Significance and molecular detection of noroviruses in fresh produce

Dragoslava Radin[1]

[1]Department of Food Microbiology, Faculty of Agriculture, University of Belgrade, 11080 Belgrade, Serbia

Human noroviruses (HuNoVs) are a primary cause of epidemic and sporadic cases of non-bacterial gastroenteritis; easily transmitted by the fecal-oral route which involves food as a frequent vehicle causing HuNoVs to be the major agent of food-borne illness worldwide. Primary factors contributing to the food contamination are contacts with infected food handler, cross-contamination during preparation, contaminated raw product and insufficient heating. Fresh produce are typically eaten raw with no thermal processing, therefore they are considered as high risk food because contamination can occur at the pre-harvest stage due to faecally polluted irrigation water and possible internalization; during harvest/post-harvest stage and at the point of sale/consumption due to contact with human faeces/faecally soiled materials and infected food handlers; cross-contamination from polluted working equipment/surfaces. Detection of food-borne HuNoVs in fruits and vegetables has become important based on the increasing number of reported outbreaks. Real-time RT-PCR is considered the gold standard detection method due to its high sensitivity and specificity, as well as for possibility of quantification and multiplexing.

Keywords noroviruses; fresh produce; transmission; molecular detection

1. Introduction

Human noroviruses (HuNoVs) are the leading causative agent of foodborne disease outbreaks and the predominant etiological viral agent of acute gastroenteritis worldwide. At the present, NoVs are even considered the second most important agent of severe childhood diarrhea after rotavirus [1, 2]. NoVs are present year-round in the community, and although usually most prevalent in the winter season in temperate climates, very often increased levels of NoV activity could be detected. Frequently, increased activity is associated with emergence of a new virus variant (e.g. new genotype GII.4), as was the case in late 2012 in the United Kingdom, the Netherlands, Japan, Australia, France and New Zealand reported by epidemiological and laboratory surveillance systems [3-6].

As a primary cause of viral gastroenteritis throughout the world, HuNoVs are the principle cause of food-borne illness in Europe [7] and the United States [8]. In the US, it is estimated that NoVs are responsible for 58% of all domestically acquired foodborne illness from known agents, which amounts to 5.5 million episodes annually. Generally, NoVs are easily transmitted from person to person by the fecal-oral route, either directly or indirectly via fecal contaminated surfaces, food or water and aerosols of vomits. Food is a common vehicle for HuNoVs transmission due to contamination with human fecal material that may occur at any step during the production [9, 10]. Fresh produce, mollusks, and ready-to-eat foods are identified as the primary vehicles of foodborne NoV disease. In viral foodborne outbreaks attributed to a single commodity, leafy vegetables, fruits/nuts, and mollusks are implicated most commonly [11]. Outbreaks attributed to the category “complex food”, involve most frequently ready-to-eat foods like sandwiches, salads, food eaten raw or lightly cooked. Main sources of outbreaks are infected food handlers and pre-harvest / postharvest food handling and management.

The rise in incidences of human NoVs outbreaks, particularly those attributed to fresh produce may be the result of increased consumption of fresh produce, with the consumer desire/demand for fresh produce year around, and their export/import and transport around the globe [12]. Globally, fruit and vegetables consumption increased by an average of 4.5% per annum between 1990 and 2004 (FAO statistical database – FAOSTAT, 2007). In some European countries 43.5% and 46.1 % of population consume fresh fruit and vegetables daily, respectively [13]. Other factors that contribute to the increase of food-borne illnesses associated with fruit and vegetables include availability of new commodities, changes in production practices, improved reporting and detection methods, etc.

2. Taxonomy and characteristics of noroviruses

2.1. Taxonomy and morphology

NoVs virions are small 27-32 nm non-enveloped particles with icosahedral symmetry and single-stranded (ss) positive sense RNA. Genome of 7.4-8.3 kb is organized into three major open reading frames (ORFs): ORF1 encodes the non-structural polyprotein of six/seven protein; ORF2 encodes the major structural capsid protein (VP1) and ORF3 the small minor structural virion-associated protein (VP2). Norovirus genome is covalently linked to a viral protein called VPg (virion protein, genome-linked) [14].

Among human enteric viruses, the NoVs have a major epidemiological significance as a common cause of both epidemic and sporadic non-bacterial gastroenteritis in humans. They represent a genetically diverse and heterogeneous

group of viruses belonging to the genus *Norovirus* of the family *Caliciviridae*. The first norovirus discovered and associated to a gastroenteritis outbreak in an elementary school in Norwalk, Ohio, USA in 1968 was the Norwalk virus [15] and today considered the prototype of the genus *Norovirus*. According to *International Committee on Taxonomy of Viruses* [16] the family *Caliciviridae*, besides the genus *Norovirus*, comprises four accepted (*Vesivirus, Lagovirus, Sapovirus* and *Nebovirus*) and two tentative genera (*Recovirus, Valovirus*). The only other genus that includes human pathogenic agents, causing acute gastroenteritis is *Sapovirus* (SaV). The other genera include important animal pathogens such as the rabbit hemorrhagic disease virus in the genera *Lagovirus*, the feline calicivirus which causes a respiratory disease in domestic and wild cat species in the genera *Vesivirus*, and the Newbury-1 virus which infect bovines in the genera *Nebovirus*. The tentative genera *Recovirus,* comprises the Tulane virus isolated from stool samples of rhesus macaques whose pathogenicity remains to be clarified [17]. The other tentative genera *Valovirus* comprises the St-Valérien-like viruses isolated from pig faeces [18].

Currently, the genus *Norovirus* consists of five genogroups (GI-V), which differ regarding the amino acid composition of the major capsid protein (VP1) [19]. HuNoV strains cluster within genogroups GI, GII, and GIV, however, GI and GII are most commonly associated with enteric disease in humans. Genogroups GIII and GV enclose bovine (BoNoVs) and murine NoVs (MNV), respectively. Porcine NoVs (PoNoVs) are genetically close to human NoV and belong to GII. Recently, NoV sequences that cluster within GIV have been detected in diarrheic feces in a lion cub and dogs [20]. Furthermore, NoVs have been detected in sheep and were shown to cluster within GIII.

Each genogroup is further divided into genotypes, defined by strains with a higher level of homology across the VP1 coding region [19]. Genogroups share > 60% amino acid identity in the VP1 and each genetic cluster or genotype shares > 80% identity in amino acid sequence of VP1. NoV genogroup GI contains eight (GI.1 to GI.8) and GII seventeen (GII.1 to GII.17) different genotypes that account for most human NoV illness cases. Also, GII contains 3 PoNoV genotypes (GII.11, GII.18 and GII.19), GIII two genotypes (GIII.1 and GIII.2), and GIV and GV one genotype.

2.2. Clinical disease and epidemiology

Clinical symptoms of acute NoV-associated gastroenteritis are characterized by the sudden onset of vomiting, watery diarrhea, or both. Additional symptoms that can be seen include nausea, abdominal cramping and pain, malaise, anorexia, fever, chills, headache, and myalgias. After an incubation period of 24–48 h, there is an acute onset of symptoms which usually resolves in 2– 3 days. The human enteric viruses, including NoVs, replicate in the intestines of infected human host and are excreted via faeces and vomit, starting during the incubation period and lasting up to 10 days and longer. In persons who had been experimentally infected with NoVs, virus shedding was first detected by RT-PCR 18 hours after inoculation and lasted a median of 28 days (range 13–56 days) [21]. NoVs shed at high level in faeces (median 95×10^9 genomic copies/g) are highly contagious, resulting in a high rate of transmission to contacts, partially due to very low infectious dose that is estimated to be ≈ 18 virus particles [22].

Gastroenteritis caused by the NoVs is generally acute and self-limited; however NoVs have been recently associated with severe clinical features other than gastroenteritis, including acute renal failure, arrhythmia and signs of acute graft organ rejection in renal transplant patients [23]. Moreover, NoVs were estimated to be the second cause (26%) of hospitalizations after *Salmonella* spp. (35%) [8]. The most important at-risk groups are infants, elderly, and immunosuppressed persons since they are more susceptible to complications due to dehydration [1, 14]. The duration of illness can be longer, lasting up to six weeks in infants and young children [24, 25] or even over two years in transplant patients and other immunosuppressed individuals [26]. NoVs infections associated with deaths have been most commonly reported among the elderly population (>65 years) acquired in health care facilities [27]. An estimated 800 deaths in the US [28] and 80 deaths in England and Wales [29] annually maybe associated with NoVs infection.

NoV outbreaks are notably extensive and often occur in semi-closed environments (nursing homes, hospitals, day-care centers, schools, cruise ships and restaurants) that favor person-to-person transmission [30]. Moreover, modern lifestyles make people more vulnerable to NoV. More elderly people and infants live in communal settings, people eat more food outside the household (handled by potentially infected workers), consume more imported fresh fruit and vegetables from countries where crops are still irrigated with sewage-contaminated water and also more people are travelling and being exposed to NoV in hotels, airplanes and cruise ships [26].

Presumptive diagnosis of NoV infection can be done during an outbreak based on the Kaplan criteria: (i) no bacteria or parasites agent is found; (ii) mean (or median) duration of illness of 12 to 60 hours; (iii) mean or (median) incubation period of 24 to 48 hours; and (iv) vomiting occurs in more than half of cases [31]. However, the criteria lack sensitivity and about 30 % of NoV outbreaks do not meet these criteria. Consequently, a viral etiology should not be excluded if the criteria are not met and any diagnosis based on clinical signs should be further confirmed in the laboratory.

Outbreaks of NoV infection are considered laboratory confirmed if stool or vomits specimens from >2 ill persons are positive for NoV by reverse transcription PCR, enzyme immunoassay, or electron microscopy [32]. The development of sensitive molecular diagnostic techniques has great impact on NoV epidemiology understanding. Laboratory confirmed, primarily on the basis of reverse transcription PCR, foodborne NoV outbreaks reported to Centers for Disease Control and Prevention (CDC) since 2006 shown that 17% of outbreaks were caused by genogroup I (GI) and 80% by genogroup II (GII); 3% involved both GI and GII NoV. Despite the great genomic variability between human genogroup GII NoVs, GII.4 has been by far the most detected genotype being responsible for 60 to 70% of the

outbreaks reported to the Foodborne Viruses in Europe (FBVE) network in the period 2002-2007. In NoV epidemiology it is common the emergence of new GII.4 variant that replace previously predominant ones. Since 1995, new epidemic variants of GII.4 have emerged every two to three years, with population immunity and genetic drift as major evolutionary driving forces [33]. The distribution of GII.4 variants over the last decade was dominated by successive circulation of GII.4/2002, GII.4/2004, GII.4/2006a, GII.4/2006b, GII.4/2007EU, and GII.4/2009. The newly found NoV GII.4 Sydney 2012 variant has evolved from previous variants and has a common ancestor with the dominant NoV GII.4 variants Apeldoorn 2007 and New Orleans 2009, but is phylogenetically distinct [6].

Same pattern has been noticed for NoVs as a cause of sporadic acute gastroenteritis in children (≤18 years of age) worldwide. A systematic review of genotypic distribution studies of noroviruses performed after 2000 reviled that Genogroup GII NoV was the most prevalent, accounting for 96% of all sporadic infections. GII.4 was the most prevalent genotype, accounting for 70% of the capsid genotypes and 60% of the polymerase genotypes, followed by the capsid genotype GII.3 (16%) and the polymerase genotype GII.b (14%) [34]. Nevertheless, unexpected high prevalence of NoV GI in the environment have been reported [35, 36] with assumption that NoV GI is more prevalent in the environment due to its greater resistance to inactivation [37].

2.3. Stability and persistence

In general, virus survival in the environment is influenced by parameters such as moisture, temperature, association with solids and exposure to UV light. Enteric viruses including caliciviruses (NoV, SaV), hepatitis A virus (HAV) and hepatitis E virus (HEV) can survive for a very long time (even years) at temperatures below 5 °C and especially in the absence of UV light. These viruses can resist complete inactivation in the environment showing long-term stability and persistence [38]. HuNoV, like other enteric viruses transmitted via the faecal–oral route are non-enveloped viruses, and once present in the environment, they have higher resistance to drying or desiccation methods and spread more easily than enveloped viruses, which are less stable [39]. Generally, as non-enveloped viruses can survive the low pH and enzymes present in the human gastrointestinal tract, they are considered to be stable in faeces and in the environment [40]. Therefore, these viruses cannot always be effectively eliminated by current methods of sewage treatment [41, 42] and this cause viral contamination of the environment from treated as well as untreated wastewater.

One of the reasons why NoVs are easily transmitted and spread is their high resistance to disinfection [43] and possible stability and persistence on contaminated surfaces used in food preparation areas up to 50 days and 28 days on stainless steel at 7°C and 20°C, respectively [44]. Although NoVs show stability in relatively high concentrations of chlorine, there are strong evidences that it is effective in treatment of different surfaces, but used concentration have to be adjusted following recommendation of CDC and World Health Organization (WHO). As Table 1 shows recommended concentrations of chlorine for NoVs disinfection are in a wide range depending on applications and surface characteristics.

Table 1 Recommended concentrations of chlorine for Norovirus disinfection.

Chlorine (ppm)	Application
200	Stainless steel Food/mouth contact items
1000	Non-porous surfaces Tile floors, counter-tops Sinks, toilets
5000	Porous surfaces Wooden floors

The inactivation of viruses in the environment is less effective if they are absorbed onto or embedded within suspended solid matter that is not dried out. Furthermore, NoVs are resistant to many industrial food preservation methods and can survive wide range of temperatures (from freezing, chilling, to 60°C), acidification, reduced water activity and modified atmosphere packaging [45].

3. Transmission of Noroviruses

3.1. Foodborne virus outbreaks

The incidence of outbreaks of foodborne viral disease has increased significantly during the last decades, possibly due to the rapid globalization of the food market, the increase in personal travel and food transportation, and the profound changes in food consumption habits. According to the FBVE network report for over 10,000 viral gastroenteritis outbreaks between 2001 and 2007, proportion of food borne outbreaks varied greatly between countries. When looking at a longer period (1990 – 2008) and only the outbreaks for which sufficient epidemiological information was available,

10% were reported as foodborne, 2% as waterborne, and the rest as person-to-person outbreaks [46]. However, this is probably underestimated due to significant issues of NoV detection methods in food and linking food items to the outbreaks. Therefore, using predictive models, food-borne outbreaks were estimated to be 50% [47] which confirms the fact that from all NoV outbreaks reported in US, 40 to 57% were food-borne [48]. During 2001–2008, total of 2.922 foodborne disease outbreaks for which NoV was the suspected/confirmed cause had been reported to the Foodborne Disease Outbreak Surveillance System of the CDC. On average, 365 foodborne norovirus outbreaks were reported annually, resulting in an estimated 10.324 illnesses, 1.247 health care provider visits, 156 hospitalizations, and 1 death. In 364 outbreaks attributed to a single commodity, leafy vegetables (33%), fruits/nuts (16%), and mollusks (13%) were implicated most commonly. Infected food handlers were the source of 53% of outbreaks and may have contributed to 82% of outbreaks [28]. Most foods were likely contaminated during preparation and service (except for mollusks) and occasionally, the produce was contaminated during production and processing.

Food can be contaminated after contact with: (i) faeces or faecally contaminated water; faecally soiled materials including hands, (ii) vomit or water contaminated by vomit, (iii) contaminated environments and (iv) aerosols generated by infected people [49]. Primary factors contributing to the food contamination are (a) food handler contact, (b) cross-contamination during preparation, (c) contaminated raw product and (d) inadequate cooking and/or heating. As Figure 1 shows transmission route of NoV is clear but complex with multiple overlapping path if an emphasis is on humans as principal source of viral particles shed by faeces and vomit and feature role of food handlers in transmitting of NoV since they have been consistently identified as the source of the food contamination along the food chain [50].

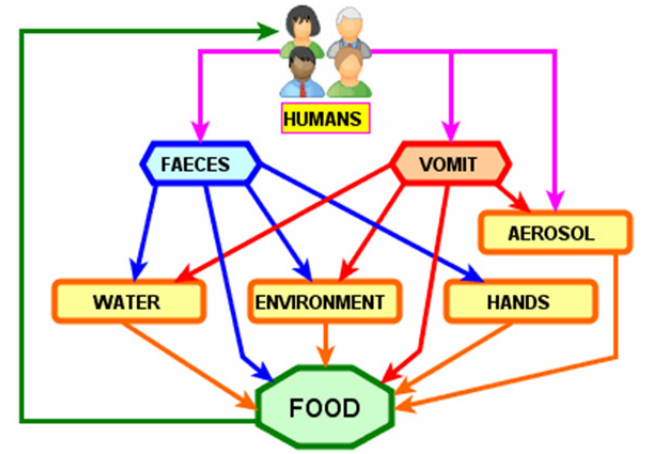

Fig. 1 Transmission route of human Noroviruses to the food, with an emphasis on humans as one of the principal source of viral particles, shed in faeces and vomit.

Many different food items such as minimally-processed fruits and vegetables, shellfish, a great variety of ready-to-eat foods like deli meat, desserts, salads and sandwiches have been associated with NoV foodborne outbreaks [50, 51]. One of the well established vehicles of transmission and principal source of food-borne virus in outbreaks are bivalve shellfish such as oysters, mussels, clams and cockles responsible for large, occasionally international, outbreaks [52]. There are several reasons why shellfish are at risk, but primarily due to their natural habitat and filter-feeding large volumes of water (up to 24 l of water/h) what enables the accumulation and concentration of viral NoV particles from polluted water in the digestive glands. The filtration can lead to virus concentrations in shellfish 100–1000 times higher than that in the surrounding water [52]. Another reason is that people in their diet frequently consume uncooked or only with a light heat treatment prepared shellfish which is not sufficient to guarantee viral safety.

3.2. Fresh produce

It is very important to emphasize that any food that has been handled manually and is not (or insufficiently) subjected to subsequent preservation and/or cooking is susceptible to be a source of transmission of enteric viruses and cause viral infection. Fresh produce are important category to which this can be applied, as raw and minimally processed fruits and salad vegetables are typically consumed in a ready-to-use or ready-to-eat form and rarely undergo any heat processing prior to consumption. Because fresh produce has high water content, absorbed from groundwater during growth may be eaten raw and without peeling, what exclude procedures that may potentially remove external contamination.

In the US, the proportion of all food-borne outbreaks associated with raw produce increased from 0.7% in the 1970s to 6% in the 1990s, while outbreak-associated illnesses cases increased from < 1% to 12% [51]. There has been an increase in reported infectious disease risks associated with consumption of fresh produce, particularly in North America linking salad and leafy greens with NoV, VTEC and *Salmonella* spp. [28, 54]. From a database of food-borne illnesses outbreaks with an identified etiology and associated food as pathogen vehicle; The Center for Science in the Public Interest (CSPI) has exposed the most common food - pathogen combinations in the US: green-based salads and

lettuce contaminated with human NoV [55]. Identified priority groups of food/pathogen combinations in EU countries based on criteria related to outbreaks, pathogen prevalence, food/pathogen interaction, are berries (raspberries, strawberries, blackberries) / NoV and lettuce / NoV, *E.coli* (EHEC), *Salmonella* spp. [56].

The European Union Summary Report on foodborne outbreaks in 2010 reported an increase in the number of outbreaks caused by vegetables in general, with significant share of NoV outbreaks attributed to vegetables [57]. The specific characteristics of the reporting practices for foodborne outbreaks in the EU (e.g. food categorization) do not allow for a comparison with similar data from other regions. However, reported outbreaks to the European Food Safety Authority (EFSA) during 2007-2011 attributed to the food of non-animal origin showed that NoV was the second causative agent, after pathogenic *Escherichia coli.* Data from 2011 were strongly influenced by the 2011 VTEC 0104 outbreak in Germany associated with sprouted seed consumption and consequently there was a considerable increase in number of human cases involved. Also, most frequent combinations of foodborne pathogen/food were NoV/raspberries and NoV/leafy greens eaten as salads (Table 2).

As the data in the Table 2 show an increased number of food-borne NoV outbreaks have been linked to fruits and vegetables worldwide. Different produce were involved, like leafy greens eaten as salads, lettuce, tomatoes, raspberries, fruit salads, carrots, melons, fresh-cut fruits, green onion, including recent large strawberry outbreak in Germany first noted in late September 2012. The outbreak led to more than 11.000 people, most of them schoolchildren, falling ill as a result of acute gastroenteritis. The officials declared an apparent failure by catering operators to prepare frozen strawberries properly prior to their consumption. NoV epidemics in Finland associated with imported frozen berries, mainly raspberries resulted in a ban to serve unheated frozen berries in catering and large-scale kitchens [58].

Table 2 Selected norovirus foodborne outbreaks attributed to fresh produce (amended from Radin, 2012; *Arch.Biol.Sci.*).

Product	Year	No. of cases	Country	Reference	
Green salad	1979	63	USA	Tauxe et al. 1997	*J. Food Prot.*
Melon	1987	-			
Raspberries	1988	108	Finland	Ponka et al. 1999	*Epidemiol. Infect.*
Celery	1991	1440	USA	Tauxe et al. 1997	*J. Food Prot.*
Raspberries	1997	300	Canada	Gaulin et al. 1999	*Canadian J.Public Health*
Carrot, tomato	2000	-	UK	ACMSF, 2000	www.food.gov.uk/acmsf
Raspberries	2001	30	Sweden	Le Guyader et al. 2004	*Int. J. Food Microbiol.*
Raspberries	2005	75	France	Cotterelle et al. 2005	***Euro Surveill.* 10, 2690**
Raspberries	2005	1043	Denmark	Korsager et al. 2005	***Euro Surveill.*10, 2729**
Blackberries	2005	241	Germany	Fell et al. 2007	*Bundesgesundheitsblatt*
Raspberries	2006	4 outbreaks/43	Sweden	Hjertqvist et al. 2006	***Euro Surveill.* 11, 3038**
Vegetables	2006	400	Finland	Makary et al., 2009	*Epidemiol. Infect.*
Salad	2006	182	Austria	Schmid et al. 2007	*Infection*
Tomatoes	2007	400	Sweden	Zomer et al. 2010	*Epidemiol. Infect.*
Coleslaw	2007	60	UK	Vivancos et al. 2009	*Int. J .Infect. Diseases*
Radish	2008	117	Korea	Yu et al. 2010	*J. Korean Med. Sci.*
Raspberries	2009	200	Finland	Maunula et al. 2009	*Euro Surveill.* 14, 19435
Salad	2009	101	Germany	Wadl et al. 2010	*BMC Infect. Diseases*
Onion	2009	52	Finland	ECDC, 2009	www.efsa.europa.eu
Lettuce	2010	22 outbreaks/423	Denmark	Ethelberg et al. 2010	*Euro Surveill.* 15, 19484
Lettuce	2010	157	Norway	ECDC, 2010	www.efsa.europa.eu
Potatoes	2010	41	Germany	ECDC, 2010	www.efsa.europa.eu
Strawberries	2012	11000	Germany	RKI, 2012	*www.rki.de*
Greens Salad	1998-2005	139 outbreaks/5,139	USA	CSPI, 2007	Outbreaks by the numbers: fruits and vegetables 1990-2005 IAFP 94th Annual Meeting
Lettuce		29 outbreaks/949			
Fruit		18 outbreaks/1,147			
Vegetables		13 outbreaks/521			
Fruit Salad		12 outbreaks/355			
Raspberries	2007-2011	27 outbreaks/913	EU	EFSA, 2013	Scientific Opinion on the risk posed by pathogens in food of non-animal origin.
Leafy greens		24 outbreaks/657			
Bulb/stem vegi		2 outbreaks/18			

Fresh produce are considered as high risk food commodities because they can be contaminated at various points along the food supply chain. Contamination of fruits and vegetables with HuNoVs can occur:

a) while growing in the fields during primary production at the pre-harvest stage due to faecally polluted irrigation water, organic-based fertilizers and potential root uptake of enteric pathogens and subsequent internalization;
b) during harvest due to contact with human faeces or faecally soiled materials and poor hygiene practice by food handlers;
c) at the post-harvest stage by inappropriate practices (contamination in food plant by infected food handlers or by spraying with contaminated water) during handling, processing, preparation, storage, distribution;
d) at the point of sale/consumption by infected food handlers who do not follow proper/adequate hygienic practices;
e) due to cross-contamination from polluted working instruments or surfaces, which have been contaminated previously by infected food handlers or contaminated food items.

Viruses can survive on the surface of fresh produce once they have been harvested [53] and remain infectious for several days or weeks, for up to 5 weeks during commercial and household storage [59]. These data are supported by virus persistence on finger pads and food preparation surfaces, which can act as vehicles for human NoV transmission long after the initial contamination event has occurred [60, 61]. Additionally, mechanical or machine harvest has become increasingly prevalent in lettuces (in general for all type of leafy greens) and this may provide increased cross-contamination with human pathogens.

Significant conclusions and parallels can be drawn from a systematic review of risk factors for contamination of fruits and vegetables with *Listeria monocytogenes*, *Salmonella*, and *Escherichia coli* O157:H7 at the pre-harvest level. Growing produce on clay-type soil, the application of contaminated or non–pH-stabilized manure, and the use of spray irrigation with contaminated water, with a particular risk of contamination on the lower leaf surface have been identified as primary risk factors. Authors suggested that reducing microbial contamination of irrigation water and soil are the most effective targets for the prevention and control of produce contamination [62].

Fresh leafy vegetables are grown and harvested under a wide range of climatic and geographical conditions, using various agricultural inputs and technologies, and on farms of diverse sizes, including field production (open field or under cover) and greenhouse production (in the soil or hydroponically). Recent studies have reported internalization and transport of enteric viruses in lettuce plants grown under hydroponic conditions or during irrigation [63, 64]. When comparing the ability of HAV and the HuNoV surrogate, murine norovirus (MNV) to internalize in spinach and green onions through root uptake in both soil and hydroponic systems a drastic difference was observed. HAV and MNV were not detected in plants (except HAV in only 1 of 64 spinach plants) grown in contaminated soil substrate systems while in hydroponic systems were internalized up to 4 log RT-qPCR units and internalized MNV remain infectious [65]. In hydroponically grown romaine lettuce inoculated with HuNoVs GII.4 viral-genome RNA was detected in leaves, shoots, and roots at day 1 post-inoculation and remained stable over the 14-day study period [66]. These findings indicate possible route of contamination by uptake of virus through the root system and subsequent transport of the virus into edible portions of the plant via vascular tissue. Internalization may be food safety concern in connection with inappropriate irrigation practices, wastewater treatment and reuse, sewage overflows, and wastewater releases from polluted sources which are all the direct causes of viral environmental contamination and food-borne outbreaks [67, 68]. NoV GI was detected on irrigated, field grown strawberries, with suspected irrigation water, only 1 h after irrigation [36].

In the production of fresh fruits and vegetables even contaminated water (including well water, river or lake water) used to dilute pesticides could be a source of HuNoV. Infectivity of the NoV was unaffected when combined with diluted pesticide which did not neutralize the effects of the contaminated water [69]. As authors concluded the application of pesticides on fresh produce may not only be a chemical hazard, but also a microbiological risk factor; both having consequences on public health.

3.3. Virus inactivation in fresh produce

Since there is no available method for HuNoV cultivation in laboratory practice, the most research on virus inactivation and removal from produce has been done using NoV surrogates, murine norovirus (MNV) and feline calicivirus (FCV). As has already been said fruits and vegetables are major vehicles for transmission of food-borne enteric viruses since they are easily contaminated at pre- and postharvest stages. Depending on the produce postharvest processing can involve spraying, washing, scrubbing, peeling or immersion into water with disinfectants to eliminate pathogens. Physical actions can result in removal at some extent of MNV from contaminated produce like honeydew melon, cantaloupe, carrot, and celery. However, the equipment cross-contamination issue occurs, as in some cases it can result in virus transfer on seven successively prepared produce items [70]. Also, the commonly used sanitizers are relatively ineffective for removing HuNoV surrogates from fresh produce. Washing with tap water and chlorine solution (200 ppm) provides on average 1log reductions in virus titer in fresh produce. Enhanced efficiency in removing a MNV-1 from fresh produce (strawberries, raspberries, lettuce and cabbage) is achieved by combining chlorine with surfactants like sodium dodecyl sulfate (SDS), Nonidet P-40, Triton X-100, and polysorbates [71].

Since effects of disinfectants, including chlorine, on viruses is unreliable (due to the concentrations, freshness of solutions, produce surface, etc.) there are attempts to use ozone as a disinfectant in produce washes. It showed, as for many other virus properties that the food matrix plays the largest role in protection against inactivation, since the ozone inactivation of FCV and MNV on produce (green onions and lettuce) was less effective than in sterile water [72]. But, bearing in mind that some disinfectants have harmful by-products that pose a concern, ozone may be an alternative method to partially reduce viral contamination on the surface of fresh produce.

Even the applications of new technologies such as high pressure processing (HPP) as an alternative to thermal processes for the destruction of food pathogens does not give a complete solution for NoV inactivation. For complete disruption of HuNoVs virus-like particles (VLPs) higher level of pressurization have to be administrated then the level sufficient to completely inactivate MNV and FCV [73]. While HuNoVs VLPs may not fully represent viable HuNoVs, indicate that HuNoVs capsid is highly resistant to HPP and what to expect as potential response of virus particles.

3.4. Prevalence of viruses in fresh produce

It is well recognized that foodborne outbreaks caused by virus have been associated with the consumption of fresh produce. However, establishing the presence of the viruses in the food is very scarce comparing with the routine detection of bacterial food pathogens. Table 3 summarizes the reported occurrence for most foodborne pathogen reported via EFSA's Zoonoses web-based application in food of non-animal origin for the period from 2004 to 2011 in EU countries including Norway and Switzerland [74]. It should be mentioned that monitoring and surveillance schemes are not harmonized between Member States, and therefore results are generally not directly comparable.

Table 3 Reported occurrence of *Salmonella* spp., pathogenic *Escherichia coli, Campylobacter* spp., n *Listeria monocytogenes, Staphylococcus aureus, Yersinia* spp., *Cronobacter* spp., and viruses in food of non-animal origin in the EU countries, 2004-2011 (adopted from EFSA Journal 2013;11:3025.).

Foodborne pathogen	Number of reporting countries	Total number of samples	Total number of positive samples	Prevalence (%)
Salomonella spp.	26	121.869	584	0.48
Pathogenic *Escherichia coli*	21	11.240	31	0.28
Campylobacter spp.	13	4.631	34	0.73
Listeria monocytogenes	24	32.988	884	2.68
Staphylococcus aureus	4	703	12	1.71
Staphylococcal enterotoxins	4	43	3	6.98
Yersinia spp.	4	1.000	236	23.60
Cronobacter spp.	1	25	0	0
Viruses	1	88	0	0

On the other hand, in a recent study of NoV prevalence in Belgian, Canadian and French fresh produce viral RNA was detected in 28%–50% of samples from leafy green vegetables and in 7%–34% of samples from soft red fruit (e.g., strawberries and raspberries) obtained from retail markets or directly from processing companies [75]. In fresh produce sampled from field and packinghouse facilities from the northwestern part of Mexico, NoV, HAV and rotavirus were detected in 28.2%, 32.6%, and 13.0% of the samples, respectively [76]. The occurrence of human enteric viruses in the leafy green vegetable supply chain (production, processing and pint-of-sale) has been monitored in three European countries (Greece, Serbia and Poland) and human (20.09%) and porcine (5.53%) adenovirus, hepatitis A (1.32%) and E (3.42%) virus, norovirus GI (2%) and GII (2.95%) and bovine polyomavirus (0.82%) were detected [77].

Viruses can be detected in fresh produce, but prevalence studies are still limited, and quantitative data on viral load are insufficient for establishment of microbiological criteria (food safety criteria) for these food categories. There have been suggestions that faecal coliforms on fresh produce may be an indicator of the probable presence of enteric viruses. However, no significant correlation have been found and in studies that have been investigated the prevalence of NoV in fruit and vegetables, despite the good bacteriological quality, an unexpected high prevalence of NoV was observed by RT-qPCR particularly in raspberries, strawberries, and cherry tomatoes [78]. Because the available detection methods cannot distinguish infectious virus from noninfectious genomic material, the specific risks posed to public health from contaminated produce remains unclear.

4. Molecular detection of noroviruses

4.1. General consideration

As NoVs cannot be cultivated to date due to absence of available cell-culture based systems for virus propagation, laboratory diagnostic methods for NoV infections rely on:

(i) virus particle observation by electron microscopy (EM), which historically, allowed the first identification of NoVs and remains a diagnostic tool for clinical samples but requires the presence of high viral loads in the analyzed sample, additionally sensitivity can be improved by the technique of immune-EM;
(ii) antigen identification by immunological assays - enzyme-linked immune sorbent assay (ELISA), based on the use of antisera raised against recombinant NoV VLPs, for the detection of NoV antigens in clinical samples, as often detects only genetically similar strains these assays should be used for screening purposes;
(iii) genomic amplification by reverse transcriptase-polymerase chain reaction (RT-PCR) or real-time RT-PCR which is nowadays the most widely used molecular technique for the detection of NoV in human and animal clinical samples (faeces and/or vomits), in food, water and environmental samples.

Major disadvantages for the use of EM and ELISA are low sensitivity (detection limits range between 10^4 and 10^6 virus particles per gram of sample) and long duration of analysis. Immunological and molecular methods share limitation from the fact that NoVs are genetically and antigenetically highly diverse. Due to their extreme genetical heterogeneity the challenge remains in detecting many diverse strains of human NoV genogroups, complicating the design of protocols in attempt to detect multiple strain variants. Furthermore, one of the major limitations of PCR method is inability to differentiate between infectious and noninfectious viruses.

Generally, detection of viral pathogens regardless of the samples matrix comprises several consecutive steps (reviewed elsewhere [79-82]):
(1) Sample preparation in order to extract and concentrate the agents from the matrix, and to remove the inhibitors;
(2) Nucleic acid (RNA) extraction and purification;
(3) Molecular detection which is in some cases followed by molecular typing.

4.2. Molecular detection by real-time RT-PCR

Molecular method real-time RT-PCR is considered to be the "gold standard" for detection of NoVs in clinical, food and environmental samples for several reasons: (i) a lower detection limit in comparison to conventional RT-PCR and other molecular methods [83]; (ii) the amplification of small PCR products and no need for post-PCR amplicon confirmation; (iii) confirmation of the identity and quantification of the product by the use of fluorescent probe-based chemistries such as Taqman probes [84]; (iv) the possibility of multiplexing of virus-specific-primers and probes detecting NoVs from different genogroups [85]. Detection of NoVs based on molecular methods, target genomic regions located at the ORF1-ORF2 junction that are highly conserved sequences among NoVs [86]. The selected targets have to guarantee an absolute specificity and must reach equilibrium between high sensitivity, broad reactivity, and reliability of quantification [82]. An evaluation of different RT-PCR NoV detection assays targeting the ORF1/ORF2 junction using a variety of human infective NoV strains circulating between 2007 and 2009 in Canada confirmed that this region still allowed amplification of a broad range of human infective NoV genotypes [87]. The European Committee for Standardization/Technical Committee 275/Working Group 6/Task Group 4 for detecting NoV and hepatitis A virus in foods and bottled water (CEN/TC275/WG6/TAG4 group) has also selected ORF1/ORF2 genomic junction region as target for RT-PCR detection of NoV [88]. To date, numerous real-time RT-PCR assays have been described for NoV detection [85-88], especially in food samples where the low concentration of HuNoV is commonly found. For these samples sensitivity of the assays is an essential issue because, as mentioned earlier NoVs have very low infectious dose. Theoretically, sensitive detection method means that it should be sensitive enough to detect a single viral particle per sample; in practice the low detection limits refer to ≤ 10 target copies.

Although it is difficult to generalize due to large differences in composition between soft fruit and hard surface vegetables, real-time RT-PCR assay has been efficient and sensitive for the rapid detection of human NoVs from different fresh produce such as lettuce, tomatoes, green onions, raspberries, strawberries, fruit salad, etc. [89-92].

4.3. Inhibitors and controls for real-time PCR

Virus detection from food includes diverse challenges, such as typically low viral load in fruits and vegetables however, sufficient to cause illness; extremely genetic heterogeneity; and presence of food components for example bioactive compounds such as phenolics, anthocyanins, organic acids and minerals from raspberries, that are able to inhibit molecular assays. Although detection of NoV relies on molecular methods such as real-time RT-PCR due to high sensitivity and specificity, it is well known that PCR reactions are susceptible to various inhibitors (e.g., humics, complex polysaccharides, microorganism debris, metal ions, organics, and nucleases) ubiquitous in environmental samples [93]. Though the identities and biochemical mechanisms of action of many inhibitors remain unclear, bile salts and complex polysaccharides in feces, heme in blood, and urea in urine have all been shown to inhibit PCR, probably through interference with binding and/or polymerization activity of DNA polymerases [94]. Carryover of reagents used for isolation of nucleic acids from samples can also inhibit amplification reactions. Generally, inhibitory factors that have been identified in PCR reactions include organic and inorganic chemicals, detergents, antibiotics, buffers, enzymes, polysaccharides, fats, and proteins [95]. The vast number of PCR inhibitors can lead to unacceptable high number of false negatives in real-time PCR NoV detection. Other causes of false-negative results include target nucleic acid degradation, sample processing errors, thermal cycler malfunction, and in reverse transcription-PCR, failure of the

reverse transcription step. To eliminate the risk of false negative results it is recommended that all PCR assays should include adequate controls shown in Table 4 to assure reliable detection of NoVs in different samples [96, 97].

Table 4 Controls for real-time RT-PCR detection of viruses in food (adopted from Rodriguez-Lazaro et al., 2012; *FEMS Microb. Rev.)*.

Control	Name	Description	Result
Process	Positive (PPC)	A negative sample spiked with sufficient viral target and processed throughout the entire protocol	A positive signal should be obtained indicating that the entire process was correctly performed
	Negative (PNC)	A negative sample spiked with sufficient amount of non-target or water and processed throughout the entire protocol	A negative signal should be obtained indicating the lack of contamination throughout the entire protocol*
Environmental		A tube containing the master mixture or water left open in the PCR set-up room to detect possible contaminating nucleic acids in the environment	
Amplification	Positive PCR	A viral template known to contain the target sequence	Positive amplification indicates that amplification was performed correctly**
	Negative PCR***	Including all reagents used in the amplification except the template nucleic acids****	A negative signal indicates the absence of specific contamination in the amplification assay
	External (EAC)	An aliquot of a solution of control DNA, containing a defined quantity or copy number added to an aliquot of the nucleic acid of the extracted sample and analyzed in a separate reaction tube	A positive signal indicates that the sample nucleic acid extract did not contain any inhibitory substances
	Internal (IAC)	Chimerical non-target nucleic acid added to the master mix to be co-amplified with the same primer set as the viral target but with an amp icon size visually distinguishable or different internal sequence from the target amp icon	The amplification both in the presence and in the absence of the target indicates that amplification conditions are adequate

*For example, the inclusion of encapsulated RNA (or DNA) or bacteriophages
**It could be used a natural virus or chimerical nucleic acids
***or No Template Control -NTC- or Reagent Control or Blank
****Usually, water is added instead of the template.

5. Conclusion

The very low infectious dose of NoVs, their long persistence in the environment, resistance to sanitary measures effective against other microorganisms (freezing, heating and chlorination) combined with prolonged asymptomatic shedding through faeces and vomit make this pathogen extremely infectious and contributes to the extensiveness of outbreaks. Attribution of NoV disease to specific foods and increased understanding of the various contamination pathways that result in disease can help identify potential targets for interventions. Undoubtedly, fresh produce contribute to the transmission of NoVs infections. The foodborne illness outbreaks linked to fresh produce such as lettuce, green leafy based salads, raspberries, strawberries, fruit salads, etc continues to occur. Food-borne outbreaks related to fruit and vegetables are mainly caused via contamination by pre-harvest manipulations (polluted irrigation water) or by post-harvest contamination (infected food handler, contaminated equipment, and used process water). To address the food safety issues linked to fresh produce the focus should be on the implementing on-farm controls (water sanitation, certified manures, biosolids and fertilizers, etc.). Although prevention is the ideal control strategy, there are major challenges given the open nature of fresh produce production. Consequently, there remains a reliance on the post-harvest control measures and ensuring proper worker hygiene. NoV diagnostics have become incorporated more routinely into public health outbreak investigations, resulting in markedly increased recognition of NoV illnesses and outbreaks. In addition, contemporary molecular detection methods, as it is for example real-time RT-PCR enable not only the qualitative but also the quantitative detection, which opens the possibility of quantitative microbial risk assessment important for public health or food regulations. Still further work is needed in detection methods to be able to differentiate between infectious and noninfectious viruses, to resolve the threat of NoV prevalence in food to public safety.

Acknowledgements The support by Ministry of Education, Science and Technological Development (project III 46009) is gratefully acknowledged.

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