T. A., & Goswami, B. B. (2004). Use of reverse transcription and PCR to discriminate between infectious and non-infectious hepatitis A virus. *Journal of Virological Methods, 116*(2), 181–187.

Breindl, M. (1971). The structure of heated poliovirus particles. *The Journal of General Virology, 11*(3), 147–156.

Chapron, C. D., Ballester, N. A., & Margolin, A. B. (2000). The detection of astrovirus in sludge biosolids using an integrated cell culture nested PCR technique. *Journal of Applied Microbiology, 89*(1), 11–15.

Diez-Valcarce, M., Kovač, K., Cook, N., Rodriguez-Lázaro, D., & Hernandez, M. (2011). Construction and analytical application of Internal Amplification Controls (IAC) for detection of food supply chain-relevant viruses by Real-Time PCR-based assays. *Food Analytical Methods, 4*(3), 437–445.

Duizer, E., Bijkerk, P., Rockx, B., De Groot, A., Twisk, F., & Koopmans, M. (2004). Inactivation of caliciviruses. *Applied Environmental Microbiology, 70*(8), 4538–4543.

Eischeid, A. C., Meyer, J. N., & Linden, K. G. (2009). UV disinfection of adenoviruses: molecular indications of DNA damage efficiency. *Applied and Environmental Microbiology, 75*(1), 23–28.

Ethelberg, S., Lisby, M., Bottiger, B., Schultz, A. C., Villif, A., Jensen, T., Olsen, K. E., Scheutz, F., Kjelso, C., & Muller, L. (2010). Outbreaks of gastroenteritis linked to lettuce, Denmark, January 2010. *Euro Surveillance*, *15*(6).

Grotto, I., Huerta, M., Balicer, R. D., Halperin, T., Cohen, D., Orr, N., et al. (2004). An outbreak of norovirus gastroenteritis on an Israeli military base. *Infection, 32*(6), 339–343.

Hijnen, W. A., Beerendonk, E. F., & Medema, G. J. (2006). Inactivation credit of UV radiation for viruses, bacteria and protozoan (oo)cysts in water: a review. *Water Research, 40*(1), 3–22.

Jiang, Y. J., Liao, G. Y., Zhao, W., Sun, M. B., Qian, Y., Bian, C. X., et al. (2004). Detection of infectious hepatitis A virus by integrated cell culture/strand-specific reverse transcriptase-polymerase chain reaction. *Journal of Applied Microbiology, 97*(5), 1105–1112.

Kärber, G. (1931). Beitrag zur kollektiven behandlung pharmakologische reihenversuche [A contribution to the collective treatment of a pharmacological experimental series]. *Archiv fur Experimentelle Pathologie und Pharmakologie, 162*, 480–483.

Kovač, K., Diez-Valcarce, M., Hernandez, M., Raspor, P., & Rodriguez-Lázaro, D. (2010). High hydrostatic pressure as emergent technology for the elimination of food borne viruses. *Trends in Food Science and Technology, 21*(11), 558–568.

Kovač, K., Diez-Valcarce, M., Hernández, M., & Rodríguez-Lázaro, D. (2011). Effect of high hydrostatic pressure processing on norovirus infectivity and genome stability in strawberry puree and mineral water. *International Journal of Food Microbiology*. doi:10.1016/j.ijfoodmicro.2011.10.005.

Linden, K. G., Shin, G. A., Faubert, G., Cairns, W., & Sobsey, M. D. (2002). UV disinfection of *Giardia lamblia* cysts in water. *Environmental Science and Technology, 36*(11), 2519–2522.

Maunula, L., Roivainen, M., Keränen, M., Mäkela, S., Söderberg, K., Summa, M., et al. (2009). Detection of human norovirus from frozen raspberries in a cluster of gastroenteritis outbreaks. *Euro Surveillance, 14*(49), 19435.

Nuanualsuwan, S., & Cliver, D. O. (2002). Pretreatment to avoid positive RT-PCR results with inactivated viruses. *Journal of Virological Methods, 104*(2), 217–225.

Nuanualsuwan, S., & Cliver, D. O. (2003). Capsid functions of inactivated human picornaviruses and feline calicivirus. *Applied Environmental Microbiology, 69*(1), 350–357.

Rodríguez, R. A., Pepper, I. L., & Gerba, C. P. (2009). Application of PCR-based methods to assess the infectivity of enteric viruses in environmental samples. *Applied Environmental Microbiology, 75*(2), 297–307.

Rodríguez-Lázaro, D., Hernández, M., Scortti, M., Esteve, T., Vázquez-Boland, J. A., & Pla, M. (2004). Quantitative detection of *Listeria monocytogenes* and *Listeria innocua* by real-time PCR: assessment of *hly*, *iap* and *lin02483* targets and AmpliFluor technology. *Applied Environmental Microbiology, 70*(3), 1366–1377.

Rodríguez-Lázaro, D., Pla, M., Scortti, M., Monzó, H. J., & Vázquez-Boland, J. A. (2005). A novel quantitative real-time PCR for *Listeria monocytogenes* that evaluates the analytical performance via an internal amplification control. *Applied Environmental Microbiology, 71*(12), 9008–9012.

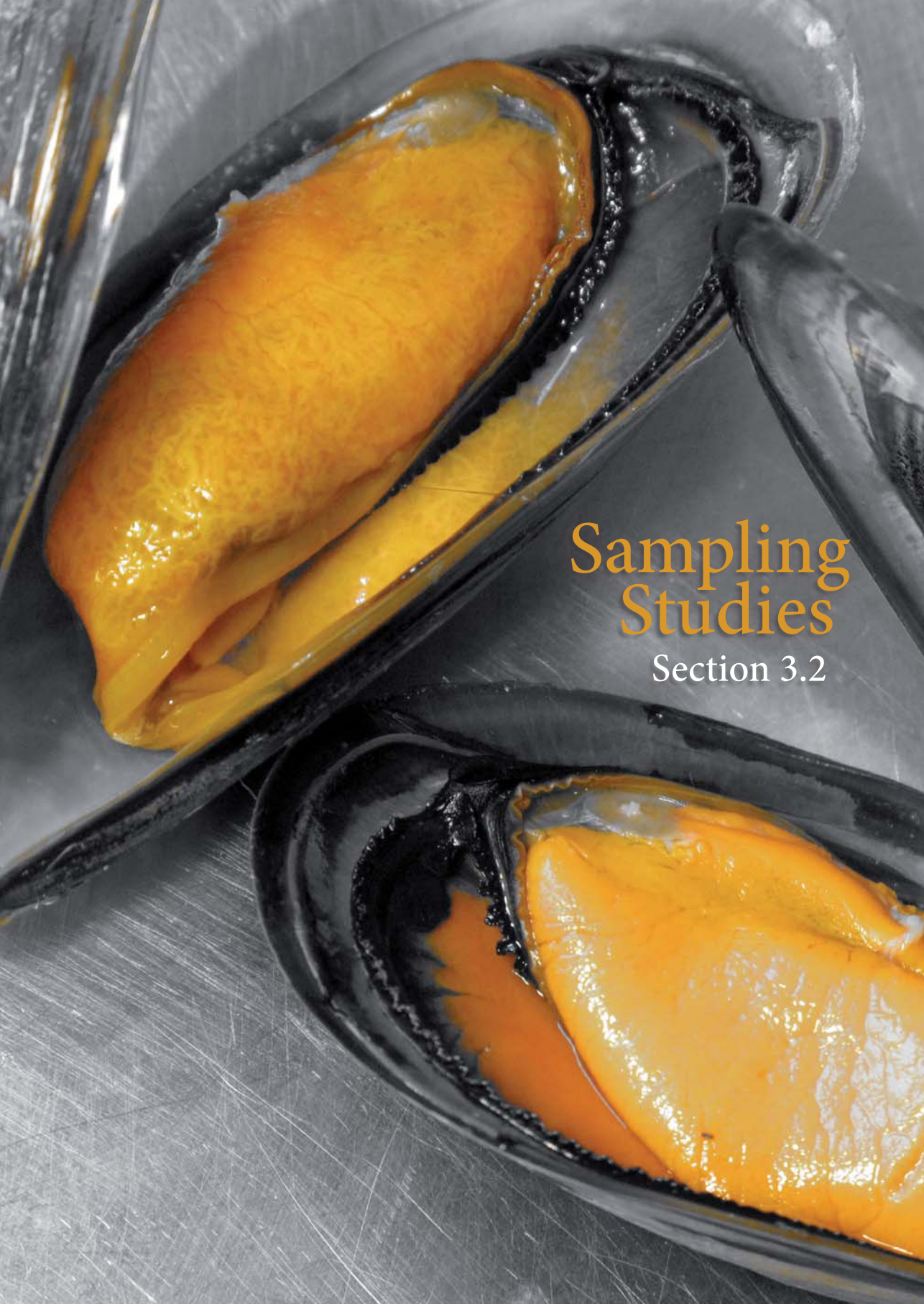
Scientific Committee on Veterinary Measures Relating to Public Health: opinion on norwalk-like viruses (adopted on 30–31 January 2002).

Shin, G. A., Lee, J. K., & Linden, K. G. (2009). Enhanced effectiveness of medium-pressure ultraviolet lamps on human adenovirus 2 and its possible mechanism. *Water Science and Technology, 60*(4), 851–857.

Sobsey, M. D., Battigelli, D. A., Shin, G.-A., & Newland, S. (1998). RT-PCR amplification detects inactivated viruses in water and wastewater. *Water Science and Technology, 38*(12), 91–94.

Tang, Q., Li, D., Xu, J., Wang, J., Zhao, Y., Li, Z., et al. (2010). Mechanism of inactivation of murine norovirus-1 by high pressure processing. *International Journal of Food Microbiology, 137*(2–3), 186–189.

Topping, J. R., Schnerr, H., Haines, J., Scott, M., Carter, M. J., Willcocks, M. M., et al. (2009). Temperature inactivation of Feline calicivirus vaccine strain FCV F-9 in comparison with human noroviruses using an RNA exposure assay and reverse transcribed quantitative real-time polymerase chain reaction—A novel method for predicting virus infectivity. *Journal of Virological Methods, 156*(1–2), 89–95.



Sampling Studies

Section 3.2

3.2.1

Hepatitis E Virus in Pork Production Chain in Czech Republic, Italy, and Spain, 2010

Ilaria Di Bartolo[1], Marta Diez-Valcarce[2], Petra Vasickova[3], Petr Kralik[3], Marta Hernández[2], Giorgia Angeloni[1], Fabio Ostanello[4], Martijn Bouwknegt[5], David Rodríguez-Lázaro[2], Ivo Pavlik[3], and Franco Maria Ruggeri[1]

[1] Istituto Superiore di Sanità, Rome, Italy [2] Instituto Tecnológico Agrario de Castilla y León (ITACyL), Valladolid, Spain [3]Veterinary Research Institute, Department of Food and Feed Safety, Brno, Czech Republic [4]Università di Bologna, Facoltà di Medicina Veterinaria, Bologna, Italy [5]National Institute for Public Health and Environment (RIVM), Bilthoven, Netherlands

Published in Emerging Infectious Diseases, Vol. 18, 1282-1289

RESEARCH

Hepatitis E Virus in Pork Production Chain in Czech Republic, Italy, and Spain, 2010

Ilaria Di Bartolo,[1] Marta Diez-Valcarce,[1] Petra Vasickova,[1] Petr Kralik, Marta Hernandez, Giorgia Angeloni, Fabio Ostanello, Martijn Bouwknegt, David Rodríguez-Lázaro, Ivo Pavlik, and Franco Maria Ruggeri

We evaluated the prevalence of hepatitis E virus (HEV) in the pork production chain in Czech Republic, Italy, and Spain during 2010. A total of 337 fecal, liver, and meat samples from animals at slaughterhouses were tested for HEV by real-time quantitative PCR. Overall, HEV was higher in Italy (53%) and Spain (39%) than in Czech Republic (7.5%). HEV was detected most frequently in feces in Italy (41%) and Spain (39%) and in liver (5%) and meat (2.5%) in Czech Republic. Of 313 sausages sampled at processing and point of sale, HEV was detected only in Spain (6%). HEV sequencing confirmed only g3 HEV strains. Indicator virus (porcine adenovirus) was ubiquitous in fecal samples and absent in liver samples and was detected in 1 slaughterhouse meat sample. At point of sale, we found porcine adenovirus in sausages (1%–2%). The possible dissemination of HEV and other fecal viruses through pork production demands containment measures.

Human hepatitis E is endemic worldwide, particularly in Asia, where large waterborne outbreaks have been reported (*1*). Seroprevalence of hepatitis E virus (HEV) is >60% in rural southern People's Republic of China (*2*) and 4%–10% in western Europe (*3*) and the United States (*4*). In these areas, hepatitis E occurs mostly as sporadic cases (*5*–*7*), but epidemics also have been described (*8*). Most cases in Europe have been linked to genotype 1 (g1) virus and associated with travel to g1-endemic areas. However, autochthonous human infections are increasing in Europe and in other industrialized countries (*5*,*6*,*9*). Of the 4 genotypes affecting humans, genotype 3 (g3) is the main HEV genotype also circulating among pigs in Europe (*10*) and human infections are observed sporadically worldwide (*11*,*12*).

Several reports indicate that HEV can be transmitted through zoonotic and foodborne pathways, including through consumption of raw and undercooked liver, meat, or sausages from domestic pigs, wild boar, and deer (*8*,*13*,*14*). Several investigations have shown that farmed domestic pigs are widely infected with and shed g3 HEV in Europe. Studies conducted in Spain (*8*,*13*,*14*), Italy (*15*), and France (*16*) have detected HEV genomic RNA in livers of pigs of slaughtering age, indicating that HEV-contaminated food might reach supermarkets (*17*). In butcher shops in the Netherlands (*18*) and Germany (*19*), ≈6.5% and ≈4%, respectively, of pork livers contained HEV, which raises concern about the potential for direct transmission through contact with or consumption of contaminated food.

Despite the large widespread distribution of HEV-shedding pigs and the possible role of farmed pigs as the main virus reservoir, the number of human hepatitis E cases in Europe remains low, suggesting inefficient virus transmission or lower pathogenicity of swine g3 strains than of g1 strains for humans. Because g3 HEV is common in pigs but rare in humans, humans are postulated to not be a main host for g3 virus replication (*11*,*20*). Nonetheless, a possibly large underestimation of HEV spread in humans cannot be excluded because of asymptomatic cases, inadequate diagnostics, and scarce medical attention (*21*). Mansuy et al. suggested inadequacy of previous diagnostic methods and recently found unprecedentedly high HEV seroprevalence among blood donors in France (*22*).

Author affiliations: Istituto Superiore di Sanità, Rome, Italy (I. Di Bartolo, G. Angeloni, F.M. Ruggeri); Instituto Tecnológico Agrario de Castilla y León, Junta de Castilla y León Valladolid, Spain (M. Diez-Valcarce, M. Hernandez, D. Rodríguez-Lázaro); Veterinary Research Institute, Brno, Czech Republic (P. Vasickova, P. Kralik, I. Pavlik); University of Bologna, Bologna, Italy (F. Ostanello); and National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands (M. Bouwknegt)

DOI: http://dx.doi.org/10.3201/eid1808.111783

[1]These authors contributed equally to this article.

Relatively few studies of foodborne human hepatitis E are available (*8*,*21*,*23*), making evaluation of HEV-associated risks difficult. Investigation of HEV throughout the pork production chain from farm to point of sale is needed to highlight areas of risk and proper control. A recent report from European Food Safety Authority biohazard experts (*24*) underscored an urgent need for integrated studies on HEV circulation, performing farm-to-table integrated risk assessment. For other foodborne pathogens, such studies comprise quantitative microbial risk assessment on the basis of exposure and dose-response models (*25*). Unfortunately, for HEV, quantitative approaches are hardly accessible because of the absence of reliable cell culture systems for viral infectivity titration.

We aimed to assess HEV prevalence in the pork production chain from slaughterhouse to point of sale in Czech Republic, Italy, and Spain during 2010 in the framework of the FP7 VITAL (Integrated Monitoring and Control of Foodborne Viruses in European Food Supply Chains) project (www.eurovital.org/). This systematic multicountry investigation of domestic swine HEV was conducted by using standardized molecular approaches, including reverse transcription quantitative PCR (RT-qPCR) detection, process, and internal amplification controls (IACs) and proper fecal viral indicators (porcine adenovirus [PAdV]).

Materials and Methods

Sampling Strategy

Samples were taken at perceived critical points for virus contamination. They were identified from Hazard Analysis and Critical Control Point System audit principles-based questionnaires (K. Willems and R. Moloney, pers. comm.) completed in each premise and analyzed by VITAL food-safety management and risk assessment experts (M. Bouwknegt and A. De Roda Husman, pers. comm.).

Samples

A total of 113 fecal, 112 liver, and 112 meat (lingual muscle) samples from 113 healthy pigs (*Sus scrofa* subsp. *domestica*) were collected in slaughterhouses from Czech Republic, Italy, and Spain during 2010 (Table 1). Samples originated from 4 pig farms per country. Packaged sausages were sampled in processing sites and supermarkets in Italy and Spain (128 and 93 samples, respectively) and in 8 supermarkets in Czech Republic (92 samples).

Additional ad hoc samples were collected during fact-finding visits to production farms, processing plants, and points of sale (Table 2). Briefly, 73 samples were collected from working surfaces and cutting tools (swabs from knife, belt surface, and meat mincer) from slaughtering areas (10 samples), processing areas (19 samples), and points of sale (12 samples) and from workers' hands (20 samples) and workers' toilets (12 samples). In Czech Republic, 6 effluent water samples from slaughterhouses also were examined.

Sample Process Control Virus

Murine norovirus 1 (MNV-1) was used as sample process control virus (SPCV). A single batch with MNV-1 at the concentration of 4.7×10^7 PFU/mL was prepared and used by all collaborating institutes throughout the study (*26*).

Virus Concentration and Nucleic Acid Isolation

Pig Feces

Feces (>1 g) were collected aseptically. A total of 250 mg of sample in 15-mL centrifuge tubes were suspended in 2.25 mL phosphate-buffered saline containing gentamycin (10 mg/mL), and 10 μL SPCV (4.7×10^5) was added. Suspensions were vortexed for 60 s and centrifuged at 3,000 × *g* for 15 min. Supernatants were immediately used for nucleic acid isolation or stored at –70°C. Nucleic acid was extracted by using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Final elution was performed 2× with 50 μL elution buffer, resulting in a 100-μL nucleic acid extract, for immediate testing or –70°C storage.

Pork Liver, Meat, and Sausages

Liver and meat or sausage samples were collected (1 cm^3 from 3 different locations) and stored in sterile plastic bags. According to the method of Bouwknegt et al. (*18*), samples were finely chopped and homogenized in an RNase-free mortar with 4 mL of Buffer RLT (RNeasy Midi Kit, QIAGEN) containing 1:100 β-mercaptoethanol. A total of 250 mg homogenate was transferred into microcentrifuge tubes containing 1 mL RLT buffer, 2.5 g sterile 1-mm zirconia beads (BioSpec Products, Inc., Bartlesville, OK, USA), and 10 μL SPCV (4.7×10^5). Tubes were applied to a mechanical disruptor (Ribolyser-Cell-Disrupter, Hybaid Ltd., Ashford, UK) for two 40-s/4-m/s cycles. After centrifugation (10,000 × *g*, 20 min, 2×), 800 μL of resulting supernatants were immediately processed by RNeasy Midi Kit or freeze-stored. Nucleic acid extracts (300 μL) were assayed immediately or freeze-stored.

Workers' Hands and Surfaces

Workers' hands and surfaces were sampled by using sterile moistened swabs, and samples were stored in 5 mL of 10 mg/mL gentamicin-containing phosphate-buffered saline in plastic tubes. Unwashed hands were sampled immediately before lunch or afternoon coffee break. For surfaces, 10-cm^2 areas were rubbed. Liquids were decanted from swab containers into 50-mL centrifuge

Table 1. Detection of HEV and indicator virus PAdV in samples from the pork production chain, Czech Republic, Italy, and Spain, 2010*

Production stage and sample source	Virus	Czech Republic		Italy		Spain		All	
		No. tested	No. (%) positive	No. tested	No. (%) positive	No. tested	No. (%) positive	No. tested	No. (%) positive
Slaughterhouse									
Feces	HEV	40	1 (3)†	34	14 (41)	39	15 (38)†	113	30 (27)
	PAdV	40	39 (98)	34	31 (91)	39	35 (90)	113	105 (93)
Liver	HEV	40	2 (5)†	33	2 (6)	39	1 (3)†	112	5 (4)
	PAdV	40	0	33	0	39	0	112	0
Meat	HEV	40	1 (3)	33	2 (6)	39	0	112	3 (3)
	PAdV	40	0	33	1 (3)‡	39	0	112	1(1)
Processing/points of sale: sausage	HEV	92	0	128	0	93	6 (6)	313	6 (2)
	PAdV	92	1 (1)	128	1 (1)	93	2 (2)‡	313	4 (1)

*HEV, hepatitis E virus; PAdV, porcine adenovirus.
†Samples originated from the same animal.
‡Sample negative for HEV.

tubes containing 10 µL SPCV (4.7×10^5). Suspensions were vortexed and centrifuged (3,000 × *g*, 5 min), and supernatants were used immediately or freeze-stored. Nucleic acids were extracted by NucliSENS miniMAG Kit (bioMérieux, Marcy l'Etoile, France) and eluted 2× with 50 µL elution buffer.

RT-qPCR

Nucleic acids were assayed undiluted and diluted 10-fold by performing RT-qPCRs in duplicate. All reaction mixes included an IAC (*27*). All RT-qPCRs were in duplex format, targeting specific viruses (MNV-1, HEV, PAdV) and IACs labeled with FAM (6-carboxy fluorescein) and VIC (Applied Biosystems, Foster City, CA, USA) probes, respectively. All tests included virus- and IAC-negative controls.

PAdV RT-qPCR

A duplex RT-qPCR was used as described (*28*), including IACs and a carryover contamination prevention system using uracil-N-glycosylase (Roche Molecular Diagnostics, Manheim, Germany). Reactions contained 1× TaqMan Universal PCR Master-Mix (Life Technologies, Branchburg, NJ, USA), 0.9 µM primers, 0.225 µM PAdV TaqMan probe (FAM-labeled), 50 nM IAC probe (VIC-labeled), and 100 copies of PAdV IAC. Ten microliters of nucleic acid extract were added to 25-µL final reaction volumes. Thermocycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C.

HEV RT-qPCR

A 1-step duplex RT-qPCR was used (*29*) and included IACs. Reactions contained 1× RNA UltraSense reaction mix (Life Technologies), 0.25 µM primers, 0.1 µM probe HEV-P (FAM-labeled), 50 nM IAC probe (VIC-labeled), 1× ROX reference dye, 1 µL RNA UltraSense enzyme mix, and 300 HEV IAC copies. Ten microliters of nucleic acid extracts were added to 20 µL final volumes. Thermocycling conditions were 15 min at 50°C and 2 min at 95°C, followed by 45 cycles of 10 s at 95°C, 20 s at 55°C, and 15 s at 72°C.

MNV-1 RT-qPCR

A 1-step duplex RT-qPCR was adopted (*30*), including IACs. Reaction contained 1× RNA UltraSense reaction mix, 0.2 µM primers, 0.2 µM probe minor groove binder–open reading frame (ORF) 1/ORF2 (FAM-labeled), 50 nM IAC probe (VIC-labeled), 1× ROX reference dye, 1 µL RNA UltraSense enzyme mix, and 600 MNV-1 IAC copies. Ten microliters of nucleic acid extract were added

Table 2. Detection of HEV and indicator virus PAdV in swabs in the pork production chain, Czech Republic, Italy, and Spain, 2010*

Production stage (area), sample type	No. tested	Positive, no. (%)	
		HEV	PAdV
Production (slaughterhouse: carcass dissection and liver removal)			
Water effluents	6	0	0
Workers' hands and aprons	7	4 (57)	5 (71)
Working surfaces	10	6 (60)	6 (60)
Processing (skin removal and sausage preparation)			
Workers' hands	7	2 (29)	1 (14)
Working surfaces	19	4 (21)	0
Points of sale			
Workers' hands and gloves	6	1 (17)	0
Working surfaces	12	1 (8)	0
Hand wash basin tap and toilet edge	12	1 (8)	1 (8)
All samples	79	19 (24)	13 (16)

*HEV, hepatitis E virus; PAdV, porcine adenovirus.

(final reaction volume 20 μL). Thermocycling conditions were 15 min at 50°C and 2 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C.

Data Reporting and Interpretation

For proper results interpretation, we considered 4 signals: 1) target virus, 2) SPCV, 3) target IAC, and 4) SPCV IAC (*31*). With cycle threshold (C_t) ≤45, independently of corresponding IAC C_t, the PCR result was considered positive. With C_t≥45 and corresponding IAC C_t≤45, results were interpreted as negative. When both targets and corresponding IACs showed C_t≥45, reactions were considered failed. When ≥1 replicate target assay (HEV or PAdV) was positive, the sample was considered positive. Absence of SPCV and its IAC signals indicated preamplification processes (virus concentration and extraction) failure (*31*). In the presence of SPCV, SPCV IAC, and target IAC signals, target virus signal absence was conclusively indicating test negative result.

HEV Genotyping

Positive HEV samples were sequence-analyzed amplifying 2 ORF2 regions (348- and 121-bp fragments) (*32*,*33*). Sixteen sequences obtained were examined in GenBank (www.ncbi.nlm.nih.gov/genbank). The 5 shorter 100-bp fragments (3 fecal samples in Italy and 1 liver and meat sample in Czech Republic) were used only to identify genotype or confirm longer sequences. The 11 longer sequences (300 bp) from 4 fecal samples in Italy (GenBank accession nos. JN861803, JN861804, JN861805, JN861806) and 7 sequences from 5 sausages and 2 environmental swabs in Spain (GenBank accession nos. JN903913, JN903914, JN903915, JN903916, JN903917, JN903918, JN903919) also were used for HEV genotyping and subgenotyping. We performed phylogenetic analyses with Bionumerics v6 (Applied Maths, Kortrijk, Belgium) by using the neighbor-joining method with 1,000 replicates with Kimura-2 correction factor.

Results

HEV in Pork Products

We detected HEV RNA in all pork production chain sites in investigated countries, with some differences (Table 1). Overall, HEV RNA was detected in ≥1 samples (feces, liver, meat) from 36 (32%) of 113 pigs examined at slaughterhouses for which all sample types were collected (Table 1). HEV RNA was detected frequently in slaughterhouse samples in Italy and Spain, i.e., 18 (53%) positive samples from 34 animals and 15 (38%) of 39, respectively (Table 1), whereas in Czech Republic, HEV RNA prevalence at slaughterhouses was remarkably lower, i.e., 3 (8%) positive samples from 40 animals. Pig feces showed highest HEV RNA presence (27%), followed by liver (4%) and meat (3%) (Table 1).

Sausage samples from Italy and Spain were collected from processing plants of the same company slaughtering animals or from same company products in local supermarkets. Sausages sampled in Czech Republic were obtained from randomly chosen supermarkets. HEV was detected in 6 (6%) of 93 samples in Spain, whereas 0 of 220 sausages in Czech Republic or Italy were positive.

PAdV in Pork Products

To evaluate possible fecal contamination, PAdV DNA presence (*34*) was determined for all samples assayed for HEV. PAdV was highly prevalent in feces (90%–98%) in investigated countries (Table 1). None of 112 liver samples were PAdV positive, and only 1 of 112 meat samples was PAdV positive, in Italy. In addition, 4 (1%) of 313 sausages (2 from Spain, 1 each from Czech Republic and Italy) were positive for PAdV (Table 1).

Environmental Samples

We collected 41 surface swabs from working surfaces, meat mincers, knives, and other working items at the 3 pork production chain sites. Overall, swab samples were positive for either HEV (11 [27%] of 41) or PAdV (6 [15%] of 41) (Table 2). HEV-positive samples were found more frequently at slaughterhouse (6 of 10) than at processing and points of sale (4 [21%] of 19 and 1 [8%] of 12, respectively) sites, and PAdV was found only in slaughterhouse samples (6 of 10). At slaughterhouses, positive swabs (3 knives, 2 floor, 1 belt surface) contained both HEV and PAdV, indicating potential fecal contamination during slaughtering steps, whereas 0 of 5 HEV-positive samples at processing and points of sale sites was positive for PAdV, disproving possible fecal cross-contamination during later production phases (Table 2). A total of 20 swab samples were taken from workers' hands, gloves, or aprons along the production chain. Overall results were similar to those for working surfaces in slaughtering premises; in fact, 5 (71%) of 7 samples were positive for both HEV and PAdV. Moreover, PAdV was detected in 1 of 2 HEV-positive samples at processing sites (Table 2). Finally, HEV or PAdV was detected in 1 (8%) of 12 toilet swab samples collected at points of sale. The 6 Czech Republic slaughterhouse effluent samples were negative for both PAdV and HEV.

Sequence Analysis

HEV-positive samples were genotyped and sequenced to determine possible animal or human origin of the virus. A total of 9 samples (4 from Italy, 5 from Spain) yielded ≈300-bp sequences and were compared with HEV sequences in public databases. All HEVs belonged to g3.

The 4 HEV-positive samples (HEVSwITFAE09BO10, HEVSwITFAE18BO10, HEVSwITFAE22BO10, HEVSwITFAE11BO10) in feces from slaughterhouses in Italy originated from the same herd and belonged to subtype g3c, sharing 99.4%–100% identical nucleotides. Three of 5 sequences from sausage in Spain belonged to subtype g3f (HEVSwESSAU56, HEVSwESSAU57, HEVSwESSAU60), whereas 2 additional g3 strains (HEVSwESSAU64, HEVSwESSAU66; 99.5% identity) could not be assigned to specific subtypes (online Appendix Figure, wwwnc.cdc.gov/EID/article/18/8/11-1783-FA1.htm), although their sequences were closer to subtype g3c. Two sequences from swabs collected in Spain (HEVSwESADHOC4A, HEVSwESADHOC5A) also belonged to g3f, showing 100% reciprocal nucleotide identity and 89% identity with g3f strains from sausages. Sequences for g3 subtypes from Italy and Spain exhibited ≤85% nucleotide identity, suggesting circulation of different strains in these countries.

Shorter sequences (121 bp, ORF2) also were obtained (*33*) from 3 fecal samples in Italy (100% identity), and 2 additional identical sequences were obtained from liver and meat at a slaughterhouse in Czech Republic. All were confirmed as g3 swine HEV, but further subtyping was not possible because of short sequence length.

Discussion

Pork is a major food source worldwide (*10*), and HEV is widespread among farmed swine and can be transmitted zoonotically, including through pork products (*5*,*10*). We investigated HEV presence throughout the pork production chain in 3 European countries from pigs entering slaughterhouses through processing to retail stores.

To optimize detection sensitivity, in our sampling strategy we assumed low HEV prevalence in pork products and environmental surfaces (*17*,*35*) and involved 3 laboratories. In addition to liver, we selected sausage because it is handled by consumers and is a blend of different meat and slaughtered animals. To maintain consistent results among countries and sample treatment, we validated standardized sampling and molecular procedures by ring test (*36*), including IAC and sample process controls (*26*,*27*,*31*).

Samples analyzed throughout the pork production chain in Italy and Spain were from the same herds from farm to retail sale. In Czech Republic, more points of sale were sampled, thus representing a larger animal population. HEV prevalence in pig feces was similar in Italy and Spain (41% and 38%, respectively), reflecting previous data in these and other European countries (*10*). Conversely, only 3% of pigs from Czech Republic shed HEV. Because of shared protocols and controls, this difference cannot be attributed to different diagnostic sensitivity among partners, which otherwise detected HEV in similar numbers of liver and meat samples.

Lower HEV shedding by pigs in Czech Republic might reflect different farming methods, such as animal housing and separation, herd size, slaughtering age, and/or environmental factors that possibly influence infectious HEV persistence, spread, and transmission. Previous data from Czech Republic (*37*) showed up to 40.0% HEV-positive bile samples from piglets, suggesting infection rates close to shedding rates reported for Italy and Spain. However, that study did not examine HEV fecal shedding, and pigs were only 2–3 months of age. Furthermore, varying prevalence of HEV in pig feces also has been reported in Italy and Spain (*15*,*38*), possibly reflecting differences in farm selection.

The absence of fecal HEV in pigs with HEV-positive liver or bile in Czech Republic suggests that bile concentration in the fecal mass was lower when samples were taken, as might be expected if pigs were fed long before reaching the slaughterhouse. This finding might also help explain the different fecal HEV positivity among countries.

We confirm broad HEV circulation within pig farms and HEV RNA in livers and other pork products (*8*,*17*,*18*). We found HEV prevalence in 3%–6% of liver samples at slaughter, similar to findings in the Netherlands (*18*) but somewhat less than in the United States (11%) (*17*). HEV RNA was present in meat samples only in Czech Republic and Italy (3% and 6%, respectively), whereas sausages were HEV positive only in Spain (6%). This finding might result from low sample numbers but also could reflect different methods for final product preparation by using different meat blends, fat, or liver intentionally or after unintentional cross-contamination.

HEV positivity markedly decreased from feces (27%) to liver (4%), meat (3%), and sausage (2%) but never disappeared during production. However, detection of HEV by RT-qPCR did not conclusively demonstrate viable virus and thus risks to consumers.

PAdV has been confirmed as a suitable indicator of swine fecal contamination during pork production (*28*). Although most pig feces in our study were PAdV positive (90%–98%), PAdV was never detected in liver and detected only occasionally in pork meat (1/33 samples in Italy) or sausage (4/313 samples, all 3 countries). Comparing HEV and PAdV findings, risks for cross-contamination of pork products with swine feces during preparation appear to be low but not absent.

Three of 112 pork meat samples tested were positive for HEV and 1 for only PAdV (Table 1). We have no proof of HEV replication in muscle, and finding HEV RNA in pork products probably reflects endogenous HEV particles in infected liver and/or viremic blood (*39*). Although liver

and bile are usually removed before processing, the HEV genome sporadically detected in meat most likely represents cross-contamination of carcasses during slaughtering, which suggests a need for worker training.

PAdV detection in 1 meat sample and 4 sausages also indicates some fecal contamination during slaughtering, which was, however, similarly low in all countries. PAdV and HEV were not present in the same sausage samples from Spain, and PAdV was detected in fewer samples than was HEV (2 vs. 6), which argues against potential higher risks for fecal contamination in the food chain in Spain. The higher HEV prevalence in sausage in Spain than in Italy or Czech Republic is unclear and deserves further investigation.

The samples from food handlers and the environment in Italy and Spain also identified areas where procedures and information could be implemented. Detection of HEV and PAdV in 60% of floor and working surfaces and 57%–71% of hands and aprons of workers dissecting pigs indicates that the initial production areas (bleeding to evisceration) are at higher risk for fecal contamination and highlight possible hazards to workers.

In the cutting/slicing/chopping areas, we did not detect PAdV in fecal samples. However, HEV detection on hands and surfaces indicates that endogenous HEV can be spread during cutting of liver and meat in industrial premises, requiring cross-contamination control measures. Limited handling might instead explain the single detection of HEV on a butcher's bench at point of sale.

The HEV detected from a supermarket personnel toilet was not genotyped. Thus, its possible origin, i.e., pig versus human, cannot be confirmed.

Our analysis of short sequences confirms presence of only g3 HEV. Sixteen sequences from Italy and Spain were subtyped; g3c was identified as the prevalent strain in Italy, and the less common g3f was noted only in Spain. Two identical HEV sequences in sausage from Spain might represent a novel g3 subtype, similar to a deer g3 HEV strain found in Spain in 2010 (*40*).

In conclusion, our study indicates that HEV is present throughout the pork production chain and that processing does not substantially abate endogenous virus. Consequently, consumers might purchase pork products that contain detectable HEV genome in up to 6.0% of instances, independent of source and country of origin, probably unrelated to fecal contamination during pork processing.

We cannot exclude the possibility that in some pork products HEV was infectious. However, HEV infectious dose for humans is unknown, and viral load in pork might not be sufficient to infect humans efficiently. Storage, processing, and blending of meat from HEV-positive and -negative animals (e.g., sausage) might substantially decrease risks for foodborne infection, possibly explaining why HEV food transmission in Europe seems relatively inefficient. However, consumers should eat only pork that has been thoroughly cooked, particularly liver, and avoid cross-contamination of surfaces and other food by handling pork products, especially offal.

This study addressed only fresh meat or sausage sold within few days of preparation. Future studies should be extended to other pork products, such as salami, which are eaten after short periods of curing and might still contain residual infectious virus.

Acknowledgments

We thank Edoardo Vignolo for his excellent technical assistance. We also thank H.W. Virgin for providing murine norovirus isolates.

This work was supported by the European Commission Framework Program 7 project "Integrated Monitoring and Control of Food-borne Viruses in European Food Supply Chains (VITAL)" (grant no. KBBE 213178), led by the coordination team of Nigel Cook, Martin D'Agostino, and Franco M. Ruggeri. This work was partially supported by the Ministry of Education, Youth and Sports (AdmireVet, CZ 1.05/2.1.00/01.0006; ED0006/01/01) of Czech Republic. M.D.-V. received a PhD studentship from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria of Spain. G.A. received a PhD fellowship under the Strategic Program 2007, by Ministry of Health, Italy: "Preparedness and Response to Emerging Zoonoses and Exotic Viral Infection through an Integrated Medical and Veterinary Approach."

Dr Di Bartolo is a researcher at the Department of Veterinary Public Health and Food Safety of the Istituto Superiore di Sanità in Rome, Italy. Her primary research interest is emerging zoonotic viruses, including HEV and norovirus.

References

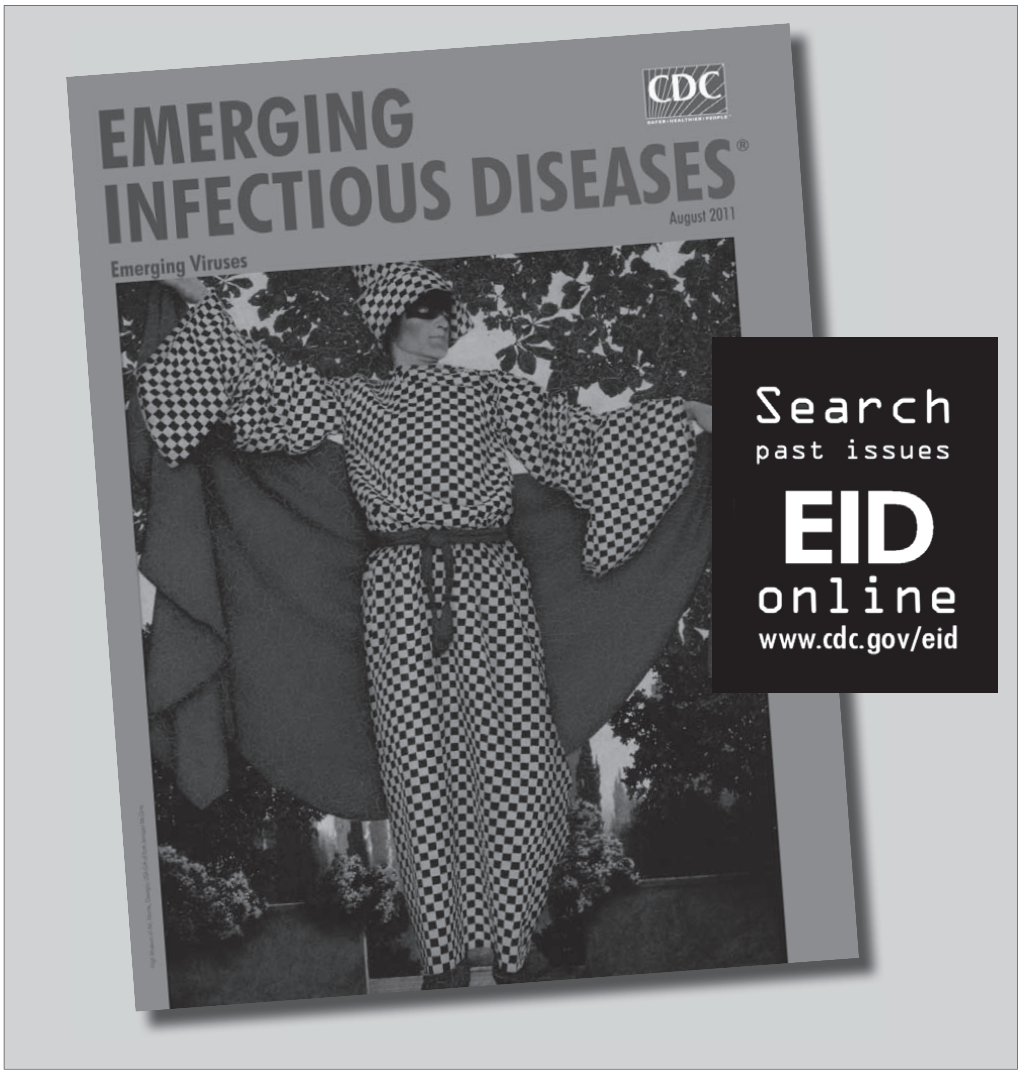
1. Guthmann JP, Klovstad H, Boccia D, Hamid N, Pinoges L, Nizou JY, et al. A large outbreak of hepatitis E among a displaced population in Darfur, Sudan, 2004: the role of water treatment methods. Clin Infect Dis. 2006;42:1685–91. http://dx.doi.org/10.1086/504321
2. Li RC, Ge SX, Li YP, Zheng YJ, Nong Y, Guo QS, et al. Seroprevalence of hepatitis E virus infection, rural southern People's Republic of China. Emerg Infect Dis. 2006;12:1682–8. http://dx.doi.org/10.3201/eid1211.060332
3. Bendall R, Ellis V, Ijaz S, Ali R, Dalton H. A comparison of two commercially available anti-HEV IgG kits and a re-evaluation of anti-HEV IgG seroprevalence data in developed countries. J Med Virol. 2010;82:799–805. http://dx.doi.org/10.1002/jmv.21656
4. Kuniholm MH, Purcell RH, McQuillan GM, Engle RE, Wasley A, Nelson KE. Epidemiology of hepatitis E virus in the United States: results from the Third National Health and Nutrition Examination Survey, 1988–1994. J Infect Dis. 2009;200:48–56. http://dx.doi.org/10.1086/599319

5. Vasickova P, Slany M, Chalupa P, Holub M, Svoboda R, Pavlik I. Detection and phylogenetic characterization of human hepatitis E virus strains, Czech Republic. Emerg Infect Dis. 2011;17:917–9.
6. La Rosa G, Muscillo M, Vennarucci VS, Garbuglia AR, La Scala P, Capobianchi MR. Hepatitis E virus in Italy: molecular analysis of travel-related and autochthonous cases. J Gen Virol. 2011;92:1617–26. http://dx.doi.org/10.1099/vir.0.031278-0
7. Brost S, Wenzel JJ, Ganten TM, Filser M, Flechtenmacher C, Boehm S, et al. Sporadic cases of acute autochthonous hepatitis E virus infection in southwest Germany. J Clin Virol. 2010;47:89–92. http://dx.doi.org/10.1016/j.jcv.2009.10.011
8. Colson P, Borentain P, Queyriaux B, Kaba M, Moal V, Gallian P, et al. Pig liver sausage as a source of hepatitis E virus transmission to humans. J Infect Dis. 2010;202:825–34. http://dx.doi.org/10.1086/655898
9. Dalton HR, Stableforth W, Thurairajah P, Hazeldine S, Remnarace R, Usama W, et al. Autochthonous hepatitis E in southwest England: natural history, complications and seasonal variation, and hepatitis E virus IgG seroprevalence in blood donors, the elderly and patients with chronic liver disease. Eur J Gastroenterol Hepatol. 2008;20:784–90. http://dx.doi.org/10.1097/MEG.0b013e3282f5195a
10. Pavio N, Meng XJ, Renou C. Zoonotic hepatitis E: animal reservoirs and emerging risks. Vet Res. 2010;41:46. http://dx.doi.org/10.1051/vetres/2010018
11. Purcell RH, Emerson SU. Hidden danger: the raw facts about hepatitis E virus. J Infect Dis. 2010;202:819–21. http://dx.doi.org/10.1086/655900
12. Dell'Amico MC, Cavallo A, Gonzales JL, Bonelli SI, Valda Y, Pieri A, et al. Hepatitis E virus genotype 3 in humans and swine, Bolivia. Emerg Infect Dis. 2011;17:1488–90.
13. Matsuda H, Okada K, Takahashi K, Mishiro S. Severe hepatitis E virus infection after ingestion of uncooked liver from a wild boar. J Infect Dis. 2003;188:944. http://dx.doi.org/10.1086/378074
14. Takahashi K, Kitajima N, Abe N, Mishiro S. Complete or near-complete nucleotide sequences of hepatitis E virus genome recovered from a wild boar, a deer, and four patients who ate the deer. Virology. 2004;330:501–5. http://dx.doi.org/10.1016/j.virol.2004.10.006
15. Di Bartolo I, Ponterio E, Castellini L, Ostanello F, Ruggeri FM. Viral and antibody HEV prevalence in swine at slaughterhouse in Italy. Vet Microbiol. 2011;149:330–8. http://dx.doi.org/10.1016/j.vetmic.2010.12.007
16. Rose N, Lunazzi A, Dorenlor V, Merbah T, Eono F, Eloit M, et al. High prevalence of hepatitis E virus in French domestic pigs. Comp Immunol Microbiol Infect Dis. 2011;34:419–27. http://dx.doi.org/10.1016/j.cimid.2011.07.003
17. Feagins AR, Opriessnig T, Guenette DK, Halbur PG, Meng XJ. Detection and characterization of infectious hepatitis E virus from commercial pig livers sold in local grocery stores in the USA. J Gen Virol. 2007;88:912–7. http://dx.doi.org/10.1099/vir.0.82613-0
18. Bouwknegt M, Lodder-Verschoor F, van der Poel WH, Rutjes SA, de Roda Husman AM. Hepatitis E virus RNA in commercial porcine livers in the Netherlands. J Food Prot. 2007;70:2889–95.
19. Wenzel JJ, Preiss J, Schemmerer M, Huber B, Plentz A, Jilg W. Detection of hepatitis E virus (HEV) from porcine livers in southeastern Germany and high sequence homology to human HEV isolates. J Clin Virol. 2011;52:50–4. http://dx.doi.org/10.1016/j.jcv.2011.06.006
20. Bouwknegt M, Engel B, Herremans MM, Widdowson MA, Worm HC, Koopmans MP, et al. Bayesian estimation of hepatitis E virus seroprevalence for populations with different exposure levels to swine in the Netherlands. Epidemiol Infect. 2008;136:567–76. http://dx.doi.org/10.1017/S0950268807008941
21. Lewis HC, Wichmann O, Duizer E. Transmission routes and risk factors for autochthonous hepatitis E virus infection in Europe: a systematic review. Epidemiol Infect. 2010;138:145–66. http://dx.doi.org/10.1017/S0950268809990847
22. Mansuy JM, Bendall R, Legrand-Abravanel F, Saune K, Miedouge M, Ellis V, et al. Hepatitis E virus antibodies in blood donors, France. Emerg Infect Dis. 2011;17:2309–12. http://dx.doi.org/10.3201/eid1712.110371
23. Wichmann O, Schimanski S, Koch J, Kohler M, Rothe C, Plentz A, et al. Phylogenetic and case-control study on hepatitis E virus infection in Germany. J Infect Dis. 2008;198:1732–41. http://dx.doi.org/10.1086/593211
24. European Food Safety Authority Panel on Biological Hazard. Scientific opinion on an update on the present knowledge on the occurrence and control of foodborne viruses. EFSA Journal. 2011;9:2190:1–96 [cited 2012 May 17]. http://www.efsa.europa.eu/en/efsajournal/doc/2190.pdf
25. Havelaar AH, Rutjes SA. Risk assessment of viruses in food: opportunities and challenges. In: Koopmans MP, Cliver DO, Bosch A, editors. Food-borne viruses: progress and challenges Washington (DC): ASM Press; 2008. p. 221–36.
26. Diez-Valcarce M, Cook N, Hernandez M, Rodriguez-Lazaro D. Analytical application of a sample process control in detection of foodborne viruses. Food Analytical Methods. 2011;4:614–8. http://dx.doi.org/10.1007/s12161-011-9262-9
27. Diez-Valcarce M, Kovac K, Cook N, Rodriguez-Lazaro D, Hernandez M. Construction and analytical application of internal amplification controls (IAC) for detection of foodborne viruses by (RT) real-time PCR. Food Analytical Methods. 2011;4:437–45. http://dx.doi.org/10.1007/s12161-011-9224-2
28. Hundesa A, Maluquer de Motes C, Albinana-Gimenez N, Rodriguez-Manzano J, Bofill-Mas S, Sunen E, et al. Development of a qPCR assay for the quantification of porcine adenoviruses as an MST tool for swine fecal contamination in the environment. J Virol Methods. 2009;158:130–5. http://dx.doi.org/10.1016/j.jviromet.2009.02.006
29. Jothikumar N, Cromeans TL, Robertson BH, Meng XJ, Hill VR. A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. J Virol Methods. 2006;131:65–71. http://dx.doi.org/10.1016/j.jviromet.2005.07.004
30. Baert L, Wobus CE, Van Coillie E, Thackray LB, Debevere J, Uyttendaele M. Detection of murine norovirus 1 by using plaque assay, transfection assay, and real-time reverse transcription–PCR before and after heat exposure. Appl Environ Microbiol. 2008;74:543–6. http://dx.doi.org/10.1128/AEM.01039-07
31. D'Agostino M, Cook N, Rodriguez-Lazaro D, Rutjes SA. Nucleic acid amplification–based methods for detection of enteric viruses: definition of controls and interpretation of results. Food Environ Virol. 2011;3:55–60. http://dx.doi.org/10.1007/s12560-011-9063-8
32. Huang FF, Haqshenas G, Guenette DK, Halbur PG, Schommer SK, Pierson FW, et al. Detection by reverse transcription–PCR and genetic characterization of field isolates of swine hepatitis E virus from pigs in different geographic regions of the United States. J Clin Microbiol. 2002;40:1326–32. http://dx.doi.org/10.1128/JCM.40.4.1326-1332.2002
33. Erker JC, Desai SM, Mushahwar IK. Rapid detection of hepatitis E virus RNA by reverse transcription–polymerase chain reaction using universal oligonucleotide primers. J Virol Methods. 1999;81:109–13. http://dx.doi.org/10.1016/S0166-0934(99)00052-X
34. Hundesa A, Maluquer de Motes C, Bofill-Mas S, Albinana-Gimenez N, Girones R. Identification of human and animal adenoviruses and polyomaviruses for determination of sources of fecal contamination in the environment. Appl Environ Microbiol. 2006;72:7886–93. http://dx.doi.org/10.1128/AEM.01090-06
35. Li TC, Chijiwa K, Sera N, Ishibashi T, Etoh Y, Shinohara Y, et al. Hepatitis E virus transmission from wild boar meat. Emerg Infect Dis. 2005;11:1958–60. http://dx.doi.org/10.3201/eid1112.051041
36. D'Agostino M, Cook N, Ruggeri FM, Martelli F, Banks M, et al. Multicenter collaborative trial evaluation of a method for detection of human adenoviruses in berry fruit. Food Analytical Methods. 2012;5:1–7. http://dx.doi.org/10.1007/s12161-011-9287-0

37. Vasickova P, Psikal I, Widen F, Smitalova R, Bendova J, Pavlik I, et al. Detection and genetic characterisation of hepatitis E virus in Czech pig production herds. Res Vet Sci. 2009;87:143–8. http://dx.doi.org/10.1016/j.rvsc.2008.10.013
38. de Deus N, Seminati C, Pina S, Mateu E, Martin M, Segales J. Detection of hepatitis E virus in liver, mesenteric lymph node, serum, bile and faeces of naturally infected pigs affected by different pathological conditions. Vet Microbiol. 2007;119:105–14. http://dx.doi.org/10.1016/j.vetmic.2006.08.027
39. Bouwknegt M, Rutjes SA, Reusken CB, Stockhofe-Zurwieden N, Frankena K, de Jong MC, et al. The course of hepatitis E virus infection in pigs after contact-infection and intravenous inoculation. BMC Vet Res. 2009;5:7. http://dx.doi.org/10.1186/1746-6148-5-7
40. Boadella M, Casas M, Martin M, Vicente J, Segales J, de la Fuente J, et al. Increasing contact with hepatitis E virus in red deer, Spain. Emerg Infect Dis. 2010;16:1994–6.

Address for correspondence: Franco Maria Ruggeri, Department of Food Safety and Veterinary Public Health, Istituto Superiore di Sanità, V.le Regina Elena, 299, 00161 Rome, Italy; email: franco.ruggeri@iss.it

All material published in Emerging Infectious Diseases is in the public domain and may be used and reprinted without special permission; proper citation, however, is required.



3.2.2

Prevalence and Transmission of Hepatitis E Virus in Domestic Swine Populations in Different European Countries

Alessandra Berto[1,7], Jantien A. Baker[7], Joao R. Mesquita[2,3], Maria S.J. Nascimento[2], Malcom Banks[1], Francesca Martelli[1], Fabio Ostanello[4] Giorgia Angeloni[4], Franco M. Ruggeri[4], Ilaria Di Bartolo[4], Petra Vasickova[5], Marta Diez-Valcarce[6], Marta Hernández[6], David Rodríguez-Lázaro[6] and Wim H.M. van der Poel[7]

[1] Veterinary Laboratories Agency, Weybridge, New Haw, Surrey, United Kingdom [2] Faculty of Pharmacy of Porto University, Department of Biological Sciences. Porto, Portugal [3] Agrarian Superior School of the Polytechnic Institute of Viseu, Department of Animal Science, Rural Engineering and Veterinary Science, Viseu, Portugal [4] Istituto Superiore di Sanità, Rome, Italy [5] Veterinary Research Institute, Department of Food and Feed Safety, Brno, Czech Republic [6] Instituto Tecnológico Agrario de Castilla y León (ITACyL), Valladolid, Spain [7] Central Veterinary Institute, Wageningen University and Research Centre, Lelystad, The Netherlands

Published in BMC Research Notes, Vol. 5, 190

BMC Research Notes

BioMed Central
The Open Access Publisher

This Provisional PDF corresponds to the article as it appeared upon acceptance. Fully formatted PDF and full text (HTML) versions will be made available soon.

Prevalence and transmission of hepatitis E virus in domestic swine populations in different European countries

BMC Research Notes 2012, **5**:190 doi:10.1186/1756-0500-5-190

Alessandra Berto (alessandra.berto@live.com)
Jantien A Baker (Jantien.Backer@wur.nl)
Joao R Mesquita (joaomeskita@hotmail.com)
Maria SJ Nascimento (saojose@ff.up.pt)
Malcolm Banks (Malcolm.Banks@ahvla.gsi.gov.uk)
Francesca Martelli (Francesca.martelli@ahvla.gsi.gov.uk)
Fabio Ostanello (fabio.ostanello@unibo.it)
Giorgia Angeloni (g85.g@libero.it)
Franco M Ruggeri (franco.ruggeri@iss.it)
Ilaria di Bartolo (ilaria.dibartolo@iss.it)
Petra Vasickova (vasickova@vri.cz)
Marta Diez-Valcarce (ita-dievalma@itacyl.es)
Marta Hernandez (ita-herperma@itacyl.es)
David Rodriguez-Lazaro (ita-rodlazda@itacyl.es)
Wim H.M. van der Poel (Wim.vanderPoel@wur.nl)

ISSN 1756-0500

Article type Short Report

Submission date 12 January 2012

Acceptance date 25 April 2012

Publication date 25 April 2012

Article URL http://www.biomedcentral.com/1756-0500/5/190

This peer-reviewed article was published immediately upon acceptance. It can be downloaded, printed and distributed freely for any purposes (see copyright notice below).

Articles in *BMC Research Notes* are listed in PubMed and archived at PubMed Central.

For information about publishing your research in *BMC Research Notes* or any BioMed Central journal, go to

© 2012 Berto *et al.* ; licensee BioMed Central Ltd.
This is an open access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

BMC Research Notes

BioMed Central
The Open Access Publisher

http://www.biomedcentral.com/info/instructions/

© 2012 Berto *et al.* ; licensee BioMed Central Ltd.
This is an open access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Prevalence and transmission of hepatitis E virus in domestic swine populations in different European countries

Alessandra Berto[1,2*]
[*] Corresponding author
Email: alessandra.berto@live.com

Jantien A Backer[1]
Email: Jantien.Backer@wur.nl

Joao R Mesquita[3,4]
Email: joaomeskita@hotmail.com

Maria SJ Nascimento[4]
Email: saojose@ff.up.pt

Malcolm Banks[2]
Email: Malcolm.Banks@ahvla.gsi.gov.uk

Francesca Martelli[2]
Email: Francesca.martelli@ahvla.gsi.gov.uk

Fabio Ostanello[5]
Email: fabio.ostanello@unibo.it

Giorgia Angeloni[6]
Email: g85.g@libero.it

Ilaria Di Bartolo[6]
Email: ilaria.dibartolo@iss.it

Franco M Ruggeri[6]
Email: franco.ruggeri@iss.it

Petra Vasickova[8]
Email: vasickova@vri.cz

Marta Diez-Valcarce[7]
Email: ita-dievalma@itacyl.es

Marta Hernandez[7]
Email: ita-herperma@itacyl.es

David Rodriguez-Lazaro[7]
Email: ita-rodlazda@itacyl.es

Wim HM van der Poel[1,9]
Email: Wim.vanderPoel@wur.nl'

[1] Central Veterinary Institute of Wageningen Univerisity and Research Centre, Lelystad, The Netherlands

[2] Animal Health and Veterinary Laboratories Agency, Adllestone, Surrey, United Kingdom

[3] Agrarian Superior School of the Polytechnic Institute of Viseu, Department of Animal Science, Rural Engineering and Veterinary Science, Viseu, Portugal

[4] Faculty of Pharmacy of Porto University, Department of Biological Sciences, Porto, Portugal

[5] Department of Veterinary Medical Sciences, University of Bologna, Ozzano Emilia, Bologna, Italy

[6] Department of Veterinary Public Health & Food Safety, Istituto Superiore di Sanita', Rome, Italy

[7] Instituto Tecnológico Agrario de Castilla y León (ITACyL), Junta de Castilla y León, Valladolid, Spain

[8] Veterinary Research Institute, Brno, Czech Republic

[9] National Centre for Zoonoses research, University of Liverpool, Liverpool, United Kingdom

Abstract

Background

Hepatitis E virus (HEV) genotype 3 and 4 can cause liver disease in human and has its main reservoir in pigs. HEV investigations in pigs worldwide have been performed but there is still a lack of information on the infection dynamics in pig populations.

Findings

The HEV transmission dynamics in commercial pig farms in six different European countries was studied. The data collected show prevalence in weaners ranging from 8% to 30%. The average HEV prevalence in growers was between 20% and 44%. The fatteners prevalence ranged between 8% and 73%. Sows prevalence was similar in all countries. Boar faeces were tested for HEV only in Spain and Czech Republic, and the prevalence was 4.3% and 3.5% respectively. The collected data sets were analyzed using a recently developed model to estimate the transmission dynamics of HEV in the different countries confirming that HEV is endemic in pig farms.

Conclusions

This study has been performed using similar detection methods (real time RT-PCR) for all samples and the same model (SIR model) to analyse the data. Furthermore, it describes HEV prevalence and within-herd transmission dynamics in European Countries (EU): Czech Republic, Italy, Portugal, Spain, The Netherlands and United Kingdom, confirming that HEV is circulating in pig farms from weaners to fatteners and that the reproductive number mathematical defined as R_0 is in the same range for all countries studied.

Keywords

Hepatitis E virus, Foodborne disease, Pork, Foodchain, PCR, Modeling, Prevalence, European countries

Findings

Background

Hepatitis E virus (HEV) is a hepatotropic virus, causative agent of hepatitis E that has clinical and morphological characteristics of acute viral hepatitis [1,2]. In humans, the infection may vary in severity from inapparent to fulminant. The mortality is between 1% and 4%, and in pregnant women this can reach 25% [1].

Genotypes 1 and 2 appear to be mainly anthroponotic whereas genotypes 3 and 4 can be also zoonotic [3,4].

In resource-limited countries, HEV infection is endemic and spreads mainly through contamination of water supplies. Autochthonous cases have been reported in the USA, Europe, industrialized countries of the Asia–Pacific area and South America. Since the first description of a swine HEV strain in 1997 [5], swine HEV has been detected all over the world and in several animal species (e.g. wild boar, mongoose and deer). In developed regions, human and swine strains show sympatric distribution [6].

Genotype 3 has been identified in humans and animals in developed countries in almost all continents.

Higher HEV seroprevalence is detected in slaughterhouse workers and veterinarians [7,8], and it is evaluated that one third of the worldwide population has been in contact with the virus since HEV antibodies have been detected in serum [9,10].

In 2008 Di Bartolo et al. [11] investigated the prevalence of swine HEV in 274 pigs from six different swine farms of Northern Italy. Viral RNA was tested in faeces and HEV RNA was detected in 42% of the samples. All farms tested positive for HEV, with a prevalence ranging between 12.8% and 72.5%. All age groups tested HEV-RNA positive, although infection was more prevalent in weaners than in the older fatteners (42.2% vs. 27.0%).

Fernandez-Barredo et al. [12] et al. in 2006, tested 146 faecal samples of pigs from 21 farms. HEV RNA was detected in faecal samples from 34 pigs (23.29%). Pigs in the first month of feeding (60%) and weaners (41.7%) presented higher HEV prevalence.

De Deus *et al.* [13] conducted a prospective study, where 19 sows and 45 piglets were tested for antibodies to HEV. HEV IgG and IgM antibody was detected in 76.9% and 15.4% of sows, respectively. HEV RNA was also detected in serum at all analysed ages with the highest prevalence at 15 weeks of age. HEV was detected in faeces and lymph nodes for the first time at 9 weeks of age and peaked at 12 and 15 weeks of age. This peak coincided with the occurrence of hepatitis as well as with HEV detection in bile, liver, mesenteric lymph nodes and faeces, and with highest IgG and IgM OD values at 15 weeks.

Few HEV transmission dynamics studies have been performed so far. The common aim of those studies was evaluating the R_0 that represents the number of infections that one infectious animal can cause in a fully susceptible population [14] [15]. Backer et al. estimated transmission parameters to explain the prevalence pattern in pigs of different age groups. Briefly, the model describes how soon after exposure a susceptible animal can be infected (expressed by the transmission rate parameter) and how long an infectious animal excretes virus (expressed by the average infectious period).

Satou et al. [15] tried to clarify the mechanisms of transmission within farms in order to facilitate an understanding of the age-specific patterns of infection, especially just prior to slaughter.

Many HEV prevalence studies have been performed [12,16] but none of them compared HEV prevalence in different EU countries as well as in different age groups.

Hence, the aim of the present study was to evaluate HEV prevalence and HEV transmission rates in different pig age groups in different countries. For this work, results from pig samples obtained from farms in Czech Republic, Italy, Portugal, Spain, The Netherlands and United Kingdom were used. For comparison of HEV transmission rates and HEV infectious periods, the model developed by Backer *et al.* was used.

Methods

Consensus from all farm owners was obtained previous the sample collection.

All the faeces collection was performed in conformity with standard guides, since that only faces were collected in the floor of the pigs pen and the animal were no touched at all an ethical consensus was not requested and necessary for this study.

Samplings

The UK data sets (UK2007 and UK2008) consisted of 10 herds sampled by age class: weaners (6–9 weeks of age), growers (10–12 weeks of age), fatteners (13–22 weeks of age) and sows. Pig stool samples were collected from 10 different pig farms in 2007 and 10 pig farms in 2008. Five stool samples were obtained from each age group.

In the Portugal data set, each herd was tested at entering (weaning age of 3 weeks), growing (7 weeks) and at departure (slaughtering age of 21 weeks). A total of 200 pig stool samples were collected from 5 industrial pig farms (40 samples per farm) between December 2010 and February 2011. From each farm a total of 10 stool samples were obtained from each age group.

The data sets of Italy and The Netherlands comprised test results of one fattening group (21 weeks) of one single farm, whereas the data set obtained from Spain comprised of one group of sows in one single farm, where 144 faeces were tested for HEV RNA.

Ten pig farms were selected in Czech Republic, and a total of 200 pigs of different age groups: weaners, growers, fatteners, sows and boar where faeces were tested for HEV.

In all farms, samples of a minimum of 1 g of faeces were collected aseptically in a sterile plastic bag and maintained at 4°C (max. 24 h) or frozen at −20°C until processing.

RNA extraction and RT-PCR procedures

UK 2007, 2008 and Italy

RNA extraction and PCR is detailed by McCreary *et al.* 2008 [16]. Briefly 0.·2 g of faeces were suspended in 1.·8 ml phosphate-buffered saline, and 140 µl of the supernatant was used to extract RNA, using the QIAamp Viral RNA mini kit (Qiagen) according to the manufacturer's instructions. The first round of the PCR used 2 µl of RNA. The reaction conditions were 96°C for five minutes, then 35 cycles of 96°C for five seconds, 55°C for five seconds and 75°C for 30 sec, followed by 72°C for one minute. A second round was carried out with a nested PCR, using a fast cycling PCR (Qiagen). These primers of the ORF-2 region are 3158 N (forward): 5′ GTT(A)ATGCTT(C)TGCATA(T)CATGGCT-3′ and 3159 N (reverse): 5′-AGCCGACGAAATCAATTCTCTC-3′ [17]. The products of the amplification process were electrophoresed, and visualised with UV light. For confirmation, the amplicons were sequenced, and the sequences obtained were assembled by using SEQMAN or DNAStar.

In Italy, RNA was processed by a reverse transcription (RT)-nested-PCR using protocol by Huang *et al.*, 2002 [17] with SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen; Carlsbad, CA, USA) kit, as described in Di Bartolo et al. [18].

The Netherlands, Portugal, Spain and Czech Republic

Two hundred and fifty mg of soft faecal contents was suspended in 2.25 ml of gentamycin-containing PBS solution and centrifuged at 3000 *g* for 15 min. Nucleic acid was extracted from 140 µl of the supernatant using the QIAamp® viral RNA mini kit (QIAGEN), according to manufacturer's instructions.

Jothikumar's primers and probes were used and they were designed on a multiple sequence alignment of HEV genome sequences in the ORF3 region available in GenBank [19]. Real time RT-PCR was performed using RNA Ultrasense™ One-Step Quantitative RT-PCR System (Invitrogen) and primers and probe: JHEV-F (5′- GGT GGT TTC TGG GGT GAC -3′); JVHEV-R (5′- AGG GGT TGG TTG GAT GAA -3′); JHEV-P (Taqman probe) (5′- FAM- TGA TTC TCA GCC CTT CGC –BHQ1-3′) [19]. Ten µl of RNA were added to a mix

containing buffer RNA Ultrasense reaction mix (5x), ROX reference dye (50x) and RNA Ultrasense enzyme mix.

The real time RT-PCR was carried out at 50°C for 15 min, 95°C for 2 min, and 45 cycles at 95°C for 10 s, 55°C for 20 s and 72°C for 15 s.

The sensitivity of the set primers used for the HEV detection between all countries was comparable; positive (RNA of HEV positive liver) and negative (water) controls were used during the RNA extraction and during the PCRs and they worked as expected. Primers sensitivity of Huang *et al.* was 3.2 PID_{50} while Jotikimuar *et al.* sensitivity was 1.2 PDI_{50} but usually HEV in pig faeces is detectable above this values.

HEV transmission modelling

The model used to describe HEV transmission in a pig herd is an age-structured SIR model (Backer et al.[20]). Each age group was subdivided in three distinct compartments consisting of pigs that are susceptible (S), infectious (I) or recovered (R) [21]. For the analyses, it was assumed that each susceptible animal can be infected by an infectious animal in its own group or any other group with the same probability. The sample sizes in each data set were assumed to represent 5% of the total group size.

The transmission dynamics are characterized by the average infectious period μ and the transmission rate parameter β that signifies the number of infections one infectious animal can cause per time unit. The product of these two parameters is the reproductive number $R_0 = \beta \mu$ that expresses the number of infections one infectious animal can cause during its entire infectious period in a fully susceptible population. When the reproductive number is larger than unity, $R_0 > 1$, an outbreak can grow exponentially. Otherwise, when $R_0 < 1$ the outbreak will die out. Our model assumes the HEV transmission to be in endemic equilibrium, i.e. the disease can sustain itself in the regenerating pig population. For this reason, we have omitted the herds with few or only negative results, as for these endemic equilibrium could not be justified.

The UK data sets (UK2007 and UK2008) consisted of herds subdivided into three groups: weaners (6–9 weeks of age), growers (10–12 weeks of age) and fatteners (13–26 weeks of age). Animals entering the weaning group were assumed to be uninfected [20]. In the Portugal data set, the herds were assumed to consist of one group that was tested at entering (weaning age of 3 weeks) and at departure (slaughtering age of 21 weeks) [20]. The test results of the growers (age of 7 weeks) were used as proxy for the infection pressure in the entire herd [20]. The data sets of Italy and The Netherlands comprised test results of just one fattening group. For this reason, we could not estimate the transmission rate parameter and the average infectious period separately, but only their product, the reproductive number [20]. For both data sets the total residence time is assumed to be 20 weeks from weaning to slaughtering age [20]. Data sets of Spain and Czech Republic were almost completely negative. For this reason, we could not estimate the reproductive number for these sets.

Results

HEV prevalence in different age groups in the UK (2007, 10 farms and 2008, 10 farms), in Portugal (2011, 5 farms), Italy (2010, 3 farms), The Netherlands (2011, 1 farm), Czech Republic (2010, 10 farms), Spain (one farm between 2010 and 2011) are depicted in Figure 1. Briefly prevalence of weaners, grower's fatteners and sows in UK 2007 was 26%, 44%, 10% and 6% respectively. Prevalence of prevalence of weaners, growers, fatteners and sows in UK 2008 was 8%, 22%, 8.8%and 2%. Prevalence of weaners, growers, fatteners and sows in Portugal was 30%, 20%, 30% and 4% respectively. Prevalence of fatteners in Italy was 23%. Prevalence of fatteners in The Netherlands was 73% meaning that 44 out of 60 pigs were shearing virus in the faeces on the day of the sample collection. The data set is similar between the age groups and prevalence match with other studies. The prevalence in The Netherlands was relatively higher in the fattening groups compared to the other European fattening groups.One hundred and forty-four faecal samples from sows collected in Spain and tested by real time RT-PCR were found to be HEV negative, while 4.3% of the boars (1 positive out of 23) were positive. In none of the weaners and fatteners tested in the Czech Republic, HEV RNA was detected. Only one grower out of 32 (3.1%), 5 sows out of 103 (5%) and 1 boar (3.5%) out of 28 tested HEV positive by real time RT-PCR.

Figure 1 Mean HEV prevalence in six different EU countries. HEV prevalence plotted for six countries and 5 pig age groups. Error bars denote the standard error of the mean

Table 1 shows the transmission rate parameter β, average infectious period μ and reproductive number R_0 of UK 2007 and 2008 and Portugal and the reproductive number R_0 for Italy and The Netherlands. The data set from Spain and Czech Republic could not be used in this study since all or almost all animal tested were HEV negative and we could not apply the model to those data.

Table 1 Estimated transmission rate parameters

Dataset	transmission rate parameter β (day^{-1})		average infectious period μ (days)		reproductive number R_0	
UK 2007 (10 herds)	0.11	(0.070 – 0.17)	43	(33 – 59)	4.7	(3.6 – 6.4)
UK 2008 (8 herds)	0.071	(0.041 – 0.13)	43	(29 – 73)	3.1	(2.5 – 4.1)
Portugal (6 herds)	0.037	(0.0035 – 0.16)	101	(70–403)	3.7	(1.2 – 14)
Italy (3 herds)	-		-		2.0	(1.4 – 3.6)
Netherlands (1 herd)	-		-		8.4	(5.3 – 15)
Spain	-		-		-	-
Czech Republic	-		-		-	-

Median maximum likelihood estimates and 5% – 95% credible interval between brackets

Discussion

The HEV transmission dynamics in commercial pig farms in six different European countries (UK, Portugal, Italy, The Netherlands, Spain and Czech Republic) was studied.

The data collected show prevalence in weaners ranging from 8% to 30%. The average HEV prevalence in growers was between 20% and 44%. The fatteners prevalence ranged between 8% and 73%. Sows prevalence was similar in all countries. Boar faeces were tested for HEV only in Spain and Czech Republic, and the prevalence was 4.3% and 3.5% respectively.

Overall, Figure 1 describes HEV prevalence comparing Czech Republic, Italy, Portugal, Spain, The Netherlands and UK 2007, 2008. The data set is similar between the age groups and prevalence matches with other studies [12,16]. The prevalence in the fattening groups in Italy and The Netherlands was relatively higher compared to other European fattening groups [21].

These data are similar to previously published Italian [11] and Spanish [12] data, confirming that HEV prevalence during time is constant and HEV is circulating in all farms in all age groups, from weaners to fatteners and that pigs close to the slaughter age can still be HEV infected.

The collected data sets were analyzed using a recently developed model to estimate the transmission dynamics of HEV in the different countries [20].

Satou *et al.* in 2007 [15] studied HEV transmission in 6 different Japanese provinces and found the reproductive number in the order of 4.02 – 5.17, which agrees with our estimated reproductive numbers ranging from 2.0 to 8.4. The study by Satou et al. [15] was the first report on HEV transmission estimated from field data. Bouwknegt *et al.* in 2008 performed the first HEV transmission dynamics study in an animal experiment [22]. In this study, the R_0 was found to be 8.8 and 32 in two separate experiments, much higher than 1.0 indicating that swine could be assumed to be a true reservoir of HEV. The R_0 values calculated by us are lower than the R_0 values calculated by Bouwknegt et al. [22]. This is because the infectious periods are comparable, but the transmission rate parameters for the experimental and field situation are different.

The average infectious period μ in UK 2007 data was for instance estimated to be 43 (33 – 59) days, whereas Bouwknegt *et al.* [22] estimated average infectious periods of 49 (17–141) days and 13 (11 – 17) days.

The transmission rate parameter in our study was 0.11 (0.070 – 0.17) day^{-1} for UK 2007, meaning that one infectious animal infects another animal every 10 days. The transmission rate parameters were 0.071 (0.041-0.13) day^{-1} for UK 2008 and 0.037 (0.0035-0.16) day^{-1} for Portugal 2011. In the animal experiments, Bouwknegt et al. [22] estimated a higher rate of transmission, i.e. 0.66 (95% CI: 0.32–1.35) day^{-1}. The difference can be explained by the closer proximity of animals in an experimental setting compared to a farm situation and by the fact that contact animals in a transmission experiment encounter only animals that are in the early and possibly more infectious stages of virus shedding.

The transmission rate parameters for the other EU countries could not be estimated because either only one age group was tested or the majority of the animals were negative and the model was not applicable.

This study gave a genuine contribution to better understand HEV prevalence in six different European countries by a mathematical model.

We would like to highlight that HEV is highly circulating in many pig farms in Europe and can be present in fattening pigs, where usually this age group is the one arriving to the table. In industrialized regions, although the incidence of clinical hepatitis E in humans is low, the seroprevalence is relatively high [22], indicating a high proportion of subclinical disease and/or underdiagnosis [8]. It is likely that a small proportion of this exposure to HEV results from travel to endemic regions, or migration from endemic regions [23], this still leaves a substantial level of exposure to HEV that appears to have an indigenous source.

HEV positive fatteners were found in all European countries studied. This may pose an important risk for public health especially in those countries where pork products are eaten undercooked or raw.

Competing interests

There are no competing interests relating to any of the authors.

Author's contribution

First author biographical: Ms Alessandra Berto is a final year PhD student on the VITAL project and is based at the AHVLA Weybridge, UK and the Wageningen University Research Institute in The Netherlands. She tested and collected some of the data and she helped JB to set up the model. JA Backer was the modeler. JR Mesquita MSJ Nascimento provided the Portuguese samples. M. Banks, F. Martelli provided the Uk data set. F. Ostanello,G. Angeloni, I. Di Bartolo, F. M. Ruggeri : provided the Italian data set. P. Vasickova provided the Cechz republic data set. Diez-Valcarce, M. Hernandez, D. Rodriguez-Lazaro: provided the Spainish data set. W.H.M. van der Poel was involved in drafting the manuscript or revising it critically for important intellectual content and gave final approval of the version to be published. This manuscript has been read and approved by all authors.

Acknowledgment

The authors are grateful to the owners of the farms and Dr. Bas Swildens of the Utrecht University who took part in this study and for the kind and skilful assistance of veterinarians, who helped with collection of the samples.
This work was supported by the EPIZONE and the European Commission Framework Program 7 project "Integrated monitoring and control of foodborne viruses in European food supply chains (VITAL)" (Grant No. KBBE 213178) led by the coordination team of Nigel Cook (FERA, UK), Martin D'Agostino (FERA, UK) and Franco Ruggeri (ISS, Italy).

References

1. Purcell RH, Emerson SU: **Hepatitis E: an emerging awareness of an old disease.** *J Hepatol* 2008, **48**(3):494–503.

2. Meng XJ: **Recent advances in Hepatitis E virus.** *J Viral Hepat* 2010, **17**(3):153–161.

3. Wei H, Zhang JQ, Lu HQ, Meng JH, Lu XX, Xie W: **Construction and screening of hepatitis E virus-specific phage antibody combinatorial library.** *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi* 2003, **19**(5):473–475, 485.

4. Schlauder GG, Mushahwar IK: **Genetic heterogeneity of hepatitis E virus.** *J Med Virol* 2001, **65**(2):282–292.

5. Meng XJ, Purcell RH, Halbur PG, Lehman JR, Webb DM, Tsareva TS, Haynes JS, Thacker BJ, Emerson SU: **A novel virus in swine is closely related to the human hepatitis E virus.** *Proc Natl Acad Sci U S A* 1997, **94**(18):9860–9865.

6. Banks M, Heath GS, Grierson SS, King DP, Gresham A, Girones R, Widen F, Harrison TJ: **Evidence for the presence of hepatitis E virus in pigs in the United Kingdom.** *Vet Rec* 2004, **154**(8):223–227.

7. Vulcano A, Angelucci M, Candelori E, Martini V, Patti AM, Mancini C, Santi AL, Calvani A, Casagni L, Lamberti A: **HEV prevalence in the general population and among workers at zoonotic risk in Latium Region.** *Ann Ig* 2007, **19**(3):181–186.

8. Meng XJ, Wiseman B, Elvinger F, Guenette DK, Toth TE, Engle RE, Emerson SU, Purcell RH: **Prevalence of antibodies to hepatitis E virus in veterinarians working with swine and in normal blood donors in the United States and other countries.** *J Clin Microbiol* 2002, **40**(1):117–122.

9. Yazaki Y, Mizuo H, Takahashi M, Nishizawa T, Sasaki N, Gotanda Y, Okamoto H: **Sporadic acute or fulminant hepatitis E in Hokkaido, Japan, may be food-borne, as suggested by the presence of hepatitis E virus in pig liver as food.** *J Gen Virol* 2003, **84**(Pt 9):2351–2357.

10. Colson P, Borentain P, Queyriaux B, Kaba M, Moal V, Gallian P, Heyries L, Raoult D, Gerolami R: **Pig liver sausage as a source of hepatitis E virus transmission to humans.** *J Infect Dis* 2010, **202**(6):825–834.

11. Martelli F, Caprioli A, Zengarini M, Marata A, Fiegna C, Di Bartolo I, Ruggeri FM, Delogu M, Ostanello F: **Detection of hepatitis E virus (HEV) in a demographic managed wild boar (Sus scrofa scrofa) population in Italy.** *Vet Microbiol* 2008, **126**(1–3):74–81.

12. Fernandez-Barredo S, Galiana C, Garcia A, Vega S, Gomez MT, Perez-Gracia MT: **Detection of hepatitis E virus shedding in feces of pigs at different stages of production using reverse transcription-polymerase chain reaction.** *J Vet Diagn Invest* 2006, **18**(5):462–465.

13. de Deus N, Casas M, Peralta B, Nofrarias M, Pina S, Martin M, Segales J: **Hepatitis E virus infection dynamics and organic distribution in naturally infected pigs in a farrow-to-finish farm.** *Vet Microbiol* 2008, **132**(1–2):19–28.

14. Greenhalgh D, Diekmann O, de Jong MC: **Subcritical endemic steady states in mathematical models for animal infections with incomplete immunity.** *Math Biosci* 2000, **165**(1):1–25.

15. Satou K, Nishiura H: **Transmission dynamics of hepatitis E among swine: potential impact upon human infection.** *BMC Vet Res* 2007, **3:**9.

16. McCreary C, Martelli F, Grierson S, Ostanello F, Nevel A, Banks M: **Excretion of hepatitis E virus by pigs of different ages and its presence in slurry stores in the United Kingdom.** *Vet Rec* 2008, **163**(9):261–265.

17. Huang FF, Haqshenas G, Guenette DK, Halbur PG, Schommer SK, Pierson FW, Toth TE, Meng XJ: **Detection by reverse transcription-PCR and genetic characterization of field isolates of swine hepatitis E virus from pigs in different geographic regions of the United States.** *J Clin Microbiol* 2002, **40**(4):1326–1332.

18. Di Bartolo I, Ponterio E, Castellini L, Ostanello F, Ruggeri FM: **Viral and antibody HEV prevalence in swine at slaughterhouse in Italy.** *Vet Microbiol* 2011, **149**(3–4):330–338.

19. Jothikumar N, Cromeans TL, Robertson BH, Meng XJ, Hill VR: **A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus.** *J Virol Methods* 2006, **131**(1):65–71.

20. JA Backer AB, C McCreary, F Martelli, WHM van der Poel: **Transmission dynamics of Hepatitis E virus in pigs: estimation from field data and effect of vaccination**. *submitted to Epidemics* 2012.

21. Di Bartolo I, Martelli F, Inglese N, Pourshaban M, Caprioli A, Ostanello F, Ruggeri FM: **Widespread diffusion of genotype 3 hepatitis E virus among farming swine in Northern Italy.** *Vet Microbiol* 2008, **132**(1–2):47–55.

22. Bouwknegt M, Frankena K, Rutjes SA, Wellenberg GJ, de Roda Husman AM, van der Poel WH, de Jong MC: **Estimation of hepatitis E virus transmission among pigs due to contact-exposure.** *Vet Res* 2008, **39**(5):40.

23. Peron JM, Mansuy JM, Poirson H, Bureau C, Dupuis E, Alric L, Izopet J, Vinel JP: **Hepatitis E is an autochthonous disease in industrialized countries. Analysis of 23 patients in South-West France over a 13-month period and comparison with hepatitis A.** *Gastroenterol Clin Biol* 2006, **30**(5):757–762.

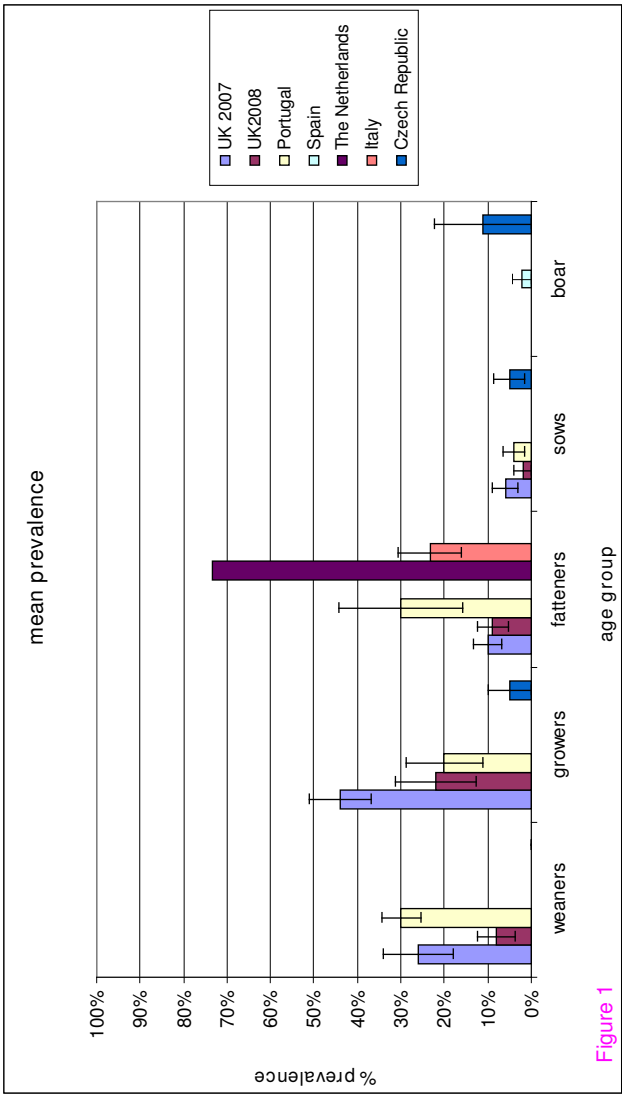

mean prevalence
UK 2007
UK2008
Portugal
Spain
The Netherlands
Italy
Czech Republic
% prevalence
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
weaners
growers
fatteners
sows
boar
age group

Figure 1

3.2.3.

Occurrence of Human Enteric Viruses in Commercial Mussels at Retail Level in Three European Countries

Marta Diez-Valcarce[1], Petros Kokkinos[2], Kirsi Södeberg[3], Martijn Bouwknegt[4], Kris Willems[5], Ana Maria de Roda-Husman[4], Carl-Henrik von Bonsdorff[3], Maria Bellou[2], Marta Hernández[1], Leena Maunula[3], Apostolos Vantarakis[2] and David Rodríguez-Lázaro[1]

[1] Instituto Tecnológico Agrario de Castilla y León (ITACyL), Valladolid, Spain [2] University of Patras, Patras, Greece [3] University of Helsinki, Helsinki, Finland [4] National Institute for Public Health and the Environment (RIVM), Bilthoven, Netherlands [5] KU Leuven University, Leuven, Belgium

Published in Food and Environmental Virology, Vol. 4, 73-80

Food Environ Virol (2012) 4:73–80
DOI 10.1007/s12560-012-9078-9

ORIGINAL PAPER

Occurrence of Human Enteric Viruses in Commercial Mussels at Retail Level in Three European Countries

Marta Diez-Valcarce · Petros Kokkinos · Kirsi Söderberg · Martijn Bouwknegt · Kris Willems · Ana Maria de Roda-Husman · Carl-Henrik von Bonsdorff · Maria Bellou · Marta Hernández · Leena Maunula · Apostolos Vantarakis · David Rodríguez-Lázaro

Received: 5 January 2012 / Accepted: 16 April 2012 / Published online: 12 May 2012
© Springer Science+Business Media, LLC 2012

Abstract In this study, the prevalence of different enteric viruses in commercial mussels was evaluated at the retail level in three European countries (Finland, Greece and Spain). A total of 153 mussel samples from different origins were analysed for human norovirus (NoV) genogroups I and II, hepatitis A virus (HAV) and hepatitis E virus (HEV). Human adenovirus (HAdV) was also tested as an indicator of human faecal contamination. A full set of controls (such as sample process control, internal amplification controls, and positive and negative controls) were implemented during the process. The use of a sample process control allowed us to calculate the efficiencies of extraction, which ranged from 79 to 0.5 %, with an average value of 10 %. Samples were positive in 41 % of cases, with HAdV being the most prevalent virus detected (36 %), but no significant correlation was found between the presence of HAdV and human NoV, HAV and HEV. The prevalences of human norovirus genogroup II, HEV and human NoV genogroup I were 16, 3 and 0.7 %, respectively, and HAV was not detected. The estimated number of PCR detectable units varied between 24 and 1.4×10^3 g^{-1} of digestive tract. Interestingly, there appeared to be a significant association between the type of mussel species (*M. galloprovincialis*) and the positive result of samples, although a complete overlap between country and species examined required this finding to be confirmed including samples of both species from all possible countries of origin.

Keywords Enteric viruses · Mussels · Hepatitis A · Norovirus · Human adenovirus · Real-Time PCR

Introduction

Due to their filtre-feeding nature, bivalve molluscs tend to accumulate human pathogens (Rippey 1994) in their stomach and their digestive glands (Schwab et al. 1998; Rodríguez-Lázaro et al., in press). In one study, Lees (2000) observed that shellfish grown in sewage polluted waters tend to bioaccumulate environmentally stable enteric viruses, such as norovirus (NoV), hepatitis A virus (HAV) and enterovirus (EV). Processing interventions such as depuration do not completely eliminate viral particles (Loisy et al. 2005; Schwab et al. 1998) and the habit of

Marta Diez-Valcarce, Petros Kokkinos and Kirsi Söderberg contributed equally to the work.

M. Diez-Valcarce · M. Hernández · D. Rodríguez-Lázaro (✉)
Instituto Tecnológico Agrario de Castilla y León (ITACyL), Junta de Castilla y León, Ctra. Burgos, km 119, 47071 Valladolid, Spain
e-mail: ita-rodlazda@itacyl.es

P. Kokkinos · M. Bellou · A. Vantarakis (✉)
Department of Public Health, Medical School, University of Patras, University Campus, 26500 Patras, Greece
e-mail: avantar@med.upatras.gr

K. Söderberg · C.-H. von Bonsdorff · L. Maunula (✉)
Department of Food and Environmental Hygiene, University of Helsinki, Agnes Sjöberginkatu 2, PO Box 66, 00790 Helsinki, Finland
e-mail: leena.maunula@helsinki.fi

M. Bouwknegt · A. M. de Roda-Husman
National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands

K. Willems
Department of Microbial and Molecular Systems (M2S), Katholieke Universiteit Leuven, Leuven, Belgium

eating bivalve mollusks raw or slightly cooked increases the health risk related to shellfish consumption (Butt et al. 2004; Rippey 1994).

For the detection of enteric viruses in shellfish, molecular methods such as reverse transcription-polymerase chain reaction (RT-PCR) are widely used (Le Guyader et al. 2000; Bosch et al. 2011). However, the low quantity of virus in environmental samples such as shellfish renders them a difficult and variable matrix that is also known to cause amplification inhibition (Lowther et al. 2008) increasing the risk of false negative results. For this reason, effective preliminary sample treatment steps such as elution and concentration of viruses from the shellfish tissue and RNA extraction and purification are essential for final PCR accuracy and reproducibility (Le Guyader et al. 2000). To overcome those issues the utilization of several controls throughout the process is necessary (Rodríguez-Lázaro et al. 2007, in press; Bosch et al. 2011; D'Agostino et al. 2011). Sample process controls (SPCV) and internal amplification controls (IAC) must be used to verify the accuracy of the results obtained (D'Agostino et al. 2011; Diez-Valcarce et al. 2011a, b). An SPCV is used to verify whether the sample treatment has operated correctly and also allow us to estimate the efficiency of extraction for each individual sample analysed. The IAC is used to monitor the possible inhibition of the reaction due to inhibitory compounds in the sample, avoiding any false negative interpretation of the analysis.

The increasing amount of data on virus detection in shellfish (Le Guyader et al. 2000.; Formiga-Cruz et al. 2002; Myrmel et al. 2004; Croci et al. 2007) and shellfish-borne viral outbreaks (Svraka et al. 2007; Le Guyader et al. 2008; Vilariño et al. 2009; Pintó et al. 2009; Baker et al. 2011) points out the necessity of a constant surveillance system in European countries. The European FP7 project VITAL (Integrated Monitoring and Control of Foodborne Viruses in European Food Supply Chains), which ran from 2008 to 2011, aimed to gather data on virus contamination of food sources for quantitative viral risk assessment and development of virus-specific guidance for food supply chain operators. In this project, different European laboratories have investigated the shellfish supply chain for NoVGI, NoVGII, HAV and hepatitis E virus (HEV). Human adenovirus (HAdV) was also investigated to demonstrate the potential existence of a route of viral faecal contamination from human sources to the sampling point within the food supply chain. In this study a survey was performed to acquire information on viral prevalence in mussels across Europe at the retail level. Methods used have been previously validated through ring trials in order to have comparable quantitative data (D'Agostino et al. 2012).

Materials and Methods

Sampling Strategy

This study was conducted in three European countries (Spain, Greece and Finland) during the period of summer–winter 2010 (from May to December). Mussel species collected were *Mytilus galloprovincialis* in Spain and Greece (102 samples) and *Mytilus edulis* in Finland (51 samples). The origin of samples was also different: in Finland they were imported from Denmark; in Spain, all samples were locally collected in the Galicia region; while in Greece, samples were imported from Chile (40 samples), New Zealand (5 samples), Spain (4 samples) and also collected locally (2 samples).

In each country, a total of 51 mussel samples were taken at local retail stores during ten independent sampling times separated by at least 1 week. On each sampling occasion, five mussels (six on one sampling occasion) were randomly selected for subsequent analysis. One hundred and two samples were purchased fresh and 51 were purchased frozen, all samples being cultured mussels. The sampling plan was developed on a rationale assuming that if a batch of mussels was contaminated, it was likely that the growing waters were contaminated and that a large proportion of the batch would carry at least 1 virus particle. With the detection system used in this study we were able to detect contamination in retail stores with 95 % confidence when 50 % or more of the mussels were contaminated. This strategy increases the probability of detecting virus when low virus concentrations were expected since extraction and inhibition controls were used, and analyses were performed in duplicate and in serial dilutions of nucleic acids. Aiming for most accurate estimates of prevalence given the total fixed number of samples, the priority was to detect the virus when 50 % or more of the samples in a batch were contaminated.

Sample Process Control Virus

The SPCV was murine norovirus 1 (MNV-1) (Diez-Valcarce et al. 2011b), which had been propagated in RAW264.7 cells to a concentration of 10^8 plaque forming units (pfu) ml^{-1}. MNV-1 stocks were kindly provided by the group of Dr. Franco Ruggeri at the Istituto Superiore de Sanità, Rome, Italy by agreement with Washington University, St. Louis, MO, USA.

Positive Controls

Positive controls were nucleic acids extracted from the target viruses or chimerical standards provided in the project (Martínez-Martínez et al. 2011). Nucleic acid

sequences of these chimerical standards were identical to the sequence of the target viruses.

Virus Concentration and Extraction from Shellfish

Mussels were selected and any mud from the shell was washed off using tap water. The sample was then processed using the method of Henshilwood et al. (2003). Briefly, one shellfish was placed on a rubber shucking block and the shells opened with a clean shucking knife. The digestive gland was dissected out aseptically using scissors and forceps (or equivalent tools), transferred to a clean Petri dish, and chopped finely with a razor blade to homogenate the sample. The chopped glands were then weighed, and transferred into a centrifuge tube. The SPCV (10 µl; ca. 10^6 pfu) was added. One ml of 3 U ml^{-1} proteinase K solution (prepared in molecular grade water) was added and mixed well. The sample was incubated at 37 °C in a shaking incubator or equivalent for 60 min, ensuring that the speed setting for the shaker induced continual gentle movement of the enzyme/gland mixture. A second incubation was carried out by placing the tube in a water bath at 65 °C for 15 min with shaking. The sample was then centrifuged at 3,000×*g* for 5 min, and 500 µl of supernatant was transferred to a clean microcentrifuge tube and was immediately used for nucleic acid extraction or stored at −20 °C. Nucleic acids (500 µl) were extracted using a NucliSENS® miniMAG® kit (bioMérieux) according to the manufacturer's instructions. The final elution was performed twice with 150 µl elution buffer, resulting in a 300 µl nucleic acid extract. The nucleic acid extract was assayed immediately or stored at −70 °C.

Detection of Viruses

The presence of enteric pathogenic viruses—HEV, HAV, NoVGI and NoVGII—were evaluated using reverse transcription real-time PCR (RT-qPCR). Detection of SPCV was also conducted by RT-qPCR. In addition, the presence of HAdV was also evaluated using real-time PCR (qPCR) in Spain and Greece. In all the cases, a neat and a 10-fold dilution of the virus nucleic acid extract were tested; all samples were tested in duplicate (two neat and two diluted). An internal amplification control (IAC) (Diez-Valcarce et al. 2011a) and its probe labelled with VIC (50 nM) were included in every assay.

All RT-qPCR assays were performed using the RNA Ultrasense reaction mix (Invitrogen), the qPCR assays were performed using TaqMan Universal PCR Master Mix (Applied Biosystems) and a carry-over contamination prevention system, uracil N-glycosylase. In each assay, 10 µl sample of nucleic acid extract was added, to make a final reaction volume of 20 µl, except in case of HAdV in which the final reaction volume was 25 µl. All oligonucleotides were purchased from MWG Biotech AG (Ebersberg, Germany) except the minor-groove binder (MGB) TaqMan probes HAV150(-) and MGB-ORF1/2 that were acquired from Applied Biosystems (Warrington, UK) and NV1LCpr that was acquired from Sigma-Aldrich (St. Louis, MO, USA). Virus assays were performed using the oligonucleotides and the conditions described in Table 1.

Extraction and Theoretic Efficiencies

The SPCV was employed as a control of the virus concentration and nucleic acid extraction. Prior to virus recovery from the mussel homogenates, the samples were spiked with a known quantity (ca. 10^6 pfu) of MNV-1. Viral RNA extracted from mussels was tested for target viruses undiluted and 10-fold diluted to evaluate the effect of potential qRT-PCR inhibitors. If MNV-1 signal was negative for a sample, it was retested from the beginning due to the PCR inhibition or the sample inhibition of the process. The extraction efficiency value was calculated by comparing the C_q value (quantification cycle, previously known as the threshold cycle) for the 10-fold dilution of MNV-1 (not extracted) with that obtained for the SPCV in the tested samples. The result was classified as poor (extraction efficiency <1 %), acceptable (1–10 %), or good (>10 %) (da Silva et al. 2007).

The theoretic efficiency was calculated by comparing the C_q value of a mussel sample containing the control (SPCV) with the C_q value of the SPCV alone, just spiked in the reagents used for concentration and extraction of the sample but without any matrix (chopped mussel); the formula used was: $2^{C_q\ \mathrm{SPCV} - C_q\ \mathrm{sample}} \times 100$. This efficiency was also classified in the same three categories (poor, acceptable and good).

Reporting and Interpretation of Data

For a proper interpretation of the results, four different signals were assayed: The target virus, the SPCV control, the target IAC and the SPCV IAC (D'Agostino et al. 2011). When at least one of the two replicate targets (for HAV, HEV, NoVGI, NoVGII and HAdV) was detected, these mussel samples were considered to be positive. Twelve of the 153 (7.8 %) samples were inhibited when neat samples were assayed, but diluted samples amplified for the target. When an assay showed a C_q value ≤45, independently of the corresponding IAC C_q value, the result was interpreted as positive. When an assay showed no C_q value for the target with the corresponding IAC C_q value ≤45 and at least one of the four replicates of MNV-1 (two neat and two diluted) assayed positive, the result was interpreted as

Table 1 Primers, probes and amplification conditions of the virus systems used in this study

Target	Name	Sequence (5′–3′)	Amplification conditions	References
HAV	HAV68	TCACCGCCGTTTGCCTAG	1 cycle: 15 min 50 °C	Costafreda et al. (2006)
	HAV240	GGAGAGCCCTGGAAGAAAG	1 cycle: 2 min 95 °C	
	HAV150(−)	6FAM-CCTGAACCTGCAGGAATTAA-MGBNFQ	45 cycles: 15 s 95 °C + 1 min 60 °C	
HEV	JVHEVF	GGTGGTTTCTGGGGTGAC	1 cycle: 15 min 50 °C	Jothikumar et al. (2006)
	JVHEVR	AGGGGTTGGTTGGATGAA	1 cycle: 2 min 95 °C	
	JVHEVP	6FAM-TGATTCTCAGCCCTTCGC-BHQ	45 cycles: 10 s 95 °C + 20 s 55 °C + 1 min 60 °C	
NoVGI	QNIF4	CGCTGGATGCGNTTCCAT	1 cycle: 15 min 50 °C	Svraka et al. (2007)
	NV1LCR	CCTTAGACGCCATCATCATTTAC	1 cycle: 2 min 95 °C	
	NV1LCpr	6FAM-TGGACAGGAGAYCGCRATCT-BHQ	45 cycles: 15 s 95 °C + 1 min 60 °C	
NoVGII	QNIF2d	ATGTTCAGRTGGATGAGRTTCTCWGA	1 cycle: 15 min 50 °C	da Silva et al. (2007)
	COG2R	TCGACGCCATCTTCATTCACA	1 cycle: 2 min 95 °C	
	QNIFS	6FAM-AGCACGTGGGAGGGCGATCG-BHQ	45 cycles: 15 s 95 °C + 1 min 60 °C	
HAdV	AdF	CWTACATGCACATCKCSGG	1 cycle: 2 min 50 °C	Hernroth et al. (2002)
	AdR	CRCGGGCRAAYTGCACCAG	1 cycle: 10 min 95 °C	
	AdP1	6FAM-CCGGGCTCAGGTACTCCGAGGCGTCCT-BHQ	45 cycles: 15 s 95 °C + 1 min 60 °C	
MNV-1	Fw-ORF1/2	CACGCCACCGATCTGTTCTG	1 cycle: 15 min 50 °C	Baert et al. (2008)
	Rv-ORF1/2	GCGCTGCGCCATCACTC	1 cycle: 2 min 95 °C	
	MGB-ORF1/2	6FAM-CGCTTTGGAACAATG-MGB-NFQ	45 cycles: 15 s 95 °C + 1 min 60 °C	

negative. When an assay showed both the target and its corresponding IAC C_q values absent, the reaction was considered to have failed.

Virus Quantification

The number of viruses per gram of mussel tissue was estimated using the most probable number-like approach (Teunis et al. 2005; De Roda Husman et al. 2009). Presence/absence profiles for target viruses were generated per mussel by examining neat and serial 10-fold dilutions of nucleic extracts of samples until the end-point dilution, in duplicate. It was assumed that viruses, if present, were distributed homogeneously in samples. The unit of quantification was a PCR detectable unit (PDU), which represents an unknown number of target genomes (under ideal amplification conditions and a perfect assay, a single PDU would represent a single virus genome).

Statistical Analysis

Statistical analysis was performed by Pearson's Chi-square test to test the significance between various categorical variables: mussel species and presence rate and fresh or frozen mussel and presence rate. $p < 0.05$ was considered significant and $p < 0.001$, highly significant. Odds ratios were also calculated. Statistical analysis was performed by using SPSS software version 17 (SPSS Inc., Chicago, IL, USA).

Results

Efficiencies of Extraction

The mean virus extraction efficiency of the process was 10 % with a standard deviation of 22. Values ranged from 79 to 0.5 %, and the mean virus theoretic efficiency was 6 % with a standard deviation of 15, with values ranging from 51 to 0.3 %. Overall: 92 % of the samples showed acceptable or good extraction efficiency (45 and 47 %, respectively) and only 8 % showed poor extraction efficiency (<1 %). Similarly, most of the samples (88 %) showed acceptable or good theoretic efficiency (55 and 33 %, respectively) and only 8 % showed poor theoretic efficiency (<1 %).

Detection of Viruses

Enteric viruses were detected in 41 % of the tested samples (62/153): only one type of enteric virus was detected in

Table 2 Prevalence of hepatitis A virus (HAV), hepatitis E virus (HEV), human norovirus genogroups I and II (NoVGI and NoVGII, respectively), and human adenovirus (HAdV) in mussels, in specified mussel species sampled in Finland, Greece and Spain

Mussel species	Country	Virus				
		HAV	HEV	NoV GI	NoV GII	HAdV
M. edulis	Finland	0/51	0/51	1/51 (2 %)	2/51 (4 %)	NT
M. galloprovincialis	Greece	0/51	NT	0/51	0/51	34/51 (67 %)
	Spain	0/51	3/51 (6 %)	0/51	23/51 (45 %)	3/51 (6 %)
	Subtotal	0/102	3/51 (6 %)	0/102	23/102 (23 %)	37/102 (36 %)
Overall		0/153	3/102 (3 %)	1/153 (0.7 %)	25/153 (16 %)	37/102 (36 %)

NT not tested samples

38 % of samples (58/153), and two types of enteric viruses were detected in 3 % (4/153) of the samples (Table 2). HAdV was the most prevalent virus, detected in 36 % of the samples (37/102), followed by NoVGII (16 %; 25/153), HEV (3 %; 3/102) and NoVGI (0.7 %; 1/153). However, HAV was not found in any of the samples analysed. Interestingly, none of the samples tested positive for HAdV was positive for any of the other human pathogenic viruses, indicating no significant correlation between the presence on HAdV and any of the pathogenic viruses studied. No significant differences were observed in the sensitivity of the assays among the three laboratories, since previous ring trials tests were done in the laboratories involved in the study to overcome these possible issues before the actual study was performed. The most likely estimates for PDU concentrations ranged between 24 and 1.4×10^3 g^{-1} of mussel tissue for NoV GII, between 127 and 348 for HEV and was estimated to be $\sim$260 for NoV GI (Table 3).

A high percentage of the 102 *M. galloprovincialis* were positive for enteric viruses compared to the 51 *M. edulis* (Table 2). There was a highly significant association between the type of mussel species and the analytical outcome of the sample ($p < 0.001$): a sample was $\sim$25 times more likely to be positive if the shellfish species was *M. galloprovincialis* than if it was *M. edulis*. Among the 102 fresh mussels 25 % (25/102), 6 % (3/51), 3 % (3/102) and 0.98 % (1/102) were positive for NoVGII, HAdV, HEV and NoVGI, respectively, whereas only HAdV was detected in 34 samples (67 %) of the frozen mussels samples (Table 4). Therefore, no significant association was found between the storage conditions of the mussels and whether or not samples were positive ($p > 0.05$).

Table 3 Estimated number of PCR detectable units (PDU) per g of mussel digestive tract and associated 95 % confidence interval (CI)

Virus	Estimated PDU	
	Mean	95 % CI
NoV GII	24	1–104
	33	2–144
	35	2–154
	37	2–172
	40	2–176
	40	2–178
	54	9–167
	61	10–188
	68	4–314
	86	14–283
	89	14–294
	126	17–684
	197	27–1,064
	262	15–1,214
	348	55–1,570
	413	65–1,870
	423	67–1,914
	439	69–1,982
	453	73–2,047
	545	86–2,461
	701	102–3,638
	804	117–4,175
	970	142–5,032
	1066	156–5,528
	1463	214–7,588
NoV GI	262	15–1,214
HEV	127	18–685
	177	25–955
	348	55–1,570

Discussion

The results obtained showed that 41 % (62/153) of samples were contaminated with at least one of the enteric viruses studied. This percentage rose up to 59 % (60/102) if we consider only *Mytillus galloprovincialis* species. In studies conducted in countries close to those of this study, the prevalence of enteric viruses varies from around 15 % for NoV (Terio et al. 2010), 34.4 % for NoV (Suffredini et al. 2011) in Italy, 4.5 % for NoVGI in Turkey (Yilmaz et al.

Table 4 Number of samples showing the presence of hepatitis A virus (HAV), hepatitis E virus (HEV), human norovirus genogroups I and II (NoVGI and NoVGII, respectively) and human adenovirus (HAdV) in fresh and frozen mussels sampled in Finland, Greece and Spain

VIRUS	Fresh	Frozen
HAV	0	0
HEV	3/51 (6 %)	0
NoVGI	1/102 (1 %)	0
NoVGII	25/102 (24 %)	0
HAdV	3/51 (6 %)	34/51 (67 %)
TOTAL	32/102 (31 %)	34/51 (67 %)

2010), 6.8 % for NoV and 18.6 % for HAdV (Myrmel et al. 2004) in Norway and 37 % for NoV and 33 % for HAV in Portugal (Mesquita et al. 2011). Factors such as decreased shellfish activity at lower temperature and differential retention of viruses by distinct mollusc species cannot be overlooked (Lees 2000). *M. galloprovincialis* was harvested in areas from Spain, Greece, Chile and New Zealand, whereas *M. edulis* was harvested in Denmark, so factors such as mussel species may have influence the final prevalence observed. Despite the highly significant association between the mussel species and the analytical outcome, the origin of the mussels can also play a part in this association, more samples of both species and from all different origins would be required to more deeply understand this association. A similar result was found regarding the storage conditions of samples: we found pathogenic viruses only in fresh purchased mussels whereas all frozen samples were negative for the pathogenic viruses analyzed (Table 4). But as all those negative samples were from the same species (*M. edulis*), more samples of different species also stored frozen are needed to get any conclusion about the possible effect of freezing in elimination of pathogenic viruses in shellfish.

The effectiveness of monitoring programmes based on bacteriological indicators such as *Escherichia coli* to determine the sanitary quality of molluscs and their harvesting areas (Council Directive 91/492/EEC, EC Reg No 854/2004) has been previously questioned (Mesquita et al. 2011; Silva et al. 2010). Consequently, we evaluated the use of HAdV as indicator of faecal contamination and to link its presence to that of other enteric pathogenic viruses such as human NoV as previously suggested (Silva et al. 2011; Wyn-Jones et al. 2011). Our results show that HAdV was the virus most frequently detected (36 %; 37/102). This could indicate that the shellfish, independently of the species and the country of origin, were in contact with waters polluted with human faeces during their production. However, there was not a direct relationship between the presence of HAdV and the detection of the pathogenic viruses assayed (NoV, HAV and HEV), this finding being in accordance with previous results (Myrmel et al. 2004).

An interesting result from our study is the total absence of HAV in the tested samples. Shellfish is considered a main route of contamination for enteric viruses (Rodíguez-Lázaro et al., in press), but HAV is not as commonly detected as NoV (Vilariño et al. 2009). Rotaviruses and astroviruses were also analysed in the molluscs collected in Spain in this study, but none of the samples were positive (data not shown), similar to other studies (Vilariño et al. 2009). One explanation may be that the bioaccumulation of NoVs is not only based on passive filtration but also an active process of fixation on shellfish tissues (Maalouf et al. 2011; Le Guyader et al. 2006).

Simultaneous presence of different viruses or virus strains could lead to more severe symptoms, the occurrence of two episodes of the same or different diseases, and also potentially facilitate emergence of new recombinant strains (Lees 2000). In this study, the simultaneous presence of two or more enteric viruses was found in four samples (3 %), but only one (0.7 %), was contaminated with both human NoV genogroups (NoVGI and NoVGII). Interestingly, the possibility has also been suggested that coexistence of NoV genogroups in an outbreak could be a good indicator for a shellfish-related origin of the outbreak (Hamano et al. 2005). However, due to the lack of information on potential outbreaks originated from the batches of samples analysed in the current study, this hypothesis cannot be corroborated here. No actions were taken when positive samples were found since this was out of the scope of this study, and no current legislation applies for enteric viruses in shellfish.

In conclusion, this study provides relevant information on the presence of potentially pathogenic enteric viruses in shellfish, especially NoVGII. Regarding the potential value of HAdV as indicator virus in routine screening, there was no significant correlation between the viral indicator HAdV and the target viruses.

Acknowledgments This work was supported by the European Commission Framework Program 7 project "Integrated monitoring and control of foodborne viruses in European food supply chains (VITAL)" (Grant No. KBBE 213178) led by the coordination team of Nigel Cook (FERA, UK), Martin D'Agostino (FERA, UK) and Franco M. Ruggeri (ISS, Italy). MD-V received a Ph.D. studentship from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Spanish Ministry of Science and Innovation.

References

Baert, L., Wobus, C. E., Van Coillie, E., Thackray, L. B., Debevere, J., & Uyttendaele, M. (2008). Detection of murine norovirus 1 by using plaque assay, transfection assay, and real-time reverse

transcription-PCR before and after heat exposure. *Applied and Environmental Microbiology, 74*(2), 543–546.

Baker, K., Morris, J., McCarthy, N., Saldana, L., Lowther, J., Collinson, A., et al. (2011). An outbreak of norovirus infection linked to oyster consumption at a UK restaurant, February 2010. *Journal of Public Health (Oxford), 33*(2), 205–211.

Bosch, A., Sánchez, G., Abbaszadegan, M., Carducci, A., Guix, S., Le Guyader, F. S., et al. (2011). Analytical methods for virus detection in water and food. *Food Analytical Methods, 4*(1), 4–12.

Butt, A. A., Aldridge, K. E., & Sanders, C. V. (2004). Infections related to the ingestion of seafood Part I: Viral and bacterial infections. *The Lancet Infectious Diseases, 4*(4), 201–212.

Costafreda, M. I., Bosch, A., & Pintó, R. M. (2006). Development, evaluation, and standardization of a real-time TaqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples. *Applied and Environmental Microbiology, 72*(6), 3846–3855.

Croci, L., Losio, M. N., Suffredini, E., Pavoni, E., Di Pasquale, S., Fallacara, F., et al. (2007). Assessment of human enteric viruses in shellfish from the northern Adriatic sea. *International Journal of Food Microbiology, 114*(2), 252–257.

D'Agostino, M., Cook, N., Di Bartolo, I., Ruggeri, F. M., Martelli, F., Banks, M., et al. (2012). Multicenter collaborative trial evaluation of a method for detection of human adenoviruses in berry fruit. *Food Analytical Methods, 5*(1), 1–7.

D'Agostino, M., Cook, N., Rodríguez-Lázaro, D., & Rutjes, S. (2011). Nucleic acid amplification-based methods for detection of enteric viruses: Definition of controls and interpretation of results. *Food and Environmental Virology, 3*(2), 55–60.

da Silva, A. K., Le Saux, J. C., Parnaudeau, S., Pommepuy, M., Elimelech, M., & Le Guyader, F. S. (2007). Evaluation of removal of noroviruses during wastewater treatment, using real-time reverse transcription-PCR: different behaviors of genogroups I and II. *Applied and Environmental Microbiology, 73*(24), 7891–7897.

De Roda Husman, A. M., Lodder, W. J., Rutjes, S. A., Schijven, J. F., & Teunis, P. F. (2009). Long-term inactivation study of three enteroviruses in artificial surface and groundwaters, using PCR and cell culture. *Applied and Environmental Microbiology, 75*(4), 1050–1057.

Diez-Valcarce, M., Cook, N., Hernández, M., & Rodríguez-Lázaro, D. (2011a). Analytical application of a sample process control in detection of foodborne viruses. *Food Analytical Methods, 4*(4), 614–618.

Diez-Valcarce, M., Kovac, K., Cook, N., Rodríguez-Lázaro, D., & Hernández, M. (2011b). Construction and analytical application of internal amplification controls (IAC) for detection of foodborne viruses by (RT) real-time PCR. *Food Analytical Methods, 4*(3), 437–445.

EU. 1991. Council Directive 91/492/EEC of 15 July 1991 laying down the health conditions for the production and the placing on the market of live bivalve mollusks. *Official Journal L268*, 1–14.

EU. 2004. Commission regulation (EC) No. 854/2004 of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. *Official Journal L139*, 83–127.

Formiga-Cruz, M., Tofiño-Quesada, G., Bofill-Mas, S., Lees, D. N., Henshilwood, K., Allard, A. K., et al. (2002). Distribution of human virus contamination in shellfish from different growing areas in Greece, Spain, Sweden, and the United Kingdom. *Applied and Environmental Microbiology, 68*(12), 5990–5998.

Hamano, M., Kuzuya, M., Fujii, R., Ogura, H., & Yamada, M. (2005). Epidemiology of acute gastroenteritis outbreaks caused by noroviruses in Okayama. *Japan Journal of Medical Virology, 77*(2), 282–289.

Henshilwood, K., Dore, W. J., Anderson, S., & Lees, D. N. (2003). Investigation of Norwalk like virus elimination during depuration using a real time quantitative PCR. In A. Villalba, J. L. Romalde, B. Reura, & R. Beiras (Eds.), *Molluscan shellfish safety* (pp. 451–465). Paris: UNESCO.

Hernroth, B. E., Conden-Hansson, A. C., Rehnstam-Holm, A. S., Girones, R., & Allard, A. K. (2002). Environmental factors influencing human viral pathogens and their potential indicator organisms in the blue mussel, *Mytilus edulis*: the first Scandinavian report. *Applied and Environmental Microbiology, 68*(9), 4523–4533.

Jothikumar, N., Cromeans, T. L., Robertson, B. H., Meng, X. J., & Hill, V. R. (2006). A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. *Journal of Virological Methods, 131*(1), 65–71.

Le Guyader, F., Haugarreau, L., Miossec, L., Dubois, E., & Pommepuy, M. (2000). Three-year study to assess human enteric viruses in shellfish. *Applied Environmental Microbiology, 66*(8), 3241–3248.

Le Guyader, F. S., Le Saux, J. C., Ambert-Balay, K., Krol, J., Serais, O., Parnaudeau, S., et al. (2008). Aichi virus, norovirus, astrovirus, enterovirus, and rotavirus involved in clinical cases from a French oyster-related gastroenteritis outbreak. *Journal of Clinical Microbiology, 46*(12), 4011–4017.

Le Guyader, F., Loisy, F., Atmar, R. L., Hutson, A. M., Estes, M. K., Ruvoën-Clouet, N., et al. (2006). Norwalk virus-specific binding to oyster digestive tissues. *Emerging Infectious Diseases, 12*(6), 931–936.

Lees, D. (2000). Viruses and bivalve shellfish. *International Journal of Food Microbiology, 59*(1–2), 81–116.

Loisy, F., Atmar, R. L., Guillon, P., Le Cann, P., Pommepuy, M., & Le Guyader, F. S. (2005). Real-time RT-PCR for norovirus screening in shellfish. *Journal of Virological Methods, 123*(1), 1–7.

Lowther, J. A., Henshilwood, K., & Lees, D. N. (2008). Determination of norovirus contamination in oysters from two commercial harvesting areas over an extended period, using semiquantitative real-time reverse transcription PCR. *Journal of Food Protection, 71*(7), 1427–1433.

Maalouf, H., Schaeffer, J., Parnaudeau, S., Le Pendu, J., Atmar, R. L., Crawford, S. E., et al. (2011). Strain-dependent norovirus bioaccumulation in oysters. *Applied and Environmental Microbiology, 77*(10), 3189–3196.

Martínez-Martínez, M., Diez-Valcarce, M., Hernández, M., & Rodríguez-Lázaro, D. (2011). Design and application of nucleic acid standards for quantitative detection of enteric viruses by real-time PCR. *Food and Environmental Virology, 3*(2), 92–98.

Mesquita, J. R., Vaz, L., Cerqueira, S., Castilho, F., Santos, R., Monteiro, S., et al. (2011). Norovirus, hepatitis A virus and enterovirus presence in shellfish from high quality harvesting areas in Portugal. *Food Microbiology, 28*(5), 936–941.

Myrmel, M., Berg, E. M., Rimstad, E., & Grinde, B. (2004). Detection of enteric viruses in shellfish from the Norwegian coast. *Applied and Environmental Microbiology, 70*(5), 2678–2684.

Pintó, R. M., Costafreda, M. I., & Bosch, A. (2009). Risk assessment in shellfish-borne outbreaks of hepatitis A. *Applied and Environmental Microbiology, 75*(23), 7350–7355.

Rippey, S. R. (1994). Infectious diseases associated with molluscan shellfish consumption. *Clinical Microbiology Reviews, 7*(4), 419–425.

Rodríguez-Lázaro, D., Cook, N., Ruggeri, FM., Sellwood, J., Nasser, A., Nascimento, MS., D'Agostino, M., Santos, R., Saiz, JC., Rzeżutka, A., Bosch, A., Gironés, R., Carducci, A., Muscillo, M., Kovač, K., Diez-Valcarce, M., Vantarakis, A., von Bonsdorff, CH., de Roda Husman, A. M., Hernández, M., van der

Poel, WH (2011). Virus hazards from food, water and other contaminated environments. FEMS Microbiology Reviews. doi: 10.1111/j.1574-6976.2011.00306.x.

Rodríguez-Lázaro, D., Lombard, B., Smith, H., Rzezutka, A., D'Agostino, M., Helmuth, R., et al. (2007). Trends in analytical methodology in food safety and quality: monitoring microorganisms and genetically modified organisms. *Trends in Food Science & Technology, 18*(6), 306–319.

Schwab, K. J., Neill, F. H., Estes, M. K., Metcalf, T. G., & Atmar, R. L. (1998). Distribution of Norwalk virus within shellfish following bioaccumulation and subsequent depuration by detection using RT-PCR. *Journal of Food Protection, 61*(12), 1674–1680.

Silva, H. D., García-Zapata, M. T. A., & Anunciação, C. E. (2011). Why the use of adenoviruses as water quality virologic marker? *Food and Environmental Virology, 3*(3-4), 138–140.

Silva, A. M., Vieira, H., Martins, N., Granja, A. T., Vale, M. J., & Vale, F. F. (2010). Viral and bacterial contamination in recreational waters: a case study in the Lisbon bay area. *Journal of Applied Microbiology, 108*(3), 1023–1031.

Suffredini, E., Pepe, T., Ventrone, I., & Croci, L. (2011). Norovirus infection in shellfish using two Real-Time PCR methods. New Microbiologica, 34(1), 9-16

Svraka, S., Duizer, E., Vennema, H., de Bruin, E., van der Veer, B., Dorresteijn, B., et al. (2007). Etiological role of viruses in outbreaks of acute gastroenteritis in The Netherlands from 1994 through 2005. *Journal of Clinical Microbiology, 45*(5), 1389–1394.

Terio, V., Martella, V., Moschidou, P., Di Pinto, P., Tantillo, G., & Buonavoglia, C. (2010). Norovirus in retail shellfish. *Food Microbiology, 27*(1), 29–32.

Teunis, P. F. M., Lodder, W. J., Heisterkamp, S. H., & de Roda Husman, A. M. (2005). Mixed plaques: statistical evidence how plaque assays may underestimate virus concentrations. *Water Research, 39*(17), 4240–4250.

Vilariño, M. L., Le Guyader, F. S., Polo, D., Schaeffer, J., Kröl, J., & Romalde, J. L. (2009). Assessment of human enteric viruses in cultured and wild bivalve molluscs. *International Microbiology, 12*(3), 145–151.

Wyn-Jones, A. P., Carducci, A., Cook, N., D'Agostino, M., Diviza, M., Fleischer, J., et al. (2011). Surveillance of adenoviruses and noroviruses in European recreational waters. *Water Research, 45*(3), 1025–1038.

Yilmaz, H., Bostan, K., Turan, N., Muratoglu, K., Yilmaz, A., Ozkul, A., et al. (2010). Real-time PCR detection of norovirus in mussels collected from the Bosphorus in Istanbul, Turkey. *Food and Environmental Virology, 2*(2), 64–68.

Inactivation Studies

Section 3.3

3.3.1

Effect of High Hydrostatic Pressure Processing on Norovirus Infectivity and Genome Stability in Strawberry Puree and Mineral Water

Katarina Kovač[1], Marta Diez-Valcarce[1], Peter Raspor[2], Marta Hernández[1] and David Rodríguez-Lázaro[1]

[1] Instituto Tecnológico Agrario de Castilla y León (ITACyL), Valladolid, Spain [2] Biotechnology, Microbiology and Food Safety, Biotechnical Faculty, University of Ljubljana, Slovenia

Published in International Journal of Food Microbiology, Vol. 152, 35-39

International Journal of Food Microbiology 152 (2012) 35–39

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Short communication

Effect of high hydrostatic pressure processing on norovirus infectivity and genome stability in strawberry puree and mineral water

Katarina Kovač [a,1], Marta Diez-Valcarce [a,1], Peter Raspor [b], Marta Hernández [a,*], David Rodríguez-Lázaro [a,**]

[a] InstitutoTecnológicoAgrario de Castilla y León (ITACyL), Valladolid, Spain
[b] Chair of Biotechnology, Microbiology and Food Safety, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia

ARTICLE INFO

Article history:
Received 6 May 2011
Received in revised form 4 October 2011
Accepted 8 October 2011
Available online 14 October 2011

Keywords:
Foodborne viruses
Norovirus
Inactivation
HHP
Real-time PCR

ABSTRACT

We report an evaluation of the effect of various combinations of pressures and times on the inactivation of norovirus (NoV) in two types of matrices that are important in NoV transmission: water and soft fruits. The human NoV surrogate murine norovirus was used as the model virus. The effect of HHP on the viral genome was evaluated by using RT real-time PCR (RT-qPCR), and infectivity assay was used to assess effects on the ability of the virus to attach to and replicate in cells. HHP treatments of 400 MPa for 2.5 min proved to be sufficient for efficient inactivation of NoV (>99.9% reduction). The efficacy of viral inactivation was highly dependent on the matrix in which the virus was present. Therefore, the effect of HHP should be carefully studied in all matrices to which HHP could potentially be applied. Finally, we found no consistent correlation between RT-qPCR and virus infectivity results, and consequently RT-qPCR is not a satisfactory tool for predicting risks to human health.
© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Noroviruses (NoVs) are the most common cause of both outbreaks and sporadic cases of acute gastroenteritis worldwide (Noda et al., 2008). They are estimated to cause 58% and 30% of all foodborne illnesses involving known pathogens annually in the USA and Australia, respectively (Scallan et al., 2011; Hall et al., 2005). NoV outbreaks are often the result, initially, of exposure to contaminated food or water, and then usually spread further by contact with primary cases (Becker et al., 2000). Foodborne outbreaks reported in recent years have been associated with shellfish (Baker et al., 2010) and fresh produce including raspberries (Maunula et al., 2009), vegetable salads (Grotto et al., 2004) and lettuce (Ethelberg et al., 2010). Waterborne outbreaks have also been well documented and have been caused by contaminated drinking water (ter Waarbeek et al., 2010) or recreational waters (Sartorius et al., 2007).

Efficient food processing technologies are needed to improve the safety of food products and water. Thermal treatment is the most widely used procedure for microbial inactivation in foods but can cause undesired side effects on the sensory, nutritional and functional properties of foods, and consequently alternative improved food processing techniques are being developed (Mañas and Pagán, 2005). One promising alternative is high hydrostatic pressure (HHP) technology. It preserves the organoleptic characteristics of food products, while rendering them microbiologically safe with an extended shelf life (Fonberg-Broczek et al., 2005; Patterson, 2005). However, although the effects of HHP on foodborne pathogenic bacteria have been extensively studied, less is known about pressure inactivation of enteric viruses (Kovač et al., 2010). Furthermore, the mechanism by which virus may be inactivated by HHP is not yet well understood.

An important aspect of studies on virus inactivation is that the rate of inactivation is determined by the reduction of the ability of the virus to attach to and replicate in cell culture (Kovač et al., 2010). However, human NoVs have not been reliably propagated in cell cultures, and therefore the information concerning the survival and inactivation are mainly obtained by using surrogate viruses, principally feline calicivirus (FCV) and murine norovirus (MNV-1) (Doultree et al., 1999; Hewitt et al., 2009). MNV-1 shares biochemical and molecular similarities, an identical route of infection with NoV (Wobus et al., 2006) and it is more acid-tolerant than FCV (Cannon et al., 2006), which is a characteristic probably shared with human NoV as they survive in the acidic gastric tract (Wobus et al., 2006). Therefore it is recognised to be more suitable model of human NoV.

In general, the degree of virus inactivation by HHP increases as pressure and/or time of treatment increase. However, effect of HHP on enteric viruses is extremely diverse. Poliovirus (oral poliovirus vaccine, serotype 1) survives treatments with 600 MPa for 2 h (Oliveira et al.,

* Correspondence to: Instituto Tecnológico Agrario de Castilla y León (ITACyL), Ctra. Burgos, km,119. 47071 Valladolid, Spain. Tel.: +34 983 41 5287; fax: +34 983 410462.
** Correspondence to: Instituto Tecnológico Agrario de Castilla y León (ITACyL), Ctra. Burgos, km,119. 47071 Valladolid, Spain. Tel.: +34 983 317383; fax: +34 983 410462.
E-mail addresses: ita-herperma@itacyl.es (M. Hernández), ita-rodlazda@itacyl.es, davidrl@ibgm.uva.es (D. Rodríguez-Lázaro).
[1] These authors contributed equally to the work.

0168-1605/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2011.10.005

1999), while hepatitis A virus (HAV) titres can be reduced for more than 6 $\log_{10}$ by a 450 MPa treatment for 5 min (Kingsley et al., 2002). An important factor that can affect viral inactivation by HHP is the local environment or substrate in which the virus is found (Kovač et al., 2010). For example, increasing the concentration of sugar or salt protects viruses against HHP (Kingsley and Chen, 2008; 2009; Kingsley et al., 2002). This effect has also been described for MNV-1 suspensions supplemented with $CaCl_2$ (Sánchez et al., 2011). Foods are highly complex matrices, and their components therefore significantly affect the extent of viral inactivation. Diverse effects have been observed in different types of foods or even in the same food contaminated with different viruses. For example, oysters provide baroprotective effect for FCV and MNV-1 against HHP (Kingsley et al., 2007; Murchie et al., 2007; Li et al., 2009). However, discrepancies have been observed in the results of HAV inactivation by HHP in oysters and in buffers with similar pH and NaCl concentration: Kingsley and Chen (2009) observed that HAV was more resistant in oyster homogenates suggesting that some oyster components are baroprotective, while Grove and co-workers (Grove et al., 2009) observed a greater HAV inactivation in oyster homogenate than in buffered medium for several pressure and salt combinations. HAV was also found to be more pressure sensitive in strawberries and green onions than in cell culture medium probably due to lower pH of real food (Kingsley et al., 2002, 2005). However, MNV-1 was shown to be more sensitive to pressure at neutral than acidic pH (Lou et al., 2011). The diversity among these results indicates that more studies are needed to evaluate the effect of foods on virus inactivation by HHP.

The aim of this study was to examine and compare the effect of different combinations of pressures and times on MNV-1 genome and infectivity by RT real-time PCR (RT-qPCR) and $TCID_{50}$ (50% Tissue Culture Infectious Dose) assay, respectively in two types of matrices, water and strawberry puree, important in NoV transmission. Our results provide the first accurate and precise description of the relationship between the reduction of virus infectivity and its genome degradation after HHP treatment of foods and water.

2. Materials and methods

2.1. Food and water samples

Bottled mineral water and fresh strawberries were obtained from local markets. To prepare the strawberry puree, strawberries were homogenised with a mechanical household blender (Bosch, Munich, Germany). Bottled mineral water contained 105 mg/L bicarbonate, 17.3 mg/L sulphate, 2.8 mg/L chloride, 5.8 mg/L nitrate, 27.2 mg/L calcium, 8.8 mg/L of magnesium, 4.8 mg/L of sodium, <1 mg/L of potassium, and 0.2 mg/L of fluor. The conductivity of water was 204 μS/cm.

2.2. Virus and cells

Murine norovirus (MNV-1) was propagated on confluent monolayers of RAW 264.7 cells, a mouse macrophage cell line. RAW 264.7 cells were grown in Dulbecco's modified Eagle medium (DMEM) (Gibco-Invitrogen, NY, USA) supplemented with 10% heat-inactivated foetal bovine serum (Gibco-Invitrogen), 1× antibiotic/antimycotic suspension (Gibco-Invitrogen) and 2 mM L-Glutamine (Gibco-Invitrogen) at 37 °C and 5% CO_2. After appearance of cytopathic effect, cell lysates were freeze-thawed three times and centrifuged at 1300×*g* for 25 min at 4 °C, to remove the cell debris. Aliquots of supernatant containing the virus were stored at −80 °C.

2.3. High hydrostatic pressure treatment

Fifteen milliliter samples of bottled mineral water or 15 g samples of strawberry puree were introduced into pressure resistant multilayered bags and inoculated with 100 μL of MNV-1 stock (~1×10^7 $TCID_{50}$/mL). Samples were gently mixed and the bags were heat-sealed using a vacuum packaging EU-7 apparatus (Tecnotrip, Barcelona, Spain). Strawberry puree samples were then incubated for ~12 h at 4 °C to facilitate attachment of the viruses to the puree before the HHP treatment. Samples were placed in another bag containing an ice-water mixture to keep them at low temperature (~4 °C), heat-sealed and introduced into the HHP device. As adiabatic heating is approximately 3 °C/100 MPa (Jung et al., 2010) the temperature of the sample did not rise over 25 °C. The samples were pressurised in a WAVE 6000/135 High Pressure Processing apparatus (NC Hyperbaric, Burgos, Spain), using water at ~15 °C as the hydrostatic medium, to one of various pressures (200, 300, 400 and 600 MPa) for various times (2.5, 5 and 10 min). The pressure come up rate was ~120 MPa/min. Pressure release time was in all cases less than 10 s. The treatment times described in the paper do not include come-up and come-down times. Control samples (non-pressurised) were processed in a similar way to the HHP-treated samples, except that they did not undergo the HHP treatment. Virus infectious particle counts and virus genome equivalents were determined after the HHP treatment and the results are reported as $\log_{10}$ (N_t/N_0) where N_0 and N_t are the infectious virus titres or the virus genome equivalents of the untreated and the HHP-treated samples, respectively.

2.4. Concentration of MNV-1 from matrices

After HHP treatment MNV-1 was concentrated from samples of bottled mineral water and strawberry puree using the following methods.

2.4.1. Concentration of MNV-1 from bottled mineral water

Fifteen milliliter samples were transferred to Amicon Ultra-15 Ultracel-100K Centrifugal Filter devices (Millipore, Molsheim, France) and centrifuged for 20 min at 3220×*g* (Kovač et al., 2009). Immediately after centrifugation, the concentrated samples (~140 μL) were decontaminated by sequential filtering through 0.45 μm and 0.22 μm pore-size Costar® Spin-X® Centrifuge Tube Filters (Corning, NY, USA) for 2 min at 10000×*g* (Butot et al., 2008). Filtered samples were then subjected to RNA extraction or infectivity assay.

2.4.2. Concentration of MNV-1 from strawberry puree

The protocol described by Dubois et al. (2002) was used to concentrate MNV-1 from strawberry puree. Briefly, 40 mL of Tris Glycine 1% Beef Extract (TGBE) buffer pH 9.5 including 6500 U of pectinase produced from *Aspergillus aculeatus* (Sigma-Aldrich, St. Louis, MO, USA) was added to the samples and the samples were agitated by rocking for 20 min at 50 rpm to elute the virus from strawberry puree. The eluate was collected and the pH was determined and adjusted with sodium hydroxide (1 N) to pH 9.4. The samples were transferred to Bag® Page F filter stomacher bags (Interscience, Saint Nom la Bretèche, France) to remove particles of puree. The resulting filtrate was transferred to 50 mL Corning centrifuge tubes (Corning, NY, USA) and centrifuged at 10,000×*g* for 30 min at 4 °C. The supernatant was decanted into a sterile beaker and the pH was adjusted with hydrochloric acid (1 N) to 7.2. The samples were transferred into 50 ml centrifuge tubes and 0.25 volumes of 5× PEG/NaCl solution (Sigma-Aldrich) were added, and the solution was mixed and incubated with gentle rocking at 4 °C for 60 min. After centrifugation at 10000×*g* for 30 min at 4 °C the supernatant was discarded and the sample was centrifuged again at 10000×g for 5 min and 4 °C. The pellet was resuspended in 500 μL of PBS and the suspension transferred to a chloroform-resistant tube. Then 500 μL of chloroform:butanol solution (1:1) was added, and the sample mixed by vortexing, left to stand for 5 min and centrifuged at 10000×*g* for 15 min at 4 °C. The aqueous phase was transferred into a 1.5 ml tube and the concentrated samples were decontaminated as described above.

2.5. RNA extraction, RT real-time PCR and virus genome quantification

RNA was extracted with the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Virus RNA eluates were stored at −80 °C until use. One-step RT real-time PCR (RT-qPCR) assays were performed and analysed essentially as described by Diez-Valcarce et al. (2011) using the Light Cycler 480 RNA Master Hydrolysis Probes Kit (Roche Diagnostics, Mannheim, Germany) in a 20 μL reaction volume containing 1× Light Cycler® 480 RNA Master Hydrolysis Probes kit buffer, 3.25 mM Activator, 200 nM ORF1/ORF2 primers and 200 nM of MGB-ORF1/ORF2 probe (Baert et al., 2008) and 10 μL of the RNA solution. Reactions were run on a Light Cycler® 480 II apparatus (Roche Diagnostics, Mannheim, Germany) using the following programme: 30 min at 63 °C, 30 s at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. One-step RT-qPCR assays were analysed using SW 1.5 software (Roche Diagnostics, Mannheim, Germany). Virus genome equivalents were quantified by interpolation in a standard regression curve of Cq (quantification cycle; Bustin et al., 2009) values generated from 10-fold serial dilutions of RNA from virus with known titre. Unless otherwise stated, all reactions were performed in triplicate.

2.6. Infectivity assay

$TCID_{50}$ assay was used to determine virus titre before and after HHP treatments. Freshly prepared RAW 264.7 cells were diluted in complete DMEM to a concentration of 2×10^5 cells/mL and 100 μl was seeded into each well in a 96-well plate. After 4 h of incubation at 37 °C under 5% CO_2, 100 μL aliquots of ten-fold serial dilutions of concentrated treated or untreated virus, prepared in DMEM (Gibco-Invitrogen) supplemented with 2% heat-inactivated foetal bovine serum (Gibco-Invitrogen), 1× antibiotic/antimycotic suspension (Gibco-Invitrogen) and 2 mM L-Glutamine (Gibco-Invitrogen), were added to eight wells on the plate per dilution. Plates were incubated at 37 °C under 5% CO_2 and checked every 2 days for the presence of cytopathic effect. The wells with cytopathic effect were recorded as positive. After 1 week the final reading was performed and the tissue culture infectious dose ($TCID_{50}$/mL) was calculated using the Kärber formula (Kärber, 1931):

$$\log TCID_{50} = L - d \times (S - 0.5)$$

where L is the log starting dilution, d is difference between log dilutions and S is the sum of the proportion of positive replicates.

2.7. Statistical analysis

The significance of differences between the means for virus inactivation determined by the infectivity assay and RT-qPCR, and between the different treatments in each matrix were evaluated by the Student's t-test with a significance level of $P<0.05$. The SPSS 16.0 Statistical Analysis software (SPSS Inc., Chicago, IL, USA) was used.

3. Results and discussion

3.1. Effect of HHP on MNV-1 infectivity in bottled mineral water and strawberry puree

Mineral water and strawberry puree samples were experimentally contaminated with MNV-1 and the effect of HHP treatment on the infectivity of virus was determined (Fig. 1). As expected, the reduction in MNV-1 infectivity increased as the pressure of the treatment increased. At pressures of 200 MPa, MNV-1 inactivation was approximately 1 $\log_{10}$ or below for all treatment times and in both matrices, except for strawberry puree samples treated with 200 MPa for 10 min (3.21 $\log_{10}$ reduction). Similar inactivation rates (~1 $\log_{10}$) were also observed in water samples for HHP treatments at 300 MPa. Inactivation rates in strawberry puree samples treated with 300 MPa were higher (1.21, 2.63 and 2.75 $\log_{10}$ for treatments at 300 MPa for 2.5, 5 and 10 min, respectively). A previous study reported a greater reduction of FCV titres in mineral water with a treatment of 200 MPa for 6 min: a 5 FCV $\log_{10}$ reduction (Buckow et al., 2008) compared to almost no MNV-1 reduction (0.17 $\log_{10}$) observed in our study. This discrepancy reveals that inactivation results of model virus must be interpreted with caution, and that the selection and suitability of models of human NoV for studies on elimination and survival is a critical issue.

At higher pressures (400 and 600 MPa), MNV-1 was inactivated to undetectable levels in both matrices at all treatment times, i.e. $\log_{10}$ reductions of more than 5.13 in water and 3.33 in strawberry puree (Fig. 1). Similar results were obtained in a previous study in which treatment at 400 MPa for 5 min was sufficient to inactivate MNV-1 in oysters to undetectable levels (>4.1 PFU - plaque forming units) (Li et al., 2009).

The effectiveness of HHP treatment at low pressures was clearly dependent on the matrix (Fig. 1). At 200 MPa for 2.5 min or 5 min and 300 MPa for 2.5 min, there was no significant difference ($P>0.05$) in the reduction of MNV-1 titres between strawberry puree and mineral water. However, after longer treatment times (200 MPa for 10 min and 300 MPa for 5 or 10 min) MNV-1 inactivation in strawberry puree was significantly better than that in water. The reductions were 1.17 and 1.33 $\log_{10}$ in mineral water but 2.63 and 2.75 $\log_{10}$ in strawberry puree following treatment at 300 MPa for 5 and 10 min, respectively. These differences may be consequences of the acidic pH of strawberry puree (~3.5) as low pH significantly enhances pressure inactivation of virus (Kingsley and Chen, 2009). Similar results have also been reported previously for comparisons of HAV infectivity reduction in two different matrices with different pHs – strawberry puree (pH 3.67) and green onions (pH 5.12) (Kingsley et al., 2005) – and for various foodborne bacteria including *Salmonella* spp., *Staphylococcus aureus*, *Escherichia coli* (Alpas et al., 2000) and *Listeria monocytogenes* (Alpas et al., 2000; Stewart et al., 1997). Interestingly an opposite effect was observed by Lou and co-workers (Lou et al., 2011): MNV-1 was less sensitive to HHP when treated in strawberry puree or in cell culture medium with acidic pH compared to treatment at neutral pH. The reason for this discrepancy in the results is not clear, and more detailed studies on the effect of combination of pH and HHP should be undertaken.

3.2. Effect of HHP on MNV-1 genomes in bottled water and strawberry puree

It has been suggested that HHP does not affect viral RNA (Kingsley et al., 2002; Li et al., 2009; Lou et al., 2011, Tang et al., 2010). However, previous studies only used conventional RT-PCR and therefore viral genomes were not quantified. We demonstrate that HHP reduced the numbers of MNV-1 genomes significantly ($P<0.05$) in all treatment protocols, except for the 200 MPa 2.5 and 5 min treatment of virus in mineral water (Fig. 1). $\log_{10}$ genome reductions in mineral water samples increased with increasing pressure: the reductions were 0.49, 1.05, 2.78 and 3.25 $\log_{10}$ for 200, 300, 400 and 600 MPa treatments, respectively (mean results for all the times at each pressure). Note, however, that the differences between the 400 and 600 MPa treatments were not significant. In contrast, the reduction in genome copy numbers in strawberry puree was in the range from 0.40 to 1.67 $\log_{10}$ regardless of the treatment pressure and time; there were no significant differences between the different pressures using the average results of all treatment times for each pressure. A previous study using pressures higher than 300 MPa for 15 min at 25 °C described significant reductions in genome copy number in contaminated cell culture samples (Sánchez et al., 2011), but the reductions were smaller than those we report for mineral

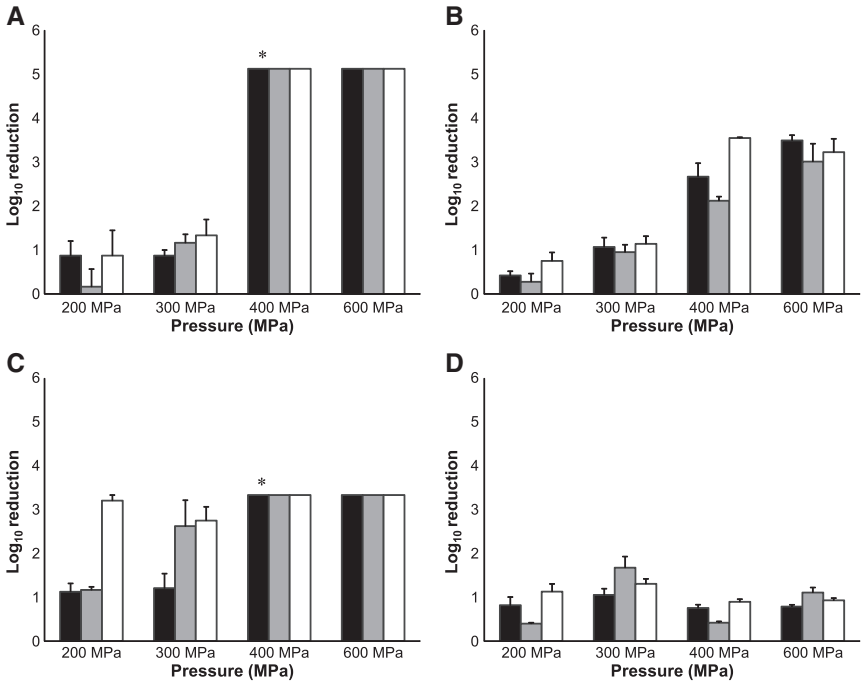


Fig. 1. Effect of HHP treatment on MNV-1 in bottled mineral water (A, B) and strawberry puree (C, D) samples. Columns present $\log_{10}$ reductions of virus infectivity (A, C) and virus genome counts (B, D) after treatments with 200, 300, 400 and 600 MPa for 2.5 min (black), 5 min (grey) and 10 min (white). Each reported value is the mean of three replicates ± standard deviations, calculated as $\log_{10}(N_t/N_0)$, where N_0 is the titre of the virus in untreated controls and N_t is the titre after the HHP treatment. Virus infectivity was effectively eliminated by 400 and 600 MPa treatments. Asterisks (*) indicate the combination of lowest treatment pressure and time, sufficient for complete inactivation of MNV-1 in both matrices.

water samples. If $CaCl_2$ was added to the cell culture solution, HHP had no significant effect on genome counts (Sánchez et al., 2011). Our results and previous findings clearly indicate that the effect of HHP on the viral load and the $\log_{10}$ genome counts depends significantly on the composition of the matrix in which the virus is assayed.

3.3. Comparison of genome degradation and virus infectivity results after treatment with HHP

Virus inactivation is generally determined by measuring infectivity on cell cultures (Baert et al., 2009). However, this is a time consuming procedure and some enteric viruses are not able to replicate in cell cultures. Consequently, new alternative molecular methods for determination of viral infectivity are being developed (Rodríguez et al., 2009), but such methods have not been extensively evaluated for different types of foodborne viruses. Although PCR does not differentiate between infective and noninfective viruses (Richards, 1999), it may be a valuable technique if a relationship could be established between loss of infectivity as assessed with cell cultures and genome degradation as determined by RT-qPCR. We therefore investigated the relationship, if any, between the results of these two methods after HHP treatment of MNV-1 in food matrices.

Following HHP treatment of MNV-1 in strawberry puree the reductions of virus infectivity were significantly greater ($P<0.05$) than the reduction of virus genome counts as measured by PCR, except for 200 MPa 2.5 min treatment and 300 MPa 2.5 and 5 min treatment (Fig. 1). By contrast, for low pressure (200 and 300 MPa) treatments of the virus in mineral water there were no significant differences ($P>0.05$) between the reduction of genome number and that of virus infectivity: both quantification methods indicated inactivation of between 0.17 and 0.88 $\log_{10}$ for 200 MPa and between 0.88 and 1.33 $\log_{10}$ for 300 MPa (Fig. 1). However, at higher pressures, reduction of the virus infectivity was significantly greater ($P<0.05$) than that of virus genome number for MNV-1 in water (Fig. 1). Similar results were obtained in the study of Sánchez et al. (2011) in which the reduction of the number of virus genomes and of infective viruses following treatment at 300 (at 25 and 45 °C) and 350 MPa (at 25 °C) were comparable whereas no correlation was found for treatments at higher pressures.

In conclusion, we demonstrate that treatments of 400 MPa for 2.5 min are sufficient for effective (>99.9% reduction) inactivation of MNV-1. Therefore, HHP is a promising technique for inactivating NoV in foods as the pressures currently used in commercial HHP applications (up to 600 MPa) (Tonello, 2009) may be sufficient to inactivate low levels of norovirus contamination of produce and water. However, our results also demonstrate that the effectiveness of viral inactivation is highly dependent on the matrix in which the virus is present. Therefore, the effect of HHP should be rigorously evaluated in any matrix to which HHP may be applied before industrial exploitation. Finally, our results did not show a consistent relationship between RT-qPCR and virus infectivity results, and consequently RT-qPCR is not an appropriate tool for predicting viral infectious risks to human health.

Acknowledgments

We thank Dr. Herbert W. Virgin IV, Washington University School of Medicine (US) for providing the virus strain murine norovirus 1.

Katarina Kovač was supported by a FEMS Research Fellowship 2009–2, a short-term scientific mission of COST Action Number 929

(COST-STSM-929-05122) and a PhD student fellowship from the University of Leon, (Spain).

References

Alpas, H., Kalchayanand, N., Bozoglu, F., Ray, B., 2000. Interactions of high hydrostatic pressure, pressurization temperature and pH on death and injury of pressure-resistant and pressure-sensitive strains of foodborne pathogens. International Journal of Food Microbiology 60 (1), 33–42.

Baert, L., Wobus, C.E., Van Coillie, E., Thackray, L.B., Debevere, J., Uyttendaele, M., 2008. Detection of murine norovirus 1 by using plaque assay, transfection assay, and real-time reverse transcription-PCR before and after heat exposure. Applied and Environmental Microbiology 74 (2), 543–546.

Baert, L., Debevere, J., Uyttendaele, M., 2009. The efficacy of preservation methods to inactivate foodborne viruses. International Journal of Food Microbiology 131 (2–3), 83–94.

Baker, K., Morris, J., McCarthy, N., Saldana, L., Lowther, J., Collinson, A., Young, M., 2010. An outbreak of norovirus infection linked to oyster consumption at a UK restaurant, February 2010. Journal of Public Health 33 (2), 205–211.

Becker, K.M., Moe, C.L., Southwick, K.L., MacCormack, J.N., 2000. Transmission of Norwalk virus during football game. The New England Journal of Medicine 343 (17), 1223–1227.

Buckow, R., Isbarn, S., Knorr, D., Heinz, V., Lehmacher, A., 2008. Predictive model for inactivation of feline calicivirus, a norovirus surrogate, by heat and high hydrostatic pressure. Applied and Environmental Microbiology 74 (4), 1030–1038.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical Chemistry 55 (4), 611–622.

Butot, S., Putallaz, T., Sánchez, G., 2008. Effects of sanitation, freezing and frozen storage on enteric viruses in berries and herbs. International Journal of Food Microbiology 126 (1–2), 30–35.

Cannon, J.L., Papafragkou, E., Park, G.W., Osborne, J., Jaykus, L.A., Vinjé, J., 2006. Surrogates for the study of norovirus stability and inactivation in the environment: a comparison of murine norovirus and feline calicivirus. Journal of Food Protection 69 (11), 2761–2765.

Diez-Valcarce, M., Kovač, K., Cook, N., Rodriguez-Lazaro, D., Hernandez, M., 2011. Construction and analytical application of internal amplification controls (IAC) for detection of food supply chain-relevant viruses by real-time PCR-based assays. Food Analytical Methods 4 (3), 437–445.

Doultree, J.C., Druce, J.D., Birch, C.J., Bowden, D.S., Marshall, J.A., 1999. Inactivation of feline calicivirus, a Norwalk virus surrogate. The Journal of Hospital Infection 41 (1), 51–57.

Dubois, E., Agier, C., Traore´, O., Hennechart, C., Merle, G., Crucie`re, C., Laveran, H., 2002. Modified concentration method for the detection of enteric viruses on fruits and vegetables by reverse transcriptase–polymerase chain reaction or cell culture. Journal of Food Protection 65 (12), 1962–1969.

Ethelberg, S., Lisby, M., Bottiger, B., Schultz, A.C., Villif, A., Jensen, T., Olsen, K.E., Scheutz, F., Kjelso, C., Muller, L., 2010. Outbreaks of gastroenteritis linked to lettuce, Denmark, January 2010. Euro Surveillance 15 (6).

Fonberg-Broczek, M., Windyga, B., Szczawiński, J., Szczawińska, M., Pietrzak, D., Prestamo, G., 2005. High pressure processing for food safety. Acta Biochimica Polonica 52 (3), 721–724.

Grotto, I., Huerta, M., Balicer, R.D., Halperin, T., Cohen, D., Orr, N., Gdalevich, M., 2004. An outbreak of norovirus gastroenteritis on an Israeli military base. Infection 32 (6), 339–343.

Grove, S.F., Lee, A., Stewart, C.M., Ross, T., 2009. Development of a high pressure processing inactivation model for hepatitis A virus. Journal of Food Protection 72 (7), 1434–1442.

Hall, G., Kirk, M.D., Becker, N., Gregory, J.E., Unicomb, L., Millard, G., Stafford, R., Lalor, K., OzFoodNet Working Group, 2005. Estimating foodborne gastroenteritis, Australia. Emerging Infectious Diseases 11 (8), 1257–1264.

Hewitt, J., Rivera-Aban, M., Greening, G.E., 2009. Evaluation of murine norovirus as a surrogate for human norovirus and hepatitis A virus in heat inactivation studies. Journal of Applied Microbiology 107 (1), 65–71.

Jung, S., Tonello Samson, C., de Lamballerie, M., 2010. High hydrostatic pressure food processing. In: Proctor, A. (Ed.), Alternatives to Conventional Food Processing. Royal Society of Chemistry, London, pp. 254–306.

Kärber, G., 1931. Beitrag zur kollektiven behandlung pharmakologische reihenversuche [A contribution to the collective treatment of a pharmacological experimental series]. Archiv fur Experimentelle Pathologie und Pharmakologie 162, 480–483.

Kingsley, D.H., Chen, H., 2008. Aqueous matrix compositions and pH influence feline calicivirus inactivation by high pressure processing. Journal of Food Protection 71 (8), 1598–1603.

Kingsley, D.H., Chen, H., 2009. Influence of pH, salt, and temperature on pressure inactivation of hepatitis A virus. International Journal of Food Microbiology 130 (1), 61–64.

Kingsley, D.H., Hoover, D.G., Papafragkou, E., Richards, G.P., 2002. Inactivation of hepatitis A virus and calicivirus by high hydrostatic pressure. Journal of Food Protection 65 (10), 1605–1609.

Kingsley, D.H., Guan, D., Hoover, D.G., 2005. Pressure inactivation of hepatitis a virus in strawberry puree and sliced green onions. Journal of Food Protection 68 (8), 1748–1751.

Kingsley, D.H., Holliman, D.R., Calci, K.R., Chen, H., Flick, G.J., 2007. Inactivation of a norovirus by high-pressure processing. Applied and Environmental Microbiology 73 (2), 581–585.

Kovač, K., Gutierrez-Aguirre, I., Banjac, M., Peterka, M., Poljšak-Prijatelj, M., Ravnikar, M., Mijovski, J.Z., Schultz, A.C., Raspor, P., 2009. A novel method for concentrating hepatitis A virus and caliciviruses from bottled water. Journal of Virological Methods 162, 272–275.

Kovač, K., Diez-Valcarce, M., Hernandez, M., Raspor, P., Rodriguez-Lazaro, D., 2010. High hydrostatic pressure as emergent technology for the elimination of foodborne viruses. Trends in Food Science & Technology 21 (11), 558–568.

Li, D., Tang, Q., Wang, J., Wang, Y., Zhao, Q., Xue, C., 2009. Effects of high-pressure processing on murine norovirus-1 in oysters (*Crassostrea gigas*) in situ. Food Control 20 (11), 992–996.

Lou, F., Needoo, H., Chen, H., Li, J., 2011. Inactivation of human norovirus surrogate by high pressure processing: effectiveness, mechanism and potential application in fresh produce industry. Applied and Environmental Microbiology 77 (5), 1862–1871.

Mañas, P., Pagán, R., 2005. Microbial inactivation by new technologies of food preservation. Journal of Applied Microbiology 98 (6), 1387–1399.

Maunula, L., Roivainen, M., Keränen, M., Mäkela, S., Söderberg, K., Summa, M., von Bonsdorff, C.H., Lappalainen, M., Korhonen, T., Kuusi, M., Niskanen, T., 2009. Detection of human norovirus from frozen raspberries in a cluster of gastroenteritis outbreaks. Euro Surveillance 14 (49).

Murchie, L.W., Kelly, A.L., Wiley, M., Adair, B.M., Patterson, M., 2007. Inactivation of a calicivirus and enterovirus in shellfish by high pressure. Innovative Food Science & Emerging Technologies 8 (2), 213–217.

Noda, M., Fukuda, S., Nishio, O., 2008. Statistical analysis of attack rate in norovirus foodborne outbreaks. International Journal of Food Microbiology 122 (1–2), 216–220.

Oliveira, A.C., Ishimaru, D., Goncalves, R.B., Smith, T.J., Mason, P., Sa´-Carvalho, D., Silva, J.L., 1999. Low temperature and pressure stability of picornaviruses: implications for virus uncoating. Biophysical Journal 76 (3), 1270–1279.

Patterson, M.F., 2005. Microbiology of pressure-treated foods. Journal of Applied Microbiology 98 (6), 1400–1409.

Richards, G.P., 1999. Limitations of molecular biological techniques for assessing the virological safety of foods. Journal of Food Protection 62 (6), 691–697.

Rodríguez, R.A., Pepper, I.L., Gerba, C.P., 2009. Application of PCR-based methods to assess the infectivity of enteric viruses in environmental samples. Applied and Environmental Microbiology 75 (2), 297–307.

Sánchez, G., Aznar, R., Martínez, A., Rodrigo, D., 2011. Inactivation of human and murine norovirus by high-pressure processing. Foodborne Pathogens and Disease 8 (2), 249–253.

Sartorius, B., Andersson, Y., Velicko, I., De Jong, B., Löfdahl, M., Hedlund, K.O., Allestam, G., Wångsell, C., Bergstedt, O., Horal, P., Ulleryd, P., Soderstrom, A., 2007. Outbreak of norovirus in Västra Götaland associated with recreational activities at two lakes during August 2004. Scandinavian Journal of Infectious Diseases 39 (4), 323–331.

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.A., Roy, S.L., Jones, J.L., Griffin, P.M., 2011. Foodborne illness acquired in the United States—major pathogens. Emerging Infectious Diseases 17 (1), 7–15.

Stewart, C.M., Jewett, F.F., Dunne, C.P., Hoover, D.G., 1997. Effect of concurrent high hydrostatic pressure, acidity and heat on the injury and destruction of *Listeria monocytogenes*. Journal of Food Safety 17 (1), 23–36.

Tang, Q., Li, D., Xu, J., Wang, J., Zhao, Y., Li, Z., Xue, C., 2010. Mechanism of inactivation of murine norovirus-1 by high pressure processing. International Journal of Food Microbiology 137 (2–3), 186–189.

ter Waarbeek, H.L., Dukers-Muijrers, N.H., Vennema, H., Hoebe, C.J., 2010. Waterborne gastroenteritis outbreak at a scouting camp caused by two norovirus genogroups: GI and GII. Journal of Clinical Virology 47 (3), 268–272.

Tonello, C., 2009. Commercial applications including processing capacities and costs, Proceedings of Workshop on Ultra Fresh and Chilled Meals. Wageningen, The Netherlands.

Wobus, C.E., Thackray, L.B., Virgin IV, H.W., 2006. Murine norovirus: a model system to study norovirus biology and pathogenesis. Journal of Virology 80 (11), 5104–5112.

3.3.2

Evaluation of High Hydrostatic Pressure Effect on Human Adenovirus Using Molecular Methods and Cell Culture

Katarina Kovač[1], Martijn Bouwknegt[2], Marta Diez-Valcarce[1], Peter Raspor[3], Marta Hernández[1] and David Rodríguez-Lázaro[1]

[1] Instituto Tecnológico Agrario de Castilla y León (ITACyL), Valladolid, Spain [2]National Institute for Public Health and the Environment (RIVM), Bilthoven, Netherlands [3] Biotechnology, Microbiology and Food Safety, Biotechnical Faculty, University of Ljubljana, Slovenia

Published in International Journal of Food Microbiology, Vol. 157, 368-374

International Journal of Food Microbiology 157 (2012) 368–374

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Evaluation of high hydrostatic pressure effect on human adenovirus using molecular methods and cell culture

Katarina Kovač [a], Martijn Bouwknegt [b], Marta Diez-Valcarce [a], Peter Raspor [c], Marta Hernández [a,*], David Rodríguez-Lázaro [a,**]

[a] Instituto Tecnológico Agrario de Castilla y León (ITACyL), Junta de Castilla y León, Valladolid, Spain
[b] Laboratory for Zoonoses and Environmental Microbiology, Centre for Infectious Disease Control Netherlands, National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands
[c] Chair of Biotechnology, Microbiology and Food Safety, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia

ARTICLE INFO

Article history:
Received 11 April 2012
Received in revised form 1 June 2012
Accepted 7 June 2012
Available online 15 June 2012

Keywords:
Foodborne viruses
Adenovirus
Inactivation
HHP
Cell culture
Real-time PCR

ABSTRACT

Human adenoviruses (HAdV) are shed in human faeces and can consequently contaminate environmental waters and possibly be transferred to foods by irrigation. Therefore, efficient inactivation technologies for water and foods are needed. High hydrostatic pressure (HHP) processing is a non-thermal, energy-efficient and rapid emergent inactivation technology, which has been widely studied to eliminate pathogenic micro-organisms in foods. We have applied HHP to HAdV-2 in water and cell culture medium (CCM) and measured the effect on virus infectivity and genome and capsid integrity, by using infectivity assay, real-time PCR (qPCR) and qPCR with prior enzymatic treatment (ET-qPCR) with Proteinase K and DNase I. While lower pressures did not provide satisfactory inactivation levels, 400 and 600 MPa treatments were estimated to reduce virus infectivity by approximately 6 $\log_{10}$ units when effectively applied for 93 s and 4 s, respectively (i.e., excluding come up times of the pressure unit). However, virus genome remained intact even when higher pressures were applied. While acidic pH protected HAdV-2 from inactivation with HHP, no baroprotective effect was observed when 1% sucrose was added to the CCM. On the other hand, 10 mM $CaCl_2$ added to the CCM was estimated to protect HAdV-2 from HHP with longer treatment times (>10 min). When virus was treated in bottled mineral water, significantly higher infectivity reduction was observed compared to the same treatment in CCM. In conclusion, HHP was shown to effectively reduce HAdV-2 infectivity up to 6.5 $\log_{10}$ units within 4 s and can thus contribute to public health protection for food- and water-borne virus transmission. However, its precise effect is matrix dependent and therefore matrix-specific evaluations need to be considered for assuring reliable inactivation in practice.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Human adenoviruses (HAdV) are non-enveloped viruses with double-stranded DNA genome. They can cause a wide range of diseases from gastroenteritis, respiratory tract or eye infections to haemorrhagic cystitis or meningoencephalitis (Mena and Gerba, 2009). Gastroenteritis is caused particularly by serotypes 40/41, however almost all serotypes can replicate in the gastrointestinal tract. They can establish persistent infections and therefore viral particles may be shed in faeces for months or even years (Rodríguez-Lázaro et al., 2012). Consequently, HAdV have been found in sewage, and in river, coastal, swimming pool and drinking waters (Haramoto et al., 2007; Kokkinos et al., 2011). Adenovirus infection can be caused by consumption of contaminated water or inhalation of aerosolized droplets during water recreation (Dong et al., 2010) which can lead to waterborne outbreaks with both, respiratory (e.g. serotypes 1, 2, 5, 6) and enteric (e.g. serotypes 40, 41) human adenovirus serotypes (Jiang, 2006). Even though no foodborne outbreak with adenovirus has been documented so far, potential viral transmission by foods is possible as they were already detected in raw vegetables (Cheong et al., 2009) and shellfish (Umesha et al., 2008). Consequently, efficient inactivation technologies for water and foods are needed. Different strategies have been developed to eliminate HAdV, mostly based on inactivation of virus by UV technologies (e.g. Baxter et al., 2007; Meng and Gerba, 1996; Thurston-Enriquez et al., 2003b), ozone (Thurston-Enriquez et al., 2005), or chemical disinfectants such as free chlorine (Baxter et al., 2007; Cromeans et al., 2010; Thurston-Enriquez et al., 2003a), monochloramine (Baxter et al., 2007; Cromeans et al., 2010; Sirikanchana et al., 2008) or by combination of these technologies (Lee and Shin, 2011; Shin and Lee, 2010).

High hydrostatic pressure (HHP) processing is a non-thermal, energy-efficient and emergent inactivation technology, which has

* Corresponding author. Tel.: +34 983 41 5287; fax: +34 983 410462.
** Corresponding author. Tel.: +34 983 415233; fax: +34 983 410462.
E-mail addresses: ita-herperma@itacyl.es (M. Hernández), ita-rodlazda@itacyl.es (D. Rodríguez-Lázaro).

0168-1605/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2012.06.006

been widely studied to eliminate pathogenic microorganisms in foods (Bermúdez-Aguirre and Barbosa-Cánovas, 2011). HHP-treated foods maintain most of their natural characteristics like colour, flavour and health promoting substances (Kingsley et al., 2005). The effect of HHP has been already studied for inactivation of enteric viruses, especially norovirus and hepatitis A virus (reviewed in Kovač et al., 2010). However, there is little information of the effect of HHP on HAdV inactivation (Leonard et al., 2007; Wilkinson et al., 2001). Therefore, the aim of this study was to dissect the general effect of HHP on HAdV. We evaluated the inactivation capacity of the HHP on HAdV, and characterized its effect on HAdV infectivity, and genome and capsid integrity using $TCID_{50}$ (50% Tissue Culture Infectious Dose) assay, real-time PCR (qPCR), and qPCR with a previous enzymatic treatment (ET-qPCR) with proteinase K and DNase I. We also aimed to evaluate the suitability of the two molecular methods to quantify infective virus after the treatment with HHP, and finally we evaluated the applicability of this methodology in bottled water.

Chemical composition of the substrate can drastically affect the severity of HHP treatment on microorganisms (Patterson, 2005). For example, pH and water activity of foods can significantly influence the inactivation of microorganisms by HHP (Patterson, 2005). Low pH, which is characteristic for several fruits (e.g. berries) and vegetables (e.g. tomato), and pressure can act synergistically leading to enhanced microbial inactivation (Kingsley and Chen, 2009; Patterson, 2005). However, protection effect of lower pH in murine norovirus (MNV-1) was also previously observed (Lou et al., 2011). Furthermore, some food components such as proteins, lipids, carbohydrates or cations can confer a protective effect (Patterson, 2005). Sucrose, an important natural component (~1%) of berries (Souci et al., 2008) which have been linked to virus-borne outbreaks (Hjertqvist et al., 2006; Maunula et al., 2009), was recognized to protect feline calicivirus (FCV) when treated with HHP (Kingsley and Chen, 2008). Ca^{2+} is a cation, known to be baroprotective (Patterson, 2005) and found in several foods in different concentrations. Calcium treatments, particularly addition of $CaCl_2$ to foods, maintain or improve tissue firmness and crispness (Sánchez et al., 2011). Therefore, we also evaluated the effect of pH and the presence of $CaCl_2$ or sucrose as potentially protective agents on inactivation of virus when treated with HHP. Finally, as FCV, an RNA virus, has been reported to be more efficiently inactivated in mineral water than in cell culture medium (Buckow et al., 2008), our third objective was to evaluate if this same effect of water as a matrix is also observed in HAdVs.

2. Materials and methods

2.1. Virus and cells

Human adenovirus serotype 2 (HAdV-2) was propagated on confluent monolayers of human lung carcinoma A-549 cells (European Collection of Cell Cultures, Salisbury, UK). Cells were grown in cell culture medium (CCM) consisting of Dulbecco's modified Eagle medium (DMEM) (Gibco-Invitrogen, NY, USA) supplemented with 10% heat-inactivated foetal bovine serum (Gibco-Invitrogen), 1× antibiotic/antimycotic suspension (Gibco-Invitrogen) and 2 mM L-glutamine (Gibco-Invitrogen) at 37 °C and 5% CO_2. After appearance of cytopathic effect, cell lysates were freeze-thawed three times and centrifuged at 1300×*g* for 25 min at 4 °C to remove the cell debris. Aliquots of virus supernatant were stored at −80 °C.

2.2. High hydrostatic pressure treatment

HAdV-2 stocks, prepared in CCM, were used for HHP treatment. To check the effect of pH on HAdV-2 inactivation, the pH of virus stock was adjusted to 4.0 using hydrochloric acid (1 N). The effect of $CaCl_2$ or sucrose on HAdV-2 inactivation was evaluated by adjusting the virus stock to final concentration of 10 mM $CaCl_2$ or 1% sucrose, respectively before HHP treatment. Effect of a real matrix, bottled mineral water, on virus inactivation was evaluated by diluting 100 μL of HAdV-2 in 900 μL of water. One-ml aliquots of HAdV-2 in CCM (~7×10^9 $TCID_{50}$ mL^{-1}) or 1-mL samples of bottled mineral water containing HAdV-2 (~7×10^8 $TCID_{50}$ mL^{-1}) were introduced in Pasteur pipettes, which were heat-sealed using a vacuum packaging EU-7 apparatus (Tecnotrip, Tarrasa, Spain). Each Pasteur pipette was placed in pressure resistant multilayered bag containing water. Bags were heat-sealed and overlaid with another bag containing water and again heat sealed. In order to keep virus at low temperature (~4 °C) and thus minimize the effects of adiabatic heating, which is around 3 °C/100 MPa (Jung et al., 2010), the bags were stored on ice until the HHP treatment. The samples were pressurised in a WAVE 6000/135 High Pressure Processing Equipment (NC Hyperbaric, Burgos, Spain). Different combinations of time (1, 2.5, 5 and 10 min) and pressure (200, 250, 300, 350, 400 and 600 MPa) were applied. Water at ~4 °C was used as the hydrostatic medium. The pressure come up rate was ~120 MPa min^{-1}. Pressure release time was in all cases less than 10 s. The treatment times described in this study do not include come-up and come-down times. Control samples (non-pressurised) were processed in a similar way, except that they did not undergo the HHP treatment. Each experiment was performed in triplicate.

2.3. Enzymatic treatment

The HAdV-2 samples subjected to HHP and without HHP treatment (controls) were treated with a combination of Proteinase K (Sigma-Aldrich, St. Louis, MO, USA) and DNase I (Roche Diagnostics, Mannheim, Germany) as follows: 20 U of Proteinase K was added per 100 μL of sample and the sample incubated at 37 °C for 30 min. Then 1 U of DNase I was added per 100 μL of sample and the sample was incubated at 37 °C for 1 h. The reaction was stopped by incubation of samples for 30 min at 75 °C and DNA was extracted immediately.

2.4. DNA extraction and qPCR

DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Virus DNA eluates were stored at −80 °C until use. qPCR assays were performed and analysed as described previously (Diez-Valcarce et al., 2011a) using the Light Cycler 480 Probes Master (Roche Diagnostics, Mannheim, Germany) in a 25 μL reaction volume containing 1× Light Cycler 480 Probes master kit buffer, 900 nM Ad-F/R primers and 225 nM of AdP1 probe (Hernroth et al., 2002) and 5 μL of the DNA solution. Reactions were run on a Light Cycler 480 II apparatus (Roche Diagnostics, Mannheim, Germany) using the following programme: 10 min at 95 °C and 45 cycles of 15 s at 95 °C and 1 min at 60 °C. qPCR assays were analysed using SW 1.5 software (Roche Diagnostics, Mannheim, Germany). Virus genome equivalents were quantified by interpolation in a standard regression curve of Cq (quantification cycle; Bustin et al., 2009) values generated from 10-fold serial dilutions of DNA from virus with known titres. All reactions were performed in triplicate.

2.5. Infectivity assay

$TCID_{50}$ assay was performed as described previously (Kovač et al., 2012) using A-549 cells. Plates were checked for the presence of cytopathic effect on the days 10 and 14. Wells with cytopathic effect were recorded as positive. The tissue culture infectious dose ($TCID_{50}$ mL^{-1}) was calculated after the second reading with the Kärber formula (Kärber, 1931):

$$\log_{10}TCID_{50} = L - d \times (S - 0.5) \quad (1)$$

where L is the $\log_{10}$ starting dilution, d is the difference between $\log_{10}$ dilutions and S is the sum of the proportion of positive replicates.

2.6. Statistical analysis

Decay of infectious virus was fit to different statistical models to test hypothesis regarding the inactivation rate. Firstly, constant exponential decay was modelled as (Chick, 1908):

$$\log_{10}[c_t] = \log_{10}[c_0 \exp(-\lambda t)] \quad (2)$$

with c_t being the infectious virus concentration after treatment for duration t, c_0 is the starting concentration and λ the constant inactivation rate. Secondly, a mixture of two constant inactivation rates was modelled according to De Roda Husman et al. (2009) as:

$$\log_{10}[c_t] = \log_{10}[c_0(w \exp(-\lambda_1 t) + (1-w)\exp(-\lambda_2 t)] \quad (3)$$

where w is the mixing parameter and λ_1 and λ_2 are the two constant inactivation rates. Thirdly, data were fitted to a model to test the hypothesis that inactivation rates change continuously as t increases. The first model is described by Cho et al. (2003) and involves an exponentially changing decay rate:

$$\log_{10}[c_t] = \log_{10}[c_0 \exp(-\lambda_1[1-\exp(-\lambda_2 t)]]. \quad (4)$$

In case both a shoulder and tail were observed, as was the case for pressure-dependent inactivation, the data were fitted to the model described by Geeraerd et al. (2000) with the modification that the shoulder length (ω_i) is pressure-dependent:

$$\log_{10}\left[c_p\right] = \log_{10}\left[(c_0 - c_{res}) \cdot \exp(-\lambda p) \cdot \frac{\exp(\lambda(\omega_1 - p\omega_2))}{1 + (\exp(\lambda(\omega_1 - p\omega_2)) - 1)\exp(-\lambda p)} + c_{res}\right]. \quad (5)$$

In this equation, p is the pressure in MPa and c_{res} is the residual infectivity constituting the tail. The parameter estimates were obtained by maximum likelihood estimation, and the best fitting model was chosen using the corrected Aikaike Information Criterion (lowest was considered best) (Hurvich and Tsai, 1989). Statistical tests for differences in inactivation rates between experiments for which the observed inactivation was described with the same model family were done with the likelihood ratio test ($\alpha = 0.05$). The 95% intervals for the maximum likelihood parameters were obtained by Markov Chain Monte Carlo sampling from the likelihood functions using the Metropolis–Hastings algorithm (Gilks et al., 1996). In this procedure, new values for parameters were randomly drawn from a normal distribution with $\mu = 0$ and standard deviation such that 35–40% of samples were accepted. The burn-in was 2500 iterations and the chain was stopped when 10,000 samples were accepted. The chains were explored graphically to examine its stability.

Predictions for 1 and 6 $\log_{10}$ unit reduction were based on the most likely parameter values, estimates for the 2.5% and 97.5% limits were calculated from the MCMC posterior. All analyses were done in Mathematica version 8 (Wolfram Research, Champaign, IL, USA).

3. Results and discussion

3.1. Effect of HHP on HAdV-2 infectivity

Results of the HAdV-2 infectivity reduction due to HHP for 1, 2.5, 5 and 10 min, as evaluated by cell culture assay ($TCID_{50}$), are presented in Fig. 1. The parameter estimates are shown in Table 1. Virus stocks were treated in cell culture medium (CCM) of pH = 7.8. No decay was observed for pressures of 200 and 250 MPa applied for up to 10 min. These results coincide with the study of Wilkinson et al. (2001) where with 200 MPa treatment for 15 min infectivity of HAdV-2 was unaffected. Viruses were found to be inactivated at pressures ≥ 300 MPa, with a dynamic mode of decay with increasing pressure: for 300 MPa a single (constant) decay rate explained the results, for 350 MPa a mixture of two decay rates (biphasic) and for 400 and 600 MPa a time-dependent decay rate. A possible explanation for dynamicity in decade mode is that virus particles differ in susceptibility to pressure. Low pressure affects only the most sensitive particles at a constant rate, whereas increasing pressure and time applied affect particles with lower susceptibility-levels as well, but at different decay rates. From a statistical perspective, the extremes in the observations could be explained adequately by either the absence of a decay rate (no decay) or an infinite number of decay rates (i.e., as function of time of pressure applied), with the monophasic and biphasic modes as transitory phases between these two. When analysing the inactivation as a function of pressure, an initial phase without inactivation was observed, followed by a steep decay and a subsequent tailing (Fig. 2). Using Eq. (5), with pressure and the time applied as explanatory variables, no inactivation was estimated to occur for pressures up to approximately 325 MPa when applied for 1 min, and decreased with 4.3 MPa per minute extra application to approximately 285 MPa when applied for 10 min. The tailing is estimated to occur after about 6.5 $\log_{10}$ unit reduction (from 5.6×10^9 to 1.8×10^3).

Table 2 shows the estimated time required for 1 and 6 $\log_{10}$ unit reduction in infectivity for the different pressures applied. The increased pressure applied significantly decreased time needed for the defined $\log_{10}$ reduction. No decay was observed for pressures up to 250 MPa when applied for 10 min, but we cannot predict whether HAdV-2 infectivity reduction would occur with prolonged treatment time. However, when applying only 50 MPa more, estimated mean time for 1 and 6 $\log_{10}$ reduction is 417 and 2500 s, respectively (estimated using Eq. (2)). Increasing the pressure from 350 to 400 MPa decreased the time needed for a 6 $\log_{10}$ reduction 10-fold, while a 1 $\log_{10}$ reduction is observed in approximately the same mean time with both pressures. Six hundred MPa is most efficient among the pressures tested in HAdV-2 infectivity reduction with a 6 $\log_{10}$ unit reduction estimated to occur in about 4 s (estimated using Eq. (4)). Note that these estimates do not consider the come-up time required by the pressure unit to reach the respective pressure.

3.2. Effect of HHP on HAdV-2 capsid and genome integrity in comparison to reduction of virus infectivity

To examine whether the observed loss of infectivity was caused by genome degradation, the number of genomes in samples was evaluated

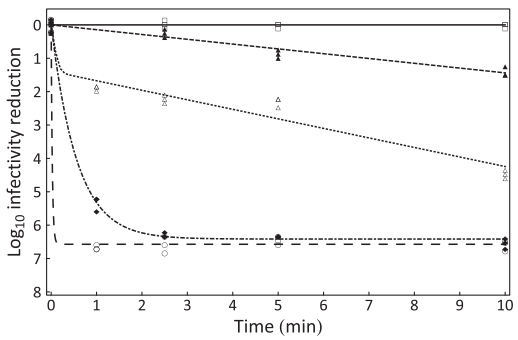


Fig. 1. Effect of HHP treatment on HAdV-2 in cell culture medium (CCM) as a function of time. Markers represent single observations of $\log_{10}$ reductions of virus infectivity after treatment with 250 (□; –), 300 (▲; ---), 350 (Δ; …), 400 (♦; -·-) and 600 (○; — — —) MPa. The lines represent the fitted models. The application of a pressure of 200 MPa did not lead to observable inactivation, similar to 250 MPa, and is therefore not depicted in the figure.

Table 1
Estimated model parameters and 95% confidence intervals. The parameters correspond to the parameters from Eqs. (2), (3) or (4).

Treatment	Parameter											
	c_0		w		λ_1		λ_2		κ_1		κ_2	
	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI
200 MPa	6×10^9	5×10^9–8×10^9	–	–	0	–	–	–	–	–	–	–
250 MPa	7×10^9	6×10^9–8×10^9	–	–	0	–	–	–	–	–	–	–
300 MPa, CCM	7×10^9	5×10^9–1×10^{10}	–	–	0.32	0.25–0.40	–	–	–	–	–	–
350 MPa, CCM	7×10^9	3×10^9–2×10^{10}	0.96	0.89–0.99	10^4	6–10^8	0.63	0.48–0.78	–	–	–	–
350 MPa, pH 4	2×10^9	2×10^9–4×10^{10}	0.95	0.91–0.97	31	5–4000	0.42	0.35–0.48	–	–	–	–
350 MPa, sucrose	4×10^9	2×10^9–9×10^{10}	0.98	0.95–0.99	757	4–6×10^5	0.60	0.45–0.74	–	–	–	–
350 MPa, $CaCl_2$	3×10^9	2×10^9–4×10^9	–	–	–	–	–	–	10.7	10.0–11.5	0.36	0.30–0.42
350 MPa, water	4×10^8	2×10^8–6×10^8	–	–	–	–	–	–	16.0	15.2–16.6	0.54	0.48–0.60
400 MPa	7×10^9	4×10^9–1×10^{10}	–	–	–	–	–	–	14.8	14.2–15.3	1.8	1.6–2.0
600 MPa	7×10^9	4×10^9–1×10^{10}	–	–	–	–	–	–	15.2	14.5–15.8	11.2	4.1–27

by qPCR. Some studies have quantified the genome integrity of RNA viruses after HHP treatment (Kovač et al., 2012; Sánchez et al., 2011). We aimed to quantitatively evaluate genome (DNA) integrity of HAdV-2 treated in CCM using a range of pressures from 200 to 600 MPa during 1, 2.5, 5 and 10 min. No decay was observed for all pressures except 200 MPa (p-value 0.04). However, the latter decay was minimal, with an estimated 1 $\log_{10}$ reduction after about 160 min of applying pressure. Given the absence of decay for lower and higher pressure applied in this experiment, the decay at 200 MPa is not likely to be caused by a structural effect of pressure on genome integrity. Even though only a fragment (Hernroth et al., 2002) of viral genome was amplified, our results suggest that viral DNA was not degraded by HHP. Similarly, reduction of genome counts for RNA viruses did not correlate with reduction of infectivity after HHP treatment, except in some cases when low pressures were applied (≲300 MPa) (Kovač et al., 2012; Sánchez et al., 2011).

To examine whether the observed loss of infectivity was caused by damage to the viral capsid, samples were subjected to enzymatic treatment (ET) with Proteinase K — to degrade affected capsids and release DNA and DNase — to degrade unprotected DNA of affected virus. This was done in the most severe HHP condition (600 MPa for 5 min). Similar ET approach (using RNase instead of DNase) was successfully used to differentiate between an intact virus (hepatitis A virus, poliovirus 1, feline calicivirus) and a virus inactivated by different disinfection methods (UV light disinfection, chlorine disinfection, and thermal treatment) (Nuanualsuwan and Cliver, 2002). Therefore, the concentration of Proteinase K (20 U) applied to HAdV-2 was adopted from previous experiments. As in our case DNA virus was used, DNase effectiveness on extracted DNA from HAdV-2 was evaluated firstly. One, 2 and 5 U of DNase provided ~7 log reduction of genome counts which is more than the maximum reduction of virus titre during different combinations of time and pressure applied. Therefore, the lowest concentration (1 U) was used for further experiments.

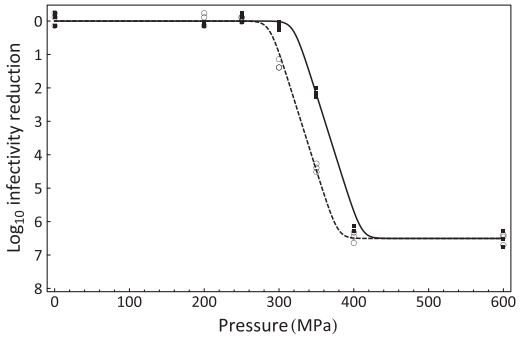


Fig. 2. Pressure-dependent inactivation when applied for 2.5 (■; –) and 10 (○; ---) minutes. The initial phase without inactivation was estimated to be 325 MPa when applied for 1 min and decreased by approximately 4.3 MPa per each extra minute of application.

Applying ET prior to qPCR reduced virus genome counts for 2.5 $\log_{10}$ whereas, in the control sample not exposed to pressure, but with ET, no reduction in genome counts was observed. These results suggest that HHP affected HAdV-2 capsid proteins to the point that they still protect DNA from degradation (no reduction of genome counts by qPCR), but made these proteins more susceptible to digestion by Proteinase K, and thus allowed DNase to eliminate genomes of affected virus particles (evident reduction of genome counts by ET-qPCR). Our results coincide with the results for MNV-1 (Diez-Valcarce et al., 2011b). Nevertheless, the difference between results of qPCR compared to ET-qPCR in the case of MNV-1 was much lower, which may suggest that the susceptibility of MNV-1 capsid proteins to HHP is lower compared to HAdV-2 and therefore Proteinase K degrades more successfully HAdV-2 than MNV-1 capsid. A possible reason for the discrepancy in results between MNV-1 and HAdV-2 could be the difference in structure and shape of these two viruses.

When comparing the infectivity reduction based on genome counts by qPCR or ET-qPCR to the infectivity reduction based on the cell culture assay using the same treatment (600 MPa, 5 min), the infectivity reduction was significantly higher with cell culture assay (6.5 $\log_{10}$). Therefore, our results indicate that quantification of virus genomes by qPCR or ET-qPCR after HHP treatment is not adequate to assess HAdV-2 infectivity reduction. They also indicate that the general effect of virus inactivation by HHP is not directly correlated

Table 2
Predicted time (in seconds or fraction thereof) required for 1 and 6 $\log_{10}$ unit reduction in HAdV-2 infectivity for different pressures applied. Viruses were present in cell culture medium (CCM) of pH 7.8, unless specified differently, with or without additives. The 'Eq.'-column specifies the best fitting model, which was also used for the prediction. Note that these estimates do not consider the come-up time required by the pressure unit to reach the respective pressure.

Treatment	Eq.	1 $\log_{10}$ unit reduction		6 $\log_{10}$ unit reduction	
		Mean	95% interval	Mean	95% interval
Up to 250 MPa in CCM	–	∞[a]	–	∞	–
300 MPa in CCM	Eq. (2)	417	347–550	2500[b]	2100–3300[b]
350 MPa, in:					
CCM	Eq. (3)	7.7	<1–59	969[b]	820–1270[b]
CCM, pH = 4	Eq. (3)	7.9	0.1–27	1547[b]	1365–1816[b]
CCM + 1% sucrose	Eq. (3)	32	1–40	1009[b]	838–1210
CCM + 10 mM $CaCl_2$	Eq. (4)	40	35–49	>3600[b]	2800[c]
Water	Eq. (4)	17	15–19	220	189–262
400 MPa in CCM	Eq. (4)	5.7	5.1–6.5	93	76–128
600 MPa in CCM	Eq. (4)	<1	<1[d]	3.7	1.6–4.6

[a] No decay observed.
[b] Note: Prediction outside of the time window observed in the experiment.
[c] Lower limit supplied only.
[d] Upper limit supplied only.

to effects on the virus genome and protein capsid integrity, but rather to effects on proteins associated with adhesion to and invasion of eukaryotic cells, which is in accordance with previous findings (Diez-Valcarce et al., 2011b; Kovač et al., 2012; Tang et al., 2010). As viruses which lose the ability to attach to the cells cannot cause infection, the only real assessment of risk to human health can be obtained by cell culture assay (e.g. $TCID_{50}$). Consequently, we only used $TCID_{50}$ assay to measure the effect of HHP on HAdV-2 in modified CCM in the subsequent experiments.

3.3. Effect of pH and the presence of $CaCl_2$ or sucrose on HAdV-2 inactivation by HHP

Virus suspensions were treated with 350 MPa for 1, 2.5, 5 or 10 min as we previously observed that HAdV-2 infectivity was affected with this pressure to the level where variation in infectivity could be measured. Results of the effect of pH, $CaCl_2$ and sucrose on HAdV-2 inactivation by HHP are presented in Figs. 3 and 4. The parameter estimates are shown in Table 1.

To determine the effect of pH on HAdV-2 inactivation with HHP, the pH of the virus solution in CCM was adjusted to 4.0 using hydrochloric acid (1 N). The infectivity reduction can be described statistically by a process involving two decay rates, for both pH levels (Fig. 3). The tailing at a pH of 4 is less steep compared to that for pH 7.8, suggesting a protective effect of an acidic environment. The time required at 350 MPa for 1 $\log_{10}$ unit inactivation was about the same at the two pH levels, but more time is required to achieve greater infectivity reductions under a pH of 4 compared to a pH of 7.8 (see also Table 2). A possible explanation for the observed effect is that a fraction is comparably susceptible to pressure for both pH (the initial decay), whereas the other fraction of acid-adapted HAdV-2 was more resistant to pressure than for HAdV-2 at higher pH. Similar results were reported in the study of Lou et al. (2011) where MNV-1 in CCM was also more efficiently inactivated at neutral (pH 7.0) than at acidic environment (pH 4.0). FCV was more efficiently inactivated when treated at pH higher than 5.2. However, reductions of FCV titers already occurred without pressure treatment at low pH (Kingsley and Chen, 2008). By contrast, significantly enhanced inactivation was found for MNV-1 (Kovač et al., 2012), and HAV (Kingsley and Chen, 2009; Kingsley et al., 2002, 2005) treated in acidic environment. It is not clear why decay rates are influenced by pH, even when applied to the same virus. However, one hypothesis for this effect may be the use of different matrices in which the viruses were treated and therefore, combination of matrix components and pH may have various effects. Alternatively, low pH might alter other characteristics of viruses, such as the isoelectric point, resulting in an observed decay that is not caused (solely) by the pressure applied. However, all

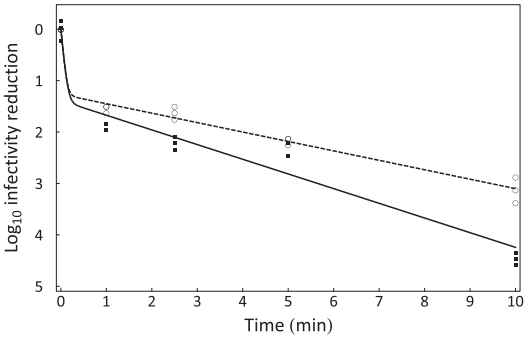


Fig. 3. Effect of HHP treatment on HAdV-2 in cell culture medium (CCM) at pH 7.8 (■; –) and CCM with pH adjusted to 4.0 (○; ---). Markers represent single observations of $\log_{10}$ reductions of virus infectivity after treatment with 350 MPa.

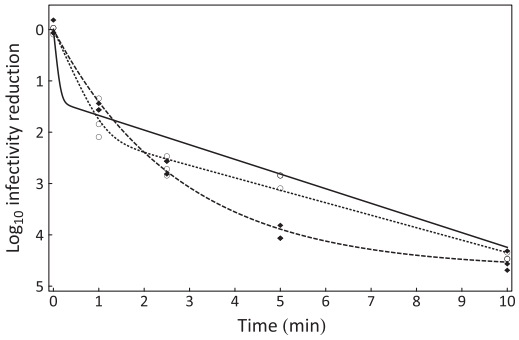


Fig. 4. Effect of HHP treatment on HAdV-2 in cell culture medium (CCM) at pH 7.8 (—) and CCM with 10 mM $CaCl_2$ (◆; ---) and CCM with 1% sucrose (○; …). Markers represent single observations of $\log_{10}$ reductions of virus infectivity after treatment with 350 MPa. The markers for CCM at pH 7.8 have been left out to improve the clarity of the figure.

these results from different studies, using different viruses, show the same tendency, suggesting that pH affects the virus inactivation with HHP.

$CaCl_2$ or sucrose was added to the virus in CCM in the concentrations usually found in foods associated with virus outbreaks (i.e., 10 mM $CaCl_2$, 1% sucrose), and the effect of these two components on HAdV-2 inactivation by HHP was evaluated (Fig. 4). Adding any of the two additives to the CCM did not alter the effect observed after a treatment of 10 min at 350 MPa. Furthermore, the decay process observed for CCM with and without sucrose was comparable (a mixture of two decay rates). An increasing protective effect of sucrose was observed for FCV when the sucrose concentration increased up to 40%, while higher concentrations (up to 70%) did not significantly enhanced this protective effect (Kingsley and Chen, 2008). As we aimed to mimic the real situation found in the food matrix, low concentration of sucrose proportional to the one present in different berries (Souci et al., 2008) normally connected to the enteric virus outbreaks, was added to the virus sample and probably therefore no protection effect was noticed. Likewise, a similar low concentration of sucrose did not provide any protection effect to the FCV (Kingsley and Chen, 2008). Therefore we can conclude that concentration of sucrose which is normally present in berries does not act baroprotective to HAdV-2.

However, the mode of decay was different for $CaCl_2$, i.e. time-dependent inactivation rate. The initial decay at 350 MPa was larger than for CCM (with or without sucrose), but greater (e.g., $\geq$6 $\log_{10}$ units) infectivity reductions were estimated to require significantly longer treatment times when $CaCl_2$ is present (Table 2). Interestingly, the same amount of $CaCl_2$ considerably increased pressure resistance of MNV-1 treated for 15 min with pressures up to 400 MPa (Sánchez et al., 2011). Given our modelling results, prolonged treatment times were expected to also show a similar baroprotective effect.

3.4. Effect of bottled mineral water as a matrix on HAdV-2 inactivation by HHP

Results of the effect of bottled mineral water on HAdV-2 inactivation compared to the virus inactivation in CCM are presented in Fig. 5. We aimed to evaluate whether virus is affected similarly when treated in CCM and a non-artificial matrix like bottled mineral water. With 350 MPa pressure applied for 1, 2.5, 5 and 10 min, the reduction of virus infectivity, measured by $TCID_{50}$ assay, was significantly higher when virus was treated in water (Fig. 5). For example, for a 6 $\log_{10}$ unit reduction in HAdV-2 infectivity when treated in water, a 4-min pressure of 350 MPa is estimated to be required. When treated in CCM, this duration was estimated to be about 16 min (Table 2). A

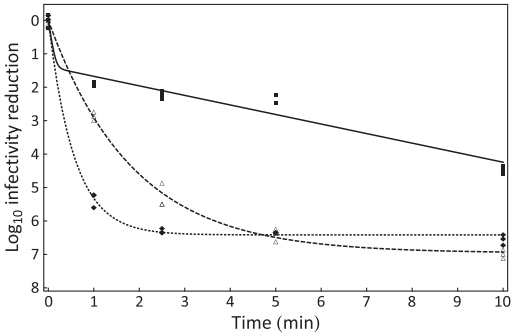


Fig. 5. Effect of HHP treatment on HAdV-2 in cell culture medium (CCM) at 350 MPa (■; –) and 400 MPa (♦; …), and HAdV-2 in bottled mineral water at 350 MPa (△; ---). Markers represent single observations of $\log_{10}$ reductions of virus infectivity after treatment with 350 or 400 MPa.

much higher reduction was also observed for FCV when treated in mineral water compared to treatment in CCM (Buckow et al., 2008). Moreover, HAdV-2 infectivity reduction by 350 MPa for ≥5 min was comparable to the maximal reductions of about 6 $\log_{10}$ units observed for HAdV-2 treated in CCM with pressures of 400 or 600 MPa for similar treatment times. These results suggest that some of the CCM components (e.g. foetal bovine serum, L-glutamine, glucose) protected virus from destruction, and therefore much lower pressure is necessary to achieve the same level of virus infectivity reduction when virus is treated in water. As most of the foods in general are complex matrices, also their components may protect viruses as observed for CCM. Therefore, CCM is a more suitable model for evaluation of virus inactivation by HHP in foods. However, studies made directly on food items (reviewed in Kovač et al., 2010) show that every single matrix, or even the same matrix in different form, acts different in terms of protection/extra reduction of the virus and therefore the real effect can be observed only when specific matrix of interest is used.

3.5. Application of HHP for inactivation of enteric viruses in the food and beverage industry

The contamination of water and food is normally with low levels of enteric viruses (Fong and Lipp, 2005; Croci et al., 2008). Consequently, a 6 $\log_{10}$ unit virus infectivity reduction is expected to reduce the food-borne viral risks substantially. Our results (see Table 2) therefore indicate that an optimal combination for inactivation of viruses by HHP in the food industry would be the use of very high pressures in combination with short treatment times (e.g. ≥400 MPa for <10 s). However, caution with predicting combination of treatment time and pressure is needed as results also show that some additives, change of pH or different matrices affect significantly the time required for effective virus infectivity reduction. While water as a matrix shortened 350 MPa treatment needed for 6 $\log_{10}$ reduction, $CaCl_2$ or lower pH prolonged it (Table 2).

Our experiments were done only using HAdV-2 as a virus model, and therefore cannot be generalized to other enteric viruses without confirmatory studies. For MNV-1, a human norovirus surrogate, for instance, HAdV-2 can serve as good proxy, since, similar to HAdV-2, a pressure of 400 MPa applied for 2.5 min completely inactivated MNV-1, treated in bottled mineral water (more than 5.13 $\log_{10}$ reduction) (Kovač et al., 2012). For hepatitis A virus (HAV) treated in CCM, however, <5.0 $\log_{10}$ reductions were observed with 400 MPa treatment even after 10 min (Kingsley et al., 2006; Grove et al., 2008, 2009), suggesting that the use of the decay rates of HAdV-2 to predict the infectivity reduction for HAV could overestimate the actual reduction.

Therefore, more inactivation studies using a range of processing and technological parameters for different food products and viruses are needed to clearly determine conditions for efficient removal of viruses and develop predictive inactivation models for practical food industry application.

In conclusion, we demonstrate that pressures of ≥400 MPa efficiently reduce the titer of infectious HAdV-2, while no reduction of virus genome counts was observed even with 600 MPa treatments. ET improved qPCR results, which means that HHP considerably affected HAdV-2 capsid. However, reductions of genome counts with or without previous ET applied did not coincide with reductions of virus infectivity and therefore these molecular methodologies are not appropriate to quantify infective virus titer after HHP treatment. Interestingly, an important effect of composition of the matrix was observed: while acidic pH protected HAdV-2 from inactivation with HHP, no baroprotective effect was observed when sucrose was added to the CCM. The addition of $CaCl_2$ to CCM showed a somewhat altered decay process: the initial decay was more rapid compared to the absence of $CaCl_2$, whereas longer treatment times were estimated to protect HAdV-2 from HHP. Finally, a significantly higher reduction was observed when viruses were treated in bottled mineral water compared to CCM, indicating therefore that the components of CCM probably act baroprotectively. Consequently, precise evaluation of the effect of different matrices on virus inactivation by HHP is needed to assure reliable inactivation of viruses in the food and beverage industry and thus contribute to public health protection for food- and water-borne virus transmission.

Acknowledgements

This study was supported by project RTA 2011-079-C02-01 of the Ministry of Economy and Competiveness, Government of Spain. Katarina Kovač was supported by a PhD student fellowship from the University of Leon, Spain.

References

Baxter, C.S., Hofmann, R., Templeton, M.R., Brown, M., Andrews, R.C., 2007. Inactivation of adenovirus types 2, 5, and 41 in drinking water by UV light, free chlorine, and monochloramine. Journal of Environmental Engineering 133 (1), 95–103.

Bermúdez-Aguirre, D., Barbosa-Cánovas, G.V., 2011. An update on high hydrostatic pressure, from the laboratory to industrial applications. Food Engineering Reviews 3 (1), 44–61.

Buckow, R., Isbarn, S., Knorr, D., Heinz, V., Lehmacher, A., 2008. Predictive model for inactivation of feline calicivirus, a norovirus surrogate, by heat and high hydrostatic pressure. Applied and Environmental Microbiology 74 (4), 1030–1038.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical Chemistry 55 (4), 611–622.

Cheong, S., Lee, C., Song, S.W., Choi, W.C., Lee, C.H., Kim, S.J., 2009. Enteric viruses in raw vegetables and groundwater used for irrigation in South Korea. Applied and Environmental Microbiology 75 (24), 7745–7751.

Chick, H., 1908. An investigation of the laws of disinfection. The Journal of Hygene 8 (1), 92–158.

Cho, M., Chung, H., Yoon, J., 2003. Disinfection of water containing natural organic matter by using ozone-initiated radical reactions. Applied and Environmental Microbiology 69 (4), 2284–2291.

Croci, L., Dubois, E., Cook, N., de Medici, D., Schultz, A.C., China, B., Rutjes, S.A., Hoorfar, J., Van der Poel, W.H.M., 2008. Current methods for extraction and concentration of enteric viruses from fresh fruit and vegetables: towards international standards. Food Analytical Methods 1 (2), 73–84.

Cromeans, T.L., Kahler, A.M., Hill, V.R., 2010. Inactivation of adenoviruses, enteroviruses, and murine norovirus in water by free chlorine and monochloramine. Applied and Environmental Microbiology 76 (4), 1028–1033.

De Roda Husman, A.M., Lodder, W.J., Rutjes, S.A., Schijven, J.F., Teunis, P.F., 2009. Long-term inactivation study of three enteroviruses in artificial surface and groundwaters, using PCR and cell culture. Applied and Environmental Microbiology 75 (4), 1050–1057.

Diez-Valcarce, M., Kovač, K., Cook, N., Rodríguez-Lázaro, D., Hernández, M., 2011a. Construction and analytical application of internal amplification controls (IAC) for detection of food supply chain-relevant viruses by real-time PCR-based assays. Food Analytical Methods 4 (3), 437–445.

Diez-Valcarce, M., Kovač, K., Raspor, P., Rodríguez-Lázaro, D., Hernández, M., 2011b. Virus genome quantification does not predict norovirus infectivity after application of food inactivation processing technologies. Food and Environmental Virology 3, 141–146.

Dong, Y., Kim, J., Lewis, G.D., 2010. Evaluation of methodology for detection of human adenoviruses in wastewater, drinking water, stream water and recreational waters. Journal of Applied Microbiology 108 (3), 800–809.
Fong, T.T., Lipp, E.K., 2005. Enteric viruses of humans and animals in aquatic environments: health risks, detection and potential water quality assessment tools. Microbiology and Molecular Biology Reviews 69 (2), 357–371.
Geeraerd, A.H., Herremans, C.H., van Impe, J.F., 2000. Structural model requirements to describe microbial inactivation during a mild heat treatment. International Journal of Food Microbiology 59 (3), 185–209.
Gilks, W.R., Richardson, S., Spiegelhalter, D.J., 1996. Markov Chain Monte Carlo in Practise. Chapman & Hall, New York, NY. 486 pp.
Grove, S.F., Forsyth, S., Wan, J., Coventry, J., Cole, M., Stewart, C.M., Lewis, T., Ross, T., Lee, A., 2008. Inactivation of hepatitis A virus, poliovirus and a norovirus surrogate by high pressure processing. Innovative Food Science & Emerging Technologies 9 (2), 206–210.
Grove, S.F., Lee, A., Stewart, C.M., Ross, T., 2009. Development of a high pressure processing inactivation model for hepatitis A virus. Journal of Food Protection 72 (7), 1434–1442.
Haramoto, E., Katayama, H., Oguma, K., Ohgaki, S., 2007. Quantitative analysis of human enteric adenoviruses in aquatic environments. Journal of Applied Microbiology 103 (6), 2153–2159.
Hernroth, B.E., Conden-Hansson, A.C., Rehnstam-Holm, A.S., Girones, R., Allard, A.K., 2002. Environmental factors influencing human viral pathogens and their potential indicator organisms in the blue mussel, *Mytilus edulis*: the first Scandinavian report. Applied and Environmental Microbiology 68 (9), 4523–4533.
Hjertqvist, M., Johansson, A., Svensson, N., Abom, P.E., Magnusson, C., Olsson, M., Hedlund, K.O., Andersson, Y., 2006. Four outbreaks of norovirus gastroenteritis after consuming raspberries, Sweden, June–August 2006. Euro Surveillance 11 (9).
Hurvich, C.M., Tsai, C.L., 1989. Regression and time series model selection in small samples. Biometrika 76 (2), 297–307.
Jiang, S.C., 2006. Human adenoviruses in water: occurrence and health implications: a critical review. Environmental Science & Technology 40 (23), 7132–7140.
Jung, S., Tonello Samson, C., de Lamballerie, M., 2010. High hydrostatic pressure food processing. In: Proctor, A. (Ed.), Alternatives to Conventional Food Processing. Royal Society of Chemistry, London, pp. 254–306.
Kärber, G., 1931. Beitrag zur kollektiven behandlung pharmakologische reihenversuche (A contribution to the collective treatment of a pharmacological experimental series). Archiv fur Experimentelle Pathologie Pharmakologie 162, 480–483.
Kingsley, D.H., Chen, H., 2008. Aqueous matrix compositions and pH influence feline calicivirus inactivation by high pressure processing. Journal of Food Protection 71 (8), 1598–1603.
Kingsley, D.H., Chen, H., 2009. Influence of pH, salt, and temperature on pressure inactivation of hepatitis A virus. International Journal of Food Microbiology 130 (1), 61–64.
Kingsley, D.H., Hoover, D.G., Papafragkou, E., Richards, G.P., 2002. Inactivation of hepatitis A virus and calicivirus by high hydrostatic pressure. Journal of Food Protection 65 (10), 1605–1609.
Kingsley, D.H., Guan, D., Hoover, D.G., 2005. Pressure inactivation of hepatitis A virus in strawberry puree and sliced green onions. Journal of Food Protection 68 (8), 1748–1751.
Kingsley, D.H., Guan, D., Hoover, D.G., Chen, H., 2006. Inactivation of hepatitis A virus by high-pressure processing: the role of temperature and pressure oscillation. Journal of Food Protection 69 (10), 2454–2459.
Kokkinos, P.A., Ziros, P.G., Mpalasopoulou, A., Galanis, A., Vantarakis, A., 2011. Molecular detection of multiple viral targets in untreated urban sewage from Greece. Virology Journal 8, 195.
Kovač, K., Diez-Valcarce, M., Hernandez, M., Raspor, P., Rodriguez-Lázaro, D., 2010. High hydrostatic pressure as emergent technology for the elimination of foodborne viruses. Trends in Food Science & Technology 21 (11), 558–568.
Kovač, K., Diez-Valcarce, M., Raspor, P., Hernández, M., Rodríguez-Lázaro, D., 2012. Effect of high hydrostatic pressure processing on norovirus infectivity and genome stability in strawberry puree and mineral water. International Journal of Food Microbiology 152 (1–2), 35–39.
Lee, J.K., Shin, G.A., 2011. Inactivation of human adenovirus by sequential disinfection with an alternative UV technology and free chlorine. Journal of Water and Health 9 (1), 53–58.
Leonard, J.N., Ferstl, P., Delgado, A., Schaffer, D.V., 2007. Enhanced preparation of adeno-associated viral vectors by using high hydrostatic pressure to selectively inactivate helper adenovirus. Biotechnology and Bioengineering 97 (5), 1170–1179.
Lou, F., Neetoo, H., Chen, H., Li, J., 2011. Inactivation of a human norovirus surrogate by high-pressure processing: effectiveness, mechanism, and potential application in the fresh produce industry. Applied and Environmental Microbiology 77 (5), 1862–1871.
Maunula, L., Roivainen, M., Keränen, M., Mäkela, S., Söderberg, K., Summa, M., von Bonsdorff, C.H., Lappalainen, M., Korhonen, T., Kuusi, M., Niskanen, T., 2009. Detection of human norovirus from frozen raspberries in a cluster of gastroenteritis outbreaks. Euro Surveillance 14 (49).
Mena, K.D., Gerba, C.P., 2009. Waterborne adenovirus. Reviews of Environmental Contamination and Toxicology 198, 133–167.
Meng, Q.S., Gerba, C.P., 1996. Comparative inactivation of enteric adenoviruses, poliovirus and coliphages by ultraviolet irradiation. Water Research 30 (11), 2665–2668.
Nuanualsuwan, S., Cliver, D.O., 2002. Pretreatment to avoid positive RT-PCR results with inactivated viruses. Journal of Virological Methods 104 (2), 217–225.
Patterson, M.F., 2005. Microbiology of pressure-treated foods. Journal of Applied Microbiology 98 (6), 1400–1409.
Rodríguez-Lázaro, D., Cook, N., Ruggeri, F.M., Sellwood, J., Nasser, A., Nascimento, M.S., D'Agostino, M., Santos, R., Saiz, J.C., Rzeżutka, A., Bosch, A., Gironés, R., Carducci, A., Muscillo, M., Kovač, K., Diez-Valcarce, M., Vantarakis, A., von Bonsdorff, C.H., de Roda Husman, A.M., Hernández, M., van der Poel, W.H., 2012. Virus hazards from food, water and other contaminated environments. FEMS Microbiology Reviews 36 (4), 786–814.
Sánchez, G., Aznar, R., Martínez, A., Rodrigo, D., 2011. Inactivation of human and murine norovirus by high-pressure processing. Foodborne Pathogens and Disease 8 (2), 249–253.
Shin, G.A., Lee, J.K., 2010. Inactivation of human adenovirus by sequential disinfection with an alternative ultraviolet technology and monochloramine. Canadian Journal of Microbiology 56 (7), 606–609.
Sirikanchana, K., Shisler, J.L., Mariñas, B.J., 2008. Inactivation kinetics of adenovirus serotype 2 with monochloramine. Water Research 42 (6–7), 1467–1474.
Souci, S.W., Fachmann, W., Kraut, H., 2008. Food Composition and Nutrition Tables, 7th revised and completed edition. MedPharm Scientific Publishers, Stuttgart, Germany.
Tang, Q., Li, D., Xu, J., Wang, J., Zhao, Y., Li, Z., Xue, C., 2010. Mechanism of inactivation of murine norovirus-1 by high pressure processing. International Journal of Food Microbiology 137 (2–3), 186–189.
Thurston-Enriquez, J.A., Haas, C.N., Jacangelo, J., Gerba, C.P., 2003a. Chlorine inactivation of adenovirus type 40 and feline calicivirus. Applied and Environmental Microbiology 69 (7), 3979–3985.
Thurston-Enriquez, J.A., Haas, C.N., Jacangelo, J., Riley, K., Gerba, C.P., 2003b. Inactivation of feline calicivirus and adenovirus type 40 by UV radiation. Applied and Environmental Microbiology 69 (1), 577–582.
Thurston-Enriquez, J.A., Haas, C.N., Jacangelo, J., Gerba, C.P., 2005. Inactivation of enteric adenovirus and feline calicivirus by ozone. Water Research 39 (15), 3650–3656.
Umesha, K.R., Bhavani, N.C., Venugopal, M.N., Karunasagar, I., Krohne, G., Karunasagar, I., 2008. Prevalence of human pathogenic enteric viruses in bivalve molluscan shellfish and cultured shrimp in south west coast of India. International Journal of Food Microbiology 122 (3), 279–286.
Wilkinson, N., Kurdziel, A.S., Langton, S., Needs, E., Cook, N., 2001. Resistance of poliovirus to inactivation by high hydrostatic pressures. Innovative Food Science & Emerging Technologies 2 (2), 95–98.

3.3.3

Natural Plant Essential Oils Do Not Inactivate Non-enveloped Enteric Viruses

Katarina Kovač[1], Marta Diez-Valcarce[1], Peter Raspor[2], Marta Hernández[1] and David Rodríguez-Lázaro[1]

[1] Instituto Tecnológico Agrario de Castilla y León (ITACyL), Valladolid, Spain [2] Biotechnology, Microbiology and Food Safety, Biotechnical Faculty, University of Ljubljana, Slovenia

Published in Food Analytical Methods, Vol. 4, No.3, 437-445

Food Environ Virol
DOI 10.1007/s12560-012-9088-7

BRIEF COMMUNICATION

Natural Plant Essential Oils Do Not Inactivate Non-enveloped Enteric Viruses

Katarina Kovač · Marta Diez-Valcarce · Peter Raspor · Marta Hernández · David Rodríguez-Lázaro

Received: 7 May 2012 / Accepted: 31 August 2012
© Springer Science+Business Media, LLC 2012

Abstract The application of plant essential oils (EOs) (hyssop and marjoram) was evaluated for inactivation of non-enveloped viruses using murine norovirus and human adenovirus as models. No significant reduction of virus titres ($TCID_{50}$) was observed when EOs were used at different temperatures and times.

Keywords Non-enveloped viruses · Essential oils · Inactivation · Enteric viruses

The main cause of human gastroenteritis in developed countries is associated with the consumption of food and water contaminated with non-enveloped enteric viruses such as human norovirus (Bosch et al. 2011; Rodríguez-Lázaro et al. 2012; Scallan et al. 2011). Consequently, high standards of food safety and different processing methods have been proposed to improve microbiological safety of food and water. However, many of these methods are efficiently used for inactivation of foodborne bacteria but have a less detrimental effect on non-enveloped enteric viruses (Grove et al. 2006; Baert et al. 2009).

K. Kovač · M. Diez-Valcarce · M. Hernández (✉) · D. Rodríguez-Lázaro (✉)
Instituto Tecnológico Agrario de Castilla y León (ITACyL), Ctra. Burgos, Km. 119, 47071 Valladolid, Spain
e-mail: ita-herperma@itacyl.es

D. Rodríguez-Lázaro
e-mail: ita-rodlazda@itacyl.es

P. Raspor
Chair of Biotechnology, Microbiology and Food Safety, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia

Nowadays consumers require foods with minimal artificial additives. Consequently, novel food preservation procedures must reduce their use to a minimum. One alternative is the use of natural antimicrobials such as essential oils (EO) which are formed by aromatic plants as secondary metabolites. Their major components with antimicrobial effects are phenolic compounds, terpenes, aliphatic alcohols, aldehydes, ketones, acids, and isoflavonoids (Tiwari et al. 2009). EOs or their components have antimicrobial, antiparasitical, and insecticidal properties (Burt 2004; Bakkali et al. 2008) and could therefore be considered suitable for application in or on foods or in cleaning products. Even though the antiviral effectiveness of EOs has been demonstrated against human-enveloped RNA and DNA viruses like herpes simplex virus type 1 and 2 (HSV-1 and HSV-2), dengue virus type 2 (DEN-2), Junin virus (JUNV), and influenza virus (Reichling et al. 2009), little information is available on EOs effect on human non-enveloped viruses (Cermelli et al. 2008; Garozzo et al. 2009).

The aim of the study was to investigate the efficacy of EOs to inactivate foodborne non-enveloped viruses (or their surrogates) in order to assess the potential of using EOs as a strategy to reduce or eliminate foodborne viruses in the food industry. EOs of two aromatic plants which are commonly used in European cuisine, *Hyssopus officinalis* (hyssop) and *Thymus mastichina* (marjoram), were selected and evaluated for their ability to inactivate two model non-enveloped viruses, a human norovirus surrogate, murine norovirus (MNV-1) with RNA genome, and a human adenovirus serotype 2 (HAdV-2) with DNA genome. Even though HAdV-2 is a respiratory strain it can be found in feces, as almost all HAdV serotypes can replicate in the gastrointestinal tract, and may therefore be transmitted to sewage and further contaminate water and food by irrigation (Kovač et al. 2012a).

Published online: 23 September 2012

Hyssopus officinalis and *Thymus mastichina* plants were produced in the central part of Spain and EOs were extracted as described previously (Moldão-Martins et al. 2004). Both EOs consist mainly of monoterpenes (Moldão-Martins et al. 2004; De Martino et al. 2009). HAdV-2 and MNV-1 were propagated on confluent monolayers of human lung carcinoma A-549 and mouse macrophage RAW 264.7 cell lines, respectively, as previously described (Kovač et al. 2012a, b). MNV-1 stock contained $\sim 1 \times 10^5$ $TCID_{50}$ ml^{-1} and HAdV-2 stock contained $\sim 1 \times 10^9$ $TCID_{50}$ ml^{-1} of viruses.

To unambiguously assess the effect of EOs on the virus particles, cytotoxicity of the EOs was determined prior to performing the antiviral assay. EOs were dissolved in ethanol and several dilutions were prepared in cell culture medium (CCM) containing 2 % fetal bovine serum (FBS) to obtain different concentrations of EOs (from 0.05 to 0.0001 %). The final concentration of ethanol added to the cells was less than 0.2 %, which did not show any toxic effect. Cytotoxicity of EOs was determined by visual cytopathic effect and by the trypan blue exclusion method (Bouslama et al. 2011). Cytotoxicity was expressed as the percentage of surviving cells to damaged cells with less than 70 % of viable cells defined as an indicator of cytotoxicity. Marjoram was found to be cytotoxic at concentrations that exceeded 0.002 and 0.006 % and hyssop at concentrations that exceeded 0.001 and 0.003 % for RAW 264.7 and A-549 cells, respectively.

An antiviral assay was performed by adding 100 µl of EO diluted in ethanol and CCM to 100 µl of virus stock. For direct treatment of both viruses 0.02 % EOs were used, and therefore the final concentration on the cells for $TCID_{50}$ assay did not exceed the non-cytotoxic concentrations of oils. However, in vitro cytotoxicity data can overestimate the toxicity of a substance in vivo. In addition, in many instances non-cytotoxic concentrations of EOs do not present any antibacterial effect (Reichling et al. 2009). For these reasons, 0.2 % EOs, which might already cause a slight cytotoxic effect on cells at the first $TCID_{50}$ assay dilution, were also used for treatment of HAdV-2. Nevertheless, this concentration did not affect the results as the cytopathic effect of HAdV-2 was obvious up to, at least the seventh $TCID_{50}$ dilution. Control samples were prepared identically but they did not contain EO. Each sample was mixed vigorously and incubated for 2 or 24 h at three different temperatures: 4 °C, room temperature, and 37 °C. Each experiment was done in triplicate. $TCID_{50}$ assay was used to determine virus titre after treatments and was performed and calculated as described before (Kovač et al. 2012a, b). Statistical analyzes were done by the Student's t test with a significance level of $P < 0.05$. The SPSS 15.0 Statistical Analyzes Software was used.

The effects of marjoram and hyssop EOs on HAdV-2 (DNA virus) and MNV-1 (RNA virus) are presented in Table 1. No significant reduction of HAdV-2 titre ($P < 0.05$) was observed except for the treatment with 0.2 % hyssop for 24 h at 4 °C. However, the reduction (i.e., difference between control and the treated samples) was only 0.29 $\log_{10}$ and therefore, it can be considered negligible. Similar results were observed for MNV-1 after application of 0.02 % marjoram or hyssop (Table 1). In accordance with our results, previous studies have also shown that some non-enveloped viruses (HAdV-2, poliovirus 1, ECHO 9, and Coxsackie B1) were not affected by tea tree oil compounds while some of these oil compounds had an inhibitory effect on influenza virus (Garozzo et al. 2009, 2011). Also eucalyptus oil did not shows any antiviral activity against adenovirus, whilst a mild effect was observed on mumps virus (Cermelli et al. 2008).

Table 1 Effect of marjoram (M) and hyssop (H) against human adenovirus (HAdV-2) and murine norovirus (MNV-1) after 2 or 24 h of incubation at 4 °C, room temperature (~20 °C), and 37 °C

Virus	Treatment	Titre ($\log_{10}$ $TCID_{50}$ ml^{-1})					
		4 °C		Room temperature		37 °C	
		2 h	24 h	2 h	24 h	2 h	24 h
HAdV-2	Control	9.50 ± 0.13	9.54 ± 0.07	9.67 ± 0.31	9.47 ± 0.13	9.67 ± 0.07	9.42 ± 0.14
	0.02 % M	9.42 ± 0.14	9.39 ± 0.03	9.63 ± 0.25	9.33 ± 0.07	9.42 ± 0.19	9.25 ± 0.13
	0.2 % M	9.50 ± 0.00	9.42 ± 0.26	9.63 ± 0.13	9.54 ± 0.19	9.42 ± 0.07	9.38 ± 0.33
	0.02 % H	9.67 ± 0.07	9.79 ± 0.14	9.58 ± 0.19	9.38 ± 0.13	9.50 ± 0.25	9.17 ± 0.26
	0.2 % H	9.38 ± 0.00	9.25 ± 0.13[a]	9.46 ± 0.19	9.33 ± 0.26	9.29 ± 0.19	9.25 ± 0.13
MNV-1	Control	4.25 ± 0.13	3.83 ± 0.19	4.21 ± 0.14	2.92 ± 0.07	2.88 ± 0.13	<1.50[b]
	0.02 % M	4.46 ± 0.14	3.67 ± 0.07	4.13 ± 0.13	3.29 ± 0.19[a]	3.00 ± 0.13	<1.50[b]
	0.02 % H	4.63 ± 0.22	3.83 ± 0.26	4.38 ± 0.22	3.13 ± 0.13[a]	3.13 ± 0.25	<1.50[b]

[a] Significant difference between treatment results and control using Student's t test ($P < 0.05$)

[b] Recovered titre below detection limit (1.50 $TCID_{50}$ ml^{-1})

Interestingly, significant variations ($P > 0.05$) of MNV-1 titer in control samples at different times and temperatures were observed (Table 1). To check if ethanol caused this effect, the same experiment was performed without ethanol. However, no significant difference was observed between the results obtained with and without ethanol (data not shown). The variation observed in the titre of the controls must thus be attributed to differences in treatment temperature and times.

EOs of hyssop and marjoram have previously been reported to act against foodborne bacteria (Friedman et al. 2002; Kizil et al. 2010) and therefore have the potential to be used as antimicrobials in the food industry. In addition, hyssop EO also showed strong inactivation activity against enveloped HSV (Schnitzler et al. 2007; Koch et al. 2008; Reichling et al. 2009). However, our results show that hyssop and marjoram EOs do not affect the two selected non-enveloped viruses (HAdV-2 and MNV-1). EOs can inactivate viruses in different ways: dissolution of the envelope (Siddiqui et al. 1996), interference with the virion envelope structure or by masking viral compounds needed for adsorption or entry into host cells (Koch et al. 2008; Schuhmacher et al. 2003). Consequently, the first two modes of inactivation can be excluded for non-enveloped viruses. As our results did not show any reduction of virus titre after treatments with EOs, the mechanism of masking viral compounds can also be excluded.

In conclusion, the results of our study, in combination with previous studies using different EOs and non-enveloped viruses, indicate that the application of EOs is not expected to be an effective alternative to reduce or eliminate the most emergent foodborne viruses in the food industry.

Acknowledgments This study was supported by Project RTA 2011-079-C02-01 of the Ministry of Economy and Competitiveness, Government of Spain. Katarina Kovač was supported by a PhD student fellowship from the University of Leon, Spain. Marta Diez-Valcarce received a PhD studentship from the Instituto Nacional de Investigacíon y Technología Agraria y Alimentaria (INIA), Spain.

References

Baert, L., Debevere, J., & Uyttendaele, M. (2009). The efficacy of preservation methods to inactivate foodborne viruses. *International Journal of Food Microbiology, 131*(2–3), 83–94.

Bakkali, F., Averbeck, S., Averbeck, D., & Idaomar, M. (2008). Biological effects of essential oils—A review. *Food and Chemical Toxicology, 46*(2), 446–475.

Bosch, A., Sánchez, G., Abbaszadegan, M., Carducci, A., Guix, S., Le Guyader, F. S., et al. (2011). Analytical methods for virus detection in water and food. *Food Analytical Methods, 4*(1), 4–12.

Bouslama, L., Hayashi, K., Lee, J. B., Ghorbel, A., & Hayashi, T. (2011). Potent virucidal effect of pheophorbide a and pyropheophorbide a on enveloped viruses. *Journal of Natural Medicines, 65*(1), 229–233.

Burt, S. (2004). Essential oils: Their antibacterial properties and potential applications in foods—A review. *International Journal of Food Microbiology, 94*(3), 223–253.

Cermelli, C., Fabio, A., Fabio, G., & Quaglio, P. (2008). Effect of eucalyptus essential oil on respiratory bacteria and viruses. *Current Microbiology, 56*(1), 89–92.

De Martino, L., De Feo, V., & Nazzaro, F. (2009). Chemical composition and in vitro antimicrobial and mutagenic activities of seven *Lamiaceae* essential oils. *Molecules, 14*(10), 4213–4230.

Friedman, M., Henika, P. R., & Mandrell, R. E. (2002). Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. *Journal of Food Protection, 65*(10), 1545–1560.

Garozzo, A., Timpanaro, R., Bisignano, B., Furneri, P. M., Bisignano, G., & Castro, A. (2009). In vitro antiviral activity of *Melaleuca alternifolia* essential oil. *Letters in Applied Microbiology, 49*(6), 806–808.

Garozzo, A., Timpanaro, R., Stivala, A., Bisignano, G., & Castro, A. (2011). Activity of *Melaleuca alternifolia* (tea tree) oil on influenza virus A/PR/8: Study on the mechanism of action. *Antiviral Research, 89*(1), 83–88.

Grove, S. F., Lee, A., Lewis, T., Stewart, C. M., Chen, H., & Hoover, D. G. (2006). Inactivation of foodborne viruses of significance by high pressure and other processes. *Journal of Food Protection, 69*(4), 957–968.

Kizil, S., Hasimi, N., Tolam, V., Kilinc, E., & Karatas, H. (2010). Chemical composition, antimicrobial and antioxidant activities of hyssop (*Hyssopus officinalis* L.) essential oil. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 38*(3), 99–103.

Koch, C., Reichling, J., Schneele, J., & Schnitzler, P. (2008). Inhibitory effect of essential oils against herpes simplex virus type 2. *Phytomedicine, 15*(1–2), 71–88.

Kovač, K., Bouwknegt, M., Diez-Valcarce, M., Raspor, P., Hernández, M., & Rodríguez-Lázaro, D. (2012a). Evaluation of high hydrostatic pressure effect on human adenovirus using molecular methods and cell culture. *International Journal of Food Microbiology, 157*(3), 368–374.

Kovač, K., Diez-Valcarce, M., Raspor, P., Hernández, M., & Rodríguez-Lázaro, D. (2012b). Effect of high hydrostatic pressure processing on norovirus infectivity and genome stability in strawberry puree and mineral water. *International Journal of Food Microbiology, 152*(1–2), 35–39.

Moldão-Martins, M., Beirão-da-Costa, S., Neves, C., Cavaleiro, C., Salgueiro, L., & Beirão-da-Costa, M. L. (2004). Olive oil flavoured by the essential oils of *Menthapiperita* and *Thymus mastichina* L. *Food Quality and Preference, 15*(5), 447–452.

Reichling, J., Schnitzler, P., Suschke, U., & Saller, R. (2009). Essential oils of aromatic plants with antibacterial, antifungal, antiviral, and cytotoxic properties—An overview. *Forschende Komplementärmedizin, 16*(2), 79–90.

Rodríguez-Lázaro, D., Cook, N., Ruggeri, F. M., Sellwood, J., Nasser, A., Sao Jose Nascimento, M., et al. (2012). Virus hazards from food, water and other contaminated environments. *FEMS Microbiology Reviews, 36*(4), 786–814.

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., et al. (2011). Foodborne illness acquired in the United States—Major pathogens. *Emerging Infectious Diseases, 17*(1), 7–15.

Schnitzler, P., Koch, C., & Reichling, J. (2007). Susceptibility of drug-resistant clinical herpes simplex virus type 1 strains to essential oils of ginger, thyme, hyssop, and sandalwood. *Antimicrobial Agents and Chemotherapy, 51*(5), 1859–1862.

Schuhmacher, A., Reichling, J., & Schnitzler, P. (2003). Virucidal effect of peppermint oil on the enveloped viruses herpes simplex virus type 1 and type 2 in vitro. *Phytomedicine, 10*(6–7), 504–510.

Siddiqui, Y. M., Ettayebi, M., Haddad, A. M., & Al-Ahdal, M. N. (1996). Effect of essential oils on the enveloped viruses: Antiviral activity of oregano and glove oils on herpes simplex virus type 1 and Newcastle disease virus. *Medical Sciences Research, 24*(3), 185–186.

Tiwari, B. K., Valdramidis, V. P., O'Donnell, C. P., Muthukumarappan, K., Bourke, P., & Cullen, P. J. (2009). Application of natural antimicrobials for food preservation. *Journal of Agricultural and Food Chemistry, 57*(14), 5987–6000.

Chapter 4

General discussion

Estimation of the biological risk associated to food is needed to prioritize interventions and allocate resources. Risk analysis (RA) is a structured process consisting of three separate but interconnected elements: (i) risk assessment, (ii) risk management and (iii) risk communication (Codex Alimentarius Commission, 1999) (Figure 5). Direct or indirect costs derived from healthcare or decrease in productivity are well studied in the case of bacteria, but less information is available regarding the burden of viral contamination in food and its economical and social implications. Aiming to fulfil this lack of information this Thesis in the framework of the EU FP7 project VITAL worked towards the establishment and public release of viral detection procedures to objectively assess the risk associated to food contaminated with virus. By developing new methods or adapting and modifying existing ones, standard operating procedures (SOPs) were produced to be used by all eleven participant laboratories in the project. A total of 23 SOPs were developed and they are currently available to the public in the VITAL website (www.eurovital.org). These SOPs constitute the needed tools to *assess the risk* associated to virus contamination of foodstuffs. In sections, 3.1.1, 3.1.2, 3.1.3 and 3.1.4, the design and implementation of a suite of analytical controls that are included in these SOPs are described, and their applicability is demonstrated under experimental conditions. To further evaluate the workability and ease to reproduce the detection methods developed, a validation of the procedures was performed by means of an international interlaboratory test. The actual viral risk associated with consumption of contaminated foods was assessed with two sampling studies in two different European food supply chains. The second element of a RA process consist of measures to *manage the risk*, i.e. the implementation of control measures to reduce the presence of virus in the food supply chains or, if elimination of the hazard is not possible, technologies that reduce viral threat to acceptable levels (at least 4 $\log_{10}$ reduction). This reduction of viral infectivity can be achieved using different principles;

high hydrostatic pressure (HHP) is a promising non-thermal technology for virus inactivation believed to physically affect the virus particle, although the complete mechanism remains unknown. Two viruses, one with DNA genome (HAdV-2) and one with RNA genome (MNV-1) were used to further understand how HHP affects virus and its infectivity. But HHP is not, by far, the only non-thermal technology for virus inactivation. Viral inactivation can be also achieved by chemically affecting the viral particles with, for example, the natural compounds present in the EO fraction of some plants. These compounds can inactivate microorganisms because they interfere with structures that are necessary for the successful interaction host-microorganism. To elucidate the mechanism with which the EO interacts with enteric viruses, a study was performed using the EO fraction of hyssop and marjoram, two aromatic plants commonly used as food condiments in European cuisine. The *communication of risks* is the third and last part of a RA process. All the data gathered within the assessment and the management parts must be transferred to consumers, to governmental policy-makers and regulatory agencies. Industry- and practitioner-directed guidance on appropriate control practices for virus contamination have been published in form of guidance sheets that are now available in the VITAL website in four different languages, including our version in Spanish (www.eurovital.org/GuidanceSheets1.htm).

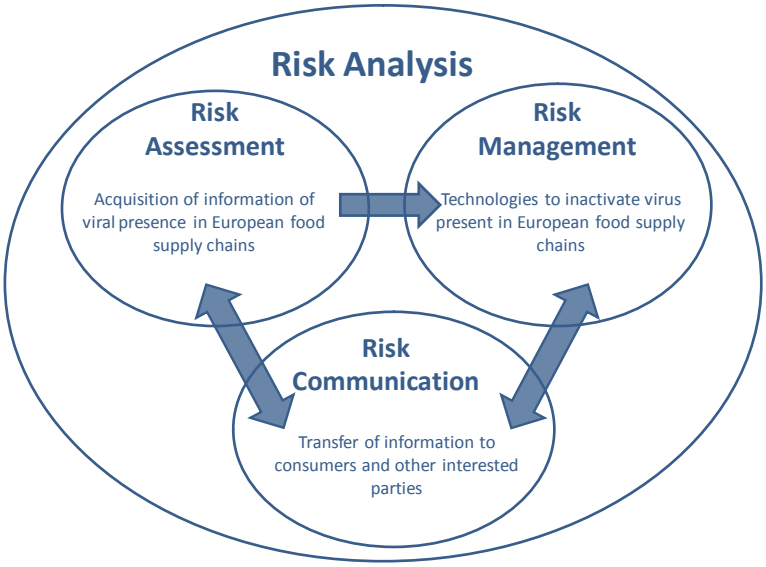


Figure 5. Structure of Risk Analysis process as defined by the Codex Alimentarius Commission (1999).

To *assess the risk associated with viral contamination of food*, specifically tailored methodology was prepared and bespoken controls were designed and implemented. Persistent inhibitory substances in the amplification reaction were detected by two different strategies; incorporating an IAC in the reaction, and loading neat and tenfold diluted samples. This approach permitted us to detect the presence of inhibition in 15 and 41 reactions in the shellfish and pork production chains, respectively. These samples would have been, otherwise, interpreted as negative. A significant increase in the prevalence of HEV has been also previously demonstrated after the incorporation of an IAC: hepatitis E virus prevalence in pig farms in The Netherlands rose from 33 to 55% by incorporating an appropriate IAC in the amplification reactions (Rutjes *et al.*, 2007).

The utilisation of SPCV to monitor the performance of the pre-amplification process is a prerequisite if a method aims to become a standard (Baert *et al.*, 2011) and

consequently, in the EU FP7 VITAL project the inclusion of a SPCV was agreed. Several virus species have been previously claimed as good candidates: MNV-1, vMC_0 and FCV (Costafreda *et al.*, 2006; Cannon *et al.*, 2006). Thereupon the main features of each are discussed. MNV-1 exhibits close similarity in structure and behaviour to human noroviruses; is non-pathogenic to humans; and can be grown in adapted cell lines (RAW 264.7). Even though the use of MNV-1 is under patent, held by the Washington University, this patent does not apply when used for research purposes. vMC_0, in turn, is a mutant virus strain of the wild type pMC_0 of mengovirus lacking the poly(C) tract, this latter feature renders it avirulent, but it also makes it a genetically modified organism (GMO). Working with GMOs would require the user laboratories to comply especial legal requirements which could hamper the general implementation of the methods. And finally FCV; it is a respiratory virus, not an enteric one, which makes it the less acceptable candidate of the three proposed. Furthermore, in some studies significant differences between FCV and MNV-1 in pH resistance and ethanol susceptibility have been observed (Cannon *et al.*, 2006; Sattar *et al.*, 2011). We experimentally demonstrate the applicability of the chosen SPCV (MNV-1) performing the process incorrectly to show the absence of SPCV signal in the PCR, which should be interpreted as a failed concentration procedure. Thus, indicating that the pre-amplification process should be repeated (section 3.1.2). To further characterize the potential use of vMC_0 or MNV-1 as SPCV an additional study was performed. No significant differences were observed regarding the time elapsed from the addition of SPCV (vMC_0 or MNV-1) to the beginning of the pre-amplification process (concentration and extraction). However, a significant difference was observed, as expected, depending on the step where the SPCV was incorporated to the process, supporting the approach of adding the SPCV at the beginning of the process to allow its complete monitoring. Considering all the information mentioned

above and the differences observed among the candidates, MNV-1 was confirmed as the best possible candidate, especially for the specific purpose of detection of human noroviruses. In fact, MNV-1 as SPCV, has been proved useful in real sampling studies since a total of 18 pre-amplification processes, in the shellfish (16) and pork (2) production chains were detected to have failed and were repeated, avoiding this way any possible underestimation of the real prevalence of enteric viruses in the food supply chain studied.

Always with a risk assessment scope, we keep on improving methodology by incorporating synthetic nucleic acid standards to be used as positive controls in the amplification reactions. These standards have not been, however, used in this Thesis with quantification purpose despite this is one of their possible usages and a major advantage as experimentally demonstrated (section 3.1.4). The method of choice for quantification was the most probable number-like approach (Teunis *et al.*, 2005; De Roda Husman *et al.*, 2009). This method has permitted us the quantification of pathogenic viruses in shellfish, as shown in the results of section 3.2.2. The possible public health implications derived from the presence and viral load in foodstuffs during the sampling studies are discussed later in this chapter.

Any good methodology must be evaluated to demonstrate its workability and ease to reproduce. With this purpose an international interlaboratory exercise was performed (section 3.1.5). To our knowledge, this is the first validation study on a virus detection method in food. In this collaborative trial all participant laboratories from nine different countries were involved and the performance of a molecular-based method to detect human adenovirus in raspberries was determined. The overall results were considered acceptably robust (trial sensitivity and specificity of 98.5% and 69.7%,

respectively). Accordingly, the specific methodology developed was incorporated into the SOPs to be used in actual sampling studies.

The detection methodologies based on molecular methods such as real-time PCR, as those described above, have however an important drawback: is not currently possible to distinguish infective from non infective particles. The inclusion of all the previously described controls, permit us to be confident that a negative result is undoubtedly negative. A positive result, however, only indicates that the sequence, and consequently the virus, was present in the sample but whether this sequence belongs to an infective virus or not remains unknown. Aiming to overcome this matter, a preenzymatic treatment of the samples prior to deliver them to the amplification step was assessed (section 3.1.6). This strategy, consisting of a treatment of the samples with proteinase K and endonucleases, has been previously proposed (Nuanualsuwan and Cliver, 2002; Topping *et al.*, 2009) and despite being theoretically correct, it cannot be implemented in a real scenario as we demonstrated. We assayed three different virus inactivation procedures (UV, HHP and temperature); each of them using different mechanisms to inactivate viruses, and the results obtained showed that inactivated viruses were still detectable by molecular methods. To confirm if the discrepancy was due to an incorrect or non-optimal experimental design, several combinations of times and different concentrations of enzymes were assayed and similar results were observed. The quantification of virus genomes, even if using a preenzymatic treatment for eliminating the genomes of structurally affected virus particles, does not seem to be a feasible approach for the estimation of virus infectivity and consequently real time PCR detection methods continue having the same drawback for distinguishing infective from non-infective viral particles. Therefore a major research effort must be dedicated to adapt and improve the current detection methodology and technology for

the acquisition of results providing evidence on the infectivity status of the detected virus.

Once the methodologies were experimentally tested, two major prevalence studies were performed in two different European food supply chains: pork and shellfish production. Within the pork production chain, HEV and PAdV were the viruses tested; HEV as an emergent zoonotic agent and PAdV as an index of faecal contamination of porcine origin. In the case of shellfish, target viruses were NoV (genogroup I and II), HEV and HAV, and HAdV as a human faecal indicator.

The herd-prevalence of HEV can be estimated analysing faecal samples. With this purpose we participate in a multinational study involving six European countries, and HEV was demonstrated to be widespread within the European farms (section 3.2.2). Similarly, and as part of a wider sampling scheme covering all the production stages (from farm to fork), faeces of animals were also collected at slaughterhouse level in three European countries: Czech Republic, Italy and Spain (section 3.2.1) and HEV was detected in the faeces of animals of all three countries. Faecal prevalence of HEV was 27%, and assuming that animals in our study were fatteners (13-22 weeks), this prevalence is similar to the one observed in samples in Portugal (30%) (section 3.2.2). The sampling strategy of taking faecal and liver samples from the same animal permit us to detect that the absence of HEV in faeces of the animals did not necessarily correspond to absence of HEV in the other targeted samples (i.e. liver). In fact, in 3 animals we found HEV RNA in liver whereas no HEV RNA was found in the faeces. A possible explanation to this phenomenon could be that bile concentration in the faecal mass might be expected to be lower at the sampling time (in the slaughterhouse) than in the liver, as pigs were probably fed long before reaching the slaughterhouse. The prevalence of HEV RNA observed in commercial livers (ranging from 3% to 6%) was

in accordance to the one found in a similar study on the prevalence of HEV in porcine liver in The Netherlands (Bouwknegt *et al.*, 2007). HEV RNA was detected in sausage samples, all from Spain (6% of Spanish sausages, representing 2% of the total sausages sampled). The interpretation of this finding is not clear and deserves further investigation. Regarding the role of PAdV as an indicator virus, most faeces were positive for PAdV, but it was never detected in liver and only occasionally in pork meat (1/112) or sausages (4/313). Comparing the prevalence of HEV and PAdV, the risks for cross-contamination of pork products with porcine faeces during slaughtering or production stages appear to be low, but not completely absent.

The HEV positive samples were sequenced and it was determined that they all belonged to genotype 3. Five of the seven Spanish sequences belonged to subtype g3f, an HEV strain that is predominant and indigenous to Europe. In a previous classification of HEV strains, subtype g3f included only sequences of Spanish or Dutch origin (Lu *et al.*, 2006). The other two Spanish sequences were not classified in any subtype but were closely related to subtype g3c, the same subtype found in the four Italian HEV sequences. These two unclassified sequences well represent a new subtype of HEV g3, or, due to their similarities to g3c, represent sequences of that subtype. Subtype g3c contains only European sequences but in 2006, when the classification was done, only Dutch sequences were included in this subtype and now this subtype has spread to other European countries (i.e. Italy, and presumably Spain) as shown in our results.

The detection of HEV sequences did not conclusively demonstrate viable virus and thus public health implications to consumers. However, we cannot exclude the possibility that in some of the products in which HEV was found the virus was infectious. To assess the risk of health events occurrence, a dose-response model was

used. This model was based on intravenous inoculation of pigs with HEV, corrected to reflect a potential infection after exposure to the agent trough the faecal-oral route and we estimated that actual health risks exist in the case of pork products containing HEV.

The sampling of the shellfish supply chain was performed simultaneously with two other European countries (Finland and Greece).Samples were purchased at retail level (point of sale), as studies of virus contamination of shellfish in growing and harvesting areas have been largely conducted and much information was already available (Le Guyader *et al.*, 2000; Formiga-Cruz *et al.*, 2002). Pathogenic enteric viruses were found in the shellfish production chain at point of sale (16% NoVGII, 3% HEV and 0.7% NoVGI), a high percentage of these positive samples were taken in Spain, only NoVGI was not found in the Spanish samples and the final percentage in the study corresponds to a different country. Additionally, in Spain we also analysed samples for other two important enteric viruses, AsV and RVGA, but none of the samples were positive. Finding pathogenic viruses in shellfish is of particular interest as this food production chain includes food items that are consumed raw or slightly cooked (i.e. mussels steamed to open). The high prevalence (36%) of HAdV, used in this study as an index virus, indicates that samples, regardless the mussel species or the country of origin, were in contact with waters polluted with faeces of human origin. However, as no significant correlation between HAdV and the pathogenic viruses was found, we cannot draw any further conclusion. Due to higher prevalence of NoV, these results were further analysed using a dose-response model based on a highly infectious variant of NoV, Norwalk virus. The response measured was infection after ingestion of the inoculum by human volunteers. Interestingly, an equal risk of health events after consumption of mussels was estimated to exist among countries, despite apparently high differences in the average concentration of virus per mussel. That is, the risk of

health events among the population was the same in a country with an average concentration of ~16 PCR detectable units (PDU) of NoV and in a country with an average concentration of ~2 PDU of NoV. The high infectivity of the NoV variant used in the dose-response model might explain this similar assessment of the risk of health events after such a different exposure to the virus.

Taking into consideration together the dose-response models and the data on virus concentrations in different foods along the food production chains studied, we can conclude that viral contamination occurs, implying potential risk of health events. The occurrence of these events though, differs between viruses and production chains. In the case of the shellfish production chain, the risk related to consumption for HAV and HEV was minimal, on the contrary actual health risks were estimated to exist for NoV. Similarly, consumption of pork products and risk of HEV appear to be linked. It must be keep in mind, however, that for calculating these estimations specific virus dose-response models were used; and a model, by definition, will never totally represent the real setting. Furthermore, a more realistic modelling of the risk of viral infection should have included the possible person-to-person spread. The inclusion of this secondary transmission between people would increase the total burden of viral foodborne disease and it must be accordingly acknowledge. However, the person-to-person transmission *per se* does not affect the estimation of the infection risks derived directly from consumption of food contaminated with viruses. The interpretation of results derived from the sampling studies using the dose-response models were performed by the group of experts in quantitative viral risk analysis (QVRA) within the VITAL project (RIVM, the Netherlands), with whom I spent a three-month research stay during my PhD to learn and collaborate in the QVRA of the VITAL

results. Therefore, some of the results discussed above were taken from the project final report.

After assessing the risk associated with viral contamination of food, this *risk must be managed*. With this purpose, studies to reduce virus contamination of food through inactivation of the viral particles were performed. Two different non-thermal inactivation technologies (HHP and natural compounds present in the EO fraction of plants) were used with two viruses, MNV-1 and HAdV-2, one surrogate of human norovirus and one pathogenic human virus, with RNA and DNA genomes, respectively. The results obtained were disparate, though. Whereas HHP has been proved as an efficient option, the application of EOs did not meet the expected levels of virus inactivation.

An HHP treatment of 400MPa resulted in an adequate reduction on viral infectivity in both viruses assayed; 2.5 min were sufficient to reach a reduction of viral infectivity in MNV-1 of at least 5.13 $\log_{10}$ and 3.33 $\log_{10}$ in water and strawberry puree, respectively. In the case of HAdV-2, under the same pressure conditions, virus infectivity was estimated to be reduced by approximately 6 $\log_{10}$ in slightly more than 1.5 min (93 sec). This reduction values meet the requirements of a food safety objective (FSO), that in the case of virus we established as at least 4 $\log_{10}$ reduction in infectivity.

It was observed that many factors modulate the virus inactivation with HHP. Some extrinsic factors are the treatment parameters themselves, time and pressure applied to the virus. The reduction in infectivity increased as the time and the intensity of treatment increased. For example in the case of MNV-1 in strawberry puree, a $\log_{10}$ reduction of 1.21, 2.63 and 2.75 was observed using 300 MPa during 2.5, 5 and 10 min, respectively. It must be noted that this observation is valid for the virus studied, since

there are some exceptions. Poliovirus, for example, does not follow this statement as it seems to be barotolerant, i.e. treatments of 600MPa during 60 min showed no significant reduction in infectivity (Wilkinson *et al.*, 2001). Virus inactivation can be also highly influenced by intrinsic factors such as the composition of the medium in which the virus is, e.g. food or cell culture medium. Foods are extremely complex matrices and different characteristics, e.g. pH, sugar content or presence of chelants can affect the extent of the viral inactivation. Those effects can be so relevant that paradoxical results can be observed; acidic environment (strawberry puree) can favour virus inactivation, as observed in MNV-1 (section 3.3.1), but it can also act as a baroprotective factor as observed elsewhere when MNV-1 was treated in an aqueous medium (Lou *et al.*, 2011). These findings must serve us to keep in mind that the real effect can be observed only when the specific matrix of interest is used. As indicated above there are even some intrinsic differences due to the particularities of each virus species; caliciviruses (including human NoV and their surrogates) are small non enveloped viruses with an extremely simple, and therefore resistant, viral capsid. HAdV-2, on the other hand, is also a non-enveloped virus but it has "spikes" or fibers associated with each penton base of the capsid that aids in the attachment to the host cell.

The quantification of virus infectivity before and after the inactivation procedure is an essential requirement for assessing the achievement of an established FSO. However, the preenzymatic treatment of samples cannot, at present, address this issue, as discussed previously. This has been demonstrated for MNV-1; and in case of an unrelated virus, HAdV, with even different nucleic acid in its genome, the same conclusions were drawn. Differences were observed when comparing molecular detection methods, (RT)-real-time PCR, with and without preenzymatic treatment but

no relationship could be established between genome quantification by these molecular methods and viral infectivity measured by cell culture assays. This lack of correlation serves as a confirmation that HHP effect on virus infectivity targets capsidic proteins responsible of virus attachment to the cell as otherwise, similar virus reduction would have been observed also by molecular methods.

The application of EOs as inactivating agent causes no significant reduction of MNV-1 and HAdV-2. Only in the case of HAdV-2 treated with 0.2% hyssop EO during 24 h at 4ºC, the difference in the virus titre was statistically significant ($p < 0.05$), but the reduction was 0.29 $\log_{10}$ and such a reduction is insufficient to considerably reduce the risk of foodborne infection. Consequently, we conclude that the application of the EOs of hyssop and marjoram under our experimental conditions do not satisfactorily inactivate non-enveloped enteric viruses.

Finally, the third and last element of a RA process consists of the *communication of the risk.* This Thesis aims to comply with part of this task serving as an instrument to transfer the information acquired and the conclusions drawn to the scientific community, by means of the published manuscripts; to the consumers and producers, by means of the guidance sheets, and to any other interested parties.

In conclusion, the research developed can contribute to (i) the improvement of a standardised virus detection methodology, an essential requisite to have comparable data between laboratories, (ii) to elucidate the presence of enteric viruses in the food chain and estimate their associated risk for the consumers, and finally, (iii) to increase our understanding on how non-thermal inactivation procedures can reduce the infectivity of enteric viruses present in food.

Chapter 5

Conclussions

1. The use of controls such as the sample process control virus (SPCV) and the internal amplification control (IAC) in the detection of viruses using molecular methods has been demonstrated to be a reliable and robust approach for assessing the results of the analysis of samples from different food supply chains, as well as for evaluating the efficiency during the extraction process.
2. No significant differences were found in the performance of the sample process controls (mengovirus and murine norovirus) up to 24 hours after their addition to the sample. Moreover, the incorporation of the SPCV at the beginning of the analysis allows a more complete monitoring of the process. Therefore, murine norovirus was considered the best possible candidate, especially for the specific purpose of detection of human noroviruses.
3. Synthetic nucleic acids can be used as standards for accurate virus quantification, and constitute an alternative source (to extracted natural virus nucleic acid) of positive controls in molecular detection methods.
4. Preenzymatic treatment prior to nucleic acid extraction does not seem to be a feasible approach to quantify virus infectivity using real-time PCR.
5. Hepatitis E virus RNA is present throughout the pork production chain in Europe (from farm to fork) and this presents a potential health risk for consumers.
6. The frequent detection of porcine adenovirus in pig faeces, along with its low presence in the pork products (i.e. meat and sausages), and complete absence in liver, indicates that risks for contamination with swine faeces during slaughtering and food manipulation appear to be low but not absent.
7. The estimation of the transmission of hepatitis E virus from an infected to a susceptible animal in farmed swine populations in Europe was calculated to

be from 10 to 27 days, on the basis of data on hepatitis E virus presence in faeces taken from different European farms.

8. Pathogenic viruses (human norovirus genogroup I and II and hepatitis E virus) were found in shellfish at point of sale. Considering that shellfish can be consumed raw or slightly cooked, dose-response models were used and only norovirus was found to represent a health risk.
9. Shellfish samples purchased at retail level showed high prevalence of human adenovirus, although no correlation with pathogenic viruses was found. Thus, this finding indicates that samples were in contact with waters polluted with human faeces but does not support the use of adenovirus as pathogenic virus indicator.
10. High hydrostatic pressure, using treatments of 400 MPa or higher, has been proven to be an efficient non-thermal inactivation technology to achieve a food safety objective of at least 4 $\log_{10}$ reduction in virus infectivity.
11. The application of essential oils of marjoram and hyssop did not meet the expected levels of virus inactivation to be considered an appropriate procedure for food decontamination.

Chapter 6

Resumen

La transmisión de enfermedades por consumo de alimentos contaminados es cada vez más frecuente, y una gran proporción de esas enfermedades es debida a agentes víricos. Es por ese motivo que la obtención de información sobre la prevalencia de determinados virus a lo largo de la cadena alimentaria es necesaria, puesto que a pesar de su importancia la prevalencia de los virus de origen alimentario está todavía relativamente poco estudiada. A la hora de suplir esa falta de información sobre la presencia de virus en los alimentos, disponer de una metodología correcta es un factor clave, implicando el desarrollo de procedimientos adecuados para la toma de muestras, la concentración, la extracción y la detección de los virus. De la misma manera que es esencial adquirir información sobre la prevalencia de los virus en la cadena alimentaria, es también muy importante avanzar en el desarrollo de procesos que puedan inactivar los virus presentes en los alimentos de una manera efectiva, consiguiendo así alimentos seguros y saludables para los consumidores. Por lo tanto, los objetivos de esta tesis han sido la detección, identificación y monitorización de virus entéricos en la cadena alimentaria así como la evaluación de la eficacia de algunas tecnologías emergentes de inactivación vírica. Para el logro de estos objetivos, (i) se han utilizado protocolos estandarizados que incluyen un conjunto de controles diseñados específicamente para la detección de los virus transmitidos por alimentos utilizando herramientas moleculares y se ha evaluado la viabilidad de los mismos por medio de un ensayo internacional de validación, (ii) se han llevado a cabo estudios de muestreo en dos cadenas alimentarias diferentes (producción de carne de cerdo y mariscos), y (iii) se ha estudiado el efecto de dos procesos de inactivación (altas presiones hidrostáticas y compuestos químicos de aceites esenciales de plantas) sobre los virus entéricos.

1. Construcción y aplicación analítica de controles internos de amplificación (IAC) para la detección de virus importantes en la cadena alimentaria mediante ensayos basados en la PCR a tiempo real.

Se construyeron controles internos de amplificación (IAC) para su incorporación en los ensayos de amplificación de ácidos nucleicos mediante PCR a tiempo real (RTi-PCR) para los siguientes virus; poliomavirus bovino (BPyV), virus de la hepatitis A (HAV), virus de la hepatitis E (HEV), adenovirus humano (HAdV), norovirus humano genogrupo I (NoVGI), y genogrupo II (NoVGII), norovirus murino (MNV-1) y adenovirus porcino (PAdV). Se optimizaron las cantidades de IAC a añadir en los ensayos de manera que los límites de detección de los sistemas no se vieran afectados. Se demostró además la eficacia de los IAC en la identificación de ensayos fallidos, mediante la realización de un experimento en el cual el procedimiento se llevó a cabo de manera incorrecta deliberadamente.

Construcción de los controles internos de amplificación

La estrategia para la construcción de los controles internos de amplificación (IAC) para la detección de virus mediante RTi-PCR y mediante RTi-PCR con transcripción inversa (RT-RTi-PCR) se puede dividir en dos fases (i) En una primera fase se produce una molécula de ADN de doble cadena mediante PCR convencional que contiene una secuencia no especifica del virus diana (en nuestro caso específica del gen *prfA* de *L. monocytogenes*), flanqueada por secuencias complementarias a los cebadores específicos de los virus, a continuación el producto quimérico obtenido se clona en un plásmido. (ii) La segunda fase consiste, en el caso de que los virus diana sean virus ARN, en la producción de moléculas de ARN mediante transcripción *in vitro* usando la polimerasa de ARN T7. La molécula de ADN (vector) o de ARN (fragmento transcrito) es el IAC que será amplificado por los cebadores específicos del virus y detectado

posteriormente usando una sonda complementaria a la secuencia interna del control, que es diferente a la secuencia diana del virus.

Optimización de la concentración de sonda del IAC en la PCR a tiempo real

Los pasos que se siguieron en el proceso de optimización fueron: (i) Verificación de que el IAC podía ser amplificado y detectado con cada par de cebadores específicos. (ii) Comprobación de que tanto la molécula del IAC como el virus diana se podían amplificar y detectar de manera simultánea, es decir, que en presencia de moléculas de IAC y del virus diana en el mismo tubo de reacción ambas señales estaban presentes. (iii) Optimización de la concentración de sonda de IAC, en el caso de los virus ADN se determinó llevando a cabo diferentes reacciones de RTi-PCR en presencia de 3.000 copias de IAC, sin ADN del virus, con la sonda específica del virus a una concentración fija de 100 nM y diversas concentraciones crecientes (25 nM, 50 nM y 100 nM) de la sonda del IAC. Cuando se trató de virus ARN se siguió la misma estrategia, se realizaron diversas RT-RTi-PCR con 2.000 copias de IAC, sin ácido nucleico del virus, sonda específica del virus a una concentración fija de 100 nM y diversas concentraciones crecientes (25 nM, 50 nM y 100 nM) de la sonda del IAC. En todos los casos la concentración de sonda del IAC elegida fue 50 nM. (iv) Determinación del límite de detección y la capacidad de inhibición del sistema por parte del IAC. Para ello se comprobó que en presencia de un determinado número de copias del IAC, las moléculas del virus se podían detectar. En el caso de BPyV, 10 copias del virus en presencia de 300 copias de IAC podían ser detectadas de forma fiable con Cp=28,79±0,06 para el IAC y Cp=36,37±0,59 para BPyV. En el caso de HAV, 10 copias del virus en presencia de 300 copias de IAC podían ser detectadas de forma fiable con Cp=30,29±0,31 para el IAC y Cp=26,28±0,61 para HAV. Para HEV, 10 copias del virus en presencia de 300 copias de IAC podían ser detectadas de forma fiable con

Cp=35,48±0,36 para el IAC y Cp=41,50±1,80 para HEV. Para HAdV, 10 copias del virus en presencia de 100 copias de IAC podían ser detectadas de forma fiable con Cp=33,69±0,25 para el IAC y Cp=34,62±0,60 para HAdV. En presencia de 300 copias de la molécula de IAC, 100 copias de NoVGI se pudieron detectar de manera robusta con unos valores de Cp=28,38±0,10 para el IAC y Cp=29,78 ±0,21 para NoVGI y en el caso de NoVGII se observó que en presencia de 300 copias del IAC, 10 copias del virus eran detectables de forma robusta con unos valores de Cp=32,65±0,03 para el IAC y Cp=27,54±0,09 para NoVGII. Para MNV-1 se observó que en presencia de 600 copias del IAC, 10 copias del virus eran detectables de forma robusta con unos valores de Cp=36,07±0,44 para el IAC y Cp=38,74±0,56 para MNV-1. Finalmente en el caso de PAdV, 10 copias del virus en presencia de 100 copias de IAC podían ser detectadas de forma fiable con Cp=34,66±0,10 para el IAC y Cp=36,54±0,56 para PAdV.

Evaluación del funcionamiento del IAC en la detección de virus de origen alimentario

Para evaluar el correcto funcionamiento del IAC se utilizaron dos virus como modelo: HAdV y MNV-1. Se preparó una muestra de 25 g de puré de fresa, negativa para estos virus, y se contaminó con aproximadamente 10^6 $TCID_{50}$ de HAdV y MNV-1. A continuación se realizó la concentración de la muestra y la extracción de ácidos nucleicos y se simuló una extracción de ácidos nucleicos inadecuada añadiendo 50 µl de puré de fresa sin extraer (conteniendo inhibidores). Los resultados de la RT-RTi-PCR fueron los esperados: en el caso de la muestra positiva, ambas señales, las del virus diana y la del IAC, fueron positivas; en el caso de la muestra negativa no se obtuvo señal para el virus pero sí para el IAC, y en el caso de la muestra positiva conteniendo sustancias inhibidoras no se obtuvo señal ni para el virus ni para el IAC, indicando que la reacción de amplificación no se llevó a cabo adecuadamente y se debía repetir.

Los IACs suponen una herramienta muy útil para la monitorización del buen funcionamiento de la PCR, puesto que se trata de los únicos controles analíticos capaces de identificar posibles problemas durante la reacción de manera individualizada en cada una de las muestras. Los IACs diseñados e implementados en este trabajo han sido optimizados individualmente para cada uno de los virus de interés de manera que el límite de detección del sistema con la adición del IAC no se viera afectado o en la menor medida posible. Queda así demostrado que los IACs construidos en este estudio se pueden utilizar de manera fiable para proporcionar un control robusto en los ensayos de detección de virus de origen alimentario y que pueden ser de aplicación rutinaria en el análisis de los alimentos.

2. Aplicación analítica de un control de procesado de muestra en la detección de virus de origen alimentario.

El objetivo de este estudio fue definir la aplicación analítica de un virus control de procesado de la muestra (SPCV) en métodos basados en la amplificación de ácidos nucleicos para la detección de virus entéricos en los alimentos. Así como evaluar su idoneidad para definir el rendimiento del tratamiento de la muestra antes de la amplificación de los ácidos nucleicos (rendimiento de extracción). Se seleccionaron el HAdV como virus diana, y el MNV-1 como SPCV. HAdV es un virus entérico que ha sido además propuesto como virus indicador de la existencia de vías de contaminación de origen humano. MNV-1 fue seleccionado como SPCV puesto que posee una estructura molecular y bioquímica similar y una vía de infección igual al NoV humano.

Con el fin de demostrar la utilidad del MNV-1 como SPCV se prepararon muestras contaminadas artificialmente con una cantidad conocida de HAdV (virus diana) y de MNV-1 (SPCV). Los procedimientos se llevaron a cabo de forma tanto correcta como incorrecta. De esta manera se obtuvieron los siguientes resultados: se obtuvo señal de amplificación en ambos virus, HAdV y MNV-1 y sus correspondientes IACs en los alimentos contaminados artificialmente en los que los protocolos se llevaron a cabo de forma correcta. Los rendimientos medios de extracción fueron 39,47%, 24,79% y 36,29% en el caso de fresas, lechuga y mejillones, respectivamente. Por el contrario, no se detectaron señales de amplificación en el caso de HAdV ni para MNV-1 en el supuesto en que los protocolos de concentración y extracción de ácidos nucleicos se realizaron de forma incorrecta.

No se encontraron diferencias significativas (p<0,05) en las señales de amplificación de los IAC, que fueron positivas en todos los casos (para ambos virus, HAdV y MNV-1, y en ambas posibles situaciones, cuando los protocolos se realizaron correcta e

incorrectamente). Este hecho nos indica que el paso de amplificación (PCR) se llevó a cabo de manera correcta y por lo tanto la falta de señal de amplificación para HAdV y MNV-1 se debió a un error durante los pasos de concentración y extracción de ácidos nucleicos, que era el supuesto que se pretendía mimetizar.

Muchas matrices alimentarias, como los vegetales para ensalada (productos de cuarta gama listos para el consumo), moluscos bivalvos y frutos rojos son susceptibles a la contaminación por virus. Estos alimentos contienen sustancias que pueden afectar (i) la elución de los virus del alimento y su posterior concentración, (ii) la extracción de ácidos nucleicos de los virus (iii) y/ o inhibir su posterior amplificación. Por lo tanto es esencial que se realice una verificación del proceso que incluya la detección de protocolos fallidos puesto que éstos podrían enmascarar la presencia de virus patógenos debido a una interpretación incorrecta de los resultados. La utilización de un SPCV puede servir para verificar los resultados de un análisis en la detección de virus entéricos en la cadena alimentaria.

Un aspecto importante del SPCV es que debe de ser un virus que comparta una estructura bioquímica y molecular muy similar al virus diana. Este es un aspecto crítico puesto que la implementación efectiva de este control dependerá de su capacidad para mimetizar al virus objeto del estudio y a todas las circunstancias a las que se verá sometido durante el proceso analítico.

En este trabajo se utilizaron tres tipos diferentes de alimentos: fresas, lechuga y mejillones, que han estado implicados en diversos brotes alimentarios de origen vírico y en los cuales se han encontrado a menudo sustancias inhibidoras de la PCR. El MNV-1 usado como SPCV demostró su efectividad para verificar el procedimiento analítico aplicado a la detección de virus entéricos (desde el paso de concentración de virus hasta la extracción de ácidos nucleicos y su posterior amplificación). La

utilización de un IAC además de un SPCV permitió una detección de problemas aún más precisa, ya que sirvió para monitorizar la presencia de posibles sustancias inhibidoras de la PCR. El principio de un SPCV es que si se detecta su señal, el método de concentración y extracción se han llevado a cabo correctamente. Si no se detecta, el método ha fallado y la matriz alimentaria debe ser analizada de nuevo. Además de esta aplicación cualitativa, un SPCV también nos permite determinar el rendimiento de extracción de cada muestra individual, mediante la comparación de los resultados de los valores de (RT)-RTi-PCR del SPCV antes y después de la adición de la muestra. Si el SPCV es un buen subrogado (modelo) del virus diana, su eficiencia de extracción reflejaría la de éste, y permitiría una determinación más precisa de la carga vírica en la muestra. Es decir, si el resultado del análisis es que se han detectado 20 copias genómicas del virus diana, 40 copias genómicas del SPCV, e inicialmente se añadieron 100 copias del SPCV, se puede inferir que la cantidad original de virus objeto del estudio que estaba contaminando la muestra era alrededor de 50 copias genómicas, puesto que el rendimiento de extracción del SPCV fue del 40%. Para concluir, con este estudio se ha demostrado la utilidad de la inclusión de un SPC para evaluar el correcto funcionamiento del proceso analítico. El SPCV descrito en este estudio supone una herramienta de control robusta y fiable que puede ser aplicada de forma rutinaria en el análisis de alimentos para la detección de virus de origen alimentario.

3. Estudio de los factores que influyen en la aplicación de los virus control de procesado de muestra para la detección de virus entéricos en los alimentos

El objetivo de este estudio fue evaluar el impacto de dos aspectos importantes en el uso de SPCVs: (i) el momento de la adición del SPCV y (ii) el tiempo transcurrido desde su adición hasta que se lleva a cabo la concentración y extracción de la muestra.

Con este propósito se inocularon artificialmente dos matrices alimentarias modelo (fresa y lechuga) con cantidades conocidas de MNV-1 y vMC_0 en tres etapas diferentes del protocolo de concentración y de extracción y se determinó el rendimiento analítico de los dos SPCVs. También se calcularon los rendimientos de extracción de los análisis cuando éstos se llevaron a cabo a diferentes tiempos después de la adición del SPCV sobre muestras de lechuga.

Evaluación del efecto sobre el rendimiento de extracción debido a la etapa en la que se añade el SPCV

Para demostrar en que paso del proceso era más apropiado añadir los SPCV se probaron tres opciones diferentes tanto en fresa como en lechuga. Se añadieron 20 µl de una mezcla que contenía ambos SPCV (vol 1:1): (i) sobre la superficie de la matriz y se dejó secar antes de continuar; o bien (ii) en una solución tampón (TGBE) o (iii) en el sobrenadante recuperado después de la primera centrifugación del procedimiento de extracción. Cada una de estas tres opciones se llevó a cabo por triplicado en tres experimentos independientes.

La adición del subrogado (SPCV) en la solución tampón TGBE mostró, en general, un mayor rendimiento de extracción. Este rendimiento de extracción osciló, en el caso de la fresa¸ entre el 40,54% y el 56,75% y el 13,40% y el 82,81% para MNV-1 y vMC_0, respectivamente. Los rendimientos de extracción promedio en el caso de MNV-1

fueron buenos (es decir, un rendimiento superior al 10%) con independencia del momento en que se añadió el subrogado. En el caso en el que el SPCV se añadió sobre la superficie de la fresa, los rendimientos de extracción no fueron significativamente diferentes ($p > 0,05$) a los observados cuando éste se añadía en una etapa posterior del proceso (tan sólo fueron significativamente diferentes los rendimientos observados cuando el SPCV se añadía en la solución tampón y después del primer paso de centrifugación, $p < 0,05$). En el caso de vMC_0 los rendimientos fueron significativamente diferentes cuando el SPCV se añadió sobre la superficie de la fresa, a los rendimientos observados cuando se añadía en la solución tampón y después de la primera centrifugación. El test Jonckheere mostró una tendencia en los resultados, el rendimiento medio de extracción aumentaba en la medida en que el subrogado se añadía a la matriz en etapas más tardías del proceso.

Los rendimientos de la reacción en el caso de la lechuga iceberg fueron del 12,54% al 21,97% y del 9,05% al 33,97% para MNV-1 y vMC_0, respectivamente. En el caso de MNV-1, el momento de adición del subrogado no afectó a los rendimientos de extracción ($p > 0,05$), e independientemente del momento de adición, los rendimientos fueron buenos (rendimiento >10%), excepto en un experimento en el que el subrogado se añadió sobre la superficie de la lechuga. Sin embargo, en el caso de vMC_0 el momento de adición del subrogado si afectó al rendimiento. Y los rendimientos de vMC_0 sólo fueron aceptables cuando el virus se añadió sobre la superficie de la lechuga (9,05% ± 0,82), y significativamente diferentes respecto a la adición en la solución tampón TGBE y después de la primera centrifugación

Evaluación del efecto sobre el rendimiento de la extracción en función del tiempo transcurrido desde la adición del SPCV y el posterior proceso analítico

Veinte µl de la mezcla de subrogados se añadieron a la superficie de la lechuga, se dejó que se secara, y se llevó a cabo el protocolo de concentración-extracción bien inmediatamente, o bien después de 1, 2, 4 o 24 horas. Los rendimientos de extracción observados en la lechuga iceberg oscilaron entre el 8,74% y el 13,60% y el 9,05% y el 20,00% en el caso de MNV-1 y vMC_0, respectivamente. Los rendimientos promedio fueron buenos, (> 10%) en casi todos los casos a excepción de MNV-1 cuando la lechuga se procesó después de 2 horas (rendimiento aceptable, 9,30 ± 0,63) y 24 horas (rendimiento aceptable, 8,74 ± 1,03) y en el caso de vMC_0 cuando la lechuga se procesó inmediatamente (rendimiento aceptable, 9,05 ± 1,50). Resulta interesante que el tiempo transcurrido desde la adición de los SPCVs hasta el inicio del proceso no mostrara, sin embargo, diferencias significativas en los rendimientos de extracción.

En conclusión, el tiempo transcurrido desde la adición del SPCV (MNV-1 o vMC_0) y el inicio del posterior proceso analítico (concentración y extracción de ácidos nucleicos) no mostró diferencias significativas. Sin embargo, si se observaron, tal y como se esperaba, diferencias significativas dependiendo de la etapa en la que el SPCV fue incorporado al proceso. Los rendimientos de extracción fueron más altos cuanto más tarde se añadía el SPCV a la muestra, lo que nos indica que durante el proceso hay una pérdida sustancial de virus, por este motivo se recomienda la adición del SPCV al principio del proceso, puesto que eso permitiría una mayor y más completa monitorización del análisis.

4. Diseño y aplicación de estándares de ácidos nucleicos para la detección y cuantificación de virus entéricos mediante PCR a tiempo real.

Se construyeron dos oligonucleótidos sintéticos (una molécula de ARN y una molécula de ADN) conteniendo múltiples secuencias diana, para ser usados como estándares de cuantificación en ensayos de amplificación de ácidos nucleicos. Las secuencias diana de fueron HAdV, PAdV y BPyV; y NoVGI, NoVGII, HEV y MNV-1 para las moléculas de ADN y ARN, respectivamente.

Construcción de los plásmidos que contenían el ADN sintético y el ADN que posteriormente se transcribió a ARN sintético

Se diseñó una molécula de ADN sintético que contenía las secuencias diana de BPyV, HAdV y PAdV para ser posteriormente utilizadas en ensayos de RTi-PCR. También se construyó una molécula de ADN sintético que contenía las secuencias diana de HEV, MNV-1, NoVGI y NoVGII para utilizarla en ensayos de PCR a tiempo real con transcripción inversa (RT-RTi-PCR) y se clonó en un plásmido. La secuencia final del vector contenía 424 pb. Puesto que se trataba de una molécula que iba a ser utilizada en ensayos de RT-RTi-PCR se llevo a cabo una transcripción *in vitro* utilizando la enzima T7 ARN polimerasa.

Evaluación de la actuación de la molécula de ARN sintético en los ensayos de RT-RTi-PCR

Se llevaron a cabo ensayos de RT-RTi-PCR de los cuatro virus (NoVGI, NoVGII, HEV y MNV-1) incluyendo diluciones decimales (10^6 a 10^1 moléculas de ARN). En base a la linearidad (R^2) y la eficiencia de la PCR (E) se calculó la capacidad de cuantificación del estándar de ARN, ambos parámetros fueron cercanos al valor óptimo en todos los experimentos ($R^2 \geq 0{,}998$ y $E \geq 0{,}89$), quedando así demostrado que el uso del ARN

sintético como estándar para construir curvas de cuantificación es una aproximación adecuada. Los límites de cuantificación (LOQ) fueron 1×10^1 copias del estándar ARN por reacción en todos los casos, con la excepción de NoVGII y HEV donde el límite de cuantificación fue de 1×10^2 copias del estándar por reacción. Además, el rango dinámico de cuantificación fue de, al menos, cinco $\log_{10}$. Se comparó el rendimiento de los ensayos de RT-RTi-PCR utilizando diluciones decimales del estándar y diluciones de ARN nativo de MNV-1 (las cantidades iban de 10^6 a 10^1 moléculas de ARN). Los resultados fueron muy similares, (R^2 de 0,998 en ambos casos y E de 0,93 y 0,89 para el ARN de MNV-1 nativo y sintético, respectivamente).

Evaluación de la actuación de la molécula de ADN sintético en los ensayos de RTi-PCR

Se llevaron a cabo ensayos de RTi-PCR de los tres virus (BPyV, PAdV y HAdV) incluyendo diluciones decimales (10^5 a 10^1 moléculas de ADN). De manera similar a los ensayos de RT-RTi-PCR, la capacidad de cuantificación también se calculó en base a la linealidad y la eficiencia de PCR. El valor de ambos parámetros fue cercano al óptimo en todos los experimentos ($R^2 \geq 0{,}96$ y $E \geq 0{,}997$), lo que demuestra que el uso del ADN sintético como estándar para construir curvas de cuantificación es también una aproximación adecuada. Los límites de cuantificación (LOQ) fueron 1×10^1 copias del estándar ADN por reacción en todos los casos. El rango dinámico de cuantificación era también de al menos cinco $\log_{10}$. Se compararon también el rendimiento de los ensayos de RTi-PCR utilizando diluciones decimales del estándar y diluciones de ADN nativo de HAdV (las cantidades iban de 10^5 a 10^1 moléculas de ADN). En este caso también la linearidad y los valores de eficiencia de la PCR fueron muy similares (R^2 de 0,999 en ambos casos y E de 1,00 y 0,96 para el ADN nativo y sintético, respectivamente).

La cuantificación precisa de los virus es importante para determinar, no sólo el nivel de contaminación de los alimentos, las superficies, las aguas, etc, sino también para determinar cualquier reducción de la contaminación por virus después de un tratamiento de desinfección. La cuantificación vírica se puede utilizar también para determinar una posible vinculación entre los niveles de virus y el riesgo de infección o de brotes asociado. Se ha sugerido que para que un ensayo de (RT)-RTi-PCR sea aceptable la curva estándar debe tener un coeficiente de correlación (R^2) ≥ 0,98 y un valor de pendiente (s) entre 3,6 y 3,1, lo que correspondería a eficacias de reacción (E) entre 0,9 y 1,1. Al comparar las curvas estándar obtenidas a partir de las diluciones decimales de los ácidos nucleicos nativos y de los estándares sintéticos se vio que en ambos estándares (ADN y ARN), todos los valores de R^2 y E se encontraban dentro de los límites aceptables. No obstante, se observó que la cuantificación de los virus ADN fue más eficiente que la cuantificación de los virus ARN. Esto es debido probablemente a la naturaleza de la molécula de ARN, más lábil, y también al paso adicional de transcripción inversa, pudiendo influir ambos factores en la eficacia de cuantificación. Finalmente, y más importante, se observó una equivalencia entre el número de copias del estándar de ARN y los equivalentes genómicos de MNV-1, y entre el número de copias del estándar de ADN y los equivalentes genómicos de HAdV. Es de esperar que esta relación de equivalencia se dé también en el resto de especies de virus presentes en estos estándares. De esta manera, la disponibilidad de estos estándares debería facilitar la generalización del uso de métodos de detección de virus basados en la amplificación de ácidos nucleicos con fines cuantitativos.

5. Ensayo internacional de validación de un método para la detección de adenovirus humano en frutos rojos

Puesto que los virus patógenos de origen alimentario pueden estar presentes en los alimentos solo de manera esporádica o durante brotes epidémicos, e incluso estando presentes raramente son detectados, su control rutinario puede ser difícil. Por este motivo la monitorización de virus indicadores podría ser un método efectivo para poner de manifiesto la existencia de una posible fuente de contaminación por la que los virus patógenos alcanzarían las cadenas de suministro de alimentos. Se ha propuesto el uso de HAdV como uno de estos virus indicadores. Mediante un ensayo de colaboración, en el que participaron once laboratorios europeos, se determinó el rendimiento cualitativo de un método basado en RTi-PCR para detectar HAdV en frambuesas. También se pretendió con este estudio evaluar si la metodología empleada, que fue común para todos los laboratorios participantes, ofrecía resultados comparables. Este método incorporaba dos controles, un control de procesado de la muestra (MNV-1) y un control interno de amplificación. La sensibilidad del ensayo, es decir, la correcta identificación de las muestras de 25 g de frambuesas contaminadas artificialmente con 5×10^2 y 5×10^4 unidades formadoras de placa (pfu), fue del 98,5%. La consistencia (*accordance*) y la concordancia (*concordance*) fueron en ambos casos del 97,0%. El valor predictivo positivo fue del 94,2%. La especificidad del ensayo, es decir, el porcentaje correcto de identificación de muestras no contaminadas artificialmente, fue del 69,7%; la consistencia fue del 80,0% y la concordancia fue del 61,7%. El valor predictivo negativo fue del 100%. Estos resultados, en conjunto, fueron considerados aceptablemente robustos y comparables.

En conclusión, la aplicación del método estudiado para la detección de HAdV en muestras de alimentos demostró que podría ser de utilidad en los programas de

control y seguridad alimentaria para identificar una vía de contaminación de origen humano en la cadena de suministro de alimentos que algunos virus patógenos como los NoV y el HAV podrían utilizar.

6. La cuantificación del genoma de norovirus no sirve para predecir su capacidad infecciosa después de aplicar tecnologías de inactivación durante la elaboración de alimentos

Para determinar la eficacia de un proceso tecnológico para reducir la capacidad infecciosa de cualquier virus contaminante durante la elaboración de alimentos es necesario distinguir inequívocamente entre virus infecciosos y no infecciosos. Esta tarea puede ser difícil, particularmente en el caso de los NoV porque no hay disponible un modelo fiable de cultivo celular. El objetivo de este estudio fue evaluar el uso de métodos moleculares – RT-RTi-PCR y el tratamiento enzimático (ET) acoplado a RT-RTi-PCR – para cuantificar la capacidad infecciosa de NoV después de la aplicación de diversas tecnologías de inactivación (HHP, UV y tratamiento térmico) que pueden ser utilizadas durante la elaboración de alimentos. En particular se centró en (i) establecer una relación entre la pérdida de la señal de RT-RTi-PCR y la pérdida de capacidad infecciosa del virus después de tratamientos de inactivación y (ii) determinar si el ET mejoraría la correspondencia entre los resultados observados por RT-RTi-PCR y los resultados de cultivo celular. Los tratamientos de inactivación utilizados en este estudio fueron altas presiones hidrostáticas (HHP), radiación ultravioleta (UV) y tratamiento térmico, y se aplicaron a un virus subrogado de los norovirus humanos, el norovirus murino (MNV-1). La comparación de los resultados en los recuentos de genoma viral por RT-RTi-PCR y ET-RT-RTi-PCR fueron significativamente diferentes (p <0,01) para todos los procedimientos de inactivación y para todas las condiciones utilizadas, excepto para el tratamiento de HHP a 600 MPa durante 5 minutos. Estos resultados indican que el ET antes de la RT-RTi-PCR puede eliminar una parte de las partículas víricas con genomas afectados, lo que repercutiría en la estimación de la reducción vírica. Sin embargo, no se encontró una correlación entre los resultados obtenidos por ET-RT-RTi-PCR y los obtenidos por cultivo celular. Por lo tanto, se

puede asumir que el efecto del ET es sólo parcial, y no resulta adecuado para estimar de una manera precisa el grado de inactivación vírica. Para confirmar si nuestros resultados se podían deber a un diseño experimental incorrecto, se analizaron varias combinaciones de tiempos y concentraciones de enzimas diferentes y no se encontró ningún error experimental. Nuestros resultados indican que la cuantificación de los genomas de los virus por PCR, independientemente de la utilización de un ET previo, no es un enfoque adecuado para establecer el grado de inactivación de un virus y / o su capacidad infecciosa. Además, nuestros resultados también ilustran que el efecto general de inactivación vírica no está directamente correlacionado con los efectos sobre la integridad del genoma del virus y las proteínas de la cápside, pudiéndose así asumir que la inactivación es debida a efectos sobre las proteínas implicadas en las etapas de adhesión e invasión.

7. Presencia del virus de la hepatitis E en la cadena de producción de carne de cerdo en tres países europeos (República Checa, Italia y España) en el año 2010

Se evalúo la presencia del virus de la hepatitis E (HEV) a lo largo de la cadena de producción de carne de cerdo en la República Checa, Italia y España. El muestreo incluyó: matadero, fase de procesado y puntos de venta. En conjunto, la presencia de HEV fue mayor en Italia (53%) y en España (39%) que en la República Checa (7,5%). Se analizaron un total de 337 muestras (heces, hígado y muestras de carne) provenientes de los mataderos mediante RT-RTi-PCR. Y también se muestrearon 313 salchichas en las instalaciones de procesado y los puntos de venta.

El hallazgo de HEV en muestras provenientes de los tres países participantes en el estudio (República Checa, Italia y España) indica que HEV está extendido de manera generalizada en Europa. La estrategia de tomar muestras fecales y el hígado del mismo animal nos permitió detectar que la ausencia HEV en las heces de los animales no se correspondía necesariamente con la ausencia de ARN de HEV en hígado. De hecho, en 3 animales encontramos ARN de HEV en el hígado, mientras que no se encontró ARN de HEV en las heces. Una posible explicación de este hecho podría ser que la concentración de bilis esperada podría ser menor en la masa fecal en el momento del muestreo (en el matadero), puesto que los cerdos se alimentaron mucho antes de llegar al matadero, que en el hígado. HEV se detectó principalmente en heces en Italia (41%) y España (38%), mientras que en la República Checa se detectó en el 3% de las muestras. En el caso de las muestras de hígado, Italia fue el país en el que con mayor frecuencia se detectó ARN de HEV (6%), seguido de la República Checa (5%) y España (3%). Sólo se detectó HEV en España (6% de las muestras españolas, 2% del total de muestras de salchicha), la interpretación de este hallazgo no está clara y merece una investigación en mayor profundidad. Respecto al uso de PAdV como indicador viral, la

mayoría de las heces fueron positivas para PAdV, pero nunca se detectó en hígado y sólo de vez en cuando en la carne de cerdo (1/112) o en las salchichas (4/313). Al comparar la prevalencia de HEV y PAdV, se puede ver que el riesgo de contaminación cruzada de los productos derivados del cerdo con heces porcinas durante el sacrificio o en etapas posteriores de la fase de producción parece ser bajo, pero no es completamente inexistente.

La secuenciación de las muestras positivas para HEV confirmó que las cepas pertenecían todas al genotipo g3. Cinco de las siete secuencias españolas pertenecían al subtipo g3f, una cepa de HEV que es predominante en Europa. Las otras dos secuencias españolas no se pudieron clasificar como pertenecientes a ningún subtipo, pero se vio que estaban estrechamente relacionadas con el subtipo g3c, el mismo subtipo al que pertenecían las cuatro secuencias de HEV italianas. Estas dos secuencias sin clasificar podrían bien representar un nuevo subtipo de HEV g3, o bien, al estar relacionadas con g3c, representar secuencias de ese subtipo. El subtipo g3c sólo contiene secuencias de origen europeo, pero en 2006, cuando se hizo la clasificación, este subtipo sólo incluía secuencias holandesas, y ahora se ha extendido a otros países europeos (Italia y, probablemente España) como se muestra en los resultados.

La detección de secuencias de ARN de HEV no demostró la capacidad infectiva del virus y por lo tanto se desconocen las implicaciones en materia de Salud Pública para los consumidores. Sin embargo, no podemos excluir la posibilidad de que en algunos de los productos en los que se encontró HEV, el virus fuera infeccioso. Para evaluar el riesgo de infección tras el consumo de un alimento (*risk of health event*), se utilizó un modelo de dosis-respuesta. Este modelo se basa en la inoculación intravenosa de HEV en cerdos, aplicando un factor de corrección para reflejar una posible infección después de la exposición al agente a través de la vía fecal-oral y se estimó que existían

riesgos reales para la salud en el caso de los productos que contenían carne de cerdo con HEV.

8. Prevalencia y transmisión del virus de la hepatitis E en poblaciones de cerdos domésticos en diversos países europeos

Los genotipos 3 y 4 de HEV pueden causar hepatitis en humanos y tienen como principal reservorio los cerdos. Existe una gran falta de información sobre la dinámica de infección en las poblaciones porcinas. Por este motivo se estudió la dinámica de infección de HEV en granjas porcinas comerciales de seis países europeos (República Checa, Italia, Portugal, España, Holanda y Reino Unido). Además, se describió también la prevalencia de HEV y la dinámica de transmisión dentro de la piara. Los datos recogidos muestran que la prevalencia en cerdos destetados va desde el 8% al 30%. La prevalencia media de HEV en los cerdos en fase de crecimiento fue de entre el 20% y el 44%. La prevalencia en los cerdos en fase de acabado o engorde varió entre el 8% y el 73%. En cerdas, la prevalencia fue similar en todos los países. Se analizaron muestras de verraco sólo en España y la República Checa, y la prevalencia fue del 4,3% y del 3,5%, respectivamente.

Para realizar este estudio se utilizaron métodos de detección similares (RT-RTi-PCR) para todas las muestras y los datos se analizaron todos con el mismo modelo (modelo SIR). El modelo que se utilizó para describir la transmisión de HEV en una piara de cerdos es un modelo estructurado por edad SIR. Cada grupo de edad se subdividió en tres compartimentos distintos que consisten en los cerdos que son susceptibles (S), infecciosos (I) o recuperados (R). Para realizar el análisis, se asumió que cada animal susceptible podía ser infectado por un animal infeccioso en su propio grupo o en cualquier otro grupo con la misma probabilidad. Se asumió que los tamaños de muestra en cada conjunto de datos representaban el 5% del tamaño total del grupo.

La dinámica de transmisión se caracteriza por el periodo infeccioso promedio (μ) y el parámetro tasa de transmisión (β): que indica el número de infecciones que puede

causar un animal infeccioso por unidad de tiempo. El producto de estos dos parámetros es el número reproductivo R_0 ($R_0 = \mu \times \beta$) que expresa el número de infecciones de un animal infeccioso puede causar durante su período infeccioso completo en una población totalmente susceptible. Cuando el número reproductivo es mayor que la unidad, $R_0 > 1$, un brote puede crecer exponencialmente. De lo contrario, cuando $R_0 < 1$, el brote se extinguirá. El modelo SIR asume que la transmisión de HEV está en equilibrio endémico, es decir, la enfermedad se puede sostener por sí misma (regeneración) en la población de cerdos. Por esta razón, en la aplicación del modelo se omitieron las piaras con resultados negativos o con tan sólo unos pocos resultados positivos, puesto que en ellos no se podía justificar el equilibrio endémico.

El parámetro tasa de transmisión (β) en nuestro estudio fue de $\beta = 0{,}11$ (IC del 95%: 0.070 - 0.17) $día^{-1}$ para los datos del Reino Unido de 2007, lo que significa que un animal infeccioso infectaría a otro animal cada 10 días. Los parámetros $\beta = 0{,}071$ (IC del 95%: 0,041 - 0,13) $día^{-1}$ para los datos del Reino Unido de 2008 y $\beta = 0{,}037$ (IC del 95%: 0,0035 - 0,16) $día^{-1}$ para los datos de Portugal 2011. En condiciones experimentales se estimó que la tasa de transmisión era mayor $\beta = 0{,}66$ (IC del 95%: 0.32 - 1,35) $día^{-1}$, esta diferencia se podría explicar por la mayor proximidad de los animales en un entorno experimental en comparación con un entorno agrícola como es una granja. Los parámetros β de los otros países participantes en el estudio no pudieron ser estimados, bien porque sólo se analizó un único grupo de edad o bien porque la mayoría de los animales fue negativo para el análisis y el modelo no era aplicable.

Este estudio contribuye a una mejor comprensión de la prevalencia y transmisión de HEV en seis países europeos mediante una modelización matemática estructurada por edad (SIR). Los resultados obtenidos confirman que el HEV está circulando en las

granjas de cerdos en Europa y puede estar presente en los cerdos en fase de engorde o acabado (13-26 semanas de edad); siendo, por lo general, este grupo de animales el que con más frecuencia se consume en los hogares. Se encontraron cerdos en fase de engorde o acabado positivos para HEV en todos los países europeos estudiados. Esto puede suponer un riesgo importante para la Salud Pública, sobre todo en aquellos países donde se consumen productos porcinos crudos o no completamente cocidos.

9. Incidencia de virus entéricos humanos en mejillones vendidos en comercios al por menor en tres países europeos

En este estudio, se evaluó la prevalencia de diferentes virus entéricos en los mejillones vendidos en comercios al por menor en tres países europeos (Finlandia, Grecia y España). Se adquirieron un total de 153 muestras de mejillones de diferentes orígenes para analizar la presencia NoVGI, NoVGII, HAV y HEV. También se analizó la presencia de HAdV como un indicador de contaminación fecal de origen humano. Durante el proceso de análisis de las muestras se utilizaron una serie de controles (SPCV, IAC, PAC and NAC). Con el fin de obtener datos cuantitativos comparables, en este estudio se utilizaron métodos previamente validados mediante ensayos inter-laboratorio y no se observaron diferencias significativas en la sensibilidad de los ensayos entre los tres laboratorios participantes.

El uso de un SPCV nos permitió calcular los rendimientos de extracción de las muestras, que variaron desde el 79% hasta el 0,5%, con un valor promedio de 10%. El rendimiento teórico promedio fue del 6%, con valores que iban desde el 51% al 0,3%. En conjunto, el 92% de las muestras tuvieron un rendimiento de extracción aceptable o bueno (45% y 47%, respectivamente) y sólo el 8% tuvo un rendimiento de extracción pobre (< 1%). Respecto al rendimiento de extracción teórico, la mayoría de las muestras (88%) tuvo un rendimiento aceptable o bueno (55% y 33%, respectivamente) y también sólo el 8% de las muestras tuvo rendimientos teóricos de extracción pobres (< 1%).

Las muestras fueron positivas en el 41% de los casos (62/153). Se detectó un único tipo de virus entérico en el 38% de las muestras (58/153), y dos tipos de virus entéricos en el 3% (4/153) de las muestras. HAdV fue el virus que se detectó con mayor frecuencia (36%, 37/102), seguido por NoVGII (16%, 25/153), HEV (3%, 3/102) y NoVGI (0,6%,

1/153). Sin embargo, no se detectó HAV en ninguna muestra. A pesar de que HAdV fue el virus más frecuentemente detectado no se encontró una correlación significativa entre la presencia de HAdV y ninguno de los virus patógenos analizados (NoVGI, NoVGII, HAV y HEV). Por lo tanto, tan sólo podemos concluir que las muestras estuvieron en contacto con aguas contaminadas con heces humanas pero no hay indicaciones que apoyen el uso de adenovirus como indicador de virus patógenos.

El número estimado de unidades detectables por PCR (PDU) en las muestras osciló entre 24 y 1,4 × 10^3 PDU g^{-1} de tejido de mejillón en el caso de NoV GII, entre 127 y 348 PDU g^{-1} de tejido de mejillón en el caso de HEV y para NoVGI se calculó que las PDU g^{-1} de tejido de mejillón fueron ~260. Un alto porcentaje de las 102 muestras de *M. galloprovincialis* fueron positivas para alguno de los virus entéricos analizados en comparación con las 51 muestras de *M. edulis*. Se vio que existía una correlación altamente significativa entre la especie de mejillón y el resultado analítico de la muestra ($p < 0{,}001$), una muestra tenía ~25 veces más probabilidades de ser positiva si la especie del mejillón era *M. galloprovincialis* que si se trataba de *M. edulis*. Sin embargo, dado el completo solapamiento entre el país donde se recogieron las muestras y las especies examinadas esta aparente correlación requiere de un estudió más extenso en el que se incluirían muestras de ambas especies y de todos los países de origen. También se estudió el posible efecto debido a la conservación de las muestras (es decir, si las muestras estaban congeladas o frescas en el momento de adquirirlas). Las 102 muestras de mejillones se adquirieron frescas y el análisis para detectar virus entéricos dio los siguientes resultados: el 25% (25/102), el 6% (3/51), el 3% (3/102) y el 0,98% (1/102) de las muestras fueron positivas para NoVGII, HAdV, HEV y NoVGI, respectivamente, mientras que, de entre los mejillones congelados, sólo se detectó HAdV en 34 muestras (67%). Por lo tanto, no se encontró una correlación significativa (p <0,05) entre las

condiciones de almacenamiento de los mejillones en el momento de la compra y si las muestras fueron positivas o no a la presencia de virus entéricos.

10. Estudios de inactivación de virus de origen alimentario mediante el uso de altas presiones hidrostáticas o aceites esenciales de plantas.

Se llevaron a cabo una serie de estudios con el fin de reducir la contaminación vírica de los alimentos mediante la inactivación de las partículas virales. Para ello se utilizaron dos tecnologías de inactivación alternativas a los tratamientos térmicos (HHP y compuestos naturales presentes en aceites esenciales, EO, de las plantas) con dos virus, MNV-1-y HAdV-2, un subrogado del norovirus humano y un virus patógeno humano, con genomas de ARN y ADN, respectivamente. Sin embargo, los resultados obtenidos fueron dispares mientras que se demostró como la aplicación de HHP era una opción eficaz, el uso de EOs no alcanzó los niveles de inactivación esperados en los virus estudiados.

Un tratamiento de HHP de 400MPa fue suficiente para alcanzar una reducción adecuada sobre la capacidad infecciosa de ambos virus (HAdV-2 y MNV-1). Únicamente 2,5 min fueron suficientes para reducir la capacidad infecciosa de MNV-1 en al menos 5,13 $\log_{10}$ y 3,33 $\log_{10}$, en agua y puré de fresa, respectivamente. En el caso de HAdV-2, aplicando las mismas condiciones de presión, se estimó una reducción de la capacidad infecciosa del virus en aproximadamente 6 $\log_{10}$ en poco más de 1,5 min (93 seg). Estos niveles de reducción en la capacidad infecciosa de los virus cumplen con los requisitos marcados para alcanzar un objetivo de seguridad alimentaria (FSO), que en el caso de los virus se estableció en, al menos, una reducción de la capacidad infecciosa de 4 $\log_{10}$.

La inactivación de virus usando HHP está modulada por muchos factores. Algunos factores extrínsecos son los propios parámetros de tratamiento, el tiempo y la presión aplicada al virus. Se observó que la reducción de la capacidad infecciosa de los virus aumentaba con el aumento en el tiempo y la intensidad del tratamiento. Por ejemplo,

en el caso de MNV-1 en puré de fresa, se observó una reducción de 1,21 log_{10}, 2,63 log_{10} y 2,75 log_{10} utilizando 300 MPa durante 2,5 min, 5 min y 10 min, respectivamente. Cabe señalar que este hecho se observó en los virus estudiados (MNV-1 y HAdV-2), pero existen algunas excepciones. En el caso de poliovirus, por ejemplo, esta afirmación no se cumple, ya que tratamientos de 600 MPa durante 60 min no causaron una reducción significativa de la capacidad infecciosa del virus. El grado de inactivación de los virus se puede ver también influido por factores intrínsecos tales como la composición del medio en el que se encuentra, los virus no se comportan igual cuando se tratan en alimentos o medio de cultivo, por ejemplo. Los alimentos son matrices muy complejas y diferentes características, como por ejemplo el pH, el contenido en azúcar o la presencia de sustancias quelantes, pueden afectar el grado de inactivación vírica. Estas diferencias pueden ser tan relevantes como para hacer que se observen resultados paradójicos; el ambiente ácido (de un puré de fresa) puede favorecer la inactivación de virus, como se observó en MNV-1, pero también puede actuar como un factor baro-protector, tal y como han descrito otros autores, cuando MNV-1 fue tratado en un medio acuoso ácido. Estos resultados demuestran que el efecto real sólo se puede observar cuando se utiliza la matriz específica en la que se quiere reducir la carga vírica. Otro tipo de factores intrínsecos que afectan el grado de inactivación de los virus al aplicar HHP se pueden deber a particularidades propias de cada especie de virus; los calicivirus (incluyendo el NoV humano y sus subrogados) son virus pequeños, sin envuelta y con un una cápside proteica extremadamente simple, y por lo tanto, resistente. El HAdV-2, por su parte, es también un virus sin envuelta, pero tiene unas estructuras en forma de "espículas" o "fibras" asociadas a la base de cada penton de la cápside. Estas estructuras intervienen en la unión a la célula huésped, por lo tanto, cualquier daño en ellas podría repercutir en la capacidad infecciosa del virus.

Los resultados obtenidos en el caso de HAdV se analizaron mediante un modelo de mejor ajuste y se vio que no había reducción en la capacidad infecciosa para presiones de 200 y 250 MPa aplicadas durante un máximo de 10 min. A presiones ≥ 300 MPa, los virus comenzaban a inactivarse, pero de un modo dinámico, es decir, a 300 MPa sólo había una tasa de inactivación, la inactivación era constante (modelo de inactivación monofásica). Sin embargo para presiones de 350 MPa, la tasa de inactivación ya no era única, sino que había dos (bifásica) y para 400 y 600 MPa la tasa de inactivación observada era dependiente del tiempo. Una posible explicación para este dinamismo observado en las tasas de inactivación podría ser que las partículas de los virus difieren en la susceptibilidad a la presión, existirían dos sub-poblaciones. A presiones más bajas sólo se verían afectadas las partículas más sensibles y a una velocidad constante (modelo de inactivación monofásica), mientras que a presiones superiores, también se verían afectadas las sub-poblaciones de partículas con menor susceptibilidad y con tasas de inactivación diferentes (modelo de inactivación bifásica).

En el caso del empleo de EOs, no se observó ninguna reducción significativa en MNV-1 y 2-HAdV. Tan sólo en el caso de HAdV-2, la diferencia observada en la concentración del virus fue estadísticamente significativa (p <0,05) cuando se trató con el EO de hisopo al 0,2% durante 24 horas a 4 ºC. Sin embargo, esta reducción fue de 0,29 $\log_{10}$, una reducción insuficiente para disminuir considerablemente el riesgo de una infección de origen alimentario. Por lo tanto, llegamos a la conclusión de que el uso de EOs de hisopo y mejorana, en las condiciones experimentales utilizadas, no sirve para alcanzar un nivel satisfactorio de inactivación de los virus entéricos sin envoltura.

Chapter 7

Conclusiones

1. Se ha demostrado que el uso de controles como el virus control de procesado de la muestra (SPCV) y el control de amplificación interno (IAC) en la detección de virus mediante métodos moleculares es una manera fiable y eficaz para evaluar los resultados del análisis de muestras provenientes de diversas cadenas de producción de alimentos, así como para evaluar el rendimiento del proceso de extracción.
2. No se encontraron diferencias significativas en el rendimiento de los controles de procesado de la muestra (mengovirus y norovirus murino) hasta 24 horas después de ser añadidos a la muestra. Se ha visto además, que la incorporación del SPCV al comienzo del análisis permite una monitorización más completa del procedimiento. Por lo tanto, se consideró el norovirus murino como el mejor candidato posible, especialmente para el propósito específico de detección de los norovirus humanos.
3. Los ácidos nucleicos sintéticos pueden ser utilizados como patrones para llevar a cabo una cuantificación precisa de los virus y constituyen además una fuente alternativa (a los ácidos nucleicos naturales extraídos de los virus) como controles positivos en los métodos de detección molecular.
4. El tratamiento preenzimático previo a la extracción de ácidos nucleicos no parece ser una opción válida para cuantificar la capacidad infecciosa de un virus mediante el uso de PCR a tiempo real.
5. Se detectó ARN del virus de la hepatitis E a lo largo de toda la cadena de producción de carne de cerdo en Europa (desde la granja hasta la mesa), lo que implica un riesgo potencial para la salud de los consumidores.
6. El adenovirus porcino se detectó frecuentemente en las heces de cerdo, este hallazgo, junto con su escasa detección en los productos derivados del cerdo (carne y salchichas), y su ausencia total en las muestras de hígado, indica que

los riesgos de contaminación con heces de cerdo durante el sacrificio y la manipulación de los alimentos de origen porcino parecen ser bajos, pero no completamente inexistentes.

7. La transmisión del virus de la hepatitis E en poblaciones porcinas en granjas europeas desde un animal infectado a uno susceptible se estimó en una cada 10 - 27 días, en base a los datos sobre la presencia del virus de la hepatitis E en heces muestreadas en diferentes granjas europeas.
8. Se detectaron virus patógenos (el norovirus humano genogrupo I y II y el virus de la hepatitis E) en mejillones muestreados en el punto de venta. Teniendo en cuenta que el marisco es un alimento que se consume crudo o poco cocido, se utilizaron modelos de dosis-respuesta y se concluyó que sólo los norovirus representaban un riesgo para la salud.
9. Las muestras de mejillones comprados al por menor tenían una alta prevalencia del adenovirus humano, aunque no se encontró ninguna correlación con los virus patógenos. Por lo tanto, este hallazgo indica únicamente que las muestras estuvieron en contacto con aguas contaminadas con heces humanas pero no apoya el uso del adenovirus humano como indicador de la presencia de virus patógenos.
10. Se ha demostrado la eficacia de las altas presiones hidrostáticas (tratamientos de 400MPa o superiores) como una tecnología de inactivación no térmica capaz de alcanzar un objetivo de seguridad alimentaria de reducción en la capacidad infectiva del virus de al menos 4 $\log_{10}$.
11. El uso de los aceites esenciales de mejorana e hisopo no alcanzó los niveles de inactivación vírica esperados para ser considerado un procedimiento adecuado en la descontaminación de alimentos.

Chapter 8

References

1. Anonymous ISO/TS 22119:2011. Microbiology of food and animal feeding stuffs-Realtime polymerase chain reaction (PCR) for the detection of foodborne pathogens-General requirements and definitions.

2. Anonymous ISO/TS 22174:2005. Microbiology of food and animal feeding stuffs-Polymerase chain reaction (PCR) for the detection of food-borne pathogens-General requirements and definitions.

3. Atmar R.L. and Estes M.K., 2001. Diagnosis of noncultivatable gastroenteritis viruses, the human caliciviruses. Clinical Microbiology Reviews 14, 15-37.

4. Atmar R.L., 2006. Molecular methods of virus detection in foods. Viruses in Foods. Food Microbiology and Food Safety Series (Goyal SM ed.), pp. 121-149 Springer, New York.

5. Baert L., Mattison K., Loisy-Hamon F., Harlow J., Martyres A., Lebeau B., Stals A., Van Coillie E., Herman L. and Uyttendaele M., 2011. Review: norovirus prevalence in Belgian, Canadian and French fresh produce: a threat to human health? International Journal of Food Microbiology 151, 261-269.

6. Bányai K., Martella V., Molnár P., Mihály I., Van Ranst M. and Matthijnssens J., 2009. Genetic heterogeneity in human G6P[14] rotavirus strains detected in Hungary suggests independent zoonotic origin. The Journal of Infection 59, 213-215.

7. Bidawid S., Farber J.M. and Sattar S.A., 2000. Contamination of foods by food handlers: experiments on hepatitis A virus transfer to food and its interruption. Applied and Environmental Microbiology 66, 2759-2763.

8. Bofill-Mas S., Albinana-Gimenez N., Clemente-Casares P., Hundesa A., Rodríguez-Manzano J., Allard A., Calvo M. and Girones R., 2006. Quantification and stability of human adenoviruses and polyomavirus JCPyV in wastewater matrices. Applied and Environmental Microbiology 72, 7894-7896.

9. Borgen K., Herremans T., Duizer E., Vennema H., Rutjes S., Bosman A., de Roda-Husman A.M. and Koopmans M., 2008. Non-travel related HEV genotype 3 infections in The Netherlands; A case series 2004-2006. BMC Infectious Diseases 8, 61-71.

10. Bosch A., Sanchez G., Abbaszadegan M., Carducci A., Guix S., Le Guyader F.S., Netshikweta R., Pintó R.M., van der Poel W., Rutjes S., Sano D., Rodríguez-Lázaro D., Kovac K., Taylor M.B., van Zyl W. and Sellwood J., 2011. Analytical methods for virus detection in water and food. Food Analytical Methods 4, 4-13.

11. Bouwknegt M,, Lodder-Verschoor F,, van der Poel W,H,, Rutjes S,A and de Roda Husman A.M., 2007. Hepatitis E virus RNA in commercial porcine livers in The Netherlands. Journal of Food Protection 70, 2889-2895.

12. CAC (Codex Alimentarius Commission), 1999. Principles and guidelines for the conduct of microbiological risk assessment, CAC/GL-30.

13. Cannon J.L., Papafragkou E., Park G.W., Osborne J., Jaykus L.A. and Vinjé J., 2006. Surrogates for the study of norovirus stability and inactivation in the environment: A comparison of murine norovirus and feline calicivirus. Journal of Food Protection 69, 2761-2765.

14. Clemente-Casares P., Pina S., Buti M., Jardi R., Martín M., Bofill-Mas S. and Girones R., 2003. Hepatitis E Virus Epidemiology in industrialized countries. Emerging Infectious Diseases 9, 448-454.

15. Cliver D.O., 2008. Historic Overview of Food Virology. Food-Borne Viruses: Progress and Challenges. Koopmans M.P.G., Cliver D.O. and Bosch A., eds. pp 1-28. ASM press, Washington, DC.

16. Coelho C., Heinert A.P., Simões C.M. and Barardi C.R., 2003. Hepatitis A virus detection in oysters (Crassostrea gigas) in Santa Catarina State, Brazil, by reverse transcription-polymerase chain reaction. Journal of Food Protection 66, 507-511.

17. Cook N., Bridger J., Kendall K., Iturriza-Gómara M., El-Attar L. and Gray J., 2004. The zoonotic potential of rotavirus. The Journal of Infection 48, 289-302.

18. Cook N. and Rzeżutka A., 2006. Hepatitis viruses. Emerging foodborne pathogens. Motarjemi Y. and Adams M. eds. pp. 282-308, Woodhead Publishing Limited, Cambridge, UK.

19. Costafreda M.I., Bosch A. and Pintó R.M., 2006. Development, evaluation, and standardization of a real-time TaqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples. Applied and Environmental Microbiology 72, 3846-3855.

20. Croci L., De Medici D., Morace G., Fiore A., Scalfaro C., Beneduce F. and Toti L., 1999. Detection of hepatitis A virus in shellfish by nested reverse transcription-PCR. International Journal of Food Microbiology. 48, 67-71.

21. Croci L., Dubois E., Cook N., de Medici D., Schultz A.C., China B., Rutjes S.A., Hoorfar J. and Van der Poel W.H.M., 2008. Current Methods for Extraction and

Concentration of Enteric Viruses from Fresh Fruit and Vegetables: Towards International Standards. Food Analytical Methods 1, 73-84

22. Cromeans T.L., Nainan O.V. and Margolis H.S., 1997. Detection of hepatitis A virus RNA in oyster meat. Applied and Environmental Microbiology 63, 2460-2463.

23. D'Agostino M., Cook N., Rodriguez-Lazaro D. and Rutjes S., 2011. Nucleic acid amplification-based methods for detection of enteric viruses: definition of controls and interpretation of results. Food and Environmental Virology 3, 55-60

24. Daniels D., Grytdal S. and Wasley A., 2009. Surveillance for acute viral hepatitis - United States, 2007. Centers for Disease Control and Prevention (CDC). Morbidity and Mortality Weekly Report Surveillance Summary 58, 1-27.

25. de Roda Husman A.M., Lodder W.J., Rutjes S.A., Schijven J.F. and Teunis P.F., 2009. Long-term inactivation study of three enteroviruses in artificial surface and groundwaters, using PCR and cell culture. Applied and Environmental Microbiology 75, 1050-1057.

26. Deboosere N., Pinon A., Caudrelier Y., Delobel A., Merle G., Perelle S., Temmam S., Loutreul J., Morin T., Estienney M., Belliot G., Pothier P., Gantzer C. and Vialette M.,2012. Adhesion of human pathogenic enteric viruses and surrogate viruses to inert and vegetal food surfaces. Food Microbiology 32, 48-56.

27. Di Bartolo I., Diez-Valcarce M., Vasickova P., Kralik P., Hernandez M., Angeloni G., Ostanello F., Bouwknegt M., Rodríguez-Lázaro D., Pavlik I. and Ruggeri F.M., 2012. Hepatitis E virus in pork production chain in Czech Republic, Italy, and Spain, 2010. Emerging Infectious Diseases 18, 1282-1289. DOI: 10.3201/eid1808.111783.

28. Diez-Valcarce M., Kovač K., Cook N., Rodríguez-Lázaro D. and Hernández M., 2011a. Construction and analytical application of internal amplification controls (IAC) for detection of Food Supply Chain-Relevant Viruses by Real-Time PCR-Based Assays. Food Analytical Methods 4, 437-445.

29. Diez-Valcarce M., Cook N., Hernández M. and Rodríguez-Lázaro D., 2011b. Analytical Application of a Sample Process Control in Detection of Foodborne Viruses. Food Analytical Methods 4, 614-618.

30. Domínguez A., Torner N., Ruíz L., Martínez A., Barrabeig I., Camps N., Godoy P., Minguell S., Parrón I., Pumarés A., Sala M.R., Bartolomé R., Pérez U., de Simón M., Montava R., Buesa J. and the Catalan Viral Gastroenteritis Study Group, 2008. Aetiology and epidemiology of viral gastroenteritis outbreaks in Catalonia (Spain) in 2004-2005. Journal of Clinical Virology 43, 126-131.

31. D'Souza D.H., Sair A., Williams K., Papafragkou E., Jean J., Moore C. and Jaykus L., 2006. Persistence of caliciviruses on environmental surfaces and their transfer to food. International Journal of Food Microbiology 108, 84-91.

32. Dubois E., Agier C., Traoré O., Hennechart C., Merle G., Crucière C. and Laveran H., 2002. Modified concentration method for the detection of enteric viruses on fruits and vegetables by reverse transcriptase-polymerase chain reaction or cell culture. Journal of Food Protection 65, 1962-1969.

33. Duizer E., Bijkerk P., Rockx B., De Groot A., Twisk F. and Koopmans M., 2004. Inactivation of caliciviruses. Applied and Environmental Microbiology 70, 4538-4543.

34. ECDC-EFSA. 2012. EU summary report on zoonoses, zoonotic agents and food-borne outbreaks 2010, EFSA Journal No.10, 2597.

35. Enriquez C.E., Hurst C.J. and Gerba C.P., 1995. Survival of enteric adenoviruses 40 and 41 in tap, sea, and waste water. Water Research 29, 2548-2553.

36. Espinosa A.C., Jesudhasan P., Arredondo R., Cepeda M., Mazari-Hiriart M., Mena K.D. and Pillai S.D., 2012. Quantifying the reduction in potential health risks by determining the sensitivity of poliovirus type 1 chat strain and rotavirus SA-11 to electron beam irradiation of iceberg lettuce and spinach. Applied and Environmental Microbiology 78, 988-993.

37. Estes M.K. and Kapikian A.Z., 2007. Rotaviruses. Fields Virology, 5th ed (Knipe D.M., Howley P.M., Griffin D.E., Lamb R.A., Martin M.A., Roizman B. and Straus S.E., eds), pp. 1917–1974. Lippincot William & Wilkins, Philadelphia, PA.

38. Feng K., Divers E., Ma Y. and Li J., 2011. Inactivation of a human norovirus surrogate, human norovirus virus-like particles, and vesicular stomatitis virus by gamma irradiation. Applied and Environmental Microbiology 77, 3507-3517.

39. Fino V.R. and Kniel K.E., 2008. UV light inactivation of hepatitis A virus, Aichi virus, and feline calicivirus on strawberries, green onions, and lettuce. Journal of Food Protection 71, 908-913.

40. Formiga-Cruz M., Tofiño-Quesada G., Bofill-Mas S., Lees D.N., Henshilwood K., Allard A.K., Conden-Hansson A.C., Hernroth B.E., Vantarakis A., Tsibouxi A., Papapetropoulou M., Furones M.D. and Girones R., 2002. Distribuion of human virus contamination in shellfish from different growing areas in Greece, Spain, Sweden and the United Kingdom. Applied and Environmental Microbiology 68, 5990-5998.

41. Gabbay Y.B., Linhares A.C., Cavalcante-Pepino E.L., Nakamura L.S., Oliveira D.S., Da Silva L.D., Mascarenhas J.D.P., Oliveira C.S., Monteiro T.A.F. and Leite J.P.G., 2007. Prevalence of human astrovirus genotypes associated with acute gastroenteritis among children in Belém, Brazil. Journal of Medical Virology 79, 530-538.

42. Geginat G., Kaiser D. and Schrempf S., 2012. Evaluation of third-generation ELISA and a rapid immunochromatographic assay for the detection of norovirus infection in fecal samples from inpatients of a German tertiary care hospital. European Journal of Clinical Microbiology and Infectious Diseases 31, 733-737.

43. Goyal S.M., 2006. Methods of virus detection in foods. Viruses in Foods (Goyal SM ed) pp. 101-119. Food Microbiology and Food Safety Series. Springer, New York, USA.

44. Green K.Y., 2007. Caliciviridae: The Noroviruses. Fields Virology (Knipe D.M., Howley P.M., Griffin D.E., Lamb R.A., Martin M.A., Roizman B. and Straus S.E. eds.) pp 949-979, Philadelphia, USA.

45. Guix S., Caballero S., Villena C., Bartolome R., Latorre C., Rabella N., Simo M., Bosch A. and Pinto R.M., 2002. Molecular epidemiology of astrovirus infection in Barcelona, Spain. Journal of Clinical Microbiology 40, 133-139.

46. Henshilwood K., Dore W. J., Anderson S. and Lees D. N., 2003 Molluscan shellfish safety. Villalba A., Romalde J. L., Reura B. and Beiras R. eds. pp. 451–465. Conselleria de Pesca e Asuntos Maritimos da Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, Galicia, Spain.

47. Herremans M., Bakker J., Duizer E., Vennema H. and Koopmans M.P.G., 2007. Use of serological assays for diagnosis of hepatitis E virus genotype 1 and 3 infections in a setting of low endemicity. Clinical and Vaccine Immunology 14, 562-568.

48. Hirneisen K.A., Markland S.M. and Kniel K.E., 2011. Ozone inactivation of norovirus surrogates on fresh produce. Journal of Food Protection 74, 836-839.

49. Hoorfar J., Malorny B., Abdulmawjood A., Cook N., Wagner M. and Fach P., 2004. Practical considerations in the design of internal amplification controls for diagnostic PCR assays. Journal of Clinical Microbiology 42, 1863-1868.

50. Huang F.F., Haqshenas G., Guenette D.K., Halbur P.G., Schommer S.K., Pierson F.W., Toth T.E. and Meng X.J., 2002. Detection by reverse transcription-PCR and genetic characterization of field isolates of swine hepatitis E virus from pigs in different geographic regions of the United States. Journal of Clinical Microbiology 40, 1326-1332.

51. Hundesa A., Maluquer de Motes C., Bofill-Mas S., Albinana-Gimenez N. and Girones R., 2006. Identification of human and animal Adenoviruses and Polyomaviruses for determination of sources of fecal contamination in the environment. Applied and Environmental Microbiology 72, 7886-7893.

52. Issa I.A. and Mourad F.H., 2001. Hepatitis A: an updated overview. Le Journal Médical Libanais-The Lebanese Medical Journal 49, 61-65.

53. Iturriza-Gómara M., Kang G. and Gray J., 2004. Rotavirus genotyping: keeping up with an evolving population of human rotaviruses. Journal of Clinical Virology 31, 259-265.

54. Jiang Y.J., Liao G.Y., Zhao W., Sun M.B., Qian Y., Bian C.X. and Jiang S.D., 2004. Detection of infectious hepatitis A virus by integrated cell culture/strand-specific reverse transcriptase-polymerase chain reaction. Journal of Applied Microbiology 97, 1105-1112.

55. Jones T.H., Houde A., Poitras E., Ward E. and Johns M.W., 2009. Development and Evaluation of a Multiplexed Real-Time TaqMan RT-PCR Assay with a Sample Process Control for Detection of F-specific RNA Coliphage Genogroups I and IV. Applied and Environmental Virology 1, 57-65.

56. Kang G., Kelkar S.D., Chitambar S.D., Ray P. and Naik T., 2005. Epidemiological profile of rotaviral infection in India: challenges for the 21st century. Journal of Infectious Diseases 192, S120-126.

57. Kapoor A., Li L., Victoria J., Oderinde B., Mason C., Pandey P., Zaidi S. and Delwart E., 2009. New species of astroviruses in human stool. Journal of General Virology 90, 2965-2972.

58. Karst S.M., Wobus C.E., Lay M., Davidson J. and Virgin H.W., 2003. STAT1-dependent innate immunity to a Norwalk-like virus. Science 299, 1575-1578.

59. Kingsley D.H. and Richards G.P., 2001. Rapid and efficient extraction method for reverse transcription-PCR detection of hepatitis A and Norwalk-like viruses in shellfish. Applied and Environmental Microbiology 67, 4152-4157.

60. Koopmans M. and Duizer E., 2004. Foodborne viruses: an emerging problem. International Journal of Food Microbiology 90, 23-41.

61. Kovac K., Diez-Valcarce M., Hernandez M., Raspor P. and Rodríguez-Lázaro D.,2010. High hydrostatic pressure as emergent technology for the elimination of foodborne viruses. Trends in Food Science and Technology 21, 558-568.

62. Kurdziel A.S., Wilkinson N., Langton S. and Cook N., 2001a. Survival of poliovirus on soft fruit and salad vegetables. Journal of Food Protection 64, 706-709.

63. Kurdziel, A.S., Wilkinson, N., Gordon, S.H., and Cook, N., 2001b. Development of methods to detect foodborne viruses. Rapid detection assays for food and water. S.A. Clark, K.C. Thompson, C.W. Keevil, and M.S. Smith, eds. pp. 175-177). Cambridge: Royal Society of Chemistry.

64. La Rosa G., Pourshaban M., Iaconelli M. and Muscillo M., 2008. Detection of genogroup IV noroviruses in environmental and clinical samples and partial sequencing through rapid amplification of cDNA ends. Archives of Virology 153, 2077-2083.

65. Le Cann P., Ranarijaona S., Monpoeho S., Le Guyader F. and Ferre V., 2004. Quantification of human astroviruses in sewage using real-time RT-PCR. Research in Microbiology 155, 11-15.

66. Le Guyader F., Haugarreau L., Miossec L., Dubois E. and Pommepuy M., 2000. Three-year study to assess human enteric viruses in shellfish. Applied and Environmental Microbiology 66, 3241-3248.

67. Le Guyader F.S., Le Saux J.C., Ambert-Balay K., Krol J., Serais O., Parnaudeau S., Giraudon H., Delmas G., Pommepuy M., Pothier P. and Atmar R.L., 2008. Aichi virus, norovirus, astrovirus, enterovirus, and rotavirus involved in clinical cases from a French oyster-related gastroenteritis outbreak. Journal of Clinical Microbiology 12, 4011-4017.

68. Leclerc H., Schwartzbrod L. and Dei-Cas E., 2002. Microbial agents associated with waterborne diseases. Critical Reviews in Microbiology 28, 371-409.

69. Lees, D. and CEN WG6 TAG4, 2010. International standardisation of a method for detection of human pathogenic viruses in molluscan shellfish. Food and Environmental Virology 2, 146-155.

70. Li D., Gu A.Z., He M., Shi H.C. and Yang W., 2009. UV inactivation and resistance of rotavirus evaluated by integrated cell culture and real-time RT-PCR assay. Water Research 43, 3261-3269.

71. Li D., Baert L., Zhang D., Xia M., Zhong W., Van Coillie E., Jiang X. and Uyttendaele M., 2012. The Effect of Grape Seed Extract on Human Noroviruses GII.4 and Murine Norovirus-1 in Viral Suspensions, on Stainless Steel Discs, and in Lettuce Wash Water. Applied and Environmental Microbiology [Epub ahead of print-doi:10.1128/AEM.01987-12].

72. Li T.C., Chijiwa K., Sera N., Ishibashi T., Etoh Y., Shinohara Y., Kurata Y., Ishida M., Sakamoto S., Takeda N. and Miyamura, T., 2005. Hepatitis E virus transmission from wild boar meat. Emerging Infectious Diseases 11, 1958-1960.

73. Lou F., Neetoo H., Chen H. and Li J., 2011. Inactivation of a human norovirus surrogate by high-pressure processing: effectiveness, mechanism, and potential application in the fresh produce industry. Applied and Environmental Microbiology 77, 1862-1871.

74. Lu L., Li C. and Hagedorn C.H., 2006. Phylogenetic analysis of global hepatitis E virus sequences: genetic diversity, subtypes and zoonosis. Reviews in Medical Virology 16, 5-36.

75. Maluquer de Motes C., Clemente-Casares P., Hundesa A., Martín M. and Girones R., 2004. Detection of Bovine and Porcine Adenoviruses for tracing the source of fecal contamination. Applied and Environmental Microbiology 70, 1448-1454.

76. Mansuy J.M., Peron J.M., Abravanel F., Poirson H., Dubois M., Miedouge M., Vischi F., Alric L., Vinel J.P. and Izopet J., 2004. Hepatitis E in the south west of France in individuals who have never visited an endemic area. Journal of Medical Virology 74, 419-424.

77. Martelli F., Caprioli A., Zengarini M., Marata A., Fiegna C., Di Bartolo I., Ruggeri F.M., Delogu M. and Ostanello F., 2008. Detection of hepatitis E virus (HEV) in a demographic managed wild boar (*Sus scrofa scrofa*) population in Italy. Veterinary Microbiology 126, 74-81.

78. Martínez-Martínez M., Diez-Valcarce M., Cook N., Hernández M. and Rodríguez-Lázaro D., 2011. Evaluation of extraction methods for efficient detection of enteric viruses in pork meat products. Food Analytical Methods 4, 13-22.

79. Matthijnssens J., Ciarlet M., Rahman M., Attoui H., Bányai K., Estes M.K., Gentsch J.R., Iturriza-Gómara M., Kirkwood C.D., Martella V., Mertens P.P., Nakagomi O., Patton J.T., Ruggeri F.M., Saif L.J., Santos N., Steyer A., Taniguchi K., Desselberger U. and van Ranst M., 2008. Recommendations for the classification of group A rotaviruses using all 11 genomic RNA segments. Archives of Virology 153, 1621-1629.

80. Maunula L., Kalso S., von Bonsdorff C.H. and Ponka A., 2004. Wading pool water contaminated with both noroviruses and astroviruses as the source of a gastroenteritis outbreak. Epidemiology and Infection 132, 737-743.

81. McMullen K.P., 2003. Inhibitory effects of food matrices on inhibition real-time reverse transcription polymerase chain reaction detection of foodborne viruses. Graduate School Theses and Dissertations. Paper 1429.

82. Mendez E. and Arias C.F., 2007. Astroviruses. Fields Virology, 5th ed (Knipe D.M., Howley P.M., Griffin D.E., Lamb R.A., Martin M.A., Roizman B. and Straus S.E., eds), pp. 981–1000. Lippincot William & Wilkins, Philadelphia, PA.

83. Moe C.L., Sair A., Lindesmith L., Estes M.K. and Jaykus L.A., 2004. Diagnosis of norwalk virus infection by indirect enzyme immunoassay detection of salivary antibodies to recombinant norwalk virus antigen. Clinical and Diagnostic Laboratory Immunology 11, 1028-1034.

84. Murchie L.W., Cruz-Romero M., Kerry J.P., Linton M., Patterson M.F., Smiddy M. and Kelly A.L., 2005. High pressure processing of shellfish: A review of microbiological and other quality aspects. Innovative Food Science and Emerging Technologies 6, 257-270.

85. Mushahwar I.K., 2008. Hepatitis E virus: molecular virology, clinical features, diagnosis, transmission, epidemiology, and prevention. Journal of Medical Virology 80, 646-658.

86. Navaneethan U., Al Mohajer M. and Shata M.T., 2008. Hepatitis E and pregnancy: understanding the pathogenesis. Liver International 28, 1190-1199.

87. Nuanualsuwan S. and Cliver D.O., 2002. Pretreatment to avoid positive RT-PCR results with inactivated viruses. Journal of Virological Methods 104, 217-225.

88. Nuanualsuwan S. and Cliver D.O., 2003. Infectivity of RNA from inactivated poliovirus. Applied and Environmental Microbiology 69, 1629-1632.

89. Ocazionez R.E., Meneses R., Torres F.A. and Stashenko E., 2010. Virucidal activity of Colombian *Lippia* essential oils on dengue virus replication in vitro. Memorias do Instituto Oswaldo Cruz 105, 304-309.

90. Parashar U.D., Gibson C.J., Bresse J.S. and Glass R.I., 2006. Rotavirus and severe childhood diarrhea. Emerging Infectious Diseases 12, 304–306.

91. Patel M.M., Hall A.J., Vinje J. and Parashar U.D., 2009. Noroviruses: a comprehensive review. Journal of Clinical Virology 44, 1-8.

92. Pintó R.M., Costafreda M.I. and Bosch A., 2009. Risk Assessment in Shellfish-Borne Outbreaks of Hepatitis A. Applied and Environmental Microbiology 75, 7350-7355.

93. Reuter G., Fodor D., Forgách P., Kátai A. and Szucs G., 2009. Characterization and zoonotic potential of endemic hepatitis E virus (HEV) strains in humans and animals in Hungary. Journal of Clinical Virology 44, 277-281.

94. Riou P., Le Saux J.C., Dumas F., Caprais M.P., Le Guyader S.F. and Pommepuy M., 2007. Microbial impact of small tributaries on water and shellfish quality in shallow coastal areas. Water Research 41, 2774-2786.

95. Rivalain N., Roquain J. and Demazeau G., 2010. Development of high hydrostatic pressure in biosciences: pressure effect on biological structures and potential applications in biotechnologies. Biotechnology Advances 28, 659-672.

96. Rodríguez-Lázaro D., Lombard B., Smith H.V., Rzeżutka A., D'Agostino M., Helmuth R., Schroeter A., Malorny B., Miko A., Guerra B., Davison J., KobilinskyA., Hernández M., Bertheau Y. and Cook N., 2007. Trends in analytical methodology in food safety and quality: monitoring microorganisms and genetically modified organisms. Trends in Food Science and Technology 18, 306-319.

97. Rodríguez-Lázaro D., Cook N., Ruggeri F.M., Sellwood J., Nasser A., Nascimento M.S., D'Agostino M., Santos R., Saiz J.C., Rzeżutka A., Bosch A., Gironés R., Carducci A., Muscillo M., Kovač K., Diez-Valcarce M., Vantarakis A., von Bonsdorff C.H., de Roda Husman A.M., Hernández M. and van der Poel W.H. 2012 Virus hazards from food, water and other contaminated environments. FEMS Microbiology Reviews 36, 786-814.

98. Rutjes S.A., Lodder W.J., Bouwknegt M. and de Roda Husman A.M., 2007. Increased hepatitis E virus prevalence on Dutch pig farms from 33 to 55% by using appropriate internal quality controls for RT-PCR. Journal of Virogical Methods 143, 112-116.

99. Rutjes S.A., Lodder W.J., Verschoor F., van den Berg H.H., Vennema H., Duizer E., Koopmans M. and de Roda Husman A.M., 2009. Sources of hepatitis E virus genotype 3 in The Netherlands. Emerging Infectious Diseases 15, 381-387.

100. Rutjes S.A., Lodder-Verschoor F., Lodder W.J., Van der Giessen J., Reesink H., Bouwknegt M. and de Roda-Husman A.M., 2010. Seroprevalence and molecular detection of hepatitis E virus in wild boar and red deer in The Netherlands. Journal of Virological Methods 168, 197-206.

101. Rzeżutka A. and Cook N., 2004 Survival of human enteric viruses in the environment and food. FEMS Microbiology Reviews 28, 441-453.

102. Sailaja B., Murhekar M.V., Hutin Y.J., Kuruva S., Murthy S.P., Reddy K.S., Rao G.M. and Gupte M.D., 2009. Outbreak of waterborne hepatitis E in Hyderabad, India, 2005. Epidemiology and Infection 137, 234-240.

103. Sair A.I., D'Souza D.H., Moe C.L. and Jaykus L.A. 2002. Improved detection of human enteric viruses in foods by RT-PCR. Journal of Virological Methods 100, 57-69.

104. Sanglay G.C., Li J., Uribe R.M. and Lee K., 2011. Electron-beam inactivation of a norovirus surrogate in fresh produce and model systems. Journal of Food Protection 74, 1155-1160.

105. Sattar S.A., Tetro J., Bidawid S. and Farber J., 2000. Foodborne spread of hepatitis A: Recent studies on virus survival, transfer and inactivation. The Canadian Journal of Infectious Diseases 11, 159-163.

106. Sattar S.A., Ali M. and Tetro J.A., 2011. In vivo comparison of two human norovirus surrogates for testing ethanol-based handrubs: the mouse chasing the cat! PLoS One 6, e17340.

107. Scallan E., Hoekstra R.M., Angulo F.J., Tauxe R.V., Widdowson M-A., Roy S.L., Jones J.L. and Griffin P.M., 2011. Foodborne Illness Acquired in the United States-Major Pathogens. Emerging Infectious Diseases 17, 7-15.

108. Scarcella C., Carasi S., Cadoria F., Macchi L., Pavan A., Salamana M., Alborali G.L., Losio M.M., Boni P., Lavazza A. and Seyler T., 2009. An outbreak of viral gastroenteritis linked to municipal water supply, Lombardy, Italy, June 2009. Euro Surveillance 14, pp. 19274.

109. Schmid D., Kuo H.W., Hell M., Kasper S., Lederer I., Mikula C., Springer B. and Allerberger F., 2011. Foodborne gastroenteritis outbreak in an Austrian healthcare facility caused by asymptomatic, norovirus-excreting kitchen staff. The Journal of Hospital Infection 77, 237-241.

110. Schwab K.J., Neill F.H., Estes M.K., Metcalf T.G. and Atmar R.L., 1998. Distribution of norwalk virus within shellfish following bioaccumulation and subsequent depuration by detection using RT-PCR. Journal of Food Protection 61, 1674-1680.

111. Smith A., Reacher M., Smerdon W., Adak G.K., Nichols G. and Chalmers R.M., 2006. Outbreaks of waterborne infectious intestinal disease in England and Wales, 1992-2003. Epidemiology and Infection 134, 1141-1149.

112. Steyer A., Poljsak-Prijatelj M., Barlic-Maganja D. and Marin J., 2008. Human, porcine and bovine rotaviruses in Slovenia: evidence of interspecies transmission and genome reassortment. Journal of General Virology 89, 1690-1698.

113. Su X., Zivanovic S. and D'Souza D.H., 2010. Inactivation of human enteric virus surrogates by high-intensity ultrasound. Foodborne Pathogens and Disease 7, 1055-1061.

114. Su X. and D'Souza D.H., 2011. Grape seed extract for control of human enteric viruses. Applied and Environmental Microbiology 77, 3982-3987.

115. Takahashi H., Tanaka T., Jirintai S., Nagashima S., Takahashi M., Nishizawa T., Mizuo H., Yazaki Y. and Okamoto H., 2012. A549 and PLC/PRF/5 cells can support the efficient propagation of swine and wild boar hepatitis E virus (HEV) strains: demonstration of HEV infectivity of porcine liver sold as food. Archives of Virology 157, 235-246.

116. Tanaka T., Takahashi M., Kusano E. and Okamoto H., 2007. Development and evaluation of an efficient cell-culture system for Hepatitis E virus. Journal of General Virology 88, 903-911.

117. Tei S., Kitajima N., Takahashi K. and Mishiro S., 2003. Zoonotic transmission of hepatitis E virus from deer to human beings. Lancet 362, 371-373.

118. Teunis P.F., Lodder W.J., Heisterkamp S.H. and de Roda Husman A.M., 2005. Mixed plaques: statistical evidence how plaque assays may underestimate virus concentrations. Water Research 39, 4240-4250.

119. Teunis P.F., Moe C.L., Liu P., Miller S.E., Lindesmith L., Baric R.S., Le Pendu J. and Calderon R.L., 2008. Norwalk virus: how infectious is it? Journal of Medical Virology 80, 1468-1476.

120. Tiwari B.K., Valdramidis V.P., O'Donnell C.P., Muthukumarappan K., Bourke P. and Cullen P.J., 2009. Application of natural antimicrobials for food preservation. Journal of Agricultural and Food Chemistry 57, 5987-6000.

121. Topping J.R., Schnerr H., Haines J., Scott M., Carter M.J., Willcocks M.M., Bellamy K., Brown D.W., Gray J.J., Gallimore C.I. and Knight A.I., 2009. Temperature inactivation of Feline calicivirus vaccine strain FCV F-9 in comparison with human noroviruses using an RNA exposure assay and reverse transcribed quantitative real-time polymerase chain reaction-A novel method for predicting virus infectivity. Journal of Virological Methods 156, 89-95.

122. Van der Poel W.H.M., Verschoor F., van de Heide R., Herrera M.I., Vivo A., Kooreman M. and de Roda-Husman, A.M., 2001. Hepatitis E Virus sequences in swine related to sequences in humans, The Netherlands. Emerging Infectious Diseases 7, 970-976.

123. Van Doorn L.J., Kleter B., Hoefnagel E., Stainier I., Poliszczak A., Colau B. and Quint W., 2009. Detection and genotyping of human rotavirus VP4 and VP7 genes by reverse transcriptase PCR and reverse hybridization. Journal of Clinical Microbiology 47, 2704-2712.

124. Wang Y., Levine D.F., Bendall R.P., Teo C.G. and Harrison T.J., 2001. Partial sequence analysis of indigenous hepatitis E virus isolated in the United Kingdom. Journal of Medical Virology 65, 706-709.

125. WHO. 2008. Viruses in Food: Scientific Advice to Support Risk Management Activities. Meeting Report. Microbiological Risk Assessment Series, No. 13. Geneva: World Health Organization.

126. Widdowson M.A., Jaspers W.J., Van der Poel W.H., Verschoor F., De Roda-Husman A.M., Winter H.L., Zaaijer H.L. and Koopmans M., 2003. Cluster of cases of acute hepatitis associated with hepatitis E virus infection acquired in the Netherlands. Clinical Infectious Diseases 36, 29-33.

127. Wilkinson N., Kurdziel A.S., Langton S., Needs E. and Cook N., 2001.Resistance of poliovirus to inactivation by high hydrostatic pressures. Innovative Food Science & Emerging Technologies 2, 95-98.

128. Wyn-Jones A.P., Carducci A., Cook N., D'Agostino M., Divizia M., Fleischer J., Gantzer C., Gawler A., Girones R., Höller C., de Roda Husman A.M., Kay D., Kozyra I., López-Pila J., Muscillo M., Nascimento M.S., Papageorgiou G., Rutjes S., Sellwood J., Szewzyk R. and Wyer M., 2011. Surveillance of adenoviruses and noroviruses in European recreational waters. Water Research 45, 1025-1038.

129. Zheng D.P., Ando T., Fankhauser R.L., Beard R.S., Glass R.I. and Monroe S.S., 2006. Norovirus classification and proposed strain nomenclature. Virology 346, 312-323.

130. Zhou Y.H., Purcell R.H. and Emerson S.U., 2004. An ELISA for putative neutralizing antibodies to hepatitis E virus detects antibodies to genotype 1, 2, 3 and 4. Vaccine 22, 2578-2585.

Agradecimientos

A Marta y a David, mis directores, sabéis mejor que nadie el esfuerzo invertido en este proyecto, gracias por todo: por los buenos momentos que hemos disfrutado y por los *menos buenos* de los que, quiero creer, hemos aprendido. Gracias por la confianza que depositasteis en mí hace ya cuatro años, por *empujarme* cuando me hizo falta, gracias a esos *empujones* esta Tesis es una realidad.

A Kata, mi compañera de fatigas en el laboratorio, sólo tú eres capaz de entender "la diversión" que hay en un laboratorio a las diez de la noche preparando placas de cultivo celular *(I got a feeling..that tonight it's gonna be a good night…).* Aunque a veces nos pareciera imposible, al final esto se termina, así que muchos ánimos, que ya solo queda el sprint final.

A Jaime, por ser el mejor compañero de mesa que se puede desear, por escucharme, por tu infinita paciencia y tus consejos, siempre tan serenos. Te deseo toda la suerte del mundo, con tu Tesis y en todo lo demás.

Al Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), por la concesión de mi beca predoctoral .A todos los miembros del proyecto VITAL, el estar involucrada en un proyecto de tal envergadura y con tantos expertos ha sido un autentico lujo, especialmente para una *novata*.

Un agradecimiento muy especial a todos los investigadores, y sus respectivos grupos, con los que he trabajado, y de los que he aprendido, durante mis estancias. A la Dra. Rosina Gironés y su grupo en la Universitat de Barcelona; Al Dr.Franco Maria Ruggeri y la Dra. Ilaria Di Bartolo, del Istituto Superiore di Sanità de Roma; a la Dra. Ana Maria de Roda Husman, a la Dra. Saskia Rutjes, al Dr. Martijn Bouwknegt y a Katharina Verhaelen, del RIVM en Bilthoven; al Dr. Jan Vinjè y a todo su grupo en el Centers for Disease Control and Prevention en Atlanta, y finalmente al Dr. Nigel Cook y a Martin D'Agostino, del FERA en York. Ha sido un privilegio.

Al Instituto Tecnológico Agrario de Castilla y León (ITACyL), por brindarme la oportunidad de realizar mis experimentos en sus instalaciones.

A las empresas y granjas que han colaborado en los estudios de muestreo.

A mis padres, porque sin vosotros no sería quien soy.

A mi familia, tenéis en vuestras manos el resultado de mis últimos cuatro años, este es el motivo por el que, en demasiadas ocasiones, no he estado para vosotros, sólo para *ella*. Esta Tesis es también vuestra.

A Aida y a Maite, mi *otra* familia, por estar ahí, siempre.

A todos, gracias.