



Tracing enteric viruses in the European berry fruit supply chain



Leena Maunula ^{a,*}, Agnieszka Kaupke ^b, Petra Vasickova ^c, Kirsi Söderberg ^a, Iwona Kozyra ^b, Sava Lazic ^d, Wim H.M. van der Poel ^e, Martijn Bouwknegt ^f, Saskia Rutjes ^f, Kris A. Willems ^{g,h}, Rita Moloney ⁱ, Martin D'Agostino ^j, Ana Maria de Roda Husman ^f, Carl-Henrik von Bonsdorff ^a, Artur Rzeżutka ^{b,**,1}, Ivo Pavlik ^{c,***,1}, Tamas Petrovic ^{d,****,1}, Nigel Cook ^j

^a Department of Food Hygiene and Environmental Health, Faculty of Veterinary Medicine, University of Helsinki, Finland

^b National Veterinary Research Institute, Puławy, Poland

^c Veterinary Research Institute, Brno, Czech Republic

^d Scientific Veterinary Institute "Novi Sad", Serbia

^e Central Veterinary Institute of Wageningen University and Research Centre, The Netherlands

^f National Institute for Public Health and the Environment, RIVM, The Netherlands

^g Centre of Industrial Microbiology and Biotechnology (CIMB), Department of Microbial and Molecular Systems (M²S), Faculty of Engineering Technology, KU Leuven, Belgium

^h Scientia Terrae Research Institute (STRI), Belgium

ⁱ Environmental Health Service, Health Service Executive, Sandfield Centre, Ennis, Co. Clare, Ireland

^j Food and Environment Research Agency (FERA), Sand Hutton, York, United Kingdom

ARTICLE INFO

Article history:

Received 25 January 2013

Received in revised form 30 August 2013

Accepted 3 September 2013

Available online 12 September 2013

Keywords:

Foodborne viruses

Berry fruit

Food monitoring

Real-time PCR

hAdV

NoV

ABSTRACT

In recent years, numerous foodborne outbreaks due to consumption of berry fruit contaminated by human enteric viruses have been reported. This European multinational study investigated possible contamination routes by monitoring the entire food chain for a panel of human and animal enteric viruses.

A total of 785 samples were collected throughout the food production chain of four European countries (Czech Republic, Finland, Poland and Serbia) during two growing seasons. Samples were taken during the production phase, the processing phase, and at point-of-sale. Samples included irrigation water, animal faeces, food handlers' hand swabs, swabs from toilets on farms, from conveyor belts at processing plants, and of raspberries or strawberries at points-of-sale; all were subjected to virus analysis. The samples were analysed by real-time (reverse transcription, RT)-PCR, primarily for human adenoviruses (hAdV) to demonstrate that a route of contamination existed from infected persons to the food supply chain. The analyses also included testing for the presence of selected human (norovirus, NoV GI, NoV GII and hepatitis A virus, HAV), animal (porcine adenovirus, pAdV and bovine polyomavirus, bPyV) and zoonotic (hepatitis E virus, HEV) viruses.

At berry production, hAdV was found in 9.5%, 5.8% and 9.1% of samples of irrigation water, food handlers' hands and toilets, respectively. At the processing plants, hAdV was detected in one (2.0%) swab from a food handler's hand. At point-of-sale, the prevalence of hAdV in fresh raspberries, frozen raspberries and fresh strawberries, was 0.7%, 3.2% and 2.0%, respectively.

Of the human pathogenic viruses, NoV GII was detected in two (3.6%) water samples at berry production, but no HAV was detected in any of the samples. HEV-contaminated frozen raspberries were found once (2.6%). Animal faecal contamination was evidenced by positive pAdV and bPyV assay results. At berry production, one water sample contained both viruses, and at point-of-sale 5.7% and 1.3% of fresh and frozen berries tested positive for pAdV.

At berry production hAdV was found both in irrigation water and on food handler's hands, which indicated that these may be important vehicles by which human pathogenic viruses enter the berry fruit chain. Moreover, both zoonotic and animal enteric viruses could be detected on the end products. This study gives insight into viral sources and transmission routes and emphasizes the necessity for thorough compliance with good agricultural and hygienic practice at the farms to help protect the public from viral infections.

© 2013 Elsevier B.V. All rights reserved.

* Correspondence to: L. Maunula, Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, P.O. Box 66, 00014 University of Helsinki. Tel.: +358 9 191 57143; fax: +358 9 191 57101.

** Correspondence to: A. Rzeżutka, National Veterinary Research Institute, Al. Partyzantów 57, 24-100 Puławy, Poland. Tel.: +48 818893037.

*** Correspondence to: I. Pavlik, Veterinary Research Institute, Hudcova 70, 721 00 Brno, Czech Republic. Tel.: +420 533331601; fax: +420 5 41211229.

**** Correspondence to: T. Petrovic, Scientific Veterinary Institute "Novi Sad", Rumunacki put 20, 21000 Novi Sad, Serbia. Tel.: +381 21518149; fax: +381 21518544.

E-mail addresses: leena.maunula@helsinki.fi (L. Maunula), arzez@piwet.pulawy.pl (A. Rzeżutka), pavlik@vri.cz (I. Pavlik), tomy@niv.ns.ac.rs (T. Petrovic).

¹ These authors contributed equally to the work.

1. Introduction

Consumption of berry fruit contaminated with norovirus (NoV) or hepatitis A virus (HAV) has been recognised as a cause of gastroenteritis and hepatitis, respectively, in humans (Fiore, 2004; Glass et al., 2009). In addition to their transmission through food, most enteric viruses are transmitted through water, through fomites, and directly from person to person (Carter, 2005). The important role of food handlers has been established in the contamination of food with human enteric viruses (Todd et al., 2008).

Infected humans can excrete large quantities of enteric viruses in their faeces (i.e. 10^6 – 10^{11} virus particles per g of faeces) and shedding may continue for several weeks (Atmar et al., 2008; Rodríguez-Lázaro et al., 2012). Untreated sewage of human origin usually has a high viral load. These viruses are neither completely removed nor totally inactivated by conventional wastewater treatment processes. For example, the reduction of only 1 to 2 \log_{10} infectious units of culturable human adenoviruses (hAdV) has been reported by Hewitt et al. (2011). Thus, viruses can be discharged into natural water sources. Non-enveloped RNA or DNA enteric viruses are persistent in water and in the environment (Glass et al., 2009). Ineffectively treated water used for drinking or irrigation may thus be contaminated with viruses. Since hAdV can be found at consistently high levels in sewage all year round, it has been proposed as an indicator virus for the presence of human faecal contamination in water (Wyn-Jones et al., 2011).

Although most enteric viruses are generally considered to be species specific, hepatitis E virus (HEV) is known to be zoonotic (Meng, 2011). HEV infections in Europe are often travel-related in humans, but infections caused by genotype 3 swine HEV are endemic (Di Bartolo et al., 2008; Vasickova et al., 2011). Porcine adenoviruses (pAdV; Hundesa et al., 2009) and bovine polyomaviruses (bPyV; Hundesa et al., 2010) have been proposed as indicator viruses for the source-tracking of animal faecal contamination in the environment.

Several NoV outbreaks related to raspberries have been reported (Ponka et al., 1999; Sarvikivi et al., 2012), but there is still a lack of data about how berries become contaminated by viruses. The present study was designed to elucidate the most significant sources of virus contamination in the raspberry (*Rubus idaeus*) and strawberry (*Fragaria vesca*) supply chains, in four European countries, namely the Czech Republic, Finland, Poland and Serbia. In this study, as in the studies of Berto et al. (2012), Di Bartolo et al. (2012), Diez-Valcarce et al. (2012), and Kokkinos et al. (2012), the presence of hAdV, pAdV and bPyV in samples (water, swabs, faeces and berries) was investigated. The presence of these viruses demonstrates that routes of contamination exist from the gastrointestinal tracts of humans or animals to those points in the food supply chain from where the samples were taken. Other enteric viruses such as NoV, HAV or HEV will be able to follow these routes, to contaminate the food supply chain. hAdV, pAdV and bPyV are referred to in this study as index viruses, to emphasize that their use was to reveal the existence of contamination routes and points of vulnerability to virus contamination, and not to indicate per se the presence of pathogenic virus types. Selected samples were also tested for NoV, HAV and HEV. The elucidation of points of vulnerability in the berry supply chain should assist food safety management practices to reduce the potential for enteric viral contamination.

2. Materials and methods

2.1. Outline of berry fruit supply chain

In berry production on-farm, food handlers picked berries with their bare hands (Poland, Serbia), with gloved hands (Finland, the Czech Republic) or mechanically (Serbia). The green parts of the fresh strawberries were kept attached until sold at retail. The berries were packed into disposable cartons or plastic boxes (variable sizes depending on the farms). After harvesting they were transported (pre-chilled at

10 °C, the Czech Republic only) to a processing plant or directly distributed to retail or point-of-sale.

At each processing plant, those berries selected to be frozen were cooled down to temperatures between 0 to +2 °C for 4–8 h and subsequently frozen in freezing tunnels. Once frozen (–25 °C), they went through a bulking and sorting process on conveyor belts. Frozen berries were stored below –20 °C at the plant until they were delivered on demand via a cold chain. No water was used in the handling of the berries during the processing.

2.2. Sampling strategy

In this EU project (“Integrated monitoring and control of foodborne viruses in European food supply chains [VITAL]”) samples were taken at perceived critical points for virus contamination monitoring. These critical points were identified according to the procedure described in Kokkinos et al. (2012). Briefly, the berry producing farms, processing plants and point-of-sale premises in the Czech Republic, Finland, Poland and Serbia from which samples were to be taken provided background information by completing questionnaires based on HACCP (hazard analysis and critical control points) audit principles. The critical control points in the premises were identified and then a sampling strategy was planned and followed.

Samples were tested initially for the index viruses hAdV, pAdV and bPyV. If positive for hAdV, then samples were considered to be contaminated with human faeces and further tested for NoV and HAV. If positive for pAdV, then samples were considered to be contaminated with pig faeces and further tested for HEV. Depending on the remaining resources per laboratory after this screening, a selection of samples that tested negatively for the index viruses was also tested for NoV, HAV and/or HEV.

2.3. Sampling

Of the total of 785 samples, 396 samples were taken from the production phase: irrigation water ($n = 95$), swabs from food handlers' hands ($n = 243$), toilets/latrines ($n = 22$), toilet door handles ($n = 22$), and animal faeces ($n = 14$). The 106 samples that represented the processing phase included swabs from food conveyor belts ($n = 55$) and food handlers' hands ($n = 51$). At point-of-sale phase, the 283 berry samples consisted of raspberries ($n = 232$) and strawberries ($n = 51$). These samples were collected at supermarkets and at a farmers' market. Berries were not sampled at berry production and processing, since the total number of samples to be taken was limited in each country ($n = 230$) and the likelihood for finding viruses on the berries was expected to be lower than on other sample matrices.

In the Czech Republic, Finland, Poland and Serbia, the 785 samples were collected (212, 166, 215 and 192 samples per country, respectively) according to the sampling guidelines tailored for each country (see sampling strategy 2.2). The sampling occurred during summers and autumns of 2009 and 2010 with several subsequent visits to each site, which included a total of 6 farms, 3 processing plants and 6 markets. At berry production, samples were taken on 6 occasions in the Czech Republic, Finland and Poland, (161, 65 and 94 samples, respectively), and on 7 occasions in Serbia (76 samples from two farms). Samples were taken from on 6 occasions from processing plants in both Finland and Poland (16 and 24 samples, respectively) and on 5 occasions in Serbia (66 samples). Point-of-sale samples were taken on 23 occasions in the Czech Republic (51 samples), on 19 occasions in Finland (85 samples), on 18 occasions in Poland (97 samples) and on 10 occasions in Serbia (50 samples).

In this study, the berry chain was followed at generic level from supplier to processing to point-of-sale. Thus, specific berry harvests were not followed directly. Production farms and processing plants with 4 to 150 food handlers were included in the study. Irrigation was used for growing berries in the Czech Republic, Serbia and Poland; no

irrigation was needed in Finland. Cattle and pigs were bred on one berry farm in Serbia.

2.4. Outline of sample analysis

From the total of 785 samples taken in the study (Table 1), 771 were analysed for hAdV (excluding 14 animal faecal samples); these samples included 382 samples from berry fruit growing farms, 106 samples from berry processing plants and 283 samples from points-of-sale. 333 of the 785 samples (326 water, environmental swab or berry samples, 7 porcine and 7 bovine faecal samples) in berry fruit chains were monitored for pAdVs and bPyVs in 3 countries in order to investigate whether there were routes of viral contamination originating from animals. Of these, 96 were obtained from the production phase, 39 during processing and 198 from the point-of-sale. HEV, HAV and NoV were analysed from 186, 333 and 333 samples, respectively. Fig. 1 describes the outline of the methods used for analyses of the samples.

2.5. Sample process control virus (SPCV)

The term SPCV denotes murine norovirus 1 (MNV-1) (Diez-Valcarce et al., 2011b), which had been propagated in RAW264.7 cells. MNV-1 stocks (10^7 pfu/ml) were kindly provided by the group led by Dr. Franco Ruggeri at the Istituto Superiore de Sanità, Rome, Italy by agreement with Washington University, USA.

2.6. Sample treatment

2.6.1. Faeces

A 250 mg quantity of faecal sample was suspended in 2.25 ml PBS containing 10 mg/ml gentamycin and 10 μ l (10^5 genomic copies) of the SPCV. The suspension was centrifuged at 3000 \times g for 15 min, and the supernatant was transferred into a clean microcentrifuge tube. The supernatant was then immediately used for nucleic acid extraction or stored at -20°C .

2.6.2. Swabs

Food handlers' hands, toilets, toilet door handles and conveyor belts were sampled using a 10 cm \times 10 cm sterile gauze swab (several manufacturers) moistened in 20 ml of PBS containing 10 mg/ml gentamycin, in a sterile plastic bag. Sampling was performed before handwashing at a pre-determined time of the day, e.g. immediately before lunch or coffee break. One food handler's hand (the dominant hand) was swabbed by rubbing the swab firmly four or five times on the back, the palm and the spaces between the fingers and all the fingertips.

After swabbing hands, the swabs of environmental surfaces (toilets and conveyor belts) were taken by wiping the relevant surface area and pressing the gauze firmly onto the surface before being directly transported to the laboratory. Subsequently, the gauze was squeezed

Table 1

Number of samples monitored for viruses in the berry fruit supply chain. Excluding 14 animal faecal samples, each sample was analysed for hAdV. The number of samples subjected for other virus analyses is indicated.

Supply chain phase (n = 785)		Number of samples analysed for					
		hAdV	pAdV	bPyV	HEV	HAV	NoV
Production (n = 396) (Swabs and water)	Production	382	96 ^a	96 ^b	60	188	188
	Processing plant (n = 106) (Swabs)	106	39	39	24	25	25
	Point-of-sale (n = 283) (Berries)	283	198	198	102	120	120

^a Includes 7 porcine faecal samples.

^b Includes 7 bovine faecal samples.

to release the contents. The liquid in the bag was decanted into a clean centrifuge tube and 10 μ l of SPCV was added. The suspension was vortex-mixed at full speed for 20 s, centrifuged at 3000 \times g for 5 min and the supernatant was transferred into a clean centrifuge tube. The supernatant was immediately used for nucleic acid extraction or stored at -20°C .

2.6.3. Irrigation water

A sample of 10 L of irrigation water was collected into an aseptic container and directly transported to the laboratory. The water sample pH was lowered to 3.5 with 1 M HCl, a final adjustment was made with 0.1 M HCl, and 10 μ l of SPCV was added. Then the sample was processed by the method described by Vilaginès et al. (1993), as modified by Wyn-Jones et al. (2011). Briefly, glass wool filters were prepared by compressing 10 g of glass wool (type 725; Rantigny, Saint-Gobain, France) into a 30 \times 3 cm polystyrene column to obtain a filter height of 6 to 8 cm. The filter was washed by passing a volume of 50 ml of 1 M HCl, tap water, 1 M NaOH, and finally tap water through it until the filtrate pH was neutral. The sample was drawn through the filter by a vacuum at a rate not exceeding 1.5 L/min. Subsequently, the viruses were eluted by a slow (20–30 min) passage of 200 ml 3% (w/v) beef extract (pH 9.5) in glycine buffer (0.05 M) through the filter. The eluate was flocculated by the addition of 1 M and 0.1 M HCl until the pH reached 3.5 to 3.0 and a visible floc was formed. The resultant protein floc was deposited by centrifugation at 7500 \times g for 30 min, dissolved in PBS and adjusted to a final volume of 10 ml. This suspension was then filtered through a 0.45 μ m filter (Sartorius Minisart) to remove the remaining particulates. The filter had been pre-treated by passing 5–10 ml 1.5% w/v beef extract at pH 7.4 through it to saturate the filter with proteins. The filtered suspension was stored at -20°C prior to analysis for nucleic acids.

2.6.4. Berry fruit

A sample of berry fruit was collected into a sterile plastic bag or other appropriate sterile container and transported in a chilled or frozen state (frozen berries were kept frozen) to the laboratory. The sample was processed using the method of Dubois et al. (2006) and according to ISO technical specifications (Norovirus and hepatitis A virus analyses from food and animal feed; ISO/TS 15216-1: 2013; ISO/TS 15216-2: 2013) with small modifications. Firstly, 25 g of whole berries was placed into a sterile beaker, 10 μ l of SPCV was pipetted onto the fruit and 40 ml of Tris glycine buffer (pH 9.5) containing 1% beef extract (TGBE), and 6500 U pectinase (Pectinex™ Ultra SPL solution, Sigma) were added to the sample. The pH of the sample was maintained at 9.0 while rocking at 60 rpm for 20 min at room temperature; if necessary, an adjustment by 1 N NaOH was performed with extension of the agitation period by 10 min after each adjustment. Subsequently, the liquid was decanted through a strainer (mesh size of 1.0 mm), and centrifuged at 10,000 \times g for 30 min at 4 $^\circ\text{C}$. The supernatant was transferred into a clean tube, its pH was adjusted to 7.2 and 0.25 volumes of 50% (w/v) polyethylene glycol (PEG) 8000/1.5 M NaCl solution were added. The suspension was mixed by shaking for 1 min, incubated with gentle rocking at 4 $^\circ\text{C}$ for 60 min and then centrifuged at 10,000 \times g for 30 min at 4 $^\circ\text{C}$. The obtained pellet was compacted by centrifugation at 10,000 \times g for 5 min at 4 $^\circ\text{C}$ then resuspended in 500 μ l of PBS and mixed with 500 μ l chloroform:butanol (1:1). The sample was incubated for 5 min at room temperature, centrifuged at 10,000 \times g for 15 min at 4 $^\circ\text{C}$. The aqueous phase was transferred to a clean tube and immediately used for nucleic acid extraction or stored at -20°C or -70°C .

2.7. Nucleic acids extraction

Nucleic acids were extracted from animal faeces using QIAamp Viral RNA Mini kit (QIAGEN) according to the manufacturer's instructions. The final elution step was performed twice with 150 μ l of elution buffer,

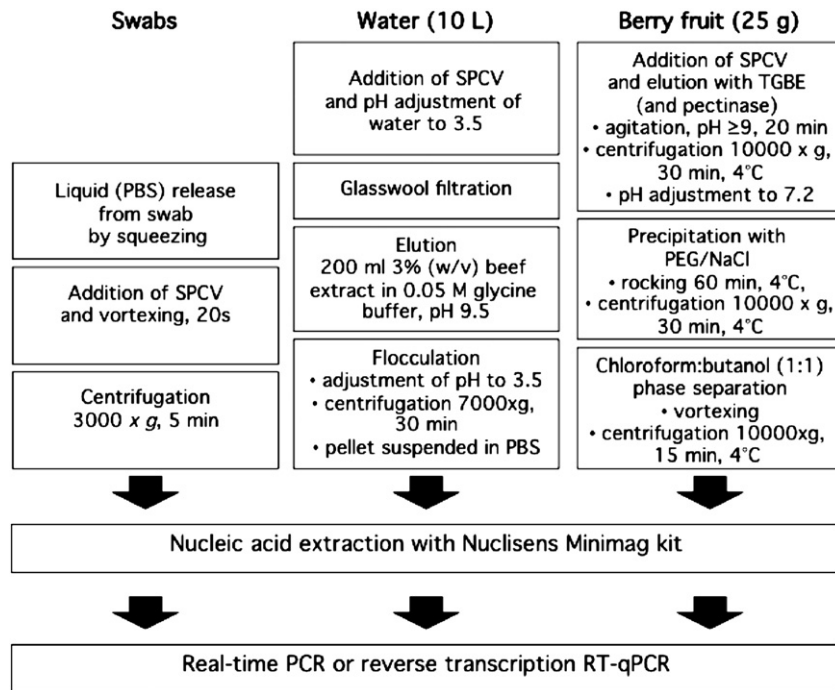


Fig. 1. Outline of the methods used for analyses of water, swabs and berry fruit.

which resulted in 300 μ l of nucleic acid extract. Nucleic acids were isolated from 5 ml of irrigation water concentrate, 5 ml of swab washes and 500 μ l of berry fruit extracts by NucliSENS miniMAG (bioMérieux) system with a final repeated elution resulting in 300 μ l of nucleic acid extract. The procedure was performed according to the manufacturer's instructions. The samples of isolated nucleic acids were assayed immediately or stored at -70°C .

2.8. Real-time PCR

Primers used for real-time PCR (qPCR), for hAdV, pAdV, bPyV, or reverse transcription real-time PCR assays (RT-qPCR), for MNV-1, NoV GI and GII, HAV and HEV, are shown in Table 2.

The detection of DNA viruses (hAdV, pAdV and bPyV) was performed using TaqMan Universal PCR Master Mix (Applied Biosystems) in a total volume of 25 μ l per reaction. The qPCR specific for adenoviruses contained 1 \times TaqMan Universal PCR Master Mix, 0.9 μ M (final concentration) forward and reverse primers, 0.225 μ M adenovirus specific probe, 50 nM internal amplification control (IAC) probe, approximately 100 copies of IAC DNA and 10 μ l of template nucleic acid. The qPCR protocol specific for hAdV was initiated by UNG treatment at 50°C for 2 min, followed by activation of AmpliTaq Gold polymerase (Applied Biosystems) at 95°C for 10 min and 45 cycles of 95°C for 15 s and 60°C for 1 min. To the contrary pAdV assay was carried out under the following conditions: UNG treatment at 50°C for 2 min, activation of AmpliTaq Gold polymerase at 95°C for 10 min and 45 cycles of 95°C for 15 s, 55°C for 20 s and 60°C for 20 s.

The bPyV specific qPCR included 1 \times TaqMan Universal PCR Master Mix (Applied Biosystems), 0.4 μ M each primer, 0.12 μ M bPyV specific probe, 50 nM IAC probe, 300 copies of IAC DNA and 10 μ l of template nucleic acid. Amplification was accomplished by the following protocol: UNG treatment at 50°C for 2 min, activation of AmpliTaq Gold polymerase at 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min.

RNA viruses (NoV GI, NoV GII, HAV, HEV and MNV-1) were detected using RNA UltraSense™ One-Step Quantitative RT-PCR System (Invitrogen) in total volume of 20 μ l. The reaction mixture specific for NoV GI, NoV GII, HAV and MNV-1 contained 1 \times RNA

Ultrasense reaction mix, 1 \times ROX reference dye, 1 μ l of RNA Ultrasense enzyme mix and 10 μ l of tested nucleic acid. RT-qPCR reaction specific for NoV GI, NoV GII and HAV also included 0.25 μ M virus specific probe, 50 nM IAC probe, 0.5 μ M forward, 0.9 μ M reverse primer and 300 copies of IAC RNA, whereas the MNV-1 assay contained 50 nM IAC probe, 0.2 μ M each MNV-1 primer and probe and 600 copies of IAC RNA. Reverse transcription reaction and amplification were performed according to the generic protocol: reverse transcription at 50°C for 15 min, preheating at 95°C for 2 min, followed by 45 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 1 min.

The presence of HEV genome was tested according to Jothikumar et al. (2006) with slight modifications; the reaction contained 1 \times RNA Ultrasense reaction mix, 0.25 μ M primers, 0.1 μ M HEV probe, 50 nM IAC probe, 1 \times ROX reference dye, 1 μ l of RNA Ultrasense enzyme mix, 300 copies of IAC RNA and 10 μ l of isolated nucleic acids. RT-qPCR was performed at 50°C for 30 min, preheating at 95°C for 2 min and 45 cycles of amplification by 95°C for 10 s, 55°C for 20 s and 72°C for 15 s.

Amplification and fluorescence detection was performed using the LightCycler 480 Instrument (Roche) using 96-well PCR plates (Czech Republic), the Rotorgene RG-3000 (Corbett/Qiagen) in Finland, and the 7500 Real-Time PCR System (Applied Biosystems) in Poland and in Serbia. Each sample was tested by qPCR or RT-qPCR in duplicates as neat samples and 1:10 diluted samples. Uracil DNA Glycosylase was used in each qPCR or RT-qPCR reaction to avoid possible carry-over contamination. Proficiency testing for the detection of adenoviruses from berry fruit was performed by participating laboratories in a pre-trial study (D'Agostino et al., 2012). Samples with a Ct value >37 in a single replicate were considered doubtful positives and the respective amplification curves were judged by both the testing laboratory and RIVM (National Institute for Public Health and the Environment, the Netherlands), not involved in the laboratory testing, to conclude on the final result. Discordant interpretations were discussed between the two laboratories.

2.9. Quality controls

Positive and negative controls were included in the entire virus detection method at all times to guarantee that the procedure was performed

Table 2
Primers and probes used in the study.

Name	Primer sequence	Reference
hAdV		Hemroth et al. (2002)
AdF	5'-CWTACATGCACATCKCSG G-3'	
AdR	5'-CRCGGGCRAAYTGACCAG-3'	
AdP1	5'-FAM-CCGGGCTCAGTACTCCGAGGCGTCT-BHQ-3'	
pAdV		Hundesda et al. (2009)
PAdVF	5'-AACGGCCGCTACTGCAAG-3'	
PAdVR	5'-AGCAGCAGGCTCTTGAGG-3'	
PAdVP	5'-FAM-CACATCCAGGTGCCCG-BHQ1-3'	
bPyV		Hundesda et al. (2010)
QB-F1-1	5'-CTAGATCTACCTCAAGGGAAT-3'	
QB-R1-1	5'-TACTTGGATCTGGACCAAC-3'	
QB-P1-2	5'-FAM-GACAAAGATGGTGTATCTGTGA-BHQ-3'	
NoV GI		da Silva et al. (2007)
QNIF4	5'-CGTGCGATCGNNTCCAT-3'	
NV1LCR	5'-CCTTAGACGCCATCATATTAC-3'	
NVG1p	5'-FAM-TGGACAGGAGAYCCRATCT-BHQ1-3'	
NoV GII		Loisy et al. (2005)
QNIF2	5'-ATGTTTCAGRTGGATGAGRTTCTCWGA-3'	
CNG2R	5'-TCGACGCCATCTTCAITCACA-3'	
QNIFS	5'-FAM-AGCACGTGGGAGGGCGATCG-BHQ1-3'	
HAV		Costafreda et al. (2006)
HAV68	5'-TCACCCCGCTTTGCC-3'	
HAV240	5'-GGAGAGCCCTGGAAGAAAG-3'	
HAV150	5'-FAM-CCTGAACCTGCAGGAATTA-MGB-NFQ-3'	
HEV		Jothikumar et al. (2006)
HEV-F	5'-GGTGGTTTCTGGGGTGAC-3'	
HEV-R	5'-AGGGGTTGGTTGGATGAA-3'	
HEV-P	5'-FAM-TGATTCACGCCCTTCGC-BHQ1-3'	
mNoV		Baert et al. (2008b)
FwORF1/2	5'-CACGCCACCGATCTGTCTG-3'	
RvORF1/2	5'-GCGCTGCCCATCACTC-3'	
Pro	5'-FAM-CGCTTTGGAACAATG-MGB-NFQ-3'	

correctly. False positive and negative results could be distinguished from true positive and negative results based on these controls. To verify that sample processing had worked correctly (virus concentration, nucleic acids isolation and sample treatment), SPCV was added to every sample prior to sample purification. Processing was valid when SPCV tested positive in the sample. A negative (for target) sample process control (NTSPC) was included within each batch of samples to detect cross-contamination during the virus concentration and nucleic acid extraction procedures (D'Agostino et al., 2011). The NTSPC did not contain any matrix or SPCV.

A target-specific IAC was included in each (RT)-qPCR reaction to control for inhibition of the RT-qPCR. All IACs and their probes were constructed as described by Diez-Valcarce et al. (2011a). An IAC 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. The IAC amplicons were detected with the specific VIC-labelled IAC probe, IACP (5'-VIC-CCA TAC ACA TAG GTC AGG-MGB-NFQ-3'). The amount of IAC added to the q(RT-)-PCR reaction was optimised in each laboratory and for each new batch of IAC. Tenfold dilutions of each new batch were tested in triplicate to determine the lowest consistent limit of detection, which was considered as the optimal working concentration. The IACs were supplied by Yorkshire Bioscience Ltd, UK.

A negative template control (NTC) and no-IAC control (NIC) were included within each (RT)-qPCR run. NTCs contained IAC and water instead of template nucleic acid, whereas the NIC contained neither IAC nor template nucleic acid. Each set of analysed samples contained

a positive amplification control, which comprised target virus nucleic acid or a plasmid standard produced by ITACyL (Instituto Tecnológico Agrario de Castilla y León, Spain; Martínez-Martínez et al., 2011). Only results of tests compliant with the full set of controls used (D'Agostino et al., 2011) were reported.

2.10. Estimation of the virus concentration

The concentration of viruses per sample was estimated as the number of PCR detectable units (PDU) using a most probable number-like approach, based on the detection probabilities given the actual portion of the samples examined. The presence or absence of target viruses was determined by RT-qPCR in neat nucleic acids solutions and sufficient serial 10-fold dilutions thereof to determine the end-point dilution, in duplicate. The higher the virus concentration, the greater the probability of multiple positive RT-qPCR results per dilution series. In contrast, the lower the concentration, the greater the probability of multiple or all negative test results. The presence/absence pattern for the j examined dilutions per sample was combined according to the following:

$$\ell = \prod_{i=1}^j \left(1 - \text{Exp}[-c \cdot V_i]^{p_i} \right) \times \text{Exp}[-c \cdot V_i]^{1-p_i}$$

where V_i is the recorded actual amount of sample examined per reaction and p_i denotes the presence ($p_i = 1$) or absence ($p_i = 0$) of ≥ 1 PDU in dilution i . The most likely PDU concentration c , is the concentration that gives the largest value for ℓ . It was assumed that when viruses were present, they were distributed homogeneously in throughout the samples.

The number of target genomes per PDU for each assay and matrix cannot be determined. Furthermore, this number is likely variable, because it depends on the efficiency of amplification. Under ideal amplification circumstances, with an exact doubling of targets per cycle, and a perfect test, 1 PDU would represent a single virus genome. The number of PDUs required for a 95% probability of detection (assuming homogeneously mixed samples) were estimated by solving the above equation for c using $p = 1$ and V_i being the total amount of sample tested. The estimate numbers for faeces, swabs, water and berries were 587 PDU/g, 27 PDU/L, 2.7PDU/L and 1.6 PDU/g, respectively. All estimates were made by the same institute, RIVM.

3. Results

3.1. Presence of hAdV in the berry fruit supply chain

Results obtained after 771 samples were analysed for hAdV are shown in Table 3. There were more hAdV-positive samples (27/382 samples) in the berry production phase than in the processing (1/106) and point-of-sale (5/283) phases.

hAdV was detected in 9.5% of the irrigation water samples, followed by toilets and door handles (9.1%) and food handlers' hands (5.8%, Table 3A). On some farms, several of the samples taken were contaminated by hAdV. For example 8/18 (44%) samples of irrigation water were contaminated by hAdV, in one strawberry-growing farm. The estimated average hAdV concentration for positive samples was 1.2×10^3 (95% interval: 760– 1.9×10^3) PDU/L water for the strawberry growing farm and 5 (0.3–23) PDU/L water for one raspberry growing farm. In toilets, an average of 96 (28–260) PDU of hAdV per toilet swab were present. hAdV was identified on the hands of seasonal workers/harvesters in 3 out of 4 countries with a hAdV concentration of 320 (220–460) PDU per food handler's hand.

Samples were analysed for hAdV contamination occurring during the raspberry processing phase in three countries. hAdV was identified once in a sample collected from a food handler's hand (Table 3B). No

Table 3
Presence of hAdV in samples taken during production, processing and point-of-sale in berry fruit chain of four countries.

Point-of-interest (n = 771)	Finland	Poland	Serbia	Czech Rep.	Total hAdV
A. Production (n = 382)	(1 farm)	(1 farm)	(2 farms)	(2 farms)	
Irrigation water	0/6 ^a	0/19	1/34 (2.9%)	8/36 (22%)	9/95 (9.5%)
Toilets	1/6 (17%)	0/6	1/4 (25%)	0/6	2/22 (9.1%)
Toilet door handles	0/8	0/5	2/3 (67%)	0/6	2/22 (9.1%)
Food handlers' hands	0/45	4/64 (6.3%)	9/21 (43%)	1/113 (0.9%)	14/243 (5.8%)
B. Processing (n = 106)	(1 plant)	(1 plant)	(1 plant)		
Conveyor belt	0/16	0/24	0/15	n.d.	0/55
Food handlers' hands	n.d. ^b	n.d.	1/51 (2.0%)	n.d.	1/51 (2.0%)
C. Point-of-sale (n = 283)	(1 market)	(2 markets)	(1 market)	(2 markets)	
Fresh raspberries	0/67	1/60 (1.7%)	0/10	n.d.	1/137 (0.7%)
Frozen raspberries	0/18	1/37 (2.7%)	2/40 (5.0%)	n.d.	3/95 (3.2%)
Fresh strawberries	n.d.	n.d.	n.d.	1/51 (2.0%)	1/51 (2.0%)

^a Number of positives/number of tested;

^b n.d. no data.

hAdV was found in samples taken from the conveyor belts at the processing plants.

Both raspberries and strawberries were shown to contain hAdV (Table 3C) at point-of-sale. The prevalence of hAdV was 1.7% (4/232) in raspberries and 2.0% (1/51) in strawberries. In farmers' markets 1.7% (1/60; versus 0/77 in supermarkets) and 2.1% (1/47; versus 0/4 in supermarkets) of fresh raspberry and strawberry samples were hAdV positive. Frozen berries contaminated by hAdV were sampled twice in supermarkets, with positives of 3.4% (2/58) in supermarket samples, and once (2.7%, 1/37) in a storehouse at point-of-sale. hAdV was detected more frequently in frozen raspberries (3.2%) than in fresh raspberries (0.7%; Fisher's exact test, $p = 0.02$). The estimated average hAdV concentration on the frozen raspberries was 0.2 (0.06–0.6) PDU per g and on the fresh raspberry 0.7 (0.04–3.4) PDU per g. The estimated concentration on the positive strawberries was 54 (15–200) PDU per g.

3.2. Animal, zoonotic and human pathogenic viruses in the berry fruit supply chain

Table 4 shows that faecal contamination of animal origin was found in the berry production phase and at point-of-sale. BPyV was present in one (1.1%) out of 89 irrigation water samples, but it was not found in processing or point-of-sale samples. PAdV was detected more often: in 4.5% of irrigation water samples, in 5.7% of fresh raspberries and in 1.3% of frozen raspberries. All pAdV-positive raspberries were collected from supermarkets: 40% (4/10; versus 0/60 in farmers' markets) of fresh raspberries and 2.5% (1/40; versus 0/47 in farmers' markets) of frozen raspberries were positive.

The presence of zoonotic and human pathogenic viruses other than hAdV in the berry fruit supply chain was analysed in 186 and 333 samples (Table 4). Although no HEV contamination was detected in the berry production phase, HEV was found once in a pack of frozen raspberries taken from a cold room at point-of-sale. The estimated PDU concentration of HEV per 1 g of raspberries was 1.1 (0.2–3.5) PDU. HAV was not detected in any of tested samples. Berries at point-of-sale did not test positive for NoV (0/120). NoV GII was detected twice in the irrigation water of a strawberry-growing farm. The average estimated NoV GII concentration was 1.1×10^3 (500–2 × 10³) PDU per 1 L of water.

4. Discussion

Berry fruit is a known vehicle for foodborne viruses. However, to our knowledge this type of large multinational study on the contamination of the berry fruit supply chain by viruses has not been carried out before. The sampling plan of the study was developed to derive optimal benefit from the number of samples determined to be taken. The strategy included increasing the probability of detecting a virus obtained from a sampling point when low virus concentrations were expected, or obtaining the most accurate estimates of prevalences given the total number of samples. The monitoring of berry producing farms, processing plants and points-of-sale for the presence of hAdV revealed that

Table 4
Distribution of animal, zoonotic and human enteric viruses in samples taken during production, processing and point-of-sale in berry-chain presented as total results from three countries (Czech Republic, Poland and Serbia).

Point-of-interest (n = 333)	Animal viruses		Zoonotic virus	Human pathogenic viruses		
	pAdV	bPyV	HEV	HAV	NoV GI	NoV GII
A. Production (n = 96)						
Irrigation water	4/89 (4.5%)	1/89 (1.1%)	0/56	0/56	0/56	2/56 (3.6%)
Toilets	n.d. ^a	n.d.	n.d.	0/9	0/9	0/9
Toilet door handles	n.d.	n.d.	n.d.	0/10	0/10	0/10
Food handlers' hands	n.d.	n.d.	n.d.	0/113	0/113	0/113
Pig faeces	4/7 (57%)	n.d.	0/4	n.d.	n.d.	n.d.
Cattle faeces	n.d.	0/7	n.d.	n.d.	n.d.	n.d.
B. Processing (n = 39)						
Conveyor belt	0/39	0/39	0/24	0/24	0/24	0/24
Food handlers' hands	n.d.	n.d.	n.d.	0/1	0/1	0/1
C. Point-of-sale (n = 198)						
Fresh raspberries	4/70 (5.7%)	0/70	0/64	0/60	0/60	0/60
Frozen raspberries	1/77 (1.3%)	0/77	1/38 (2.6%)	0/39	0/39	0/39
Fresh strawberries	0/51	0/51	n.d.	0/21	0/21	0/21

^a n.d. no data.

there are possibilities for berries to become contaminated by viruses especially at production. Zoonotic and animal viruses were also detected along the berry fruit supply chain.

4.1. Water (at berry production)

The significance of water as an efficient route of disseminating human enteric viruses has been known for a long time. Although natural waters have been shown to become contaminated by sewage or treated wastewater (Wyn-Jones et al., 2011), and drinking water containing viruses has been implicated in many waterborne outbreaks (Maunula et al., 2005), few studies about the presence of viruses in irrigation water have been published. In South Korea, Cheong et al. (2009) found adenoviruses in 4/29 and 3/30 irrigation water and vegetable samples, respectively. Our study also revealed that raspberries and strawberries were irrigated with water that contained human and animal viruses.

According to the recommendations of *Codex Alimentarius* (FAO/WHO, 2012), water of suitable quality should be used for irrigation. In Europe, regulations (Regulation (Ec) No 852/2004 of The European Parliament and of the Council on the Hygiene of Foodstuffs, and Article 5 (c) of Annex 1 of the General Hygiene Provisions for Primary Production and Associated Operations) state that potable water, or clean water be used whenever necessary to prevent contamination. However, it is not practical for all berry production farms to use potable water for irrigation. Moreover, since bacterial indicators may not be suitable for predicting some potential viral contamination and water is not regularly monitored for viruses, water that has passed the indicator test may still contain viruses. There is also a general perception that the hygienic quality of irrigation water is less important than that of drinking water. Viruses persist in water for a long time, e.g. it has been shown that NoV can persist for at least 60 days in groundwater (Seitz et al., 2011). Although drip irrigation prevents direct contact of irrigation water with berry fruit, spraying berries with pesticides shortly before harvesting might lead to the contamination of berries, since it has also been shown that viruses may persist in pesticides reconstituted in virus containing water (Verhaelen et al., 2012a).

4.2. Food handlers' hands

Our study showed that berry handlers at berry production may contaminate berries, since viruses were found in several swab samples taken from harvesters' hands. The presence of hAdV on the harvesters' hands and then also on berries at point-of-sale suggest that this is one possible route of virus transmission. The sanitary condition of water closets and handwashing facilities in the fields and in food handlers' living accommodation may not always meet the necessary standards of hygiene. This, coupled with poor hand hygiene practices, increases the risk that enteric viruses may be transferred via hands to the picked berries. It has also been experimentally shown that viruses are efficiently transferred from contaminated hands to surfaces (Barker et al., 2004; Bidawid et al., 2004). In previous studies, NoV was detected in swabs taken from food handler's hands in a gastroenteritis outbreak setting (Boxman et al., 2009). In an outbreak caused by HAV-contaminated blueberries (Calder et al., 2003), harvesters' hands were speculated to be a likely vehicle of virus transmission, although the source of contamination could not be traced.

4.3. Processing

In this study the processing site was not found to be a likely place where viruses enter the berry fruit supply chain. This finding is in agreement with previously published results that disease outbreaks caused by berry fruit had not often been traced back to processing. One reported case occurred in a tourist resort in Egypt where HAV was linked to the processing of orange juice (Frank et al., 2007). During

processing, berries are not usually manually handled but instead are processed automatically, which may diminish the likelihood of viral contamination of berries by food handlers. In the processing of lettuce and other vegetables, water is used for washing, which thereby increases the risk for viral contamination of the lettuce (Kokkinos et al., 2012). However, little or no water is used in the processing of berries, which may also be another reason why berries are not as easily contaminated during processing. Enteric viruses survive well in a cold environment, so when berries are contaminated at berry production, viruses are likely to remain infectious during processing and at point-of-sale. Experimental studies have shown that the reduction for MNV as a surrogate of human NoV during pasteurization of raspberry puree was less than 3 log-units (Baert et al., 2008a).

However, the fact that frozen berries were more often contaminated than fresh berries may suggest that freezing is in some way preferred by viruses, perhaps since it preserves the viruses on berries better than storage at higher temperatures. Future studies that focus on the processing phase and analyses of a larger number of samples may further clarify the impact of processing in viral contamination of berries.

4.4. Berries at point-of-sale

A recent publication (Tuladhar et al., 2012) found that in some circumstances hAdV may be less stable than MNV-1, a surrogate for NoV. On the other hand, Verhaelen et al. (2012b) reported that both hAdV and NoV persist for the duration of the shelf life of raspberries on contaminated berries at room temperature, which suggests that the presence of hAdV would be useful in indicating faecal contamination of berries. In our study, the prevalence of hAdV in frozen raspberries at point-of-sale was 3.2%, which was lower than those (34.5% and 6.7%, for Belgium and France, respectively) reported for NoV in the review of Baert et al. (2012). In addition to the analysis of different enteric viruses, the higher prevalence of virus in berries in Belgium may be as a result of a random selection due to a relatively low number of samples. The high prevalence may also have been influenced by place and time of the sampling. As an example, in Finland, in 2009, exceptionally high numbers of NoV outbreaks linked to berries were detected (Sarvikivi et al., 2012), but had not been detected before or since that year. Differences in the methods used for testing berries for viruses may also partially explain the differences in the prevalence values.

In the present study, the inclusion of IACs in the (RT)-qPCRs ruled out false negative interpretations of results due to the presence of RT or PCR inhibitors that are frequently found in food samples. The presence of PCR inhibitors in the samples usually leads to high ct values, the verification of which is challenging, since real-time PCR is highly sensitive as compared to confirmation tests. Inclusion of adequate controls is also necessary to exclude nucleic acid contamination in the laboratory. Furthermore, the NIC and NTC values obtained showed that positive results were not false positives due e.g. to amplicon contamination in the laboratory.

At the beginning of the study the first samples were collected before the development of the analytical methodology was complete. All these initial samples were stored frozen for several months before analysis, which may have slightly decreased the number of positive results we obtained (e.g. if viral RNA had degraded during storage). It was considered worthwhile however to ensure that the analytical methods were fully fit for purpose, and demonstrated as robust in collaborative trial (D'Agostino et al., 2012).

4.5. Zoonotic viruses

In this study the presence of human enteric viruses other than hAdV had not been confirmed either in fresh or in frozen fruits, but unexpectedly HEV was found. The insufficient amount of viral RNA did not allow us to perform further molecular analysis of this virus isolate. The potential sources of produce contamination on berry producing farms are

irrigation water, animal-based fertiliser, soil and produce handling (Beuchat, 1996; Rodríguez-Lázaro et al., 2012). In a previous study, Brassard et al. (2012) detected HEV on strawberries grown in a field irrigated with polluted river water. In our study it was not possible to definitively establish a traceable link between polluted irrigation water and HEV-contaminated produce, but it is highly likely that HEV could have originated from contaminated soil or water used for plant irrigation as some of the tested irrigation water samples were positive for the animal index viruses.

Currently, for most of the raspberry farms direct water contact with growing plants and fruits is rare due to the common use of drip irrigation systems. Natural water sources, including well water, can be exposed to enteric viruses abundantly present in agricultural run-off, leakage from sewage tanks or animal farms adjacent to the production fields. The high frequency of pAdV (57%) obtained in our study in pig faeces in a berry-growing farm and the occurrence of pAdV or bPyV in water samples supports the previous assumption that animal-based fertiliser could be considered as a vehicle for virus contamination. The significance of pig manure in the dissemination of HEV needs further investigation.

Occasional cases of hepatitis E in humans that originate from the consumption of food of animal origin have been reported (Bouwknegt et al., 2007; Colson et al., 2010). Although HEV has been found on fresh produce (Kokkinos et al., 2012) including berry fruit (Brassard et al., 2012) on other occasions, the role of this kind of food in HEV transmission to humans has not been unequivocally confirmed.

5. Conclusion

This study has demonstrated the potential for viruses to enter the berry fruit supply chain via contaminated food handlers' hands and water during the production phase. In addition, it has provided evidence for the potential for contamination of the berry fruit supply chain by HEV. Data obtained in this study will be used to estimate the prevalence of viruses in irrigation water, on food handlers' hands or berry fruit into health risks, as has previously been performed for lettuce crops (Hamilton et al., 2006; Petterson et al., 2001).

In the future, more focused studies that intensively examine berry fruit virus contamination routes should be carried out. Now that significant sources have been identified, virus-aware revisions of existing food safety management systems should be implemented. The VITAL project found more non-compliance with prerequisite food safety programmes in fresh produce production than during processing and at point-of-sale, suggesting that the virus contamination is most likely to occur at that early phase. Compliance with prerequisite programmes, such as the Codex guidelines (FAO/WHO, 2012), is essential to reduce the risk of the contamination of the food supply chains with viruses. Guidance Sheets have been developed to complement the Codex guidelines and are available in several languages at www.eurovital.org. With clear recommendations on regaining control through appropriate monitoring procedures, control of pathogenic enteric viruses in berry fruit supply chains can be fulfilled.

Acknowledgements

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). Nigel Cook and Martin D'Agostino acknowledge support from the United Kingdom Food Standards Agency.

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 the Czech Republic.

References

- Atmar, R.L., Opekun, A.R., Gilger, M.A., Estes, M.K., Crawford, S.E., Neill, F.H., Graham, D.Y., 2008. Norwalk virus shedding after experimental human infection. *Emerg. Infect. Dis.* 14 (10), 1553–1557.
- Baert, L., Uyttendaele, M., Van Coillie, E., Debevere, J., 2008a. The reduction of murine norovirus 1, *B. fragilis* HSP40 infecting phage B40-8 and *E. coli* after a mild thermal pasteurization process of raspberry puree. *Food Microbiol.* 25 (7), 871–874.
- Baert, L., Uyttendaele, M., Vermeersch, M., Van Coillie, E., Debevere, J., 2008b. Survival and transfer of murine norovirus 1, a surrogate for human noroviruses, during the production process of deep-frozen onions and spinach. *J. Food Prot.* 71 (8), 1590–1597.
- Baert, L., Mattison, K., Loisy-Hamon, F., Harlow, J., Martyres, A., Lebeau, B., Stals, A., Van Coillie, E., Herman, L., Uyttendaele, M., 2012. Review: norovirus prevalence in Belgian, Canadian and French fresh produce: a threat to human health? *Int. J. Food Microbiol.* 151 (3), 261–269.
- Barker, J., Vipond, I.B., Bloomfield, S.F., 2004. Effects of cleaning and disinfection in reducing the spread of Norovirus contamination via environmental surfaces. *J. Hosp. Infect.* 58 (1), 42–49.
- Berto, A., Martelli, F., Grierson, S., Banks, M., 2012. Hepatitis E virus in pork food chain, United Kingdom, 2009–2010. *Emerg. Infect. Dis.* 18 (8), 1358–1360.
- Beuchat, L., 1996. Pathogenic microorganisms associated with fresh produce. *J. Food Prot.* 59, 204–216.
- Bidawid, S., Malik, N., Adegunrin, O., Sattar, S.A., Farber, J.M., 2004. Norovirus cross-contamination during food handling and interruption of virus transfer by hand antisepsis: experiments with feline calicivirus as a surrogate. *J. Food Prot.* 67 (1), 103–109.
- Bouwknegt, M., Lodder-Verschoor, F., van der Poel, W.H., Rutjes, S.A., de Roda Husman, A.M., 2007. Hepatitis E virus RNA in commercial porcine livers in The Netherlands. *J. Food Prot.* 70 (12), 2889–2895.
- Boxman, I., Dijkman, R., Verhoef, L., Maat, A., van Dijk, G., Vennema, H., Koopmans, M., 2009. Norovirus on swabs taken from hands illustrate route of transmission: a case study. *J. Food Prot.* 72 (8), 1753–1755.
- Brassard, J., Gagne, M.J., Genereux, M., Cote, C., 2012. Detection of human foodborne and zoonotic viruses on irrigated, field-grown strawberries. *Appl. Environ. Microbiol.* 78, 3763–3766.
- Calder, L., Simmons, G., Thornley, C., Taylor, P., Pritchard, K., Greening, G., Bishop, J., 2003. An outbreak of hepatitis A associated with consumption of raw blueberries. *Epidemiol. Infect.* 131 (1), 745–751.
- Carter, M.J., 2005. Enterically infecting viruses: pathogenicity, transmission and significance for food and waterborne infection. *J. Appl. Microbiol.* 98 (6), 1354–1380.
- 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. *Appl. Environ. Microbiol.* 75 (24), 7745–7751.
- Colson, P., Borentain, P., Queyriaux, B., Kaba, M., Moal, V., Gallian, P., Heyries, L., Raoult, D., Gerolami, R., 2010. Pig liver sausage as a source of hepatitis E virus transmission to humans. *J. Infect. Dis.* 202 (6), 825–834.
- Costafreda, M.I., Bosch, A., Pinto, 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. *Appl. Environ. Microbiol.* 72 (6), 3846–3855.
- D'Agostino, M., Cook, N., Di Bartolo, I., Ruggeri, F.M., Berto, A., Martelli, F., Banks, M., Vasickova, P., Kralik, P., Pavlik, I., Kokkinos, P., Vantarakis, A., Söderberg, K., Maunula, L., Verhaelen, K., Rutjes, S., de Roda Husman, A.M., Hakke, R., Van der Poel, W., Kaupke, A., Kozyra, I., Rzeżutka, A., Prodanov, J., Lazic, S., Petrovic, T., Carratala, A., Gironés, R., Diez-Valcarce, M., Hernandez, M., Rodriguez-Lazaro, D., 2012. Multicenter collaborative trial evaluation of a method for detection of human adenoviruses in berry fruit. *Food Anal. Methods* 5, 1–7.
- 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. *Appl. Environ. Microbiol.* 73 (24), 7891–7897.
- D'Agostino, M., Cook, N., Rodriguez-Lazaro, D., 2011. Nucleic acid amplification-based methods for detection of enteric viruses: definition of controls and interpretation of results. In: S., R. (Ed.), *Food Environ. Virol.* 2, 55–60.
- Di Bartolo, I., Martelli, F., Inglese, N., Pourshaban, M., Caprioli, A., Ostanello, F., Ruggeri, F.M., 2008. Widespread diffusion of genotype 3 hepatitis E virus among farming swine in Northern Italy. *Vet. Microbiol.* 132 (1–2), 47–55.
- Di Bartolo, I., Diez-Valcarce, M., Vasickova, P., Kralik, P., Hernandez, M., Angeloni, G., Ostanello, F., Bouwknegt, M., Rodriguez-Lázaro, D., Pavlik, I., Ruggeri, F.M., 2012. *Emerg. Infect. Dis.* 18 (8), 1282–1289.
- 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 Anal. Methods* 4, 437–445.
- Diez-Valcarce, M., Cook, N., Hernández, M., Rodríguez-Lázaro, D., 2011b. Analytical application of a sample process control in detection of foodborne viruses. *Food Anal. Methods* 4 (4), 614–618.
- Diez-Valcarce, M., Kokkinos, P., Söderberg, K., Bouwknegt, M., Willems, K., de Roda-Husman, A.M., von Bonsdorff, C., Bellou, M., Hernández, M., Maunula, L., Vantarakis, A., Rodríguez-Lázaro, D., 2012. Occurrence of human enteric viruses in commercial mussels at retail level in three European countries. *Food Environ. Virol.* 4 (2), 73–80.
- Dubois, E., Hennechart, C., Deboosere, N., Merle, G., Legeay, O., Burger, C., Le Calve, M., Lombard, B., Ferre, V., Traore, O., 2006. Intra-laboratory validation of a concentration method adapted for the enumeration of infectious F-specific RNA coliphage, enterovirus, and hepatitis A virus from inoculated leaves of salad vegetables. *Int. J. Food Microbiol.* 108 (2), 164–171.

- FAO/WHO [Food and Agriculture Organization of the United Nations/World Health Organization], 2012. Guidelines on the Application of General Principles of Food Hygiene to the Control of Viruses in Food (CAC/GL 79 – 2012).
- Fiore, A.E., 2004. Hepatitis A transmitted by food. *Clin. Infect. Dis.* 38 (5), 705–715.
- Frank, C., Walter, J., Muehlen, M., Jansen, A., van Treeck, U., Hauri, A.M., Zoellner, I., Rakha, M., Hoehne, M., Hamouda, O., Schreier, E., Stark, K., 2007. Major outbreak of hepatitis A associated with orange juice among tourists, Egypt, 2004. *Emerg. Infect. Dis.* 13 (1), 156–158.
- Glass, R.I., Parashar, U.D., Estes, M.K., 2009. Norovirus gastroenteritis. *N. Engl. J. Med.* 361 (18), 1776–1785.
- Hamilton, A.J., Stagnitti, F.S., Premier, R., Boland, A.M., 2006. Is the risk of illness through consuming vegetables irrigated with reclaimed wastewater different for different population groups? *Water Sci. Technol.* 54 (11–12), 379–386.
- 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. *Appl. Environ. Microbiol.* 68 (9), 4523–4533.
- Hewitt, J., Leonard, M., Greening, G.E., Lewis, G.D., 2011. Influence of wastewater treatment process and the population size on human virus profiles in wastewater. *Water Res.* 45 (18), 6267–6276.
- Hundesda, A., Maluquer de Motes, C., Albinana-Gimenez, N., Rodriguez-Manzano, J., Bofill-Mas, S., Sunen, E., Rosina Girones, R., 2009. 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* 158 (1–2), 130–135.
- Hundesda, A., Bofill-Mas, S., Maluquer de Motes, C., Rodriguez-Manzano, J., Bach, A., Casas, M., Girones, R., 2010. Development of a quantitative PCR assay for the quantitation of bovine polyomavirus as a microbial source-tracking tool. *J. Virol. Methods* 163 (2), 385–389.
- 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. *J. Virol. Methods* 131 (1), 65–71.
- Kokkinos, P., Kozyra, I., Ladic, S., Bouwknegt, M., Rutjes, S., Willems, K., Moloney, R., de Roda Husman, A., Kaupke, A., Legaki, E., D'Agostino, M., Cook, N., Rzeżutka, A., Petrovic, T., Vantarakis, A., 2012. Harmonised investigation of the occurrence of human enteric viruses in the leafy green vegetable supply chain in three European countries. *Food Environ. Virol.* <http://dx.doi.org/10.1007/s12560-012-9087-8>.
- 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. *J. Virol. Methods* 123 (1), 1–7.
- Martinez-Martinez, M., Diez-Valcarce, M., Hernandez, M., Rodriguez-Lazaro, D., 2011. Design and application of nucleic acid standards for quantitative detection of enteric viruses by real-time PCR. *Food Environ. Virol.* 3 (2), 92–98.
- Maunula, L., Miettinen, I.T., von Bonsdorff, C.H., 2005. Norovirus outbreaks from drinking water. *Emerg. Infect. Dis.* 11 (11), 1716–1721.
- Meng, X.J., 2011. From barnyard to food table: the omnipresence of hepatitis E virus and risk for zoonotic infection and food safety. *Virus Res.* 161 (1), 23–30.
- Petterson, S.R., Ashbolt, N.J., Sharma, A., 2001. Microbial risks from wastewater irrigation of salad crops: a screening-level risk assessment. *Water Environ. Res.* 73 (6), 667–672.
- Ponka, A., Maunula, L., von Bonsdorff, C.H., Lyytikäinen, O., 1999. An outbreak of calicivirus associated with consumption of frozen raspberries. *Epidemiol. Infect.* 123 (3), 469–474.
- 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 and the environment. *FEMS Microbiol. Rev.* 36, 786–814.
- Sarvikivi, E., Roivainen, M., Maunula, L., Niskanen, T., Korhonen, T., Lappalainen, M., Kuusi, M., 2012. Multiple norovirus outbreaks linked to imported frozen raspberries. *Epidemiol. Infect.* 140 (2), 260–267.
- Seitz, S.R., Leon, J.S., Schwab, K.J., Lyon, G.M., Dowd, M., McDaniels, M., Abdulhafid, G., Fernandez, M.L., Lindesmith, L.C., Baric, R.S., Moe, C.L., 2011. Norovirus infectivity in humans and persistence in water. *Appl. Environ. Microbiol.* 77 (19), 6884–6888.
- Todd, E.C., Greig, J.D., Bartleson, C.A., Michaels, B.S., 2008. Outbreaks where food workers have been implicated in the spread of foodborne disease. Part 4. Infective doses and pathogen carriage. *J. Food Prot.* 71 (11), 2339–2373.
- Tuladhara, E., Bouwknegt, M., Zwietering, M.H., Koopmans, M., Duizer, E., 2012. Thermal stability of structurally different viruses with proven or potential relevance to food safety. *J. Appl. Microbiol.* 112, 1050–1057.
- Vasickova, P., Slany, M., Chalupa, P., Holub, M., Svoboda, R., Pavlik, I., 2011. Detection and phylogenetic characterization of human hepatitis E virus strains, Czech Republic. *Emerg. Infect. Dis.* 17 (5), 917–919.
- Verhaelen, K., Bouwknegt, M., Rutjes, S., de Roda Husman, A.M., 2012a. Persistence of human norovirus in reconstituted pesticides – pesticide application as a possible source of viruses in fresh produce chains. *Int. J. Food Microbiol.* 160 (3), 323–328.
- Verhaelen, K., Bouwknegt, M., Lodder-Verschoor, F., Rutjes, S.A., de Roda Husman, A.M., 2012b. Persistence of human norovirus GI.4 and GI.4, murine norovirus, and human adenovirus on soft berries as compared with PBS at commonly applied storage conditions. *Int. J. Food Microbiol.* 160 (2), 137–144.
- Vilaginès, P., Sarrette, B., Husson, G., Vilaginès, R., 1993. Glass wool for virus concentration at ambient water pH level. *Water Sci. Technol.* 27, 299–306.
- Wyn-Jones, A.P., Carducci, A., Cook, N., D'Agostino, M., Divizia, M., Fleischer, J., Gantzer, C., Gawler, A., Girones, R., Holler, C., de Roda Husman, A.M., Kay, D., Kozyra, I., Lopez-Pila, J., Muscillo, M., Nascimento, M.S., Papageorgiou, G., Rutjes, S., Sellwood, J., Szewzyk, R., Wyer, M., 2011. Surveillance of adenoviruses and noroviruses in European recreational waters. *Water Res.* 45 (3), 1025–1038.