Harmonised Investigation of the Occurrence of Human Enteric Viruses in the Leafy Green Vegetable Supply Chain in Three European Countries

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Abstract Numerous outbreaks have been attributed to the consumption of raw or minimally processed leafy green vegetables contaminated with enteric viral pathogens. The aim of the present study was an integrated virological monitoring of the salad vegetables supply chain in Europe, from production, processing and point-of-sale. Samples were collected and analysed in Greece, Serbia and Poland, from ‘general’ and ‘ad hoc’ sampling points, which were perceived as critical points for virus contamination. General sampling points were identified through the analysis of background information questionnaires based on HACCP audit principles, and they were sampled during each sampling occasion where as-ad hoc sampling points were identified during food safety fact-finding visits and samples were only collected during the fact-finding visits. Human (hAdV) and porcine (pAdV) adenovirus, hepatitis A (HAV) and E (HEV) virus, norovirus GI and GII (NoV) and bovine polyomavirus (bPyV) were detected by means of real-time (RT-)PCR-based protocols. General samples were positive for hAdV, pAdV, HAV, HEV, NoV GI, NoV GII and bPyV at 20.09 % (134/667), 5.53 % (13/235), 1.32 % (4/304), 3.42 % (5/146), 2 % (6/299), 2.95 % (8/271) and 0.82 % (2/245), respectively. Ad hoc samples were positive for hAdV, pAdV, bPyV and NoV GI at 9 % (3/33), 9 % (2/22), 4.54 % (1/22) and 7.14 % (1/14), respectively. These results demonstrate the existence of viral contamination routes from human and animal sources to the salad vegetable supply chain and more specifically indicate the potential for public health risks due to the virus contamination of leafy green vegetables at primary production.

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Introduction

In recent decades, public health promotion of healthier lifestyles has led to the increased demand for fresh produce in many industrialized nations (FAO/WHO 2008). This has led to the consumer’s demand for minimally processed, pre-packed, ready-to-eat fruit and vegetables and availability of out-of-season produce (Heaton and Jones 2008). The growing burden of the food-borne outbreaks due to viral contamination of fresh produce in many parts of the world reflects a convergence of increasing consumption of fresh produce, changes in production and distribution, and a growing awareness of the problem on the part of public health officials (Lynch et al. 2009). Vegetables are important components of a healthy and balanced diet and their consumption is encouraged in many countries by government health agencies. However, vegetables and, in particular, leafy greens that are consumed raw are increasingly being recognized as vehicles for transmission of human pathogens, including viruses (FAO/WHO 2008; Croci et al. 2008; Berger et al. 2010). Numerous food-borne virus outbreaks have been linked to the consumption of contaminated fresh produce (Lowry et al. 1989; Rosenblum et al. 1990; Kilgore et al. 1996; Hernandez et al. 1997; Pebody et al. 1998; Seymour and Appleton 2001; Holfby et al. 2001; Dentinger et al. 2001; Long et al. 2002; Wheeler et al. 2005; Widdowson et al. 2005; Heaton and Jones 2008; Makary et al. 2009; Berger et al. 2010; Scallan et al. 2011). The lessons from these outbreaks are clear, despite the uncertainties regarding the ecology of pathogens on produce. Contamination cannot be washed off; once contamination occurs, there are, at present, no points during the processing, distribution and service of fresh produce at which microbiological hazards can be effectively controlled (Lynch et al. 2009). In the food industry, the major concepts such as HACCP have been mainly concerned with bacterial and fungal pathogens. 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 (D’Agostino et al. 2012). The World Health Organisation identified NoV and HAV in fresh produce as a priority virus/commodity combination for which control measures should be considered (FAO/WHO, 2008). Despite the increased importance of fresh produce as a vehicle for human pathogens, there is currently limited knowledge about where in the supply chain contamination occurs or about the mechanism(s) by which human pathogens colonize and survive on or in fruits and vegetables (Berger et al. 2010). Enteric viruses can be introduced into the food supply chain during different stages of food production. There is no strict evidence which stage of the production process is the most vulnerable for virus contamination. However, in the majority of contamination events, produce becomes contaminated on the farm during growing or harvesting. Routes of contamination are varied and include usually application of organic wastes to agricultural land such as fertilizer, contamination of waters used for irrigation with faecal material, direct contamination by livestock, wild animals and birds and postharvest issues such as worker hygiene (Heaton and Jones 2008).

The European FP7 project VITAL (Integrated Monitoring and Control of Foodborne Viruses in European Food Supply Chains) (http://www.eurovital.org/) aimed to gather data on virus contamination with the aim of providing a basis for subsequent quantitative viral risk assessment and recommendation of control measures. This paper reports a harmonised investigation of occurrence of human enteric viruses in the leafy green vegetables supply chain in three European countries.

Materials and Methods

Sampling

The sampling and analysis strategy is presented in Fig. 1. The sampling plans were developed using background information questionnaires, based on HACCP audit principles, which were completed for each premises. The sampling was longitudinal and samples from all phases (production, processing and point-of-sale) were collected. The plans
aimed to benefit most from a fixed number of samples. The critical points in the premises where samples would be taken were termed ‘general’ sampling points. Food safety fact-finding visits were made to the premises during which, through direct observations of conditions and practices, more points were identified where contamination with viruses could potentially occur. These extra sampling points were termed ‘ad hoc’ sampling points. Conclusively, ‘general’ sampling points were determined after the analysis of the completed questionnaires by food safety management and risk assessment experts and were described in the developed guidance documents per chain phase and per participating country, while ‘ad hoc’ sampling points were determined during food safety fact-finding visits and were not the same per each premise. Samples representing the production phase were comprised of swabs from harvesters’ hands, seasonal workers’ hands, toilets/latrines and toilet door handles, irrigation water and manure. A distinction was made between harvesters and seasonal workers. Seasonal workers are randomly employed, may not follow the strict hygienic practices and may also undergo the same trainings compared to the permanently employed harvesters. Samples representing the processing phase comprised of swabs from food handlers’ hands, knives (manual or mechanical), conveyor belt and rinsing water. Lettuce heads, representing the point-of-sale phase, were collected at supermarkets and a farmers’ market. Two types of lettuce (Lactuca sativa L.) were collected and analysed; butterhead and romaine lettuce. Ad hoc samples comprised of surface swabs (toilets, toilet door handles, plastic crates with and without lettuce heads, lettuce crates supports, internal walls of a truck used for the transportation of lettuce crates, food handlers’ hands and knives), a sponge used to clean the bottom of freshly cut lettuce heads, superficial and well irrigation water and lettuce heads. A summarized description of the production process of the vegetables in the countries (A, B and C) involved in the study as well as the number of analysed samples per country is presented in Table 1.

Description of Enterprises

Food businesses in the production and processing phase were mainly small and medium enterprises (SMEs). Only large enterprises, i.e. supermarkets, were sampled at the point-of-sale with the exception of one municipal outdoor market. The majority of the food businesses at point-of-sale throughout the supply chain were more than 10-years old. Casual labour was mainly used in the primary production, mostly for manual harvesting. Good Agricultural Practice (Global GAP) was only occasionally practiced at primary production. A formal food safety system was mainly implemented at point-of-sale and in some processing food businesses. In most cases these systems were accredited. At primary production, domestic animals were regularly found to have access and/or were present on the premises posing a risk to food safety. In most of the premises at primary production no field sanitary accommodation was provided. At primary production, raw manure was occasionally stored on-site and on one site it posed a particular risk.

Most of the FBOs used their own transport vehicles, which were all structurally suitable and solely designated for transporting foodstuffs. However, refrigerated vehicles were only used at processing and point-of-sale. In these cases, temperature was monitored by means of data loggers. In most cases, the premises were regularly cleaned according to a documented cleaning plan, particularly the field/staff sanitary accommodation and the transport vehicles. In contrast with cleaning, most areas were not properly sanitized, particularly utensils, equipment and transport vehicles at production and processing. Buildings were rarely structurally pest proofed. Proper pest control measures (only rodents and flying insects) were only in place at processing and point-of-sale. Control measures against crawling insects were only in place at point-of-sale.

Throughout the supply chain, shallow or deep untreated wells were used as primary water sources. Shallow wells were sampled with satisfactory results, but in primary production water was pumped to a shallow, untreated open water basin which was used as a reservoir. Public potable water was available throughout the supply chain and was in most cases compliant with EU drinking water regulations. Public potable water was the primary water source for all activities, particularly, at point-of-sale. In all premises, the water supplied for hand washing in the staff sanitary accommodation was of potable quality. In half of the premises, soap without antimicrobials was used. Antimicrobial soap was mostly used at point-of-sale. A designated cleaning area for cleaning equipment, utensils and food crates was mostly provided in premises at point-of-sale, all of which had a designated sink. In most cases, suitable sanitizers and disinfectants were used. At primary production, no suitable protective clothing was worn by food workers, except for disposable gloves. At processing and point-of-sale, a combination of hairnets or hats, suits or aprons and boots or shoes were worn. Evidence of good practice regarding the wearing of protective clothing and hand washing was found only at processing and point-of-sale.

Throughout the food supply chain training of food workers was mostly insufficient or at best limited to basic instructions. Essential documentation on hygiene and sanitation policies and/or practices was only provided at processing and point-of-sale, which in both phases also included a training schedule.

A labeling and traceability system was in place in most premises at processing and point-of-sale. At primary production particularly and at the farmers market, labelling
was not commonly in use and consequently a traceability system or a recall procedure was implemented.

Sample Process Control Virus

The SPCV was employed as a control of the virus concentration and nucleic acid extraction. The sample process control virus was murine norovirus 1 (MNV-1), which had been propagated in RAW264.7 cells to a concentration of $10^8$ pfu ml$^{-1}$. MNV-1 stocks were kindly provided by the group of Dr. Franco Ruggeri at the Istituto Superiore di Sanità, Rome, Italy, by agreement with Washington University, USA. Before virus recovery from the analysed food and environmental matrices, the samples were spiked with a known quantity of MNV-1. Viral RNA extracted 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. 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 samples were considered to be positive. When an assay showed a Cq value $\leq 45$, ...
independently of the corresponding IAC Cq value, the result was interpreted as positive. When an assay showed no Cq value for the target with the corresponding IAC Cq 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 negative. When an assay showed both the target and its corresponding IAC Cq values absent, the reaction was considered to have failed.

**Treatment of Lettuce**

A representative sample of the lettuce was collected into a sterile plastic bag or other appropriate container and transported to the laboratory. The sample was processed by the method of Dubois et al. (2006). Approximately 25 g of sample was placed in a sterile beaker. Sample process control virus (10 μl) was inoculated onto the sample (Diez-Valcarce et al. 2011a). Tris Glycine buffer (40 ml, pH 9.5) (Sigma-Aldrich) containing 1 % Beef Extract (Difco) (TGBE) was added to the sample. The sample was then agitated at room temperature for 20 min by rocking at 60 rpm. 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 centrifuge tube, and the pH adjusted to 7.2. then 0.25 volumes of 50 % (w/v) polyethylene glycol (PEG) 8000/1.5 M NaCl (Sigma-Aldrich) were added and mixed by inversion. The suspension was then incubated with gentle rocking at 4 °C for 30 min at 4 °C 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 PBS (Invitrogen). The suspension was transferred to a chloroform-resistant tube and 500 μl chloroform : butanol was added and mixed by vortexing. The sample was allowed to stand for 5 min and 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, resulting in a 300 μl nucleic acid extract. The nucleic acid extract was assayed immediately or stored at −70 °C.

**Treatment of Faeces and Animal-derived Fertiliser**

A representative sample of faeces or animal-derived fertiliser (at least 1 g) was collected aseptically by sterile spatula into a sterile plastic bag or tube and then transported to the laboratory. 250 mg of the sample was transferred to a 15-ml centrifuge tube. 2.25 ml of 10 mg ml⁻¹ gentamycin-containing PBS solution (Invitrogen) and 10 μl of the positive process control virus were added to the sample, which was then mixed using a vortex mixer at full speed for 1–1.5 min. Subsequently, the suspension was centrifuged at 3,000×g for 15 min, and the supernatant was transferred into a clean microcentrifuge tube. The supernatant was then immediately used for nucleic acid extraction (see below) or stored at −20 °C.

**Treatment of Handlers’ Hands**

Handlers’ hands were sampled using a 10 cm × 10 cm sterile gauze swab, moistened in 20 ml of 10 mg ml⁻¹ gentamycin-containing PBS solution (Invitrogen), contained in a sterile plastic bag. Sampling was performed at a pre-determined time of day, e.g. immediately before lunch or afternoon coffee break. The workers selected for sampling were not allowed to wash hands before sampling. After opening the sterile plastic bag and removing excess buffer by squeezing the gauze swab while keeping it in the bag, one of the hands of the handler (the right hand, or left hand if left-handed) was sampled by the swab using a firm pressure rub four or five times on the back, the palm and the spaces between the fingers and all the fingertips. Subsequently, the gauze swab was returned to the plastic bag and transported to the laboratory. The gauze was squeezed in the bag to release the contents and then discarded into an appropriate receptacle. The liquid in the bag was decanted by pouring into a clean 50-ml centrifuge tube. Then, 10 μl of the sample process control virus was added and the tube vortexed at full speed for 20 s. Finally, the suspension was centrifuged at 3,000×g for 5 min and the supernatant transferred into a clean 50-ml centrifuge tube. The supernatant was immediately used for nucleic acid extraction or stored at −20 °C.

**Treatment of Irrigation and Food-processing Waters**

Ten litres (10 l) of the sample was collected into an aseptic container and then transported to the laboratory. There, 10 μl of the sample process control virus was added and the sample was conditioned to pH 3.5 with 1 M or 0.1 M HCl. Then, the sample was processed by the method of Vilagines et al. (1993), as modified by Wyn-Jones et al. (2011). A glass wool filter was made by compressing 10 g glass wool (type 725; Rantigny, Saint-Gobain, France) into a 30 cm by 3 cm polystyrene column to obtain a filter height of 6–8 cm. The filter was washed by gravity with 50 ml volumes of (in order) 1 M HCl, tap water and 1 M NaOH, followed by tap water until the filtrate pH was neutral. The sample was then passed through the filter at a rate not exceeding 1.5 l min⁻¹. When all the samples had passed through the filter, viruses were eluted from the glass
wheat by slow (20–30 min) passage of 200 ml 3 % (w/v) beef extract (Difco) at pH 9.5 in 0.05 M glycine buffer (Sigma-Aldrich) through the filter. The eluate was flocculated by the addition of 1 M and 0.1 M HCl until the pH reaches 3.5. The resultant protein floc, containing virus, was deposited by centrifugation at 7,500 g for 30 min, then dissolved in PBS, adjusting to a final volume of 10 ml. This suspension was then filtered through a 0.45-µm filter (Sartorius Minisart) to remove remaining particulates. The filter had been pre-treated to prevent viruses from attaching by passing 5–10 ml 15 % w/v beef extract at pH 7.4 through it. The filtered suspension was stored at −20 °C before nucleic acid analysis.

Nucleic Acid Extraction

Nucleic acids were extracted using a NucliSENS® mini-MAG® kit (bioMérieux) according to the manufacturer’s instructions. With samples from the soft fruit and salad vegetable supply chains, 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.

Hepatitis A Virus RT-qPCR

This assay was a one-step duplex RT-qPCR using the primers and conditions described by da Silva et al. (2007) with the inclusion of an internal amplification control (IAC) (Diez-Valcarce et al. 2011b). The reaction contained 1 × RNA Ultrasense reaction mix (Applied Biosystems), 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 300 copies of NoV GI IAC (YorBio, Yorkshire Bioscience Ltd., UK). Ten 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 RT-qPCR

This assay was a one-step duplex RT-qPCR using the primers and conditions described by Jothikumar et al. (2006) with the inclusion of an internal amplification control (IAC) (Diez-Valcarce et al. 2011b). The reaction contained 1 × RNA Ultrasense reaction mix (Applied Biosystems), 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 300 copies of HEV IAC (YorBio, Yorkshire Bioscience Ltd., UK). Ten 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.

Norovirus GI RT-qPCR

This assay was a one-step duplex RT-qPCR using the primers and conditions described by Svraka et al. (2007) with the inclusion of an internal amplification control (IAC) (Diez-Valcarce et al. 2011b). The reaction contained 1 × RNA Ultrasense reaction mix (Applied Biosystems), 0.5 µM primer QNIF4, 0.9 µM primer NV1LCR, 0.25 µM probe NVGG1p (labelled with FAM), 50 nM IAC probe (labelled with VIC), 1 × ROX reference dye (Invitrogen), 1 µl RNA Ultrasense enzyme mix (Invitrogen) and 300 copies of NoV GI IAC (YorBio, Yorkshire Bioscience Ltd., UK). Ten 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.

Norovirus GII RT-qPCR

This assay was a one-step duplex RT-qPCR using the primers and conditions described by da Silva et al. (2007) with the inclusion of an internal amplification control (IAC) (Diez-Valcarce et al. 2011b). The reaction contained 1 × RNA Ultrasense reaction mix (Applied Biosystems), 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 300 copies of NoV GII IAC (YorBio, Yorkshire Bioscience Ltd., UK). Ten 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 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. 2011b) and a carryover contamination prevention system utilising uracil N-glycosylase (UNG). 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 (YorBio, Yorkshire Bioscience Ltd., UK). Ten 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.

Porcine Adenovirus qPCR

This assay was a duplex qPCR using the primers and conditions described by Hundesa et al. (2009) with the inclusion of an internal amplification control (IAC) (Diez-Valcarce et al. 2011b) and a carryover contamination prevention system utilising uracil N-glycosylase (UNG). The reaction contained 1 × TaqMan Universal PCR Master Mix (Applied Biosystems), 0.9 μM each primer, 0.225 μM porcine adenovirus TaqMan probe (labelled with FAM), 50 nM IAC probe (labelled with VIC), and 100 copies of porcine adenovirus IAC (YorBio, Yorkshire Bioscience Ltd., UK). Ten 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.

Bovine Polyomavirus qPCR

This assay was a duplex qPCR using the primers and conditions described by Hundesa et al. (2010) with the inclusion of an internal amplification control (IAC) (Diez-Valcarce et al. 2011b) and a carryover contamination prevention system utilising uracil N-glycosylase (UNG). The reaction contained 1 × TaqMan Universal PCR Master Mix (Applied Biosystems), 0.4 μM each primer, 0.120 μM bovine polyomavirus TaqMan probe (labelled with FAM), 50 nM IAC probe (labelled with VIC), and 300 copies of bovine polyomavirus IAC (YorBio, Yorkshire Bioscience Ltd., UK). Ten 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.

Quantitation

Quantitation was performed by the most probable number approach. The nucleic acid extract was assayed neat, and in 10⁻¹ dilution, and two replicate assays were performed for each concentration. If both 10⁻¹ replicates produced a positive signal, subsequent dilutions were assayed until both replicates of a dilution were negative.

Results

General Sampling Points

Summarized results per phase, matrix and virus type are presented in Table 2. HAdVs were detected in all three studied phases of primary production, processing and point-of-sale, at 18.61 %, 2.89 % and 26.4 %, respectively. PAdVs were found only in samples from the primary production and point-of-sale phases at 15.4 % and 4.2 %, while bPyVs were detected only in samples from the primary production phase at 5.1 %.

Table 2  Summarized results of the data gathered from the ‘general’ sampling points of the leafy vegetable supply chains per phase, matrix and virus type

<table>
<thead>
<tr>
<th>Point of interest</th>
<th>hAdV</th>
<th>pAdV</th>
<th>bPyV</th>
<th>HAV</th>
<th>HEV</th>
<th>NoV GI</th>
<th>NoV GII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Production</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Irrigation water</td>
<td>17/61 (27.9 %)</td>
<td>6/39 (15.4 %)</td>
<td>2/39 (5.1 %)</td>
<td>0/35</td>
<td>1/20 (5.0 %)</td>
<td>1/35</td>
<td>1/25 (4.0 %)</td>
</tr>
<tr>
<td>Toilets/latrines</td>
<td>3/15 (20.0 %)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>1/9 (11.1 %)</td>
<td>n.d.</td>
<td>2/9 (22.2 %)</td>
<td>1/8 (12.5 %)</td>
</tr>
<tr>
<td>Toilet door handles</td>
<td>4/13 (30.8 %)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>1/10 (10.0 %)</td>
<td>0/1</td>
<td>2/10 (20.0 %)</td>
<td>2/8 (25.0 %)</td>
</tr>
<tr>
<td>Harvesters hands</td>
<td>34/209 (16.3 %)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>2/97 (2.1 %)</td>
<td>n.d.</td>
<td>0/94</td>
<td>1/101 (1.0 %)</td>
</tr>
<tr>
<td>Seasonal workers hands</td>
<td>1/30 (3.3 %)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0/1</td>
<td>n.d.</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>Manure</td>
<td>3/5 (60 %)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0/2</td>
<td>n.d.</td>
<td>n.d.</td>
<td>2/2</td>
</tr>
<tr>
<td>Processing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conveyor belt</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Rinsing water</td>
<td>2/11 (18.2 %)</td>
<td>0/5</td>
<td>0/5</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>n.d.</td>
</tr>
<tr>
<td>Knives, manual</td>
<td>0/16</td>
<td>0/16</td>
<td>0/16</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Knives mechanical</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Point-of-sale</td>
<td>70/265 (26.4 %)</td>
<td>7/166 (4.2 %)</td>
<td>0/176</td>
<td>0/149</td>
<td>4/125 (3.2 %)</td>
<td>2/149 (1.3 %)</td>
<td>1/126 (0.8 %)</td>
</tr>
</tbody>
</table>

Number of positives/number tested; n.d. no data
NoVs GI were detected at production and point-of-sale phases at 2.68 % and 1.3 %, respectively; similarly, NoVs GII was detected at 4.83 % and 0.8 %. HAV was only detected in samples from the primary production phase at 2.6 %. Finally, HEV was found in 4.76 % and 3.2 % of samples from the primary production and point-of-sale phases (no samples from the processing phase were analysed for HEV).

Manual harvesting was identified as a critical point for virus contamination and swab samples collected from harvesters’ hands were found positive for hAdV, NoV GII and HAV at 16.3 %, 1 % and 2.1 %, respectively. Swab samples collected from seasonal workers’ hands were found positive for hAdV at 3.3 %. All samples collected from food handlers’ hands of the processing phase analysed only for hAdVs were negative.

Toilet swab samples from the production phase were found positive for hAdV, NoV GI, NoV GII, and HAV at 20 %, 22.2 %, 12.5 % and 11.1 %, respectively. Similarly, swab samples from toilet door handles were found positive at 30.8 %, 20 %, 25 % and 10 %. All surface swabs from various processing equipment tested for the index viruses (hAdV, pAdV, bPyV) were negative. Irrigation water samples analysed for all target viruses were positive for all index viruses (hAdV, pAdV, bPyV at 27.9 %, 15.4 % and 5.1 %) and two pathogenic viruses (HEV, NoV GII at 5 %, 4 %). Rinsing water samples from the processing phase analysed for hAdV, pAdV, bPyV, HAV, NoV GI, were only found positive for hAdV at 18.2 %. Manure samples analysed for hAdV, HAV, NoV GII were found positive for hAdV and NoV GII at 60 % and 100 % (2/2), respectively. Lettuce heads collected at the point-of-sale were positive for both index (hAdV, pAdV at 26.4 % and 4.2 %, respectively) and pathogenic viruses (NoVGI, NoVGII, HEV at 1.3 %, 0.8 % and 3.2 %, respectively).

Ad hoc Sampling Points

HAdVs were detected in swabs: (a) of three (3) empty plastic crates which are reused by supermarkets, (b) a food worker’s knife used to cut lettuce heads from the fields, c) and from a toilet in lettuce processing and packaging facility. PAdVs were detected in two irrigation water samples. One sample of rinsing water, originating from the well on the field, was found positive for bPyV, while a swab from a toilet door handle located at point-of-sale was found positive for NoV GI. In detail, hAdV, bPyV, pAdV, HAV, HEV, NoV GI and NoV GII were detected at 9 % (3/33), 4.54 % (1/22), 9 % (2/22), 0 % (0/8), 0 % (0/8), 7.14 % (1/14) and 0 % (0/11), respectively.

Quantitation

Estimated number of PCR-detectable units for samples that contained human pathogenic viruses are presented in Table 3.

Discussion

The ability of contaminated food to serve as a vehicle of infection depends on virus stability, degree of initial contamination, the method of food processing and storage,

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Virus</th>
<th>Estimated PDU</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Hands (n per hand)</td>
<td>NoV G2</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>HAV</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>HAV</td>
<td>19</td>
</tr>
<tr>
<td>Irrigation water (n per L)</td>
<td>NoV G2</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>HEV</td>
<td>2</td>
</tr>
<tr>
<td>Lettuce heads (n per 25 g)</td>
<td>HEV (estimated for 3 samples)</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>HEV</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>NoV G1</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>NoV G1</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>NoV G2</td>
<td>10</td>
</tr>
<tr>
<td>Toilet swabs (n per swab)</td>
<td>NoV G1 &amp; NoV G2</td>
<td>280</td>
</tr>
<tr>
<td></td>
<td>NoV G1 &amp; HAV</td>
<td>96</td>
</tr>
<tr>
<td>Doorhandle swabs (n per swab)</td>
<td>NoV G1</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>NoV G1 &amp; NoV G2</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>NoV G2</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>HAV</td>
<td>280</td>
</tr>
</tbody>
</table>
viral dose needed to produce infection, and the susceptibility of the host (Koopmans et al. 2002; D’Agostino et al. 2011). Contamination of leafy green produce can occur at multiple points, including at pre-harvest stage by contaminated water sources, contaminated amendments (e.g., raw manure or compost) or wildlife intrusion and at harvest or postharvest by cross-contamination potentially by any of the multiple people who handle the produce (Gandhi et al. 2010; FAO/WHO 2008). With regards to the increasing incidence of food-borne viral infections, at time of writing, the Codex Alimentarius Committee on Food Hygiene is preparing an international draft on a Code of Hygienic Practice for the control of viruses in foods (Ambrožić et al. 2011).

We are actually moving towards a more holistic approach to food safety, where the entire food chain needs to be considered in efforts to produce safer food (FAO/WHO 2008). Such holistic approaches have been followed for bacterial agents, including Salmonella, E. coli O157 and Campylobacter, but not for viruses.

In the present study, an integrated virological monitoring of leafy green vegetables chain has been conducted in three European countries. Samples representing the production, processing and point-of-sale phases of the chain have been collected and analysed in Greece, Serbia and Poland from comparable lettuce production processes.

Of the viral agents, HAV and NoVs are most commonly documented as contaminating fruits and vegetables. In most instances, contamination of fresh produce with enteric viruses is believed to occur before the product reaches food service establishments (Koopmans et al. 2002; Croci et al. 2008). Usually, virus contamination occurs mainly on the surface of food crops although few studies have reported on the potential for uptake and translocation of virus within damaged plant tissue (Seymour and Appleton 2001). Virus adsorption to lettuce has been found to vary depending on the strain and surface properties of the virus (Vega et al. 2008). Washing does not guarantee a substantial reduction in the viral contamination (Croci et al. 2002). The presence of viruses in biosolids may increase the risk of lettuce contamination and viruses in open cuts, and stomata may be protected from sanitization (Wei et al. 2010). Recombinant Norwalk virus-like particles (rNVLP) have been recently shown to bind to the surfaces of romaine lettuce, suggesting the potential availability of specific ligands on the leaf surface (Gandhi et al. 2010).

As bacterial indicators generally fail to signal the potential for viral contamination, bacteriophages, enteroviruses and adenoviruses have all been proposed as alternative indicators (Lees 2000; Muniain-Mujika et al. 2000). 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). The hAdV qPCR assay of the present study has been designed to quantify all common human adenoviruses. This assay in environmental studies and in shellfish samples (Formiga-Cruz et al. 2002; Albina-Gimenez et al. 2009; Bofill-Mas et al. 2006, 2010) has proved to be highly specific for hAdVs. The feasibility of using hAdVs as indicators of human enteric viruses in environmental and shellfish samples was suggested by Pina et al. (1998) who reported that these viruses were easily detected and seemed to be more abundant and stable in environmental samples. HAV, HEV, NoV GI and GII have been selected as target pathogenic viruses for the present study. Vegetable food supply chains were also monitored for the presence of index viruses commonly found in faecal contamination events. To indicate the source of virus contamination (human or animal), the presence of faecally derived adenoviruses and bovine polyomavirus was monitored. The results of the present study provide validation of this approach for the detection of faecal contamination of human and/or animal origin. HAdVs were detected in all three studied phases of primary production, processing and point-of-sale. PAdVs were found in samples from primary production and point-of-sale phases, while bPyVs were detected in general samples from the primary production phase and in an ad hoc sample of rinsing water (processing phase). Collectively, the presence of the index viruses in the samples tested confirms that (a) viral contamination routes exist from source to monitoring points which pathogenic viruses could follow; and (b) the use of these index viruses is a valuable MST (Molecular Source Tracking) tool for tracing the source of faecal viral contamination.

Water has been considered as a critical control point in the farm to fork continuum. Spraying, washing or immersion of fruits and vegetables in water is a common practice during post harvest processing (Gandhi et al. 2010). Water is also likely to be an important source of contamination in the field. Possible sources are run-off from nearby animal pastures and irrigation from a contaminated source (Berger et al. 2010). The risk of disease transmission from pathogenic microorganisms present in irrigation water is influenced by the level of contamination; the persistence of pathogens in water, in soil and on crops; and the route of exposure (Steele and Odumeru 2004). AdVs were the most frequently detected viruses in both groundwater and vegetable samples that were cultivated using that groundwater (Cheong et al. 2009). The role of contaminated irrigation or washing water for viral contamination of vegetables has been underlined by the results of the present study. Interestingly, irrigation water samples analysed for all
target viruses were positive for all index viruses (hAdV, pAdV and bPyV), HEV and NoV GII. The irrigation water sample found positive for HEV was a groundwater collected from the depth of 100 m, which makes well contamination unlikely. On field investigation did not reveal the virus source for water. The farmer never used animal manure as fertilizer, and there was not any pig farm located in the neighbourhood of the lettuce plantation and even in its surrounding. One of the most relevant and frequent risk factors involved in contamination of the lettuce plantation and even in its surrounding. One of the most relevant and frequent risk factors involved in contamination with acute hepatitis E was found to be the direct or indirect consumption of water from a private well or a nearby river (Renou et al. 2008). Rinsing water samples from the processing phase were positive for hAdV and bPyV (rinsing water-ad hoc sample). These results indicate the water virus contamination by human and animal sources.

Contaminated raw manure or compost may be responsible for viral contamination of vegetables. The attachment of MNV-1 in biosolids, swine manure and dairy manure to Romaine lettuce and internalization of this virus were evaluated in a recent study of Wei and colleagues, which showed that the presence of MNV in biosolids may increase the risk of contamination in fresh produce (Wei et al. 2010). Interestingly, bovine manure samples of the present study were found positive for hAdV and NoV GII, indicating human faecal contamination. Manure was stored on the field. The possibility of faecal contamination by workers was not excluded.

Because food-borne viruses are transmitted via the faecal-oral route through contact with human faeces and because infected individuals can shed millions of virus particles in their stools, the role of infected food handlers cannot be underestimated (Koopmans et al. 2002). Indeed, any food that has been handled manually and is not further heated before consumption has the potential to be virally contaminated (Richards 2001). Touching pieces of fresh lettuce with artificially contaminated finger pads for 10 s resulted in the transfer of approximately 9.2 % of infectious HAV (Bidadid et al. 2000). Norovirus and hepatitis A outbreaks are commonly caused by the contamination of foodstuffs from the hands of infected workers (Berger et al. 2010; Heaton and Jones 2008; FAO/WHO 2008). Swab samples collected from harvesters’ hands were positive for hAdV, NoV GII and HAV, in our study, while swab samples from seasonal harvesters’ hands were positive for hAdV, underlining the role of manual handling on viral contamination. All samples collected from food handlers’ hands of the processing phase analysed only for hAdVs were negative, indicating higher levels of hygiene at the processing facilities, which has been confirmed by the food safety fact-finding missions. Toilet and toilet door handle samples from the production phase were positive for hAdV, NoV GI, NoV GII and HAV. Two additional positive samples for hAdV and NoV GI were detected in ad hoc swab samples of a toilet in lettuce processing and packaging facility and a toilet door handle located at point-of-sale. On the contrary, all surface swabs from processing equipment (manual or mechanical knives and conveyor belt) tested for the index viruses (hAdV, pAdV and bPyV) were negative. Interestingly, hAdVs were detected in two ad hoc swab samples of a worker’s knife used to cut lettuce heads from the fields and three empty plastic crates which are reused by supermarkets.

Lettuce heads collected at the point-of-sale were positive for both index (hAdV and pAdV) and pathogenic viruses (NoVGI, NoVGII and HEV). However, we were not able to unequivocally indicate whether lettuce virus contamination took place at the production phase or whether viruses were introduced at the point-of-sale phase by food handlers or customers touching produce at product display. In combination with virus prevalence data from earlier phases of the leafy vegetables chain, this finding supports the existence of routes of contamination of the final product and the potential public health risks by the consumption of lettuce.

To improve microbiological detection and monitoring and to increase insights into the contribution of fruits and vegetables to food-borne viral transmission, sensitive, reliable and standardized methods are needed (Croci et al. 2008; Fino and Kniel 2008). In the present study, advanced methods for virus detection throughout the leafy vegetable supply chains, from farm to market, have been applied. The most effective assays for detection of food-borne viruses are those based on the amplification of viral nucleic acids (Croci et al. 2008; D’Agostino et al. 2012); therefore, VITAL has applied well-confirmed RT-qPCR protocols for virus detection in food and environmental samples (Martinez-Martinez et al. 2011; Diez-Valcarce et al. 2011a, b; Costa Freda et al. 2006; Jothikumar et al. 2006; Svraka et al. 2007; Da Silva et al. 2007; Hernroth et al. 2002; Hundesa et al. 2009, 2010). The methods were based on the Standard Operating Procedures (SOPs) developed by VITAL project. Methods used have been previously validated through ring trials to have comparable quantitative data (D’Agostino et al. 2012) and the efficiencies of extraction were comparable among the three participating laboratories.

In many instances, control of viral contamination in food needs to focus on the prevention of contamination (e.g. preventive measures at source, sewage treatment or in food handling), rather than destruction of the pathogen through the use of various inactivation processes. In fact, once fresh produce is contaminated by viruses, there are no realistic post-harvest risk control measures except cooking which is not an option with ready-to-eat fresh produce. (FAO/WHO 2008; Newell et al. 2010; Lynch et al. 2009). It is essential for thorough food safety management that systems are
developed whereby viruses can be monitored at critical points throughout food supply chains. The application of a method for detection of human adenoviruses in food samples could be useful for routine monitoring for food safety management to determine if a route of contamination exists from human source to food supply chain which pathogenic viruses such as NoV and HAV could follow (D’Agostino et al. 2011). A better understanding of plant, microbiological, environmental, farm, processing and food handling factors that interact with one another to determine whether contamination occurs, and whether pathogens which survive will support the development of evidence-based policies, procedures and technologies aimed at improving the safety of fresh produce (Berger et al. 2010). The numbers of samples tested for viral contamination were relatively small in this study, especially considering the expected low prevalence. Therefore, the presented results should be interpreted as indicative and for greater confidence in the results, a greater number of samples would have to be tested (Berto et al. 2012). Furthermore, the monitoring, as applied in the current study, is more likely to detect structural contamination events rather than episodic contamination events. Therefore, sampling points that tested negative throughout the monitoring might be important for episodic viral contamination nevertheless. Information and knowledge gained from this survey should be used to direct and support future food safety research and food safety programs to manage potential risks from viral contamination of food products.

Conclusions

The current study, which was a part of the VITAL project, verified the existence of leafy vegetable virus contamination routes, demonstrated the usefulness of index viruses, as a tool for tracing the source of faecal viral contamination, and finally underlined the need for future similar studies for the integrated management of food-borne viral diseases in Europe and worldwide.

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