REVIEW ARTICLE

Virus hazards from food, water and other contaminated environments

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Abstract

Numerous viruses of human or animal origin can spread in the environment and infect people via water and food, mostly through ingestion and occasionally through skin contact. These viruses are released into the environment by various routes including water run-offs and aerosols. Furthermore, zoonotic viruses may infect humans exposed to contaminated surface waters. Foodstuffs of animal origin can be contaminated, and their consumption may cause human infection if the viruses are not inactivated during food processing. Molecular epidemiology and surveillance of environmental samples are necessary to elucidate the public health hazards associated with exposure to environmental viruses. Whereas monitoring of viral nucleic acids by PCR methods is relatively straightforward and well documented, detection of infectious virus particles is technically more demanding and not always possible (e.g. human norovirus or hepatitis E virus). The human pathogenic viruses that are most relevant in this context are nonenveloped and belong to the families of the *Caliciviridae*, *Adenoviridae*, *Hepeviridae*, *Picornaviridae* and *Reoviridae*. Sampling methods and strategies, first-choice detection methods and evaluation criteria are reviewed.

Introduction: main food and environmental virus hazards

Food and environmental virology mostly studies viruses that can be transmitted through water, sewage, soil, air, fomites (objects capable of transmitting microbial pathogens) or food (Bidawid *et al.*, 2009). Most such viruses are enteric viruses transmitted via the faecal–oral route. Infected humans can excrete large amounts of human pathogenic viruses; animal and plant material as well as other excreta and secreta can also carry high viral loads (Breitbart *et al.*, 2003; Zhang *et al.*, 2006; de Roda Husman & Bartram, 2008). Viruses transmitted via the faecal–oral route are generally nonenveloped and thus very stable in the environment (Rzeżutka & Cook, 2004) and include major aetiological agents, some of which are thought to be emerging zoonotic pathogens. These viruses cannot always be effectively eliminated by current meth-

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ods of sewage treatment (Vantarakis & Papapetropoulou, 1999; Thompson *et al.*, 2003; Van Heerden *et al.*, 2003; Van den Berg *et al.*, 2005) and consequently cause viral contamination of the environment from treated as well as untreated wastewater. Other examples of indirect routes are run-off from manure used in agriculture. There is also direct faecal contamination of the environment from humans and animals, for example by bathers or by defecation of free-range or wild animals onto soil or surface waters. The resulting viral contamination of sea and coastal water, rivers and other surface waters, groundwaters, and irrigated vegetables and fruit is associated with subsequent risks of reintroduction of the viral pathogens into human and animal populations (Yates *et al.*, 1985; Metcalf *et al.*, 1995; Muscillo *et al.*, 1997; Koopmans *et al.*, 2002; La Rosa *et al.*, 2007). Human exposure to even low levels of these pathogenic viruses in the environment, such as norovirus (NoV), can cause infection and disease (Lindesmith *et al.*, 2003; Teunis *et al.*, 2008). Individuals with an impaired immune system, including children, the elderly, pregnant women and people with HIV/AIDS, are more susceptible to such infections, and the disease outcome may be more severe. This is the case, for example, for rotavirus (RV), which is a more serious problem for young children in developing than in developed countries (Havelaar & Melse, 2003). Genetic susceptibility may also play a role in the susceptibility to infection, as in the case of NoV and the ABO histo-blood group receptor genotype (Hutson *et al.*, 2002).

Environmentally transmitted viruses include major aetiological agents of mild diseases such as gastroenteritis as well as agents of more severe diseases such as meningitis and hepatitis. Most of these viruses belong to the families *Adenoviridae*, *Caliciviridae*, *Hepeviridae Picornaviridae* and *Reoviridae* (Dubois *et al.*, 1997; Muscillo *et al.*, 2001; Lodder & de Roda Husman, 2005). The major enteric virus families include one or several types and variants of virus; the different groups may differ as concerns persistence, pathogenicity and infectivity. Some of these viruses, such as hepatitis E virus (HEV) (the sole member of the *Hepeviridae*), are thought to be zoonotic pathogens. New human pathogenic viruses that may also be transmitted via the environment emerge frequently (McKinney *et al.*, 2006). Enteric viruses are predominantly transmitted via the faecal–oral route and are present in wastewater; therefore, such water is a potential source of infection if not treated or used appropriately (Gantzer *et al.*, 1998; Baggi *et al.*, 2001; Asano & Cotruvo, 2004). These agents are adapted to the hostile environment of the gut and in most cases, can persist for a very long time in water, soil or food matrices (Raphael *et al.*, 1985; Richards, 2001; Le Cann *et al.*, 2004; Van Zyl *et al.*, 2006; Espinosa *et al.*, 2008; Hansman *et al.*, 2008).

Caliciviruses: major viral causes of gastroenteritis

NoV and sapovirus (SaV) are the most important human agents of diarrhoea worldwide (Patel *et al.*, 2009). NoVs are the leading cause of food-borne outbreaks of acute gastroenteritis and the most common cause of sporadic infectious gastroenteritis affecting people of all age group (Green, 2007; Patel *et al.*, 2008, 2009). SaVs are mainly associated with sporadic acute gastroenteritis in young children (Hansman *et al.*, 2007a; Khamrin *et al.*, 2007; Monica *et al.*, 2007) and are less commonly involved than NoV in epidemic gastroenteritis (Green, 2007), although some outbreaks have been described (Johansson *et al.*, 2005; Hansman *et al.*, 2007b, c). The burden of calicivirus (including NoV) has been clearly documented in numerous geographical areas worldwide (Hall *et al.*, 2005; EFSA, 2009; Scallan *et al.*, 2011).

NoVs and SaVs are icosaedric nonenveloped viruses with an ssRNA (+) genome of between 7.3 and 8.3 kb. They are both classified within the family of the *Caliciviridae*, as the genera *Norovirus* and *Sapovirus*, each subdivided into five genogroups (Karst *et al.*, 2003) and several serotypes. Three genogroups (GI, GII and GIV) containing more than 20 genotypes of NoV are known to infect human beings, and the intra-genotype nucleotide diversity can be as high as 15% (Zheng *et al.*, 2006). Most human infections are caused by GI and GII, whereas GIII affects swine. In the case of SaV, at least four distinct genogroups containing a number of genotypes and variants can infect humans (Farkas *et al.*, 2004). Thus, NoV and SaV detection can be difficult owing to the large number of genogroups and genotypes; furthermore, currently available detection methods are not sufficiently powerful, and indeed, the prevalence of uncommon NoV variants is probably underestimated (La Rosa *et al.*, 2008).

NoV is believed to be transmitted mainly by person-to-person contact or by aerosols after projectile vomiting (Marks *et al.*, 2000, 2003). Consumption of food or water contaminated by faecal matter or vomitus (Marks *et al.*, 2000, 2003; Rutjes *et al.*, 2006), and exposure to contaminated surfaces or fomites, are also the sources of infection (Wu *et al.*, 2005; D'Souza *et al.*, 2006). The ease with which NoV is transmitted and spread is mainly because of its infectious dose being low – fewer than 10 virus particles are required for the infection (Teunis *et al.*, 2008) – high resistance to disinfection (Duizer *et al.*, 2004a; Jimenez & Chiang, 2006; Whitehead & McCue, 2009) and possible long-term stability and persistence in the environment (Wu *et al.*, 2005; D'Souza *et al.*, 2006).

The most common cause of NoV food-borne outbreaks is the consumption of shellfish, fresh produce and ready-

to-eat food contaminated by infected, but possibly asymptomatic, food handlers (Daniels *et al.*, 2000; Cannon & Vinjé, 2008; Lamhoujeb *et al.*, 2008). The long-term stability and persistence of NoV on contaminated surfaces used in food preparation areas also make a substantial contribution to disease transmission (Cheesbrough *et al.*, 2000; Evans *et al.*, 2002; Kuusi *et al.*, 2002; Taku *et al.*, 2002; Clay *et al.*, 2006; D'Souza *et al.*, 2006; Mattison *et al.*, 2007; Lamhoujeb *et al.*, 2008, 2009). Moreover, NoV is resistant to many industrial food preservation methods and can survive chilling, freezing, acidification, reduced water activity and modified atmosphere packaging (Baert *et al.*, 2009).

NoV has also been documented as a water-borne pathogen, and numerous outbreaks have originated from sewage-polluted drinking water (Nygård *et al.*, 2003; Maunula *et al.*, 2005; Hewitt *et al.*, 2007; ter Waarbeek *et al.*, 2010) and recreational water (Hoebe *et al.*, 2004; Maunula *et al.*, 2004; Sartorius *et al.*, 2007). This may be a consequence of its suspected resistance to wastewater treatment (Lodder & de Roda Husman, 2005; Van den Berg *et al.*, 2005; da Silva *et al.*, 2007; La Rosa *et al.*, 2009; Nordgren *et al.*, 2009; Skraber *et al.*, 2009) in addition to its survival ability in aquatic settings (Kadoi & Kadoi, 2001; Allwood *et al.*, 2003; Bae & Schwab, 2008). Additionally, shellfish grown and harvested in wastewater-polluted water can concentrate NoV, which may be inadequately eliminated by standard depuration procedures (Muniain-Mujika *et al.*, 2002): the consequence is outbreaks of gastroenteritis after consumption of shellfish (Le Guyader *et al.*, 2006a; Le Guyader *et al.*, 2008; Webby *et al.*, 2007).

Hepatitis A virus: prevalent in developing countries

Hepatitis A virus (HAV) is an icosaedric nonenveloped virus species with an ssRNA (+) genome of approximately 7.5 kb and is classified in the family of the *Picornaviridae*, genus *Hepatovirus*. Approximately 1.4 million people worldwide become infected with HAV annually (Issa & Mourad, 2001). The incidence of infection varies between regions of the world, with the highest rate in developing countries where sewage treatment and hygiene practices can be poor. Conversely, the number of reported cases of HAV infection has declined substantially in countries with effective programmes of immunization with a licensed vaccine. For example, in the USA, the number of cases has been reduced by 92% to an infection rate as low as one case per 100 000 persons per year (Daniels *et al.*, 2009); similar situations now also apply to other countries including Canada, Australia, Japan and New Zealand (Jacobsen & Koopman, 2004).

HAV can, via sewage discharge, contaminate soil, food crops and natural watercourses (Bosch, 1998; Cook & Rzeżutka, 2006). Consequently, food (Pebody *et al.*, 1998; Hutin *et al.*, 1999; Lees, 2000; Dentinger *et al.*, 2001; Nygård *et al.*, 2001; Greening, 2006) and drinking water (Divizia *et al.*, 2004; Tallon *et al.*, 2008) are considered major vehicles of HAV transmission to humans. In an epidemiological investigation, 6.5% of acute cases of hepatitis A were identified as food- or water-borne; however, this figure is probably an underestimate, because a considerable proportion of cases (~68%) remain uncharacterized (Daniels *et al.*, 2009).

HAV is able to survive in several environments, notably in water, food and soil (Rzeżutka & Cook, 2004). Water is considered to be the most important source of infectious virus because it can survive for long periods in this environment. For example, the virus can survive for up to 60 days in tap water (Enriquez *et al.*, 1995), over 6 weeks in river water (Springthorpe *et al.*, 1993), over 8 weeks in groundwater (Sobsey *et al.*, 1989) and even up to 30 weeks in sea water (Crance *et al.*, 1998). HAV is also able to survive in various types of soil and remains infectious after 12 weeks (Sobsey *et al.*, 1989).

Adenoviruses: some serotypes cause gastroenteritis in children

Adenovirus (AdV) is an icosaedric nonenveloped virus with a dsDNA genome 28–45 kb long. They are classified as members of the *Adenoviridae* family, genus *Mastadenovirus,* which includes 20 known species: three bovine, five human and three porcine. Fifty-one serotypes of human AdV (hAdV) in six subgroups (A-F) have been described (Wold & Horwitz, 2007). hAdV serotypes 40/41, included in Group F, are the major causes of gastroenteritis in young children and are readily spread by the faecal–oral route. They are sensitive to chemical disinfection but are more resistant to the effects of UV light than other enteric viruses (Thurston-Enriquez *et al.*, 2003). hAdV is shed from the gut on a long-term basis regardless of the site of initial infection, although the mechanism has not been fully clarified in humans (Calcedo *et al.*, 2009; Echavarria, 2009; Roy *et al.*, 2009). A limited number of probable water-borne outbreaks of hAdV have been reported, particularly in association with conjunctivitis and swimming pools (Martone *et al.*, 1980). Chlorination failures are often cited as a major factor in outbreaks.

Enteroviruses: common viral causes of gastroenteritis

The genus *Enterovirus* (EV) comprises spherical nonenveloped viruses, with an ssRNA (+) genome of 7.2–8.5 kb,

in the family of the *Picornaviridae.* Four species have been distinguished (A, B, C and D) within which the serotypes are known by their traditional names: human EV (hEV) A includes some coxsackievirus A strains; hEV B contains coxsackievirus A9, coxsackievirus B1-6 and most of the echoviruses; and hEV C contains polioviruses 1–3 and some coxsackievirus A strains. The more recently identified hEVs have been given individual numbers, from EV68, and are classified amongst all four species (Stanway *et al.*, 2005).

These viruses may replicate in the respiratory tract and the gut and can be transmitted through aerosols and by the respiratory route or via the faecal–oral route. Many infections are asymptomatic, and as few as one in 100 may result in clinical illness. The wide range of diseases includes classical poliomyelitis, aseptic meningitis, cardiac disease, hand, foot-and-mouth disease, conjunctivitis and rashes. A common clinical picture is self-limiting fever, malaise, muscle aches and headache; diarrhoea and vomiting are present only as a part of more generalized systemic illness. Clinical illness in temperate climates is more common in the summer months; all age groups are affected, and immunity to one serotype does not protect against infection with other serotypes (Moore *et al.*, 1984). The serotypes of echoviruses and coxsackieviruses then circulate and dominate within communities change over time, and there is molecular drift within serotypes (Savolainen *et al.*, 2001). hEVs can be found in all aquatic matrices reflecting their widespread occurrence in populations (Sellwood *et al.*, 1981; Hovi *et al.*, 1996; Sedmark *et al.*, 2003). However, transmission of hEV infection through an aquatic route has been difficult to confirm as the number of asymptomatic infections is so large and the transmission by close personal contact so common.

HEV: zoonotic transmission as an emerging problem

HEV is a small, spherical and nonenveloped ssRNA (+) virus of approximately 7.2 kb. It is classified within the family of the *Hepeviridae*, genus *Hepevirus.* HEV is a major cause of acute human hepatitis in regions with inadequate water supplies and poor sanitary conditions (Purcell & Emerson, 2001; Guthmann *et al.*, 2006), and there is an increasing evidence of locally acquired HEV infections in industrialized countries (Zanetti *et al.*, 1999; Widdowson *et al.*, 2003; Buti *et al.*, 2004; Mansuy *et al.*, 2004; Ijaz *et al.*, 2005; Waar *et al.*, 2005). HEV sequences worldwide can be classified into four major genotypes (1–4) (Lu *et al.*, 2006). The relatively conserved genotypes 1 and 2 circulate primarily in humans causing the majority of HEV infections including all epidemics in Asia and Africa countries and also in Mexico. By contrast, for genotypes 3 and 4, only isolated cases of human infection have been described and only in more industrialized countries including the USA, Japan, China and countries in Europe. Although four genotypes of HEV exist, there only seems to be one serotype present (Zhou *et al.*, 2003; Herremans *et al.*, 2007; Mushahwar, 2008). Previously, HEV infections in industrialized countries were believed to be travel related, but recently an increasing number of indigenous HEV cases have been reported (Zanetti *et al.*, 1999; Widdowson *et al.*, 2003; Mansuy *et al.*, 2004; Lu *et al.*, 2006; Borgen *et al.*, 2008). Serological studies have reported the presence of HEV antibodies in a variety of animal species, notably cows, cats, dogs and rodents. However, HEV RNA has not been detected in these species, and the validity of the assays used is seldom well established owing to the lack of positive reference samples: consequently, these results must be interpreted with caution (Bouwknegt *et al.*, 2007). The presence of HEV has been reported in food, water and animals including pigs (Rutjes *et al.*, 2009a). In several animal species, HEV genotype 3 and 4 sequences have been detected, with pigs being the animal most frequently involved in countries formerly designated as nonendemic for HEV. HEV RNA has also been detected in wild boar in several countries (Takahashi *et al.*, 2004; de Deus *et al.*, 2008; Martelli *et al.*, 2008; Adlhoch *et al.*, 2009), in Sika deer (Tei *et al.*, 2003), in roe deer (Reuter *et al.*, 2009), in red deer (Rutjes *et al.*, 2010) and in mongoose (Nakamura *et al.*, 2006). Furthermore, a human HEV genotype 1 strain was detected in workhorses in Egypt (Saad *et al.*, 2007).

The non-travel-related HEV infections in industrialized countries may be of zoonotic origin. Sequences of the swine HEV genotype 3 and 4 strains closely related to human strains have been isolated in many countries worldwide (van der Poel *et al.*, 2001; Huang *et al.*, 2002; Clemente-Casares *et al.*, 2003; Lu *et al.*, 2006; Rutjes *et al.*, 2007; Reuter *et al.*, 2009), suggesting that pigs may be the reservoir of the indigenous infections in these countries. More direct evidence of zoonotic food-borne transmission of genotype 3 was obtained when four cases of hepatitis E could be linked directly to eating raw deer meat: identical HEV strains were found in the deer meat consumed and the patients (Tei *et al.*, 2003; Li *et al.*, 2005).

RV, astrovirus and other agents of gastroenteritis: water-borne pathogens affecting mostly children

Viruses of the genus *Rotavirus* are icosahedral nonenveloped nonturreted virions with a triple capsid structure and a segmented dsRNA genome of approximately

18.5 kb. They are classified in the *Reoviridae* family, and there are five major groups (A-E) (Estes & Kapikian, 2007). Group A RV (GARV) is associated with a large majority of human RV infections and represents the major cause of child mortality because of diarrhoea worldwide (Parashar *et al.*, 2006; Sánchez-Padilla *et al.*, 2009). GARV is also widespread in wild and domestic animal species, and it has been suggested that zoonotic transmission plays a substantial role in the introduction of novel strains into the human population (Cook *et al.*, 2004; Bányai *et al.*, 2009). Within GARV, at least 19 G- and 27 P-types can be distinguished on the basis of sequence diversity of the genes encoding the two outer capsid proteins (VP7 and VP4) (Matthijnssens *et al.*, 2008; Van Doorn *et al.*, 2009). The recent introduction of vaccines for human use may lead to the emergence of novel RV genotypes or the re-emergence of older strains, particularly from animal reservoirs, and such strains could displace those currently predominating (Cook *et al.*, 2004; Iturriza-Gómara *et al.*, 2004; Kang *et al.*, 2005; Steyer *et al.*, 2008).

RV persist similarly in polluted and nonpolluted fresh water (Hurst & Gerba, 1980) and even when subjected to light exposure, which can seriously affect the stability and viability of other enteric RNA viruses, for example astrovirus (Fujioka & Yoneyama, 2002; Lytle & Sagripanti, 2005). Inactivation of virus infectivity in different types of water has been consistently found to correlate with higher temperatures (John & Rose, 2005).

The genus *Mamastrovirus* (AstV) includes spherical nonenveloped viruses with an ssRNA (+) genome of between 6.8 and 7 kb. They are members of the *Astroviridae* family. There are six species affecting bovines, felines, mink, ovines, porcines and humans (HAstV). HAstV is a common cause of gastroenteritis in children and also in the elderly and immunocompromised individuals (Herrmann *et al.*, 1991; Guix *et al.*, 2002; Mendez & Arias, 2007). Eight genotypes of HAstVs have been described to date and are classified into genogroup A (HAstV-1 to 5 and HAstV-8) and genogroup B (HAstV-6 and 7) (Gabbay *et al.*, 2007). HAstVs have been occasionally found associated with gastroenteritis outbreaks involving possible water-borne or food-borne transmission (Leclerc *et al.*, 2002; Maunula *et al.*, 2004; Smith *et al.*, 2006; Domínguez *et al.*, 2008; Scarcella *et al.*, 2009), and their presence in seafood has been discussed and may depend on rainfall conditions (Le Cann *et al.*, 2004; Riou *et al.*, 2007). Recently, the possible zoonotic transmission of astroviruses from cows was proposed (Kapoor *et al.*, 2009).

Other viruses, such as kobuvirus, aichivirus, picobirnavirus and torovirus, are also found in the environment, but further epidemiological studies and wide-ranging investigations of diagnostic spectra are needed to document their distribution in the environment and impact on food safety and health.

Shedding of pathogenic viruses into the environment

Zoonotic transmission

One of the main routes of transmission of viruses to humans is zoonotic, associated with the consumption of contaminated products of animal origin, or during food manipulation by infected handlers. The other most frequent cause of virus-contaminated foods is contact with faecal-polluted waters (Fig. 1). Inadequately treated drinking water, consumption of crops contaminated after being irrigated with wastewater or fertilized with sewage and ingestion of shellfish grown in polluted waters are, therefore, common causes of food-borne viral infection of people (Bosch, 1998). Several factors affect the contamination of shellfish, vegetables, berries, fruits and herbs. Climatic variables such as season, tidal cycles, rainfall and flooding have all been implicated in viral contamination of the environment (Le Guyader *et al.*, 2000; Griffin *et al.*, 2003; Suffredini *et al.*, 2008; Guillois-Bécel *et al.*, 2009). Likewise, good livestock, agriculture and manufacturing practices are absolutely necessary to minimize the risk of viral contamination of food. Inappropriate irrigation practices, wastewater treatment and reuse, sewage overflows, and wastewater releases from polluted sources are the direct causes of viral environmental contamination and food-borne outbreaks (Le Guyader *et al.*, 2000; Griffin *et al.*, 2003; Jiménez-Clavero *et al.*, 2003; Choi *et al.*, 2004; Suffredini *et al.*, 2008; Guillois-Bécel *et al.*, 2009) (Fig. 1). Shellfish grown in areas close to intensive farming, or waste treatment plants, present a high risk of enteric virus carriage (Le Guyader *et al.*, 2000; Ley *et al.*, 2002).

There has been increasing concern about the effects on human and animal health of pathogenic viruses in animal manure. In recent years, outbreaks of food-borne diseases associated with the consumption of animal products have received much attention, leading to consumer concern about the safety of the food supply. The health risk associated with animal operations depends on diverse factors. The most important is related to the animal species being reared and the concentration of pathogenic microorganisms in animal manure. Some viruses survive both for long periods and despite treatment, and their ability to remain infectious in the environment until ingested by a human or animal host is an added concern. However, it has been difficult to determine the role of livestock in most water-borne virus outbreaks because both humans and various wildlife species can shed the

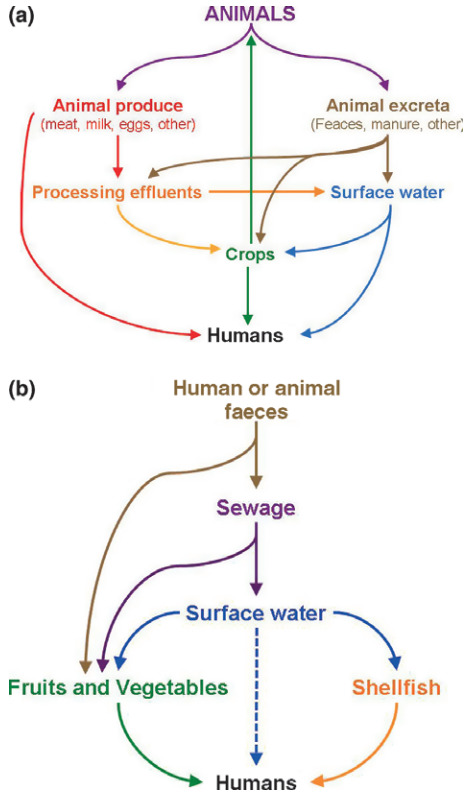

Fig. 1. Contamination routes for environmental virus hazards (a) of animal origin and (b) in foods. (a) Contamination routes of environmental virus hazards of animal origin. Zoonotic route of contamination from the original source (animal) to humans. (b) Environmental virus contamination of foods. Contamination from original source to humans using food and water as a route of transmission.

same viruses and thereby serve as sources of infection or contamination. EVs are shed in faeces and, consequently, are disseminated through contaminated soil and water; therefore, any other animal species grazing in the same pastures and/or drinking from the same water sources as infected livestock are likely to be exposed. Consequently, they may be contaminated by the same or closely related virus variants and therefore present a high risk of further disseminating the virus (Ley *et al.*, 2002; Jiménez-Clavero *et al.*, 2005).

Most pathogenic viruses emerging in human populations are of animal origin (Taylor *et al.*, 2001). There is a large spectrum of transmission modes for zoonotic viruses with domestic animal or wildlife reservoirs. They can be direct or indirect (Kruse *et al.*, 2004) and include transmission by contaminated food, water, air and soil (Fig. 1). Meat can be contaminated by excreta during processing, but may also have been contaminated earlier because of infection of the living animal. The risk of food-borne infection depends on the virus infection route, the level of contamination and the extent of inactivation during food processing. Livestock industries produce large amounts of residues that can cause substantial environmental problems. Indeed, accidental or deliberate spills, overuse of fertilizer and emissions of incorrectly, or incompletely, treated animal wastes are the major environmental risks (Jongbloed & Lenis, 1998; Jiménez-Clavero *et al.*, 2005). Cook *et al.* (2004) estimated that contamination of arable land with animal RV in spread animal waste used as fertilizer may be considerable, and similarly substantial contamination is plausible or even likely for other viruses shed in large numbers in animal excreta. As expected, detection of animal viruses in contaminated waters (groundwater, lakes, rivers, estuaries, runoffs and animal watering tanks from farms, etc.) is much more frequent in areas of intensive than less active farming (Jiménez-Clavero *et al.*, 2005). The modes and the levels of environmental contamination with viruses differ for the different types of viruses and animal species.

Occupational exposure

The working environment and procedures can be sources of viral dissemination. However, the difficulties associated with evidencing cases and relating them to possible exposure make it very complex to assess the risk of infection. Health care facilities are the most extensively studied occupational settings. In such facilities, blood-borne viruses, including human immunodeficiency virus (HIV), hepatitis B virus and hepatitis C virus, can be transmitted mainly by accidents with infected needles or sharp objects (Davanzo *et al.*, 2008). Air-borne viruses such as the influenza virus, respiratory syncytial virus, AdV, rhinovirus, coronavirus, measles, rubella, mumps viruses and parvovirus B19 are also easily spread (Aitken & Jeffries, 2001). Viral agents transmitted via the faecal–oral route, such as RV, hAdV 40 and 41 and NoV, are frequently associated with nosocomial and health care–related infections spread by contamination of air, hands and surfaces (Lopman *et al.*, 2004). Workers involved in sewage treatment and reuse for agricultural and industrial purposes can be exposed to enteric viruses. Seroepidemiological surveys show that workers in wastewater treatment plants (Clark *et al.*, 1985; Heng *et al.*, 1994; De Serres & Laliberte, 1997; Weldon *et al.*, 2000; Divizia *et al.*, 2008) and in spray irrigation activities (Katzenelson *et al.*, 1976; WHO, 2006) are at higher risk than the general population, in terms of enteric and hepatic infections. Veterinary and zootechnical jobs can also expose workers to zoonotic viruses through contact with manure and inhalation of aerosols generated by activities such as washing and cleaning (Cook *et al.*, 2004). Serological studies indicate that workers in the intensive animal husbandry sector

may be exposed to zoonotic viruses, notably H1 swine influenza virus (Olsen *et al.*, 2002). Workers in these fields of activity may therefore possibly have a role in species-jumping from animal to human populations (Baker & Gray, 2009).

Environmental matrices containing human pathogenic viruses

Human pathogenic viruses are excreted and secreted by humans into their environment through faeces, urine, saliva, sweat and tears (de Roda Husman & Bartram, 2008). The principal matrices, which can be contaminated with human viruses and represent potential sources of infection, are water, sewage, sludge, manure, air, hard surfaces, crops such as fruit and vegetables, shellfish and animal products. The range of complexity in the structure and electrostatic charge of these matrices and of the viruses is such that their interactions are extremely diverse, with corresponding differences as concerns virus inactivation and removal. In general, virus survival is influenced by parameters such as moisture, temperature, association with solids and exposure to UV.

Water and sewage

Surface waters can readily become contaminated with viruses. In the EU, guidelines for sewage discharge (Directive 91/271/EEC) concerning urban wastewater treatment were adopted in 1991 to protect the water environment from the adverse effects of discharges of urban wastewater and from certain industrial discharges. This is an important standard as it not only regulates the conditions of discharge according to the inhabitant equivalent but also stipulates requirements for corresponding collection and treatment facilities. However, the reduction values required for discharges from urban wastewater treatment plants are evaluated according to chemical and biochemical parameters, including biochemical oxygen demand, chemical oxygen demand, total suspended solids and total phosphorus and nitrogen; they do not address highly stable pathogens, like viruses. In sludge (solids remaining after wastewater treatment), viruses may be present and constitute a potential hazard.

Drinking water is abstracted from surface water in many countries and treated by sedimentation, filtration and/or disinfection, which, if done effectively, can produce a virus-free end product, although this may be dependent on the quality of the source water (Rutjes *et al.*, 2009b; Teunis *et al.*, 2009; Lodder *et al.*, 2010). The European Directive concerning quality of water intended for human consumption is Directive 98/83/EC. Monitoring should provide information about the organoleptic and microbiological quality of the water supplied as well as information concerning the effectiveness of drinking water treatment (particularly disinfection). This directive includes microbiological limits based on bacterial standards, but viruses are not considered in any of the current directives.

Manure

Manure can be defined as urine and faecal material produced by animals housed in artificial environments, such as farms and zoos. It may also contain straw bedding, is often stored for long periods and is used as a fertilizer on agricultural land. In general, enteric viruses including caliciviruses, HAV and HEV are considered to be stable in faeces (Rzeżutka & Cook, 2004). After dispersion of viruses into the environment, the inactivation rates differ substantially between types of virus and inactivation is faster in liquid manure (mixture of urine and water with less bedding material) than in solid manure. Enteric viruses can survive for a very long time (even years) at temperatures below 5 °C and especially in the absence of UV light. There is good evidence that 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. Viruses like HAV, NoV and HEV can resist complete inactivation in the environment for a very long time (Pesaro *et al.*, 1995).

Air and hard surfaces

The importance of air-borne spreading of enteric viruses is not well defined, unlike water-borne or food-borne spreading. This is largely owing to the difficulties in identifying this transmission route for single cases or outbreaks. The air-borne transmission of viruses is dependent on the likelihood of material containing viruses to form aerosols and on the survival of viruses in the air. Enteric viruses can be aerosolized by, for example, violent vomiting (as associated with NoV) (Marks *et al.*, 2000), toilet flushing (Barker & Jones, 2005), spray irrigation (Petterson *et al.*, 2001) and various processes at wastewater treatment plants (Carducci *et al.*, 1995, 2000). Some enteric viruses can cause infection by ocular contact or by inhalation and virus catchment by mucus and subsequent swallowing. Nevertheless, the most common mechanism of dissemination is the deposition of aerosol particles on surfaces, particularly food, vegetation and clothes. Surfaces such as door handles, banisters for staircases, flushing handles on toilets, toys, telephones, drinking cups and fabrics have all been implicated in the transmission of enteric viruses (Barker & Jones, 2005; Gallimore *et al.*, 2008). Faecal material or vomit may contaminate these

surfaces, and the viruses contained may then be ingested following direct contact or transfer from hands (Boone & Gerba, 2007). The characteristics of the material and the virus contribute to determining the survival rate (Abad *et al.*, 1994; Vasickova *et al.*, 2010). The detection of virus on a large variety of surfaces, like tables, door knobs, walls, toilets seats, thermometers, toys, cotton cloth, carpets, bed covers, gloves, drinking glasses, paper (Boone & Gerba, 2007) has helped to explain the routes of transmission of NoV (Wu *et al.*, 2005; Boxman *et al.*, 2009a), RV (Ansari *et al.*, 1988) and rhinovirus (Ansari *et al.*, 1991) in localized cases and outbreaks.

Food

Food and food environments are a major source of viral transmission to humans (Koopmans *et al.*, 2002; Koopmans & Duizer, 2004). Food-borne viral outbreaks are reported worldwide every year and are associated with a wide variety of foods (e.g. Verhoef *et al.*, 2008; Kuo *et al.*, 2009; Robesyn *et al.*, 2009; Vivancos *et al.*, 2009). The viruses most frequently involved in food-borne infections are NoV and HAV, but other viruses, particularly human RV, hEV, HEV and AstV, are also transmitted by food. For NoV and HAV, person-to-person spread is the most common transmission route. Secondary spread of these viruses after introduction by, for example, food-borne contamination is common and often results in larger, prolonged outbreaks (WHO and FAO, 2008). Estimates of the proportion of viral illnesses attributed to food are in the range of around 5% for HAV to 12–47% for NoV. However, all currently available estimates of food-borne illnesses make assumptions and use extrapolations from different data sources (Scallan *et al.*, 2011). Nevertheless, all essentially conclude that viruses are an important cause of food-borne illness (WHO and FAO, 2008; Scallan *et al.*, 2011). The incidence of outbreaks of food-borne viral disease has increased considerably 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 (Rodríguez-Lázaro *et al.*, 2009).

Food products can be contaminated at various points along the food supply chain. This can be because of poor practice in primary production and/or misuse of natural and environmental resources (Appleton, 2000), e.g. the irrigation of vegetables with polluted water – including contamination through roots owing to drop irrigation (Urbanucci *et al.*, 2009) – contact with human faeces or faecally soiled materials and poor hygiene practice by food handlers during the harvest of fresh produce. Furthermore, contamination may arise by inappropriate practices during processing or at the point of sale/consumption (Boxman *et al.*, 2009b). Also, there may be cross-contamination from polluted working instruments or surfaces, which have been contaminated previously by infected food handlers or contaminated food items (D'Souza *et al.*, 2006; Boxman *et al.*, 2009b; Dreyfuss, 2009). In addition, shellfish, fresh produce or ready-to-eat foods may be contaminated with human excreta, either directly or indirectly, and viral food-borne outbreaks may also originate from zoonotic viruses intrinsically present in food consumed. This has been demonstrated for HEV in raw meat and liver from wild boar and deer (Matsuda *et al.*, 2003; Tei *et al.*, 2003; Takahashi *et al.*, 2004). Moreover, the potential for food-borne transmission is a concern with every new emerging infection, even for viruses that are primarily respiratory, for example, the highly pathogenic avian influenza virus. Indeed, infectious avian influenza virus has been cultured from frozen exported meat, raising the issue of possible dissemination of such viruses via the food chain (WHO and FAO, 2008).

Foods commonly implicated in outbreaks are those that are minimally processed, such as shellfish or fresh produce, although ready-to-eat foods that have been contaminated by an infected food handler are also involved. Traditionally, bivalve mollusc shellfish such as oysters, mussels, clams and cockles have been considered as a principal source of food-borne virus that may subsequently be disseminated (Pintó *et al.*, 2009). Filter-feeding shellfish can concentrate viruses from polluted water: the filtration can lead to concentrations in shellfish 100–1000 times higher than that in the surrounding water (Carter, 2005). In addition, specific binding of NoV to the shellfish epithelia has been observed, and this may impede the release of virus during shellfish depuration (Le Guyader *et al.*, 2006b; Maalouf *et al.*, 2011). Fresh produce has high water content – absorbed from groundwater during growth – and may be eaten raw and without peeling, both procedures that may remove external contamination. Viruses can survive on their surface once harvested (Carter, 2005) and can remain infectious for several days or weeks and even during commercial and household storage for periods of up to 5 weeks (Bosch *et al.*, 2006). However, any food that has been manipulated by food-handlers and is not (or insufficiently) subjected to subsequent preservation and/or cooking is susceptible to be a source of transmission of enteric viruses.

Virus survival in foods can be affected by diverse factors. Kott & Fishelson (1974) found that poliovirus persisted longer on tomato and lettuce plants in phosphate-buffered saline than in oxidation pond effluent, possibly due to microbial activity in such effluents. Also, natural irradiation in combination with natural antiviral substances generally present in fruit may greatly reduce

virus infectivity (Konowalchuk & Speirs, 1978). However, natural or added constitutes in food such as fat, salt and sucrose may protect viruses against inactivation by heating or high hydrostatic pressure (Kovač *et al.*, 2010). Conversely, components like acids and various components of fruit juices may enhance the rate of viral inactivation (Kovač *et al.*, 2010).

Sampling strategies

Surveillance of food and environmental virus hazards

For successful public health intervention regarding food and environmental virus hazards, the early and accurate identification of infectious viral agents is of primary importance. The ability to identify quickly the causative viral pathogen of an emerging viral epidemic markedly increases the chances of success of any countermeasures for containment, prevention and control of the possible disease. Surveillance of environmental viruses can underpin the detection of both cases and outbreaks by identifying an increase in frequency of disease above its background incidence (Centers for Disease Control and Prevention, 2001) and by estimating disease impact. In addition, surveillance can help generate hypotheses and stimulate research, evaluating prevention and control measures and facilitating planning.

Many countries and international organizations, notably the World Health Organization (WHO) and the European Centre for Disease Prevention and Control (ECDC), and international research projects have devoted considerable energy to developing integrated surveillance networks; these networks are for tracking environmental viruses including food- and water-borne viral pathogens such as NoV, RV and EV and for providing information about the viruses' genetic structure and geographical distribution and about the host populations and environmental matrix involved. Recent advances in molecular biology, including DNA chip technology and whole-genome sequencing technologies, continuously improve diagnostic power to detect and characterize a wide range of pathogens and their variants. Public health surveillance systems for outbreak detection can establish the relative value of different approaches for the detection of outbreaks at the earliest stages and provide the information needed to improve their efficacy. However, substantial costs can be incurred in developing, enhancing and managing these surveillance systems and investigating false alarms (Wagner *et al.*, 2001). Furthermore, the overall economic benefits of surveillance systems for early detection and response to outbreaks have not been clearly established.

Sampling methods

A rational sampling plan is essential for the analysis of human pathogenic viruses, which may be present in small quantities and distributed heterogeneously in matrices; the plan should be established on a risk-based approach (Andrews & Hammack, 2003; Food Standard Agency, 2004a, b). Consequently, a sample or subsamples must represent the original matrix (e.g. water and food), and the sampling process (including the storage and transportation) must not alter the condition of the sample and thus not affect the subsequent analysis (Food Standard Agency, 2004a, b). Other aspects that also must be considered when developing a sampling programme are the characteristics of the matrix to be analysed (nature: solid, semi-solid, viscous or liquid; type: food, water or environmental sample; composition: rich in fat, protein or plant contents such as tannins; and amount: scarce or abundant), and the subsequent analytical method to be used (cell culture, immunological or molecular). If, for example, a sampling plan for a pâté factory is required, a balanced approach needs to be based on the observation that a sample suitable for public health (for example 25 g of a pâté) might not be suitable for subsequent analysis using a molecular method because of the heterogeneous nature and composition of the matrix. Any inadequacy concerning one of the aspects will affect the validity of the final analytical result.

Various international bodies, such as the International Organisation for Standardisation (ISO), the European Committee for the Normalisation (CEN) and the European Food Safety Authority (EFSA), and national bodies, such as the U.S. Department of Health and Human Services (USDHHS), have defined principles and/or standards for the sampling of foods and water. For example, ISO has established a series of standards for sampling (ISO 5667 series, ISO 18593:2004; ISO 8066:2004; ISO 24276:2006; ISO 7002:1986; ISO 17604:2003); however, there is no specific mention of sampling for human enteric pathogenic viruses in any of these standards. The CEN/ISO *ad hoc* expert committee for viruses in food 'CEN/TC 275/WG6/TAG4' is currently working on the first international standard for a horizontal method for the detection of HAV and NoV in food. However, the sampling process is not included in this planned standard, and the committee has decided to examine the ISO 6887 series for suitability. Similarly, the FDA's Bacteriological Analytical Manual (BAM) includes a general protocol for '*food sampling and preparation of sample homogenate*' (Andrews & Hammack, 2003), in which the scientific basis for sampling only uses previously published bacteriological criteria (ICMSF, 1986, 2002),

despite the BAM having defined a specific protocol for the detection and quantification of HAV (Goswami, 2001).

A large number of studies are related to viral food- and water-borne outbreaks, sporadic cases or studies using samples collected to determine the presence of different enteric viruses in food or the environment or to evaluate new methods for the detection of viruses in diverse matrices (Supporting Information, Tables S1 and S2). Several important lessons can be learnt from these studies. First, there is an evident lack of harmonization in the sample size, and therefore, a serious risk in the representativeness of the sampling strategies used. This is most important as most of those studies are related to viral diarrhoeal outbreaks: the consequences may include the true aetiological agent of the gastroenteritis not being found, or the infectious dose being under- or overestimated. In these studies, sizes of samples used were extremely diverse, ranging from 50 μL to 3000 L (i.e. an almost 10^8-fold difference) for water and from 1.5–200 g for food samples. Second, there is a lack of homogeneity in the selection of the animal tissues or part of the sample tested once the sample is collected. This also can affect the detection of human pathogenic viruses. For example, different shellfish tissues can be tested for human enteric viruses (i.e. the whole shellfish, the mantle, the gills, the stomach or the digestive diverticula). However, it has been demonstrated that the efficiency of recovery can differ substantially between types of sample and even that the virus may not be detectable in some (Wang *et al.*, 2008). In a study evaluating different tissues of naturally contaminated oysters to identify the most suitable for the detecting virus, the percentages of samples positive were different for the whole oyster (0.7%), mantle (2.2%), gills (14.7%), stomach (13.9%) and the digestive diverticula (13.2%), and the detection was not possible when the adductor muscles were tested (Wang *et al.*, 2008). Another important factor is the ambiguous use of individual or pooled samples for foodstuffs, especially in the case of shellfish. This affects directly both the representativeness and analytical sensitivity of the final results. For example, de Roda Husman *et al.* (2007) observed that pooling digestive glands of several oysters never resulted in a positive signal, whereas RT-PCR testing of the individual digestive glands of single oysters revealed the presence of virus RNA. This indicates that pooling can affect the final results negatively and even can produce false negative results owing to the simple mechanism of reducing the size of each individual sample used in the pool. This can be of great relevance to public health. Conversely, the use of individual samples can also affect the representativeness of the population studied. A balanced approach to difficult food matrices may therefore be to analyse a representative number of individual samples; however, this could greatly increase both the cost and the time required for the analyses and even may be unfeasible in the field. Two other important aspects also have to be considered: the period of time from the sampling to the start of the analysis in the laboratory and the conditions of storage of the sample during that period. These issues can be of particular importance if complex matrixes are analysed, as the stability of the virus may be compromised. However, they are usually not rigorously addressed during sampling, and most studies do not provide the relevant details. Even where this information is provided, the lack of uniformity is again evident. Samples are sometimes stored frozen (Loisy *et al.*, 2000; Schvoerer *et al.*, 2000, 2001; Donaldson *et al.*, 2002), refrigerated at 4 °C (Pina *et al.*, 2001; La Rosa *et al.*, 2007), at room temperature (Beuret *et al.*, 2002) or kept on ice (Noble & Fuhrman, 2001; Katayama *et al.*, 2008).

Sample representativeness

Representativeness expresses the degree to which sample data accurately and precisely reflect a characteristic or variable at a sampling point. Representativeness is a qualitative factor, which is largely dependent on the appropriate design of the sampling programme. The representativeness criterion is best satisfied by making certain that sampling locations are selected suitably and that a sufficient number of samples are collected. The sampling strategy must be unbiased, sufficient (i.e. it summarizes all relevant information about the parent population, which contained the sample, but ignoring any sample-specific information), efficient (i.e. the more the statistical values for various samples cluster around the true value and the lower the sampling error, the greater the efficiency) and consistent (the larger the sample, the closer the statistic should be to its true value) (Jarman, 1984).

Transport and storage

After sampling is completed, samples should to be transported to the laboratory facilities as soon as possible. For example, the AFNOR method XP T 90-451 '*Recherche des entérovirus*' in water (AFNOR, 1990) states that after *in situ* concentration by filtration, the sample cartridge should be removed and enclosed aseptically such that the filtration device must not be left completely dry; thereafter, samples should be transported to the laboratory within 24 h at a suitable temperature. On the other hand, the ISO method 19458 '*Water quality – Sampling for microbiological analysis*' (ISO, 2006), although not specific for mammalian virus, states that viruses should be transported and stored for a period of 24–72 h, at a tempera-

ture of 5 ± 3 °C. The guidelines '*Standard Methods for the examination of Water and Wastewater*' (Eaton *et al.*, 2005) states that samples cannot be held more than 2 h at temperatures of 25 °C or 48 h at 2–10 °C; samples have to be stored at −70 °C if not processed in this time frame. Dahling & Wright (1984) also indicate that samples stored at −70 °C are stable without virus loss for up to 4 days. Mocé i Llivina (2004) tested the stability of EV at −70 °C and demonstrated that they could infect cells after 11 months of storage at this temperature when adsorbed to cellulose ester membranes. In conclusion, transport and storage should be performed as quickly as possible, at a controlled temperature (5 ± 3 °C). In this temperature range, samples can be stored for up to 48 h. If this time cannot be respected, the samples should be frozen at −70 °C.

It is of utmost importance that laboratory personnel recognize that the safe and efficient transportation of any infectious substance is in the interest of public health generally. The packaging of infectious substances for transport must therefore be designed to minimize the risk of damage during transport. Sending or transporting infectious viruses should respect the '*Guidance on regulations for the Transport of Infectious Substances 2009–2010*' (WHO, 2008). Different forms of transportation (road, rail, sea and air) of infectious substances have different safety requirements and therefore their own international convention or code based on UN Model Regulations. As far as laboratory personnel are concerned, their responsibility lies in ensuring that the goods are packaged according to WHO regulations. Some countries have their own national regulations; when this is not the case, International Guidelines should be followed.

Safety in the laboratory

HAV and NoV are both classed as Hazard Group 2, with a vaccine currently being available for HAV. HEV is classed as Hazard Group 3 in some countries, and therefore, any intentional use of this virus in laboratories in those countries must be performed strictly in containment level 3 facilities (CL3). However, the handling of pathogenic viruses must conform with any specific national recommendations: for example, in the case of HEV, the classification differs between countries and various international bodies. Indeed, the WHO and USA recommendations for this organism is biosafety level (BSL) 2, the Spanish recommendation is generally BSL 3 but not with all BSL 3 precautions as there is no evidence of aerosol contamination, and the British recommendation is BSL 3. This should be borne in mind when sending a sample likely to contain a virus to another laboratory. Only laboratories with the available CL3 facilities should handle any package suspected of containing a CL3 microorganism. Guidance should be sought from a national body, which provides advice on best practice procedures for the safe handling and containment of Hazard Group 2, 3 and 4 organisms. Note that many national guidelines are based on EU or international guidelines. If no national regulatory body of this type exists in a country, international or European guidelines, such as the WHO Laboratory Biosafety Manual 2nd Ed. (WHO, 2003), should be followed.

Detection and identification of food and environmental virus hazards

Detection of viruses in food and environmental samples is challenging because of the large variety and complexity of samples, the possible heterogeneous distribution of a small number of viruses and the presence of components that may inhibit or interfere with virus detection (Goyal, 2006). A general flow chart for the analytical process (from sampling to final identification and characterization) for the detection of human enteric viruses is given in Fig. 2. It is necessary to separate and concentrate viruses from environmental materials before performing tests for detection (Sair *et al.*, 2002). As no standard procedure or systematic approach evaluating the adsorption of viruses onto different substrates has yet been developed, it is difficult to draw conclusions about the mechanisms involved in virus adsorption (Jin & Flury, 2002); consequently, establishing appropriate separation and concentration processes is even more demanding. Whatever the method used, the final concentrate should not be cytotoxic to cell cultures used in infectivity assays and

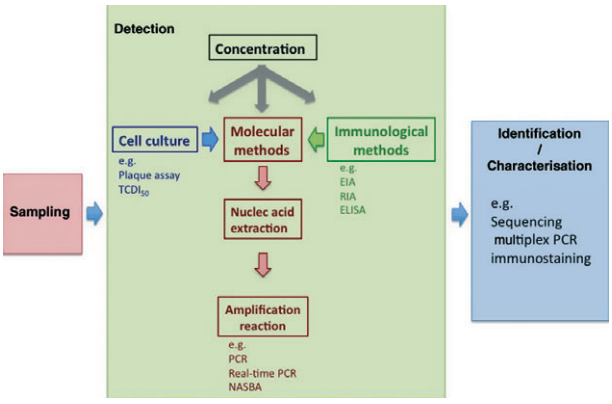

Fig. 2. Schematic diagram of the analytical process of detection and identification of environmental virus hazards. $TCDI_{50}$, median tissue culture infective dose assay; EIA, enzymatic immunoassay; RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay; NASBA, nucleic acid sequence–based amplification.

should be free of any inhibitors, which may be co-extracted or co-concentrated from environmental samples (Goyal, 2006). A variety of biological and chemical substances that are present in environmental matter or are used during sample processing have been found to act as inhibitors, including polysaccharides, haeme, phenol and cations (Atmar, 2006). Known PCR inhibitors in shellfish extracts include glycogen and acidic polysaccharides (Schwab *et al.*, 1998).

For virological analysis of aerosols, the key issue is sample collection and preparation for the different detection procedures (mainly based on cell culture and/or molecular techniques). The sample size is generally 1–3 m^3 of air. Various approaches have been developed, based on the property of air-borne particles of attaching to every surface with which they enter into contact (Verreault *et al.*, 2008). There are three different principles underlying the most commonly used air samplers: membrane filtration, impact on solid surfaces followed by elution, or impingement in a liquid medium. The eluates produced can be further concentrated (Verreault *et al.*, 2008). Other methods for the virological analysis of aerosols include cyclone or electrostatic precipitators, and in recent years, the fear of bioterrorism has triggered assessments of various new methodologies (including mass spectrometry) able to identify dangerous species in the air. However, it is unlikely that such techniques will be suitable for routine environmental analysis in the near future, and furthermore, they require the establishment of very large databases of environmental samples.

To elucidate the fate of virus dispersed through air, surface monitoring should be also performed, because larger droplets tend to settle out. Surface sampling is most extensively used in health care settings and in food production to assess not only viral contamination but also the efficacy and correct application of disinfection procedures. For hard surfaces, a defined surface area (i.e. 10 or 36 cm^2) should be swabbed; the swab is then eluted, and the elute is processed as a liquid sample. Alternative methods are contact plates, which can be similarly eluted.

Concentration of viruses

The aim of concentrating virus is to collect most of the virus present in the sample in a minimal volume (Cliver, 2008); this small sample can then be used for virus detection by molecular, immunological or cell culture–based methods (Fig. 2). Protocols for the concentration of viruses in water samples are generally based on four steps (Croci *et al.*, 2008): adsorption of viruses to a filter; elution of adsorbed viruses using a protein-rich buffer; reconcentration of viruses by flocculation, precipitation or filtration, and extraction of viruses, for example with chloroform. In solid samples (including foodstuffs), sample processing often starts with a washing step (in the case of fresh produce) or a homogenization step (in the case of, for example, shellfish); the virus is concentrated after this first step (Rodríguez-Lázaro *et al.*, 2007; Croci *et al.*, 2008). If appropriate, a minimal volume of a diluent can be added to favour dissociation of the virus from the solid matter but avoiding interference with subsequent virus concentration/extraction. For dispersion of the sample in the diluent, a suitable mixing technique is required. The following step is the removal of food solids from the extract by, for example, filtration or differential centrifugation. Concentration methods appropriate for a wide variety of matrices include adsorption elution, differential precipitation, ultracentrifugation and ultrafiltration (Rodríguez-Lázaro *et al.*, 2007).

Detection methods used for human enteric viruses

Various approaches can be used to detect human enteric viruses in concentrated samples. They range from direct observation by electron microscopy to the detection of cytopathic effects in specific cell lines and of indirect diagnostic signals using immunological or molecular methods (Fig. 2).

Direct observation by electron microscopy is a laborious, painstaking and time-consuming task, is also subjective, and has a limited sensitivity (Atmar & Estes, 2001). The observation of cytopathic effects produced in specific cell lines is not always possible as some enteric viruses, notably NoV and HEV cannot be propagated in mammalian cell lines. Even when possible, this is not a simple or cost-effective technique. It may also require the adaptation of the virus before it can grow effectively (Pintó & Bosch, 2008). There are immunological tests such as enzymatic immunoassay, radioimmunoassay or enzyme-linked immunosorbent assay (ELISA), and many are commercially available for the main enteric viruses. However, their analytical sensitivity is still too poor for effective testing of environmental samples.

To overcome these various limitations and disadvantages, molecular techniques are now being used routinely in viral laboratories, and real-time quantitative PCR (q-PCR) has become the method of choice for the detection of enteric viruses. This approach has been reinforced by the recommendation of the international ISO/CEN committee CEN/TC275/WG6/TAG 4 that real-time PCR should serve as the basis for the forthcoming international standards for the detection of NoV and HAV (Lees and CEN WG6 TAG4, 2010). A large number of scientific studies using molecular methods for the detection of enteric viruses have already been published

(see Table S3 for a representative list of the published references).

q-PCR is a molecular technique that allows the quantification of the amount of the target template (i.e. specific virus) initially present in a sample (Heid *et al.*, 1996). Other major advantages of this technique include the closed-tube format that reduces the risk of carry-over contamination, the wide dynamic range of quantification and the possibilities for automation (Rodríguez-Lázaro *et al.*, 2007). However, q-PCR also suffers from some limitations. The volume used in the amplification reaction is very small; therefore, only concentration methods that can deliver a very small volume of the resulting nucleic acid solution (i.e. in the microlitre range) from a realistic food or environmental sample can be used. In addition, the quality of the nucleic acids is an important factor that directly affects the analytical sensitivity of the assay, and diverse compounds present in samples can inhibit the amplification reaction. The standardization of inhibition tests would help overcome this limitation once appropriate synthetic standards become available (La Rosa *et al.*, 2010). Finally, definitive international standardization efforts are required to guarantee effective implementation in the real-life analytical contexts.

Other detection options include the combination of cell culture or immunological methods and a molecular technique. The combination of a cell culture step and subsequent detection by a molecular technique such as RT-PCR or nucleic acid sequence–based amplification (NASBA) reduces the incubation periods and also allows the detection of viruses that grow without causing cytopathic effects (Table S3) (Dubois *et al.*, 2002; Duizer *et al.*, 2004b).

Index viruses

Classic microbiological indicators such as faecal coliforms (*Escherichia coli* and enterococci) are the most commonly used indicators to evaluate both the level of faecal contamination and also efficiencies of the elimination of pathogens by water purification processes. However, the adequacy of these bacterial markers to indicate the presence and concentration of human viruses and protozoa cysts has been questioned in recent years (Lipp *et al.*, 2001; Tree *et al.*, 2003). EV, evaluated as cultivable enteric viruses, is the sole viral measure that has been included in past regulations. Results obtained by applying molecular techniques have shown that the presence of EVs does not significantly correlate with the presence of other pathogenic viruses that may be more abundant. Diverse groups of bacteriophages have also been suggested as indicators of viral contamination; this would allow in theory the use of simple assays for the detection of infectious viruses (Savichtcheva & Okabe, 2006; Love *et al.*, 2008), although their presence does not clearly correlate with the presence of specific viral pathogens (Formiga-Cruz *et al.*, 2003).

The improvement in molecular technologies for detecting viruses present in water and food has focused attention on new groups of DNA viruses that may be quantified with cost-effective molecular assays and are excreted in large quantities by the populations of widely divergent geographical areas. hAdV are often being detected in the environment (He & Jiang, 2005; Van Heerden *et al.*, 2005a; Katayama *et al.*, 2008; Muscillo *et al.*, 2008) and have been proposed along with human polyomaviruses as a molecular index of viral contamination of human origin (Puig *et al.*, 1994; Pina *et al.*, 1998; Bofill-Mas *et al.*, 2000). Testing for hAdV is of interest for two different reasons: both to assess the presence of this human pathogen itself and also as a more general indicator. Most of the population is seropositive for the most common AdV and also for the human polyomaviruses JCPyV and BKPyV. The presence of these viruses in water therefore presents only a low risk for healthy immunocompetent populations (Bofill-Mas *et al.*, 2001). Specific animal AdV or polyomaviruses have been also proposed as microbial source tracking tools (Hundesa *et al.*, 2006, 2009).

hAdV and JCPyV have been found in 98% of the sewage samples analysed from widely diverse geographical areas around the world (Bofill-Mas *et al.*, 2000), with concentrations of about 10^5–10^7 genome equivalents (GE) L^{-1}. The concentrations are generally higher for hAdV than for JCPyV. These viruses have also been commonly found in river water and have been used as a marker for the evaluation of the efficiency with which water treatment plants eliminate virus (Bofill-Mas *et al.*, 2006; Albinana-Gimenez *et al.*, 2009a).

q-PCR methods have been developed for the detection of hAdV in sewage, shellfish, river water and drinking water (Puig *et al.*, 1994; Pina *et al.*, 1998; Formiga-Cruz *et al.*, 2002; Albinana-Gimenez *et al.*, 2009b) and in sea water (Calgua *et al.*, 2008). hAdV has also shown to be very stable in the environment and resistant to water treatments (Thompson *et al.*, 2003; Mena & Gerba, 2009). A very high proportion of environmental or shellfish samples presenting human viral pathogens contain AdV (Formiga-Cruz *et al.*, 2002); they are the most abundant viruses, as assessed by PCR, and are regularly found in faecal contamination. In a study using q-PCR, hAdV was detected in 100% of the urban sewage samples analysed at concentrations of 10^4–10^5 GE mL^{-1}, and these viruses were still present in treated effluents at concentrations of 10^2–10^3 GE L^{-1}. The biosolids generated accumulated 10^2–10^5 AdV GE g^{-1}. JCPyV also were

quantified, and the concentrations found were 10^3–10^4 GE mL^{-1} in urban sewage, 10^2–10^3 GE L^{-1} in treated effluent and 10^3 GE g^{-1} in the biosolids generated (Bofill-Mas *et al.*, 2006).

The application of index viruses in future regulations on the microbiological quality of water should be a step forward for improving the control of the environment, food and water. However, this would require further studies, including epidemiological studies, for the definition of acceptable values of index viruses and to identify where such values would be appropriate.

Evaluation and interpretation of test results

One of the major differences between the study of the presence and enumeration of bacteria and that of viruses in food and in the environment is the availability of a "gold standard" method for detection. Classical culture-based techniques are considered the gold standard for the detection of bacteria, but the situation is exactly the opposite for the detection of viruses, since no accepted standard method exists. The lack of a defined and consensus standard method for the detection and quantification of viruses is hindering and slowing the adaptation of quantitative viral risk assessment (QVRA) models for food and food environments. Therefore, the establishment and application of a common and validated method for virus detection would make a large contribution to the effective harmonization of QVRA studies. The combination of cell culture and PCR generally produces higher viral counts than those resulting from cell culture methods (i.e. plaque-forming units or $TCID_{50}$) and could be considered a *de facto* standard (Havelaar & Rutjes, 2008).

Validity of molecular detection methods

The reliability of the results produced by molecular techniques is undermined by the lack of standard methods for the detection of viruses in environmental samples and the wide diversity of viruses, matrices, assays and recovery efficiencies described. Molecular techniques, if used with the appropriate quality controls, could allow substantial progress in the control of viral contamination of environment and food. These quality controls must include at least one negative and one positive reaction control, one negative and one positive process control and an internal or external amplification control (Hoorfar *et al.*, 2004; Costafreda *et al.*, 2006; Rodríguez-Lázaro *et al.*, 2007; Pintó & Bosch, 2008; D'Agostino *et al.*, 2011; Diez-Valcarce *et al.*, 2011a, b; Martínez-Martínez *et al.*, 2011) (Table 1). Controls for the estimation of the efficiency of the concentration and/or extraction procedures are also very important. Several approaches have suggested the use of nonpathogenic virus surrogates, with similar structural characteristics and which are not present naturally in the samples to be tested. As examples, Mengo virus MC_0 (Costafreda *et al.*, 2006) and feline calicivirus and murine NoV-1 (Cannon *et al.*, 2006) have been proposed as appropriate surrogates for HAV and human NoV, respectively.

Table 1. Analytical controls for (RT) real-time PCR-based detection of viral hazards in food matrices

Process controls
Processing Positive Control (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
Processing Negative Control (PNC): A negative sample spiked with sufficient amount of nontarget or water and processed throughout the entire protocol. A negative signal should be obtained, indicating the lack of contamination throughout the entire process. For example, the inclusion of encapsidated RNA (or DNA) or bacteriophages
Environmental Control: 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 controls
Positive PCR control: A viral template known to contain the target sequence. Positive amplification indicates that amplification was performed correctly. It could be used a natural virus or chimerical nucleic acids
Negative PCR control (or No Template Control -NTC- or Reagent Control or Blank): Including all reagents used in the amplification except the template nucleic acids. Usually, water is added instead of the template. A negative signal indicates the absence of specific contamination in the amplification assay
External Amplification control (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 analysed in a separate reaction tube. A positive signal indicates that the sample nucleic acid extract did not contain any inhibitory substances
Internal Amplification Control (IAC): Chimerical nontarget nucleic acid added to the master mix to be co-amplified with the same primer set as the viral target but with an amplicon size visually distinguishable or different internal sequence region from the target amplicon. The amplification of the IAC both in the presence and in the absence of the target indicates that the amplification conditions are adequate

Adapted from Rodríguez-Lázaro *et al.* (2007), Pintó & Bosch (2008), Bosch *et al.* (2011) and D'Agostino *et al.* (2011).

Negative results obtained using correctly designed and controlled PCR assays can provide robust evidence for the absence of pathogens or indicators in the samples analysed with strong implications for risk assessment. Such negative results from well standardized and highly sensitive PCR assays may be acceptable and may facilitate the implementation of potential regulations requiring the absence of pathogens from defined sample volumes, as is the case for food or water safety criteria. More studies are needed to evaluate the significance of positive results, because the differing sensitivities of diverse techniques, like infectivity assays if available, do not allow a definitive evaluation of the infectious capability of the viral genomes detected. Also, if viral measures are considered for regulations concerning the microbiological quality of bathing water or other environmental samples, epidemiological studies would be needed to establish acceptable limits for index viruses.

Infectious particles vs. PCR GE: implications for public health

Viral infectivity is defined as the capacity of viruses to enter the host cell and exploit its resources to replicate and produce progeny infectious viral particles (Black, 1996; Rodríguez *et al.*, 2009), which may lead to infection and subsequent disease in the human host. Therefore, the information required in risk assessment studies is the number of viral particles with infective capacity. Obviously, cell culture–based methods are the soundest methodologies for the estimation of the number of infective particles. However, as indicated earlier, there are no available culture models for some of the most significant food and environmental virus hazards, notably human NoV, HEV and even wild-type HAV. In these cases, only molecular methods are available, but although RTq-PCR is a quantitative and sensitive tool, it cannot distinguish between infective and noninfective viruses (Richards, 1999). This limits its usefulness for public health purposes. The ratio between GE and infectious particles has been reported to increase with the time, is strongly dependent upon water and climatic conditions and virus type, and can vary from 70 : 1 to 50 000 : 1 for EV in natural surface water (Rutjes *et al.*, 2005) and in artificial ground and surface waters (de Roda Husman *et al.*, 2009). For example, wastewater can contain up to 1500 GE HAstV L^{-1} but do not show any infective capacity. To overcome this limitation, several different approaches based on (RT) PCR have been assessed (reviewed in Rodríguez *et al.*, 2009; see Table 2 for examples). However, it is unclear whether any direct PCR method can satisfactorily assess viral infectivity.

Risk assessment

As stated earlier, QVRA is theoretically a powerful statistical tool for the estimation of the probability of a viral infection or disease based on exposure of the human host to the viral hazard and for establishing the dose–response relationship (Haas, 1983; Haas *et al.*, 1993). Consequently, QVRA has been used for exposure to various virus hazards in different environmental matrices, mostly for aquatic environments (e.g. Van Heerden *et al.*, 2005b).

In general, the risk analysis framework (FAO and WHO, 2006) consists of hazard identification, exposure assessment, hazard characterization and risk characterization, which should identify and preferably quantify the risk. In the case of QVRA for environmental exposure, this framework reads as follows: (1) hazard identification: the identification of viral agents that may be present in a particular environmental matrix and are capable of causing adverse health effects; (2) exposure assessment: quantitative evaluation of the likely intake of viral agents via exposure to environmental sources; (3) hazard characterization: quantitative evaluation of the nature of the adverse effects associated with the viral agents that may be present in the environment one is exposed to and; (4) risk characterization: the integration of hazard identification, exposure assessment and hazard characterization into a risk estimate of the likelihood and the severity of the adverse effects in a given population with attendant uncertainties.

Various viral characteristics, as described in this paper, are important determinants of the risk of infection or disease upon exposure: numbers (or dose), infectivity and pathogenicity to humans. Application of QVRA has been rendered difficult by the lack of culturing systems and low environmental levels of viruses that present a possible public health risk but cannot be typed or quantified. Moreover, standardized methods for quantification of virus hazards in different environmental matrixes and dose–response models for the main environmental virus hazards are not available. For reliable quantification of viruses in food and environmental matter, various factors need to be determined: the detection efficiency of the assay used, the controls appropriate for accurately measuring both the true concentration and the release of virus into the environment, and the contamination of the food (Pintó & Bosch, 2008; Pintó *et al.*, 2009). This is of the utmost importance for unculturable viruses, such as HEV and human NoV, for which only molecular quantitative detection methods are available. The raw numbers of GE, which are the data generated by such methods, must be corrected for the efficiency of the concentration and nucleic acid extraction steps and the capacity of the

Table 2. Molecular-based methods used for assessing viral infectivity

Method	Treatment	Detection	Type of sample	Target virus	References
Molecular	Proteinase and RNase	RT-PCR	Cell culture	FCV HAV, MNoV, poliovirus 1,	Nuanualsuwan & Cliver (2002, 2003); Baert *et al.* (2008)
	Proteinase and RNase	qNASBA	Stool samples and cell culture	NoV, FCV	Lamhoujeb *et al.* (2008, 2009)
	RNase protection assay	qRT-PCR	Stool samples and cell culture	NoV, FCV	Topping *et al.* (2009)
		5′ NTR RT-PCR	Cell culture	HAV	Bhattacharya *et al.* (2004); Li *et al.* (2002, 2004)
		Long target region (LTR) qRT-PCR	Cell culture	HAV, poliovirus 1, F-specific RNA phages	Li *et al.* (2002); Simonet & Gantzer (2006a, b)
Cell culture + molecular	Attachment to cell monolayer	RT-PCR	Cell culture	HAV, poliovirus 1, FCV	Nuanualsuwan & Cliver (2003)
	Virus replication in cell culture (ICC: integrated cell culture)	RT-PCR	Different types of water, sewage effluent, faecal specimens and cell culture	AdV, AstV, EV, poliovirus, RV, HAV, MS2	Blackmer *et al.* (2000); Chapron *et al.* (2000); Jiang *et al.* (2004); Ko *et al.* (2003, 2005); Lee & Kim (2002); Lee & Jeong (2004); Li *et al.* (2009); Nuanualsuwan & Cliver (2003); Reynolds *et al.* (1996); Shieh *et al.* (2008)
Immunological +molecular	Antibody capture	RT-PCR	Different types of water, faecal samples and cell culture	HAV, NoV, poliovirus 1, FCV	Gilpatrick *et al.* (2000); Myrmel *et al.* (2000); Schwab *et al.* (1996)
	Immunomagnetic separation	qRT-PCR	Artificially contaminated groundwater	HAV	Abd El Galil *et al.* (2004)

RT-PCR, reverse transcriptase PCR; qRT-PCR, reverse transcriptase real-time PCR; qNASBA, real-time nucleic acid sequence–based amplification; FCV, feline calicivirus; mNoV, murine NoV.

enzyme involved in the amplification-based detection. A formula for the estimation of exposure to viruses in food matrices has been proposed by Havelaar & Rutjes (2008).

Following exposure assessment, hazard characterization is possible using dose–response models, which describe the relationship between virus particles detected and the probability of disease. Viral dose–response models are based on three basic biological assumptions: single hit, independent action and random distribution (FAO and WHO, 2006). Using these assumptions, three different models can be applied to environmental virus hazards (Haas, 1983; Teunis & Havelaar, 2000; Zwietering & Havelaar, 2006). For example, Pintó *et al.* (2009) estimated the relationship between HAV numbers in frozen coquina shellfish involved in two hepatitis outbreaks and the risk for human health. However, for HAV, immunity needs to be taken into account. Similarly, for human NoV that only induces short-lived immunity, risk assessment should also take into account the observation that a proportion of the population is resistant to infection with NoV genogroup GI (Hutson *et al.*, 2002; Lindesmith *et al.*, 2003; Rockx *et al.*, 2005) or GII (Thorven *et al.*, 2005; Larsson *et al.*, 2006).

The viral risk can thus be estimated from the information obtained from an exposure assessment and the dose–response relationship (Zwietering & Havelaar, 2006). In addition, the estimation of the disease incidence can be also extrapolated to estimates of disease burden and costs (Havelaar & Rutjes, 2008). Published risk assessments for environmental viruses mainly concern water-borne or food-borne exposure, but other routes may be considered as well. For food-borne viruses, the EU research project '*Integrated monitoring and control of food-borne viruses in European food supply chains*' (KBBE 213178; VITAL; www.eurovital.org) has been launched to develop proactive integrated monitoring and risk management strategies for the control of viral contamination of food supply chains. Moreover, a network of food and environmental virologists, under COST Action 929, ENVIRONET (www.cost929-environet.org), has been established to improve our knowledge and the role of the environment and food in the transmission of enteric viral disease.

Concluding remarks and recommendations

Environmental virus hazards are increasingly recognized as a cause of illness in all age groups. Caliciviruses (NoV), AdV, EV, RV, HAV and HEV are the most common causes of illness because of environmental exposure. The major routes of exposure to environmental viruses involve human or animal faeces, surface water or sewage, especially irrigation waters in relation to crops, and fresh and noncooked produce along the food chain, and in particular bivalve molluscs, which filter feed in virus-contaminated waters. In addition to the risks associated with the contamination of environmental or food matrixes with viruses of human origin, there are also pathogenic viruses that are zoonotic, i.e. of animal origin and transmitted from animals.

Education of populations at risk should give particular attention to describing potential virus contamination routes, especially for those working with water, sewage, faeces and food. Education about risks is also important for health care workers and consumers. The most important preventive measures include the improvement of hygienic conditions during harvesting, processing and handling of potentially contaminated environmental matter. Legislation on handling and treatment of water, sewage and foods should be adapted as needed to reduce the risk of environmental virus contamination. The systems for sewage treatment and the codes of practice for agricultural use of sewage and surface water should be reviewed to address these issues.

Methods related to virus purification and detection of viral particles should be improved such that survival of human pathogenic viruses in the environment can be followed reliably. In parallel, techniques should be further developed for effective virus inactivation and decontamination of environmental materials suspected to pose a risk. When human disease is caused by environmental exposure to viruses, and also for the assessment of virus contamination in environmental matter, virus monitoring is required, and it may be beneficial to implement a virus surveillance strategy. Unfortunately, this is not straightforward. Samples must represent the environmental matter being studied, and tests for specific virus hazards may need specific sampling and sample processing techniques. Safe and efficient transport and laboratory practices are of utmost importance for laboratory workers and the outcomes of prevention and control measures.

The development of a suitable detection technique for a virus in an environmental sample requires a targeted specific approach. This generally starts with the separation and concentration of the virus. Appropriate concentration methods include adsorption elution, differential precipitation, ultracentrifugation and ultrafiltration. Then, various virus identification methods can be used; possible methods range from classical techniques like cell culture and electron microscopy to molecular techniques like RT-PCR and microarrays, and combinations may also be used. Development of a general method that can be applied to different matrices is difficult and, indeed, may not be feasible. Nevertheless, the CEN/TC 275- Food Analysis, Horizontal Methods; Working Group 6, Technical Advisory Group 4 (CENTAG4) is pursuing efforts for the develop-

ment of such horizontal methods for detection of viruses in foods.

To evaluate the extent of environmental virus contaminations, it can be helpful to test for particular index viruses, whose presence correlates with the presence of other pathogenic, viruses that may be more abundant. Because of their wide applicability and high level of sensitivity and specificity, molecular techniques are most commonly used for the detection of environmental virology. Powerful molecular techniques can be extremely valuable if appropriate controls are used. However, for estimation of the true virus hazard, the detection of GE, which is the output of molecular techniques, has to be related to the quantity of infectious particles present.

To estimate the probability of a viral infection, the statistical tool QVRA can be used. This involves virus hazard identification, exposure assessment, hazard characterization and risk characterization. Satisfactory exposure assessment requires a reliable quantification of the virus present in the environmental material. For reliable quantification of virus in environment, the detection efficiency of the assay used must be determined, and appropriate controls must be employed to determine accurately the true concentration and release of virus in the environment. In conclusion, the study of environmental virus hazards is extremely important to estimate the public health risks associated with viruses.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Sampling methods used for detection viral hazards in food matrices.
Table S2. Sampling methods used for detection viral hazards in water samples.
Table S3. Detection methods for viral hazards in different environmental matrices.

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