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Report of the Scientific Committee of the Spanish Agency for Food Safety and Nutrition (AESAN) on viral contamination of food, paying special attention to bivalve molluscs and control methods

Scientific Committee members

Rosaura Farré Rovira, Francisco Martín Bermudo, Ana María Cameán Fernández, Alberto Cepeda Sáez, Mariano Domingo Álvarez, Antonio Herrera Marteache, Félix Lorente Toledano, Mª Rosario Martín de Santos, Emilio Martínez de Victoria Muñoz, Mª Rosa Martínez Larrañaga, Antonio Martínez López, Cristina Nerín de la Puerta, Teresa Ortega Hernández-Agero, Perfecto Paseiro Losada, Catalina Picó Segura, Rosa María Pintó Solé, Antonio Pla Martínez, Daniel Ramón Vidal, Jordi Salas-Salvadó, Mª Carmen Vidal Carou

Secretary

Vicente Calderón Pascual

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Working Group

Rosa M. Pintó Solé (Coordinator)

Mª Rosario Martín de Santos

Albert Bosch Navarro (External consultant)

Abstract

The viruses primarily associated with food-borne illness are human Norovirus (NoV), causing gastroenteritis, and Hepatitis A virus (HAV) causing acute hepatitis. These viruses do not grow, or grow with many difficulties, *in vitro* and thus their detection in food matrices relies on molecular techniques. The major foodstuffs involved in food-borne viral infections are bivalve molluscs, salad vegetables and berries. Standardization of molecular methods is necessary before their adoption within regulatory frameworks and their routinely implementation in food analysis. A European standardization working group (CEN/TC 275/WG6/TAG4-viruses in foods) has developed standard methods for virus detection in foodstuffs. Quantitative determination of viruses in food would allow the assessment of the associated health risk after consumption of bivalves with a given number of virus genome copies since higher number of genome copies correlate with higher risk of infection.

Key words

Human Noroviruses, Hepatitis A virus, RNA, Real-time-RT-PCR, Standardization, Genome copies.

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Introduction

Enteric viruses are transmitted via the faecal-oral route, and, therefore, are potentially present in food that has come into contact with faecal matter.

Among human enteric viruses there are several agents causing gastroenteritis, such as Norovirus, Sapovirus, Astrovirus, Rotavirus and group F Adenovirus, and also agents for enteric hepatitis (A and E). Due to the number of foodborne outbreaks associated with these agents, the most important from the point of view of viral food safety are: Norovirus genogroup I (NoV GI) and II (NoV GII) and hepatitis A virus (HAV). However, the possibility must be borne in mind that future outbreaks of foodborne gastroenteritis caused by Sapovirus may appear, due to increasing incidents in the wider community; also hepatitis E, which although still not common at these latitudes, is very common in China and south-east Asia, and poses a potential hazard in the current context of global food trade. It must also be remembered that certain animals, such as pigs and wild boars, can also be infected by hepatitis E virus and act as a reservoir for the virus, as some cases have been documented of humans being infected after consuming lightly-cooked liver from infected animals.

Among the foodstuffs at greatest risk from contamination by enteric viruses are bivalve molluscs, as they have a high capacity for filtering and concentrating the viruses that may be present in their growing waters, in addition to salad vegetables and berry fruits irrigated with water containing faecal matter. These foodstuffs are usually eaten raw or lightly cooked, thus maximising the risk of infection. Additionally, food may also be infected from poor hygiene standards among food handlers who excrete the virus, symptomatically or asymptomatically.

An important contributing factor to the transmission of enteric viruses via foodstuffs is their high stability outside the body or in the environment, which is extreme in some cases, such as HAV.

Since food is one of the sources for enteric virus transmission, and as outbreaks sometimes affect closed communities at risk (hospitals, homes for the elderly), and Spain is a large producer and consumer of bivalve molluscs, which are one of the main foodstuffs involved in transmitting these viruses, the Executive Director of the Spanish Agency for Food Safety and Nutrition (AESAN) asked the Scientific Committee to draw up a report assessing the state of knowledge on virus contamination of foodstuffs, especially bivalve molluscs, and methods of control.

This report will enable the degree of knowledge on the subject in Spain to be assessed and to implement future information needs arising from the possibility that the European Union may set limits for certain viruses in foodstuffs (bivalve molluscs).

Biology of Norovirus (NoV)

Noroviruses (NoV) are members of the *Caliciviridae* family (Fauquet et al., 2005) and, therefore, are non-enveloped viruses with an icosahedral capsid, 27-40 nm in diameter, containing a single-stranded, positive RNA genome of about 7 Kb. The genome contains three open reading frames coding respectively for the non-structural proteins, including the enzymes required for genome replication as well as a protease, the main protein in the capsid or VP1, and the minor protein in the capsid or VP2. A very important characteristic of the Norovirus genome is its very high variability (Domingo and Vennema, 2008).

On the other hand, it must be emphasized that, despite a great effort, it has not been possible to replicate these viruses in cell culture (Duizer et al., 2004). Although there are references stating the opposite, the fact that the results described cannot be reproduced leads to doubts on whether Noroviruses can multiply in cell culture (Straub et al., 2007). It is thought that the inability to replicate *in vitro* is caused by the lack of suitable receptor(s) in the cell strains tested, as the transfection of the virus RNA gives rise to single-cell infectious cycles (Guix et al., 2007). Consequently, all the above leads to the use of molecular techniques to detect the virus (Kageyama et al., 2003) (Loisy et al., 2005) (Lowther et al., 2008). This, and the high variability mentioned above, means that very robust criteria must be used to choose the best-conserved regions of the genome to develop mo-lecular detection techniques (Pintó and Bosch, 2008).

NoVs are classified into five main genogroups. Human NoV belong to genogroups I (GI), II (GII) and IV (GIV). Also found within genogroup II are viruses infecting pigs, and in genogroup III, viruses infecting cattle; and although it has not been reliably proved, it has been suggested that these may be zoonotic agents. Each genogroup contains multiple genotypes that emerge and vary temporarily (Koopmans, 2005) (Siebenga et al., 2007b) (Domingo and Vennema, 2008) (Kroneman et al., 2008). Genogroups are given Roman numerals and genotypes, arabic numerals (Atmar, 2010).

Human NoVs are mainly transmitted by consumption of contaminated water or food, as well as by person-to-person contacts, or contact with contaminated fomites. Certain genotypes are associated more closely with a specific type of transmission. In this sense, genotype GII.4 has been described as being transmitted mainly from person to person, while shellfish-borne outbreaks are generally associated with genogroup GI (Le Guyader et al., 2006b) (Siebenga et al., 2007a). It is remarkable, that genotype GI.1 of human Noroviruses have been shown to internalize specifically in the cells of oyster hepatopancreas (Le Guyader et al., 2006a). In addition, differences have been described between the interactions of the different genotypes with various ligands expressed in oyster tissues. Genotype GI.1 uses a carbohydrate ligand containing an A-like antigen, and which is only present in cells in the hepatopancreas, while Genotype GII.4 not only uses the same carbohydrate, but also another containing sialic acid and is found in the vast majority of tissues (Le Guyader et al., 2006a) (Maalouf et al., 2010). It is thought that the interaction with sialic acid-containing ligands might be weaker, which would explain why genotype GII.4 viruses internalize less efficiently, than the interaction with ligands containing A-like antigens, which in turn may contribute to a greater persistence of genotype I.1 viruses and to a much more difficult removal of this latter genotype through depuration processes.

The incubation period for the illness ranges between 10 and 51 hours and the infectious is low (Glass et al., 2009). In the specific case of the Norwalk virus, Teunis et al. (2008) estimated a 50% infectious dose between 18 and 1,000 viral particles. In certain types of food, such as seafood, much higher concentrations than the above mentioned are commonly found, entailing an obvious risk (Le Guyader et al., 2003) (Le Guyader et al., 2010). On the other hand, these levels of virus are excreted by patients before the onset of symptoms and even after their cessation, which highlights the role of food handlers in transmitting NoV gastroenteritis. It is estimated that foodborne gastroenteritis via NoV accounts for 12%-47% of all gastroenteritis via NoV, and the remainder originates from person-to-person contact with contaminated fomites (FAO/WHO 2008).

Biology of hepatitis A virus (HAV)

Hepatitis A virus (HAV) belongs to the *Picornaviridae* family (Fauquet et al., 2005) and is a non-enveloped virus with an icosahedral capsid about 27 nm in diameter, containing a positive single-stranded RNA of approximately 7 Kb. Unlike NoV, the picornavirus genome contains a single open reading frame. Genetically, HAV is notable for having a high mutation rate for synonymous mutations, within the range of other picornoviruses, but a particularly low rate for non-synonymous mutations (Sánchez et al., 2003), specially at the capsid level. This characteristic evidences very strong structural constraints of the HAV capsid, which explains the low antigenic variability and the occurrence of a single serotype. However, under immune pressure escape mutants can be selected, i.e. to monoclonal antibodies *in vitro* (Aragonès et al., 2008) and even to vaccines *in vivo* (Perez-Sautu et al., 2011). The last reason for these structural constraints is found at the genome level. HAV codon usage is highly biased and antagonistic to that of the host cell, with strategically located clusters of rare codons at certain regions of the capsid coding genome, in order to slow down the speed of translation to allow an optimal protein folding (Aragonès et al., 2010). This enables the virus to have a highly cohesive capsid which associates with an extremely high stability in front of several physical and chemical agents, such as high temperatures, low pH (Hollinger and Emerson, 2007), desiccation (Abad et al., 1994); and disinfection (Abad et al., 1994). The need to maintain the clusters of rare codons, which play an important role in the structural biology of the virus capsid, explains the low variability of amino-acids, since it is not usual for a mutation to cause a change in an aminoacid compatible with the structure and at the same time keeping the degree of rarity of the codon. This special use of codons must also be placed in context with other, very particular characteristics of the molecular biology of HAV, such as the highly inefficient formation of the initiation of translation complex (Brown et al., 1994) (Whetter et al., 1994) and the inability to shut down cell protein synthesis (Ali et al., 2001). Taken all together, these contribute to very low replication rates of the virus, and so only a few strains of the virus have been able to adapt to replicate in cell culture. The wild-type strains can only be detected by molecular techniques which may be based on highly conserved regions of the genome.

In spite of the occurrence of a single serotype of the virus, six genotypes are recognised (I to VI), of which I, II and III are human strains, and IV, V and VI correspond to simian strains (Costa-Mattioli et al., 2002). There is no data showing that certain genotypes are transmitted more easily by one method or another, as already described for NoVs, and there seem to be no differences in pathogenicity among the various genotypes (Rezende et al., 2003). The genotypes generally refer to different geographical areas (Robertson et al., 1992).

Hepatitis A is a severe hepatitis that never becomes chronic. The incubation period is about three weeks, during which the virus is excreted assymptomatically in large amounts in the patient's faeces. This is because the virus is actively replicating in the liver in large amounts, but the symptoms, which include raised transaminases and liver inflammation, come more from the response of the adaptive immune response at the end of the three weeks than from replication of the virus (Hollinger and Emerson, 2007). A further problem is that, from the point of view of contaminated foodstuffs, hepatitis A is largely asymptomatic in children under 5 years old, but the virus is excreted in large amounts. The dosage required for infection is very low and has been estimated at 10-100 particles (FDA/CFSAN,

1992). As with NoVs, these extremely low levels are far exceeded in patients' stools (10^8 genome copies per gram), and therein lies the importance of persons excreting asymptomatically.

Unlike NoVs, which are viruses emerging on a global scale, HAV is only endemic in countries with low social and sanitary conditions, which makes contaminated food imports vitally important (Costafreda et al., 2006) (Pinto et al., 2009) (Polo et al., 2010). In addition, in countries where it is not endemic, such as Spain, the population does not come into contact with HAV in childhood, and so the adult population is immunologically *naïve* just at the age when the infection becomes more important clinically, with a 1% mortality rate in over 60-year-olds (Hollinger and Emerson, 2007).

It is estimated that foodborne hepatitis A accounts for only about 5% of the total, and the remainder originates from person-to-person contact with contaminated fomites (FAO/WHO 2008).

Detection and molecular quantification of human NoV and HAV in food matrices

As previously stated, wild-type strains of HAV and human NoV do not replicate in cell culture, which means that molecular techniques have to be used to detect and quantify them, since alternative methods, such as immunological detection, have the drawback of poor sensitivity mainly having in mind the low levels of viruses present in contaminated foodstuffs. Over the last few years, several methodologies have been published based on the Polymerase Chain Reaction (PCR), more specifically in the combination of the retrotranscription of RNA to cDNA, and the amplification of the cDNA by PCR (RT-PCR method), to detect HAV and NoV in food matrices. However, although these are all fairly correct, depending on a good selection of primers from highly conserved regions of the genomes, there is no harmonisation or standardisation. Therefore, since 2004, a Committee from the European Union (EU) Sanco Directorate General (CEN/TC 275/WG6/TAG4-viruses in foods) has been developing and validating a standardised double methodology (quantitative and qualitative) to detect human HAV and NoV in food matrices (salad vegetables, food surfaces, berry fruits, bivalve molluscs and bottled water), in order to establish reference methods. The first part of the standard (the quantitative method) and the second part (the qualitative method) were presented in 2009 and 2010, respectively, to the Member States of the European Committee for Standardisation (ECS) for technical and formal assessment. The final version of the methods is expected to be published in 2012.

Standardisation of the methodologies is based on three points:

- In the first place, a key point is to choose the most conserved regions of the genome of the virus to be detected, in order to apply broad-spectrum techniques. The secondary structure of the genome of RNA viruses may have a determining function in the replication cycle of viruses. Therefore, it is obvious that the regions of the genome involved in the formation of such structures must be highly conserved between strains, and their sequences should be less variable. Designing primers from these regions is a safe method to compensate the strong tendency towards variability found in RNA viruses (Pintó and Bosch, 2008). With HAV, the most highly-conserved region is located at the non-coding end 5' of the genome, as it contains a secondary structure involved in the formation of the translation initiation complex, specifically the IRES (Internal Ribosome Entry Site) structure (Costafreda et al., 2006). NoVs do not have a similar structure to the IRES found in HAV and an alternative is the region between the end of ORF1 (Open Reading Frame

coding the polymerase) and the beginning of ORF2 (Open Reading Frame coding the capsid proteins), or the joint between ORF1/ORF2, where there is a high degree of phylogenetic conservation and where, presumably, there should be an RNA structure enabling the synthesis of the subgenomic RNA necessary to produce large amounts of capsid proteins (Kageyama et al., 2003).

- Secondly, the molecular reactions for the retrotranscription of the RNA (Reverse Transcriptase, RT) to cDNA and later amplification (Thermostable polymerases) are very sensitive, especially RT, to several inhibitors. Therefore, controls must be included which, when added to the reaction tubes, enable the efficiency of enzyme activity to be checked.
- Thirdly, and very important, the most critical point of virus detection in foods that must be controlled is: the extraction of viruses and their genomes. Even the most exquisite and sensitive molecular method will dramatically fail if the nucleic acids to be detected are not extracted efficiently. Unfortunately, there are no miracle methods to extract viral nucleic acids from food matrices, and the best of these only provide efficiencies of no more than 10%. Therefore, it is vitally important to control the efficiency of the process and the best alternative is to add a model virus to the sample at a known concentration, before the extraction. This control virus must fulfil a series of requirements, such as: having a structure and physical and chemical properties similar to the target viruses, must never be present in the samples and, obviously, must have no pathogenic potential, namely an attenuated virus (Pintó and Bosch, 2008).

All these requirements are included and implemented in the protocol CEN/TC 275/WG6/TAG4-viruses in foods. In addition, the inclusion of the controls described not only enables qualitative detection of the presence of viruses in foods, but also, by providing systems to determine the efficiency at critical points, enables very precise quantification of the number of genome copies present in the sample.

The availability of a standardised method allows the comparison of data obtained by various laboratories and the analyses of contamination in products from several points of origin in the world, is an extremely important added value from the point of view of viral safety in foods. However, molecular techniques cannot provide information on the potential for infection of viruses present in a food sample, as they determine the presence of the virus genome (qualitative method) or the number of genome copies (quantitative method) and this is not the same as infectivity, as the virus capsid may be altered and not able to interact with the receptor to start its biological cycle, or the genome may be altered in regions other than that used for detection. Therefore, it is extremely important to carry out rigorous studies on the level of contamination in various types of foodstuffs from different locations and to look for correlations between these levels and the incidence and severity of outbreaks among consumers of these foodstuffs, in order to try to determine the maximum permissible level of genome copies in foods. For the quantitative methods, at least, this means trying to set limits below which the risk of infection following consumption of these foods is very low, having in mind that zero risk does not exist.

Contamination by human NoV and HAV in bivalve molluscs: review

Among different foodstuffs bivalve molluscs have been widely analyzed for enteric viruses. Therefore, this report focuses on the existing data for this type of food matrix.

Bivalve molluscs are filter feeders that concentrate the particulate material present in the large amounts of water they filter daily in order to obtain food. This particulate material may contain protozoa, bacteria and viruses that infect humans if the growing waters contain faecal matter of human origin. Although the pathogens are diluted in the water, after filtering, bivalve molluscs concentrate the viruses in their tissues, especially in the hepatopancreas. At present, there is no treatment one hundred per cent effective in eliminating viruses from bivalve mollusc tissues, and the most effective ones spoil the organoleptic properties of the bivalves. Therefore, preventing infection associated with consumption of bivalves means controlling the growing waters. However, classic microbiological criteria based on *Esterichia coli* counts in the water or in the bivalve tissue do not ensure complete absence of viruses in bivalves. Thus, it is recommended to detect and/or quantify the virus either in the growing waters or the bivalve mollusc tissues. The reference laboratory for the control of viruses in bivalve shellfish in Europe, in collaboration with reference laboratories in Member States of the EU, has chosen to control viruses in the end product.

Table 1 shows the percentages of positive samples for human NoV and/or HAV in various studies analysing the presence of these viruses in bivalve molluscs harvested and distributed across several European countries during 2001-2008. Nine studies only analyse NoV, two study only HAV, and five study both viruses. The last five, which include samples from France, Spain and Italy, show that human NoVs are more frequently detected. In addition, a strong prevalence of HAV in samples originating from Italy is shown, particularly in the Ionian and Adriatic Seas along the coast of the La Puglia region, where hepatitis A is endemic. The other studies on seafood from other European countries show a high detection rate for human NoVs from any harvesting zone, which means that there is no correlation between the positive count of human NoVs and the level of bacterial contamination in the growing waters. This observation agrees with many papers published previously (Le Guyader et al., 2000) (Romalde et al., 2002) (Vilarino et al., 2009). This provides another argument for controlling the presence of viruses in seafood, as the current standards for classifying harvest zones do not ensure the complete absence of viruses. However, this does not mean that the growing waters should not be controlled, quite the contrary; water must be controlled according to normal microbiological criteria and a virus monitoring system must also be set up.

Table 1. Percentage of human NoV and/or HAV positive seafood samples from various studies analysing seafood harvested from the coasts of Europe

Country of origin	Sampling point	Date of harvesting	No of samples	Detection method	NoV	HAV	Reference
France	Retail store:						
	Netherlands	2004-2008	58	Real time/RT-PCR	12%	NT[1]	(Boxman, 2010)
	Switzerland	2001-2002	61	RT-PCR	13%	0%	(Beuret et al., 2003)
Spain							
Galicia	Area B	2005	24	Real time/RT-PCR	58%	0%	(Vilarino et al., 2009)
	Area C	2005	17	Real time/RT-PCR	53%	0%	(Vilarino et al., 2009)
Italy							
Po Delta	Area B	2005-2006	96	RT-PCR and Real time	10%	0%	(Suffredini et al., 2008)
Adriatic Sea	Area B	2003-2004	235	RT-PCR	14%	6%	(Croci et al., 2007)
Puglia	Retail store Italy	2005-2008	116	RT-PCR	12%	NT	(Terio et al., 2010)
Ionian Sea	Area B	2002	29	RT-PCR	NT	90%	(Di Pinto et al., 2003)
Ionian Sea	Area C	2002	20	RT-PCR	NT	30%	(Di Pinto et al., 2003)
Great Britain/Ireland							
Great Britain	Area B	2004-2006	237	Real time RT-PCR	57%	NT	(Lowther et al., 2008)
Ireland	Area A	2005-2007	119	Real time RT-PCR	31%	NT	(Flannery et al., 2009)
Ireland	Area B	2005-2007	42	Real time RT-PCR	54%	NT	(Flannery et al., 2009)
Ireland	Area C	2005-2007	6	Real time RT-PCR	33%	NT	(Flannery et al., 2009)
Netherlands (NL)							
NL	Retail store Netherlands	2004-2008	126	Real time RT-PCR	9%	NT	(Boxman, 2010)
NL	Area A	2004-2008	104	Real time RT-PCR	1%	NT	(Boxman, 2010)
NL	Area A	2003-2004	21	RT-PCR	5%	NT	(Boxman et al., 2006)
Germany/Scandinavia							
Germany/ Denmark	Area A	2004-2008	36	Real time RT-PCR	11%	NT	Boxman, personal communication
Norway	Area A	2000-2003	681	Real time RT-PCR	7%	NT	(Myrmel et al., 2004)

[1]NT: Non treated. **Source:** (Boxman, 2010).

Contamination levels of human NoV and HAV in bivalve molluscs distributed in Spain

Several laboratories in Spain are already implementing the methods developed by the CEN/TC 275/WG6/TAG4-viruses in foods to control and quantify viruses in seafood.

Table 2 shows the levels of contamination for human NoVs GI and GII and HAV in several types of seafood from different points of origin. The results shown are the averages of the raw genome copies per gram of hepatopancreas, without applying correction factors for extraction efficiency and amplification, as the CEN/TC 275/WG6/TAG4-viruses in foods methodology lays down that samples with extraction and/or amplification efficiencies <1% are not quantifiable and should be viewed as merely positive. While the efficiency of the RT-PCR method is usually very good, a considerable percentage of the analyses have the drawback of inefficient extraction. Therefore, Table 2 only includes raw values, as extraction efficiency of many samples is less than the 1% cut-off level. That extraction efficiency is the most critical step in detection, and particularly in quantification of viruses in bivalve molluscs, has been widely described (Costafreda et al., 2006) (Pintó and Bosch, 2008) (Le Guyader et al., 2009). However, extraction efficiency also largely depends on experience and how familiar laboratories are with the methods used. Table 3 shows high variability among laboratories, a fact that once again justifies the need to include extraction checks in testing.

Table 2. Analysis of viral contamination levels in seafood distributed in Spain. The viruses tested are human NoVs GI and GII and HAV. The concentration of viruses is expressed as an mean ± SD of the raw genome copies per gram of hepatopancreas, i.e. without applying correction due the efficiency of extraction and/or amplification. In cases where there is a single sample, the specific result is given. Negative samples are not included in calculating averages

Food	Country of origin	NoV GI	NoV GII	HAV
Meretrix lyrata	South Korea/Vietnam	$2.5 \times 10^3 \pm 1.1 \times 10^3$	<1	1.9×10^3
Tapes decussatus	Spain	$4.5 \times 10^3 \pm 4.7 \times 10^3$	$4.8 \times 10^2 \pm 9.9 \times 10^1$	$4.5 \times 10^2 \pm 2.7 \times 10^2$
Transennella pannosa	Peru	$6.5 \times 10^5 \pm 1.1 \times 10^6$	$1.3 \times 10^3 \pm 8.7 \times 10^2$	<1
Ensis spp.	Morocco	$1.1 \times 10^4 \pm 1.4 \times 10^4$	<1	<1
Mytylus edulis	Spain	<1	$3.4 \times 10^2 \pm 4.2 \times 10^2$	<1
Callista chione	Morocco	$5.3 \times 10^5 \pm 5.8 \times 10^4$	<1	<1
Ostrea edulis	Unknown	$4.4 \times 10^3 \pm 2.1 \times 10^3$	$1.2 \times 10^2 \pm 7.6 \times 10^1$	<1
Donax spp.	Morocco/Peru	8.8×10^3	$3.4 \times 10^2 \pm 3.9 \times 10^2$	$5.6 \times 10^3 \pm 6.4 \times 10^3$

Data provided by: Dr. Susana Guix of the Enteric Virus Laboratory of the Microbiology Department and Institute of Nutrition and Food Safety at the University of Barcelona; Dr. Jesús López Romalde of the Department of Microbiology and Parasitology and Biological Research Centre (CIBUS)-Faculty of Biology at the University of Santiago de Compostela; Dr. Joana Pardos from the Health Protection Agency at the Department of Health, Regional Government of Catalonia, Girona; and Dr. David Tomás of AINIA Technology Centre, Valencia.

Table 3. Percentage of seafood samples with poor efficiency extraction for viruses and nucleic acids (<1%), and in which the analysis must be repeated. Acceptable rates (1%-10%) and good (>10%)

Extraction Efficiency	Lab. 1	Lab. 2	Lab. 3	Lab. 4	Mean ± SD
<1%	0	3	100	23	31±47
1%-10%	95	45	0	59	50±39
>10%	5	52	0	18	19±23

Table 4 shows the corrected values for all samples for which extraction efficiency rates were above the 1% cut-off level proposed by the CEN/TC 275/WG6/TAG4-viruses in foods method. It can be observed that the levels of genome copies per gram of hepatopancreas increase significantly due to correction factors.

Table 4. Analysis of viral contamination levels in seafood distributed in Spain. Tested viruses are human NoVs GI and GII and HAV. The concentration of viruses is expressed as an mean ± SD of the genome copies per gram of hepatopancreas, corrected according to the efficiency of extraction and/or amplification. Negative samples are not included in calculating averages. In cases where there is a single sample, the specific result is given

Food	NoV GI	NoV GII	HAV
Meretrix lyrata	$2.6 \times 10^7 \pm 4.4 \times 10^7$	<1	ND*
Tapes decussatus	ND	$7.7 \times 10^3 \pm 4.6 \times 10^3$	ND
Ensis spp.	$6.7 \times 10^5 \pm 8.9 \times 10^3$	ND	ND
Mytylus edulis	ND	$8.4 \times 10^3 \pm 2.8 \times 10^2$	ND
Donax spp.	2.2×10^4	ND	$2.5 \times 10^5 \pm 3.1 \times 10^5$

*ND: Not determined due to lack of data on extraction and/or amplification efficiency, or because efficiency was less than 1%.

In any case, the higher frequency in detecting NoVs rather than HAV is notable, also the higher levels of NoV GI than GII. These results agree with the bibliography (Le Guyader et al., 2006b).

Viral safety in foods

As stated previously, there are no data that can confirm that all seafood contaminated by viruses causes infection in consumers. Whether or not a viral infection is contracted after eating contaminated food will depend on a series of factors, among which are the following: 1) the number of viruses present in the food, 2) how the food is processed, which may help to inactivate the virus, 3) the stability of the virus during processing, 4) the infectious dosage of the virus, and obviously 5) the susceptibility of the consumer. The last point is individual and beyond control, the infectious dosage of human NoVs and particularly HAV is very low, as has already been described. As far as processing is concerned, most foods are eaten raw or lightly cooked, and it must also be remembered that these are highly stable agents and, therefore, the point most easily to be controlled is the level of contamination.

Due to the above, and to make the viral safety of food compatible with the economy of the producers, it is not sufficient to detect viruses in food contaminated at the point of origin, but it is recommended to quantify the number of genome copies by a standardised method and set permitted levels above which the food tested will not be deemed fit for human consumption, at least until there are effective systems to inactivate viruses that do not affect the organoleptic properties of the food in question.

However, the great unknown is still how to determine the potential the genome copies have to replicate. Putting the question another way: How many genome copies have to be ingested to cause infection in a susceptible individual? This is the key question and, as yet, there is no precise answer. With HAV, the "native" ratio, meaning no inactivation has occurred, between physical particles or genome copies and infectious viruses is approximately 60:1 (Jansen et al., 1988) (Deng et al., 1994). Also, a study carried out during research into an outbreak of hepatitis A caused by eating contaminated coquina clams (Costafreda et al., 2006) (Pintó et al., 2009) describes linearity between the infectious dosage, calculated from the genome copy: infectious virus ratio given above of 60:1 and determining the count of genome copies through a standardised methodology that includes correction factors for extraction and amplification efficiency, and the level of infection in consumers. Thus, it is estimated that 4.3×10^3 genome copies (72 infectious particles) would cause infection in 11% of consumers, 2.5×10^4 genome copies (420 infectious particles) would cause infection in 36% of consumers and 3.5×10^4 genome copies (582 infectious particles) would cause infection in 41% of consumers. With human NoVs the genome copy ratio: Infectious viruses cannot be determined, due to the lack of strains multiplying in cell culture. However, there are studies showing a positive correlation between the viral load in foods and their capacity to cause infection (Lowther et al., 2010), although since correction factors were not applied and PCR units were used instead of genome copies, it is difficult to compare the published results with others that may be generated.

In any case, implementing standardised methodologies to determine the genome copies of human NoVs and HAV in food that may be contaminated at the point of origin, even though they do not define the potential risk exactly, they do come close and therefore represent a step forward in controlling viral safety in foods and are an added value for food fit for consumption.

However, it is important to emphasise that only a small percentage of gastroenteritis caused by NoV and hepatitis A are directly related to food consumption. Most cases arise from person-to-person contact, or contact with contaminated fomites and these can be prevented through proper hygiene, such as frequent hand washing, particularly, after visiting the toilet.

Conclusions of the Scientific Committee

1. The presence of enteric viruses, especially human Norovirus and hepatitis A virus in food contaminated by faecal matter is a cause of food-borne gastroenteritis and hepatitis. Thus, their presence must be controlled by methods that are sufficiently sensitive to low levels of virus present in food, since even small doses of enteric viruses are a health hazard. However, it must be emphasised that most infections from human Norovirus and hepatitis A are caused by personal contact or with contaminated fomites, and the percentage of cases relating to food consumption is low.

2. Foods most often associated with gastroenteritis and hepatitis are those contaminated at source, such as bivalve molluscs, salad vegetables and berry fruits which are eaten raw or lightly cooked, and to a lesser extent, food handled incorrectly after cooking.
3. Human Norovirus and wild-type strains of hepatitis A virus do not replicate *in vitro* in cell culture. Therefore, the most suitable detection methods, due to their sensitivity, are based on amplification by PCR, more specifically the RT-PCR method, as they are RNA viruses.
4. Current RT-PCR techniques in real time enable the viral load of a sample to be detected and quantified. However, they require thorough standardisation and the inclusion of checks at critical steps, such as extraction of the viruses and their nucleic acids, and the molecular reactions of RT-PCR. The methodology developed by the European Committee for Standardisation CEN/TC 275/WG6/TAG4-viruses in foods complies with these requirements and will be published shortly.
5. Although molecular quantification methods (viral genome copies) lack information on the potential for infection of the samples analysed, there are data confirming a correlation between the count of genome copies and the potential for infection of the sample. From now on, the maximum permitted limits must be defined for genome copies in food samples, in order to reduce the risk to health as far as possible, always having in mind that a zero risk does not exist.

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