Food and Environmental Virology

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--Manuscript Draft--

Manuscript Number:	FAEV-101
Full Title:	Harmonised investigation of occurrence of human enteric viruses in the leafy green vegetables supply chain in three European countries
Article Type:	Original Research
Keywords:	foodborne virus; virological quality; leafy vegetables; molecular detection
Corresponding Author:	Petros A. Kokkinos, Ph.D. University of Patras Rion Patras, Achaia GREECE
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University of Patras
Corresponding Author's Secondary Institution:	
First Author:	Petros A. Kokkinos, Ph.D.
First Author Secondary Information:	
Order of Authors:	Petros A. Kokkinos, Ph.D.
	Iwona Kozyra
	Sava Lazic
	Martijn Bouwknegt
	Saskia Rutjes
	Kris Willems
	Rita Moloney
	Ana Maria de Roda Husman
	Agnieszka Kaupke
	Euaggelia Legaki
	Martin d'Agostino
	Nigel Cook
	Artur Rzezutka
	Tamas Petrovic
	Apostolos Vantarakis
Order of Authors Secondary Information:	

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Full-length article

Harmonised investigation of occurrence of human enteric viruses in the leafy green vegetables supply chain in three European countries

P. Kokkinos[1a] · I. Kozyra [2a] · S. Lazic[3a] · M. Bouwknegt[4] · S. Rutjes[4] · K. Willems[5,6] · R. Moloney[7] · AM. de Roda Husman[4] · A. Kaupke [2] · E. Legaki[1] · M. D'Agostino[8] · N. Cook[8] · A. Rzeżutka[2*] · T. Petrovic[3*] · A. Vantarakis[1*]

[1]*Environmental Microbiology Unit, Laboratory of Hygiene, University of Patras, GR 26500 Rion, Patras, Greece*
[2]*National Veterinary Research Institute, Puławy, Poland*
[3]*Scientific Veterinary Institute "Novi Sad", Serbia*
[4]*National Institute for Public Health and the Environment, RIVM, Netherlands*
[5]*Laboratory for Process Microbial Ecology and Bioinspirational Management, Consortium for Industrial Microbiology and Biotechnology, Department of Microbial and Molecular Systems, KU Leuven, Leuven, Belgium*
[6]*Scientia Terrae Research Institute, Sint-Katelijne-Waver, Belgium*
[7]*Environmental Health Service, Health Service Executive, Sandfield Centre, Ennis, Co. Clare, Ireland*
[8]*Food and Environment Research Agency (FERA), Sand Hutton, York, United Kingdom*

Running title: virus contamination of leafy greens in Europe

[a] These authors contributed equally to the work.

[*]*Corresponding authors*:
* Apostolos Vantarakis. Environmental Microbiology Unit, Department of Public Health, Medical School, University of Patras, University Campus, 26500 Patras, Greece. Tel./Fax: +30-2610-969875, e-mail: avantar@med.upatras.gr
* Tamas Petrovic. Scientific Veterinary Institute "Novi Sad", Rumenacki put 20, 21000 Novi Sad, Serbia. Tel.: +38121518149 Fax:+38121518544, e-mail: tomy@niv.ns.ac.yu
*Artur Rzeżutka. National Veterinary Research Institute. Al. Partyzantów 57, 24-100 Puławy, Poland. Tel.: +48 818893037, e-mail: arzez@piwet.pulawy.pl

Abstract

Numerous outbreaks of disease 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 taken at "general" and "ad hoc" sampling points which were perceived as critical points for virus contamination. General points were identified through the analysis of background information questionnaires based on HACCP audit principles, and ad hoc points during food safety fact-finding visits. Samples were collected and analyzed in Greece, Serbia and Poland. Sampling points representing the production and processing phases were harvesters' hands, toilets, toilet door handles, surface swabs, irrigation and rinsing water. Lettuce heads, representing the point of sale phase, were collected at supermarkets and a farmers' market. The samples were analysed for a range of viruses, including the significant foodborne agents and viruses which indicate that a contamination route exists from human or animal sources. Human (hAdV) and porcine (pAdV) adenovirus, hepatitis A (HAV) and E (HEV) virus, Norovirus GI and GII (NoV), and bovine Polyomavirus (bPyV) were detected using 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 specifically indicated the potential for virus contamination at primary production.

Keywords: foodborne virus · virological quality · leafy vegetables · molecular detection

Introduction

In recent decades public health promotion of healthier lifestyles has led to increased demand for fresh produce in many industrialized nations. (WHO/FAO 2008) This has led to consumer demand for minimally processed, prepacked, ready-to eat fruit and vegetables and availability of out-of-season produce. (Heaton 2008) The growing burden of the foodborne 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 foodborne virus outbreaks have been linked to the consumption of contaminated fresh produce. (Alexander 1986, Lowry et al. 1989, Rosenblum et al. 1990, Warner et al. 1991, Kilgore et al. 1996, Hernandez et al. 1997, Pebody et al. 1998, Seymour and Appleton 2001, Holtby et al. 2001, Nygård et al. 2001, Dentinger et al. 2001, Long et al. 2002, Koopmans and Duizer 2004, Wang and Moran 2004, Wheeler et al. 2005, Widdowson et al. 2005, Baert 2008, Heaton 2008, Warriner 2009, Mesquita 2009, Makary et al. 2009, Berger 2010, Scallan et al. 2011). The lessons from these outbreaks are clear, despite the uncertainties regarding the biology of pathogens on

produce. Contamination cannot be washed off, and 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 directed at bacterial and fungal pathogens only. Also, analyzing 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. (Rodriguez-Lazaro et al., in press) The World Health Organisation identified norovirus and hepatitis A virus 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 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 application of organic wastes to agricultural land 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 2007)

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 the data obtained from the salad vegetables chain in Europe.

Materials and Methods

Sampling strategy

To be able to estimate the infection risk for humans through consumption of the leafy vegetables by using quantitative viral risk assessment (QVRA), the levels of enteric virus contamination at several points in the food supply chain were determined. Critical points for virus contamination were identified based on virological expertise, and food safety fact-finding missions at production farms, processing plants and point of sale premises in Greece, Serbia and Poland. These points had been identified briefly as follows. Background information questionnaires, based on HACCP audit principles (K. Willems and R. Moloney; pers. comm.), were completed for each premises. Subsequently the completed questionnaires were analysed by food safety management and risk assessment experts within VITAL, to identify the critical points in the premises where samples would be taken. These points 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 were termed “ad hoc” sampling points. The outcome of the QVRA depends on data input, and therefore on the accuracy and certainty of the estimated virus concentrations. The current sampling plan was developed to optimally benefit from a fixed number of samples. The strategy included increasing the probability of detecting virus at a

sampling point when low virus concentrations were expected or aiming for most accurate estimates of *e.g.* prevalence given the total number of samples.

Sampling locations and sample types

The sampling was longitudinal and samples from production, processing and point of sale phases were collected. Food businesses in the production and processing phase were mainly small and medium enterprises (SMEs). Only large enterprises, i.e. supermarkets, were sampled with the exception of one municipal outdoor market. Samples representing the production phase were comprised of swabs from harvesters' hands, seasonal workers' hands, toilets/latrines, and toilet doorhandles, irrigation water, and manure. 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 in Greece and Serbia and a farmers' market in Poland. 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, knives), a sponge used to clean the bottom of freshly cut lettuce heads, superficial and well irrigation water, and lettuce heads.

Labelling and transport of samples

All samples taken were labelled accordingly, including, at least the following details: Analyst, date of sampling, location and reference number to ensure traceability. All samples were placed under refrigeration where possible (e.g. using a cool box) and taken to the laboratory as soon as possible for the next analytical steps.

Sample process control virus

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. 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).

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 using the method of Dubois et al. (2006). Approximately 25 g of sample was placed in a sterile beaker. 10µl of the sample process control virus was pipetted onto the sample. 40 ml of Tris Glycine pH 9.5 buffer containing 1% Beef Extract (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 50ml or two smaller centrifuge tubes, and centrifuged at 10,000 × g for 30 min at 4ºC. The supernatant(s) was decanted into a single clean tube or bottle, and the pH adjusted to 7.2. 0.25 volumes of 50% (w/v) polyethylene glycol (PEG) 8000/ 1.5 M NaCl were then added, and mixed by inversion. The suspension was then incubated with gentle rocking at 4ºC for 60 min, before centrifugation at 10,000 × g for 30 min at 4ºC. The supernatant was discarded, and the pellet compacted by centrifugation at 10,000 × g for 5 min at 4ºC before resuspension in 500µl PBS. The suspension was then transferred to a chloroform-resistant tube, and 500 µl chloroform:butanol (1:1) added and mixed by vortexing. The sample was allowed to stand for 5 min, and then centrifuged at 10,000 × g for 15 min at 4ºC. 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 and 10µl 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 minutes. Subsequently, the suspension was centrifuged at 3000 × *g* for 15 min, and the supernatant transferred into a clean microcentrifuge tube. The supernatant was then immediately used for nucleic acid extraction (see below) or stored at –20 °C. Nucleic acids were extracted using an RNeasy Midi kit (QIAGEN) according to the manufacturer's instructions. With samples from the soft fruit and salad vegetables 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.

Treatment of handlers' hands

Handlers' hands were sampled using a 10 cm x 10 cm sterile gauze swab, moistened in 20 ml of 10 mg ml^{-1} gentamycin-containing PBS solution, 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 10µl of the sample process control virus was added, and the tube vortexed at full speed for 20 seconds. Finally, the suspension was centrifuged at 3,000 × g for 5 minutes and the supernatant transferred into a clean 50 ml centrifuge tube. The supernatant was immediately used for nucleic acid extraction or stored at –20 ^{o}C. Nucleic acids were extracted using a NucliSENS® miniMAG® kit (bioMérieux) according to the manufacturer's instructions. With samples from the soft fruit and salad vegetables supply chains, the final elutions were performed with 150 µl elution buffer, resulting in a 300 µl nucleic acid extract. With samples from the pork supply chain, the final elutions were performed with 50 µl elution buffer, resulting in a 100 µl nucleic acid extract. The nucleic acid extract was assayed immediately or stored at -70 ^{o}C.

Treatment of irrigation and food-processing waters

Ten liters of sample was collected into an aseptic container, then transported to the laboratory. There 10µl of the sample process control virus was added, and the sample conditioned to pH 3.5 with 1 M or 0.1M HCl. Then the sample was processed by the

method of Vilaginès 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 sample had passed through the filter, viruses were eluted from the glass wool by slow (20-30 min) passage of 200 ml 3% (w/v) beef extract at pH 9.5 in 0.05 M glycine buffer 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 x g for 30min, 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 ml-10 ml 1.5% w/v beef extract at pH 7.4 through it. The filtered suspension was stored at -20°C prior to nucleic acid analysis. Nucleic acids were extracted using a NucliSENS® miniMAG® kit (bioMérieux) according to the manufacturer's instructions. With samples from the soft fruit and salad vegetables 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 ^{o}C.

Hepatitis A virus RT-qPCR

This assay was a one-step duplex RT-qPCR using the primers and conditions described by Costafreda et al. (2006), with the inclusion of an internal amplification control (IAC). The reaction contained 1 X RNA Ultrasense reaction mix (Applied),

0.5 μM primer HAV68, 0.9 μM primer HAV240, 0.25 μM probe HAV150(-) (labelled with FAM), 50 nM IAC probe (labelled with VIC), 1 X ROX reference dye (Invitrogen), 1 μl RNA Ultrasense enzyme mix (Invitrogen) and 300 copies of HAV IAC. 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 ^{o}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). The reaction contained 1 X RNA Ultrasense reaction mix (Applied), 0.25 μM each primer, 0.1 μM probe HEV-P (labelled with FAM), 50 nM IAC probe (labelled with VIC), 1 X ROX reference dye (Invitrogen), 1 μl RNA Ultrasense enzyme mix (Invitrogen) and 300 copies of HEV IAC. 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 ^{o}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). The reaction contained 1 X RNA Ultrasense reaction mix (Applied), 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 X ROX reference dye (Invitrogen), 1 μl RNA Ultrasense enzyme mix (Invitrogen) and 300 copies of NoV

GI IAC. 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 ^{o}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). The reaction contained 1 X RNA Ultrasense reaction mix (Applied), 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 X ROX reference dye (Invitrogen), 1 µl RNA Ultrasense enzyme mix (Invitrogen) and 300 copies of NoV GII IAC. 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 ^{o}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) and a carryover contamination prevention system utilising uracil N-glycosylase (UNG). The reaction contained 1 X TaqMan Universal PCR Master Mix (Applied Biosystems), 0.9 µM each primer, 0.225 µM adenovirus TaqMan probe (labelled with FAM), 50 nM IAC probe (labelled with VIC), and 100 copies of adenovirus IAC. Ten 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) and a carryover contamination prevention system utilising uracil N-glycosylase (UNG). The reaction contained 1 X 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. 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) and a carryover contamination prevention system utilising uracil N-glycosylase (UNG). The reaction contained 1 X 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. 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 using 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

Enterprise characterization

Enterprise type

The majority of the food businesses at point-of-sale throughout the supply chain were more than 10 years old. In the primary sector the FBO owner was also the responsible manager. The FBOs were members of a professional consortium/organisation principally in enterprises at processing and point-of-sale. Casual labour was mainly used in the primary production, mostly for manual harvesting.

Management procedures

Only a few enterprises worked under specific contract specifications. 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. Concurrently, internal and external auditing was in place. A formal quality system, i.e. ISO 9001/2008, was implemented in some enterprises throughout the food supply chain. Medical screening was in place throughout the food supply chain, particularly at processing and point-of-sale.

Environment

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. However, staff sanitary accommodation was reasonably accessible to field workers. At primary

production raw manure was occasionally stored on-site and on one site it posed a particular risk

Lay-out and design of premises

Throughout the supply chain structurally suitable designated staff sanitary accommodation was provided, and most wash hand basins were provided with a constant supply of hot and cold water. However, hand-free taps were provided only in some premises 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. At primary production unsuitable cloth towels were used for hand drying. At processing and point-of-sale paper towels were mostly used. Occasionally, hot air was provided 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 designated field hand washing facilities were provided. Designated in-process hand washing facilities were provided in all premises at point-of-sale and in only one premises at processing. At point-of-sale half of the hand washing facilities included a constant supply of cold and hot water. Paper towels were provided in all of the in-process hand drying facilities and antimicrobial soap in the majority of them.

Distribution & transport

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 using data loggers.

Cleaning & sanitation

In most case the premises were regularly cleaned according to a documented cleaning plan, particularly the field/staff sanitary accommodation and the transport vehicles. Occasionally the cleaning plan did not include all areas. In contrast with cleaning, most areas were not properly sanitized, particularly utensils, equipment and transport vehicles at production and processing.

Pest control

Buildings were rarely structurally pest proofed. Particularly, openings such as external doors, windows and air intake points were not adequately screened. Proper pest control measures were only in place at processing and point-of-sale. However, most pest control plans included only rodents and flying insects. Control measures against crawling insects were only in place at point-of-sale.

Services

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.

Personal hygiene

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.

Food workers training

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.

Monitoring

Throughout the supply chain the primary water source and the product was tested microbiologically and chemically in almost all premises. Only in one case the laboratory was not accredited. Batch samples of the product were retained for testing in almost all premises at processing and point-of-sale.

Labelling, traceability & recall

A labelling 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 nor a recall procedure was implemented.

Detection of viruses

General sampling points

Summarized results per phase, matrix and virus type are presented in Table 1. 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%, respectively, while bPyVs were detected only in samples from the primary production phase at 5.1%.

NoVs GI were detected at production and point of sale phases at 2.68% and 1.3%, respectively, and similarly, NoVs GII were 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 analyzed 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 analyzed 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 indicator 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%, 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. PAdV were detected in two irrigation water samples originating from 15 meter deep wells directly on the field. 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), 0% (0/11), respectively.

Quantitation

Estimated number of PCR detectable units for samples that contained human pathogenic viruses is presented in Table 2.

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, viral dose needed to produce infection, and the susceptibility of the host. (Koopmans et al., 2002, Warriner 2009, D'Agostino 2011) Contamination of leafy greens 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, WHO/FAO 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. (Ambrozic 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. (WHO/FAO 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.

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 2008, Dubois 2007) Usually virus contamination occurs mainly on the surface of food crops, although a 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. 2011) 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 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 et al. 2000, Muniain-Mujika et al., 2002) 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 fecal 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; Albinana-Gimenez N, 2009; Silvia Bofill-Mas, 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, and NoV GI and GII have been selected as target pathogenic viruses for the present study. Vegetables 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

an ad hoc sample of rinsing water (processing phase). Collectively, the results of the indicator viruses show that: a) route of contamination exists from source to monitoring point which pathogenic viruses could follow; 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 identified as a critical control point in the farm to fork continuum. Spraying, washing or immersion of fruits and vegetables (produce) in water is a common practice during post harvest processing. (Gandhi 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 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, 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 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 murine norovirus 1 (MNV) 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 presence of MNV in biosolids may increase the risk of fresh produce contamination. (Wei et al. 2010) Interestingly, bovine manure samples of the present study were found positive for hAdV and NoV GII at 60% (3/5) and 100% (2/2), respectively, 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 prior to 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 transfer of approximately 9.2% of infectious HAV. (Bidawid et al. 2000) Norovirus and hepatitis A outbreaks are commonly caused by the contamination of foodstuffs from the hands of infected workers (Berger 2010, Heaton 2008, WHO/FAO 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 analyzed 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 from the analysis of ad hoc swab samples of a toilet in lettuce processing and packaging facility, and a toilet door-handle located at point of sale, respectively. On the contrary, all surface swabs from processing equipment (knives -manual or mechanical-, conveyor belt) tested for the index viruses (hAdV, pAdV, bPyV) were negative. Interestingly, hAdV were detected in two ad hoc swab samples of a worker's knife used to cut lettuce heads from the fields and three (3) empty plastic crates which are reused by from supermarkets.

Lettuce heads collected at the point of sale were positive for both index (hAdV, pAdV) and pathogenic viruses (NoVGI, NoVGII, HEV). 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 foodborne 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 vegetables supply chains, from farm to market, have been applied. The most effective assays for detection of foodborne viruses are those based on amplification of viral nucleic acids (Croci et al. 2008; Bosch et al. 2011, D'Agostino 2011), therefore VITAL has applied well confirmed RT-qPCR protocols for virus detection in food

and environmental samples. The methods were based on the Standard Operating Procedures (SOPs) developed by VITAL project which will be made available by the authors on request.

In many instances, control of viral contamination in food needs to focus on 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. (WHOFAOMRA, Newell 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 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 survive will support the development of evidence-based policies, procedures, and technologies aimed at improving the safety of fresh produce. (Berger 2010)

The numbers of samples tested for viral contamination were relatively small in this study, especially considering the expected low prevalence. Therefore the results presented should be interpreted as indicative, and for greater confidence in the results, a greater number of samples would have to be tested. (Berto 2011) 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. (Arthur 2007)

Conclusions

The current study which was part of the VITAL project, verified the existence of leafy vegetables 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 foodborne viral diseases in Europe and worldwide.

Acknowledgements

This work was supported by a European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement n° KBBE-213178 (Integrated monitoring and control of foodborne viruses in European food supply chains, VITAL) led by the coordination team of Nigel Cook (FERA, UK), Martin D'Agostino (FERA, UK) and Franco M Ruggeri (ISS, Italy). The authors are grateful to the owners of the companies who took part in this study for the kind and skilful collaboration and assistance, and to all VITAL members.

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Table 1
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Table 1. Summarized results of the data gathered from the "general" sampling points of the leafy vegetable supply chains per phase, matrix and virus type.

Point of interest	hAdV	pAdV	bPyV	HAV	HEV	NoV GI	NoV GII
Production							
Irrigation water	17/61 (27.9%)	6/39 (15.4%)	2/39 (5.1%)	0/35	1/20 (5.0%)	1/35	1/25 (4.0%)
Toilets/latrines	3/15 (20.0%)	n.d.	n.d.	1/9 (11.1%)	n.d.	2/9 (22.2%)	1/8 (12.5%)
Toilet door handles	4/13 (30.8%)	n.d.	n.d.	1/10 (10.0%)	0/1	2/10 (20.0%)	2/8 (25.0%)
Harvesters hands	34/209 (16.3%)	n.d.	n.d.	2/97 (2.1%)	n.d.	0/94	1/101 (1.0%)
Seasonal workers hands	1/30 (3.3%)	n.d.	n.d.	0/1	n.d.	0/1	0/1
Manure	3/5 (60%)	n.d.	n.d.	0/2	n.d.	n.d.	2/2
Processing							
Food handlers' hands	0/33	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Conveyor belt	0/1	0/1	0/1	n.d.	n.d.	n.d.	n.d.
Rinsing water	2/11 (18.2%)	0/5	0/5	0/1	n.d.	0/1	n.d.
Knives, manual	0/16	0/16	0/16	n.d.	n.d.	n.d.	n.d.
Knives mechanical	0/8	0/8	0/8	n.d.	n.d.	n.d.	n.d.
Point of Sale							
Fresh lettuce	70/265 (26.4%)	7/166 (4.2%)	0/176	0/149	4/125 (3.2%)	2/149 (1.3%)	1/126 (0.8%)

number of positives/number tested; n.d.: no data

Table 2
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Table 2. Estimated number of PCR detectable units for samples that contained human pathogenic viruses.

		Estimated PDU	
Sample type	**Virus**	**Mean**	**95% CI**
Hands	NoV G2	84	20 – 234
(n per hand)	HAV	24	1 – 112
	HAV	19	1 – 82
Irrigation water	NoV G2	16	2 – 86
(n per L)	HEV	2	0.3 – 5
Lettuce heads	HEV (estimated for 3 samples)	6	0.3 – 28
(n per 25 g)	HEV	23	3 – 129
	NoV G1	5	0.3 – 20
	NoV G1	6	0.3 – 28
	NoV G2	10	2 – 29
Toilet swabs	NoV G1 & NoV G2	280	41 – 1308
(n per swab)	NoV G1 & HAV	96	13 – 518
Doorhandle swabs	NoV G1	84	20 – 234
(*n per swab*)	NoV G1 & NoV G2	96	13 – 518
	NoV G2	19	1 – 82
	HAV	280	41 – 1308