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Current Opinion in Virology

Transmission of viruses through shellfish: when specific ligands come into play

Françoise S Le Guyader[1], Robert L Atmar[2] and Jacques Le Pendu[3]

Shellfish are known as vectors for human pathogens and despite regulation based on enteric bacteria they are still implicated in viral outbreaks. Among shellfish, oysters are the most common vector of contamination, and the pathogens most frequently involved in these outbreaks are noroviruses, responsible for acute gastroenteritis in humans. Analysis of shellfish-related outbreak data worldwide show an unexpected high proportion of NoV GI strains. Recent studies performed *in vitro*, *in vivo* and in the environment indicate that oysters are not just passive filters, but can selectively accumulate norovirus strains based on viral carbohydrate ligands shared with humans. These observations contribute to explain the GI bias observed in shellfish-related outbreaks compared to other outbreaks.

Addresses
[1] Laboratoire de Microbiologie, IFREMER, BP 21105, 44311 Nantes Cedex 03, France
[2] Department of Medicine, Baylor College of Medicine, 1 Baylor Plaza, MS BCM 280, Houston, TX 77030, USA
[3] INSERM, U892, Université de Nantes, Institut de Recherche Thérapeutique, 8 quai Moncousu BP 70721, 44007 Nante Cedex 1, France

Corresponding author: Le Guyader, Françoise S (sleguyade@ifremer.fr, Soizick.Le.Guyader@ifremer.fr)

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Although first described ~100 years ago [1], it has only recently become very clear that food plays an important role in virus transmission. In 2007, the CDC identified viruses as the causative agent of 46% of illnesses due to food consumption in outbreaks with an identifiable etiologic agent. Noroviruses (NoVs) were the most common cause, being responsible for 193 outbreaks, while *Salmonella*, the second leading cause, was responsible for 136 outbreaks [2]. Recent estimates from the CDC are that there are 9.4 million episodes of foodborne illness caused annually by 31 major pathogens in the United States, and NoVs are responsible for 58% of these illnesses. Besides NoVs, foodborne transmission has been documented for at least 10 viral families, but only a few families have been implicated repeatedly (Table 1) [3]. If viral zoonotic transmission (e.g. hepatitis E) is not considered, the two primary routes for food contamination are infected food-handlers and the production process (such as contact of the food with sewage-contaminated waters) [4,5]. Several factors influence the transmission process, including the manner of contamination, binding or attachment of the virus to the food, survival and persistence of the virus on the food, the manner of food preparation (raw, cooked, peeled), and the susceptibility of the person eating the food to the contaminating virus [6]. The food itself also has an important role. For example, lettuce maintains a higher quantity of viable hepatitis A virus and for a longer period of time compared to fennel and carrots [7]. Recognition of foodborne illness also is influenced by public sensitivity and awareness of such illness, which can bias the tendency to report an illness. All but 3 of the 36 outbreak notifications involving viruses reported during an 11-year period (2000–2010) in the European Food Alert System for Food and Feed (RASFF) were due to NoVs. The other three were recent reports of HAV linked to dried tomatoes. Among the NoV foodborne outbreaks, 11 were associated with berries and 22 with oysters [4]. Although reporting bias may play a role in the predominance of outbreaks associated with berries and oysters, as they are known to be high-risk foods, these data also highlight the association between shellfish and viral gastroenteritis.

Norovirus

NoVs belong to the *Caliciviridae* family, a group of non-enveloped, icosahedral viruses with a single-stranded, positive sense RNA genome [8]. These viruses are highly diverse and are currently divided into 5 genogroups [9]. Genogroups I, II and IV contain human strains. Each genogroup is further subdivided into genotypes based upon analyses of the amino acid sequence of the major capsid protein, VP1. Other genotyping systems based upon shorter sequences [10] or analysis of the polymerase gene [11•] have also been described. New strains and genogroups infecting animals also have been described [12]. NoV infection causes gastroenteritis that is characterized by vomiting and diarrhea [13••]. The prevalence of vomiting along with the short incubation period (1–2 days) and short clinical illness (1–3 days) has been used epidemiologically to identify probable outbreaks of NoV-associated gastroenteritis [14,15]. The infectious dose 50% has been estimated to be as low as fewer than 20 virions [16]. NoVs bind to histo-blood group antigens

Table 1

Viruses transmitted by food.

Family	Genus (name)	Capsid	Genome	Illness and incubation	Food transmission
Adenoviridae	*Adenovirus* (type 40–41)	Icosahedral, 65–80 nm	DNA, 35 kb	Gastroenteritis (moderate)	Rare
Astroviridae	*Astrovirus*	Icosahedral, 28–30 nm	ssRNA, 6.8 kb	Gastroenteritis (moderate)	Rare
Caliciviridae	*Norovirus*	Icosahedral, 27–32 nm	ssRNA, 7.6 kb	Gastroenteritis, 1–3 days	Frequent: shellfish, berries, food handler
	Sapovirus	Icosahedral, 27–32 nm	ssRNA, 7.4 kb	Gastroenteritis, 1–3 days	Uncommon: oysters, food handler
Coronaviridae	*Coronavirus* (SARS)	Enveloped, 170 nm	ssRNA, 27–32 kb	Common cold, pneumonia, enteric disease	Suspected zoonotic, food handler
Flaviviridae	*Flavivirus*, Tick borne encephalitis virus (TBEV)	Enveloped, 40–60 nm	ssRNA, 11 kb	Fever, vomiting, fatigue, pain in the neck, back, encephalitis, 7–14 days	Rare: cow sheep goat milk
Hepeviridae	*Hepevirus* (Hepatitis E virus)	Icosahedral, 32–34 nm	ssRNA, 7.2 kb	Hepatitis, 3–8 weeks	Rare: pig meat, oyster
Orthomyxiviridae	*Influenza A* (H5N1 virus)	Enveloped, 120–300 nm	Segmented ssRNA, 13.6 kb	Flu (fever, muscle pain),	Rare: bird meat (chicken, duck, geese)
Paramyxoviridae	*Henipavirus* (Nipah virus)	Enveloped, 150–350 nm	ssRNA, 15 kb	Influenza-like illness, febrile encephalitis	Rare, food suspected in two outbreaks
Picornaviridae	*Kobuvirus* (Aichi virus)	Icosahedral, 27–32 nm	ssRNA, 8.2 kb	Gastroenteritis, 1–2 days	Uncommon: shellfish
	Enterovirus	Icosahedral, 20–30 nm	ssRNA, 7.2 kb	Diverse clinical syndromes, 3–10 days	Rare
	Hepatovirus (Hepatitis A virus)	Icosahedral, 27–32 nm	ssRNA, 7.4 kb	Hepatitis, 2–6 weeks	Frequent: shellfish, vegetables, food handler
Reoviridae	*Rotavirus*	Icosahedral, 3 layers, 70 nm	dsRNA, 11 genes 3.3–0.6 kb	Gastroenteritis, 1–3 days	Rare

Grey shading: viruses frequently transmitted via food.

(HBGAs), phylogenetically highly conserved complex glycans present on many different cell types and proposed as an attachment factor necessary to initiate infection in people [17••,18,19].

NoVs are the major cause of epidemic nonbacterial gastroenteritis worldwide and have been identified as the cause of 73% to more than 95% of outbreaks [8]. These outbreaks involve all age groups in a wide variety of settings, with a large dominance of GII strains that can constitute up to 90% of clinical strains [5,13••]. Over the past 10 years, NoV sequence analyses of outbreak strains collected from around the world show that GII.4 viruses have accounted for ~70% of all human cases [20•].

Shellfish-related NoV outbreaks

Shellfish are known to be a high-risk food for viral outbreaks but clear strain identification in shellfish is still often difficult. One of the first reports providing the sequence of a NoV strain described an outbreak in the US. A GI.4 strain was found in oyster samples, but the sequence was not identical to those detected in patients' stools [21]. At the same time in Japan, a mixture of GI and GII NoVs was detected both in stool and the related oyster samples but no sequencing was performed [22]. Since then, improvements in detection methods and the development and harmonization of molecular typing strategies have simplified data comparisons, allowing a compilation of outbreak reports that used comparable methods (Table 2).

One characteristic of shellfish-related outbreaks is their frequent association with multiple virus strains observed both in infected patients and in the involved shellfish. When a number of different virus strains are detected in patients, association of the infection with shellfish consumption can be difficult if only a few stools from an outbreak are collected. Thus, it is essential to collect as many stool samples as possible from affected individuals so that all strains that may be present can be identified. It is also important to rapidly identify the outbreak in order to trace the oyster production and to quickly collect the samples related to the outbreak. These data can be used with collected epidemiological data to fully understand the role played by shellfish in the outbreak.

Primers and probe sets specific for each NoV genogroup have been developed for detection by real time RT-PCR [23,24]. However, genotyping remains a challenge, especially in shellfish where low viral concentrations are observed and in stools containing several different strains. In addition, a cocktail of primers is often required to detect the various NoV strains because of the diversity of these viruses [11,25,26].

Table 2

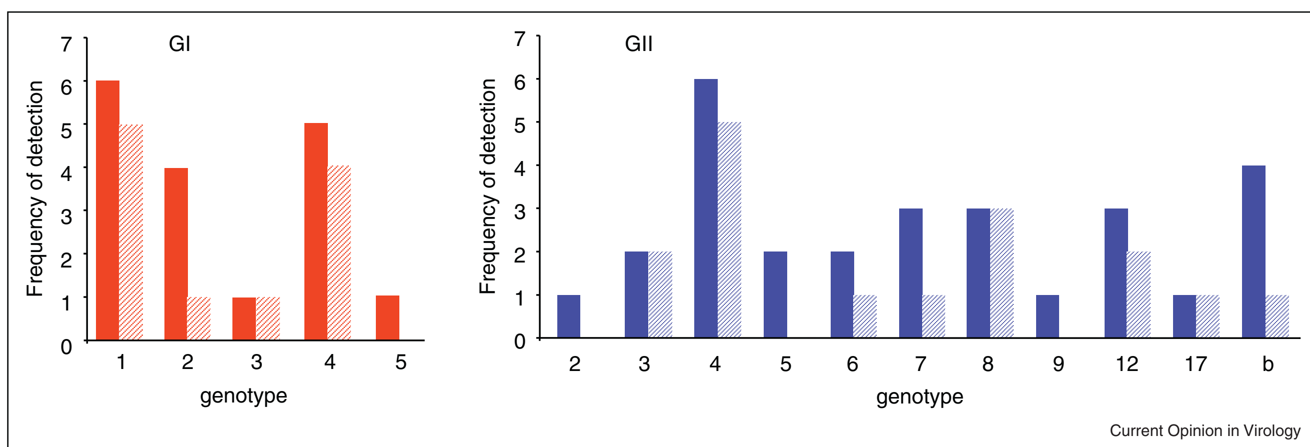
Norovirus genotypes reported from shellfish-related outbreaks.

Date	Country	Stool			Shellfish				Ref.
		# pos/# analyzed	NoV GI genotype	NoV GII genotype	Species	# pos/# analyzed	NoV GI genotype	NoV GII genotype	
May 1998	US	1/2	nd	4	Oyster	2/3	nd	4	[45]
March 2000	France	4/4	1, 2, 3	nd	Oyster	2/2	1	nd	[46]
February 2001	Netherlands	8/9	1, 4	b, 7	Oysters (France)	5/5	4	7	[47]
December 1998 to February 2002	Japan	84/108[a]	1–5, 7–9, 11–14	1, 3–12, 14, 16	Oyster — no sample				[10]
March to April 2002	Italy	24	4	8, b	Mussels	5/11	4	II, b	[48]
December 2002	France	29/53	4, 6	4, 8, b	Oysters	3/3	4	4, 8	[49]
November 2003 to January 2004	Australia	8/?	2, 4	5, 6, 7, 9, 12	Oysters (Japan)	1/1	nd	4	[50]
January 2004	UK	10/11	1, 2	3, 4	Oyster — no sample				[30]
January/March 2004	Canada	26/50	1, 2	3, 4, 5	Oysters	12/19	1	12	[51]
October 2005	Japan	18/37	nd	1, 4, 5, 6	Oyster — no sample				[52]
June 2006	New Zealand	4/4	nd	3, 6, 12	Oysters (Korea)	4/6	3	3, 6, 8, 12	[53]
February 2006	France	12/12	1, 2, 4	2, 4, 7, 17, b	Oysters	9	1, 2, 4	4, 17	[35]
January 2002 to March 2007	Japan	71[b]	1–5, 8, 10, 13–15	3–6, 8, 12	Oyster — no sample				[31••]
January 2007	Sweden	1/1	1	nd	Oysters	1/1	1	3	[54]
February 2008	France	4/5	nd	4	Oysters	4/4	nd	4	[55]
June 2008	Japan	11/24	1	4, 8	Clams	3	1	8	[56]
December 2009	US	3/6	nd	12	Oysters — no sample				[27]

nd: not detected, two manuscripts report data from 21 (a) and 11 (b) individual outbreaks.

Most outbreaks of shellfish-associated NoV disease are linked to oyster consumption, presumably because oysters are the most commonly consumed shellfish and they are usually consumed raw (although some outbreaks have been linked to cooked oysters) [27]. Overall, contamination by multiple NoV strains has been reported in 65% of reported outbreaks, with GI and GII NoVs detected, respectively, in 71% and 88% of stool samples and in 75% and 92% of shellfish samples. The frequency of each genogroup detected in shellfish-related outbreaks is clearly distinct from that of other NoV outbreaks. GI strains are more frequently encountered in shellfish-related outbreaks, and the GII.4 genotype is not as dominant (Figure 1). Among GI NoVs, the most frequently reported genotype is GI.1, followed by GI.4 and GI.2 (Figure 1). Among GII NoVs, the GII.4 genotype is the most frequently reported from both stool and shellfish samples. The GII.b variant was reported four times in patient's stool from oyster-related outbreaks, but confirmed in shellfish only once. Its frequent involvement in human to human outbreaks raises the possibility of another source of infection for these individuals involved in the alleged shellfish-related outbreaks [28•,29].

Figure 1



Genotype frequency in stool and shellfish samples. NoV GI (red) and GII (blue) genotype detected in stool (plain bar) and shellfish (striped bar) samples.

Table 3

Frequency of NoV GI and GII in shellfish contamination in non-outbreak samples from different countries.

Shellfish	Country	# samples[a]	# NoV positive	NoV GI		NoV GII		Ref.
				# positive	%	# positive	%	
Oysters	Japan	1512	75	26[b]	35	49	65	[57•]
	UK	237	139	116	83	112	80	[58]
	UK	66	55	21	38	19	34	[59]
	US	10	5	5	100	0	–	[60]
	France	100	45	19	42	36	80	[61]
	US	381	15	4	27	11	73	[62]
Clams	Spain	41	14	1	7	13	93	[63]
Mussels	Sweden	40	23	19	83	4	17	[64]
	Italy	90	31	10	32	31	100	[65]
Mollusks	Spain[b]	50	16	12	75	4	25	[66••]

[a] Individual samples consisted of pools of 4–36 individual shellfish except for the study [64] in which individual mussels were assayed.
[b] Mollusks (clams, oysters or cockles) were imported from Morocco, Peru, Vietnam and South Korea.

Some reports provide only stool analyses without shellfish data, such as the description of GI.1 and GII.3 strains implicated in an oyster-related outbreak reported from the UK [30]. In Japan GI NoVs alone were detected in four out of 11 outbreaks related to oyster consumption, with the remaining 7 outbreaks being associated with a mixture of GI and GII NoVs. In that study, GI.1 strain was detected in 3 of the 11 outbreaks [31••]. A previous study, also from Japan, reported the presence of a mixture of GI and GII NoVs in stools from 19 out of 21 oyster-outbreaks. In contrast, of 45 outbreaks not linked to shellfish consumption, all but 3 were due to GII NoVs, with both GI and GII strains being found in the remaining three [10].

Screening of shellfish not involved in outbreaks for the presence of NoVs has also been performed in several countries. Highly variable frequencies of contamination have been reported. These studies have also observed a relatively higher frequency of GI NoV contamination than seen in community outbreaks (Table 3). Both studies that reported sequencing results identified GI.1 strains in the contaminated shellfish.

Norovirus bioaccumulation and persistence in oysters

On numerous occasions viral contamination in shellfish has persisted following measures, such as depuration or relaying, that have been used successfully to remove bacterial pathogens [32]. For example, in a laboratory-based study there was only a 7% decrease in the levels of bioaccumulated Norwalk virus compared to a 95% reduction in bacterial levels following 48 hours of depuration [33]. In another study, a GII.6 NoV persisted for at least 10 days under depuration conditions while a feline calicivirus was promptly eliminated [34]. A third study reported that, after a contaminating event in a French production area, the percentage of samples positive for GI and GII NoVs, respectively, were 59% and 70%. The prevalence decreased to 41% and 17%, respectively, after 4 weeks, suggesting a greater persistence in oyster tissues of GI NoVs compared to GII strains [35].

These observations led to the hypothesis that NoVs may bind specifically to oyster tissues through carbohydrates, as observed in humans, and that this binding may facilitate bioaccumulation and increase persistence in shellfish. Using immunohistochemistry, we demonstrated that NoV VLPs specifically bind to glycans of *Crassostrea gigas* oyster tissues, and that strain-specific variation in binding occurs. GI.1 NoVs bind to the midgut and digestive diverticula but not to gills or mantle, whereas GII.3 and GII.4 NoVs bind to all of these tissues. Human saliva from type A and O secretors, but not of type B secretors, inhibited binding of the GI.1 Norwalk VLPs, in accordance with the strain HBGA binding specificity. In addition, introduction of a mutation in the virus-like particles (VLPs) glycan-binding site that abrogates glycan binding was sufficient to eliminate binding to oyster tissues, demonstrating specificity of the binding [36]. Binding was also inhibited by a lectin and anti-blood group A antibodies, indicating that the GI.1 NoV binds to *C. gigas* as well as *Crassostrea virginica* oyster tissues though an A-like antigen [37]. The A-like antigen is also implicated in the binding of GII.3 and GII.4 strains to oyster digestive tissues. Binding of these GII strains to the oyster's gills and mantle occurs through a sialic acid residue [38•].

The influence of ligand expression on NoV binding to oyster tissues was first demonstrated using VLPs. GI.1 VLPs were very efficiently bioaccumulated by *C. gigas* oysters and were detected by immunohistochemistry even at a low level of exposure, whereas a mutant VLP that was unable to recognize the A-like antigen was only detected in oyster tissues at a thousand fold higher concentration [36]. These results were confirmed using a GI.1-positive stool that bioaccumulated very efficiently

in a dose-dependant manner. When these experiments were performed at different times of the year, there was a clear seasonal impact on bioaccumulation efficiency that paralleled expression of the HBGA ligand in oyster digestive tissue [38]. The quantitative approach also showed that the GI.1 NoV directly accumulates in digestive tissues with negligible concentration in other tissues. Performing bioaccumulation using two GII NoV positive stools (one stool positive with a GII.4 and one with a GII.3 strain) led to very different results. These two strains bound to digestive tissues, gills and mantle with a similar pattern [39•]. The GII.4 strain, as well as GII.4 VLPs, was bioaccumulated at very low levels, although they were found in a number of tissues as also reported by others [40,41]. In contrast, the GII.3 strain was efficiently bioaccumulated, although less well than the GI.1 strain, with a transient retention in the gills likely due to binding to sialic acid [39]. In contrast to the findings with the GI.1 strain, no seasonal impact was observed in the bioaccumulation of the two GII NoVs or of the sialic acid containing ligand present in all tissues. Our interpretation of these data is that the GI.1 strain is efficiently accumulated and retained through an HBGA A-like ligand present in the gut. GII strains are less well accumulated because of a sialic acid containing ligand expressed in all tissues that contributes to their retention in the gills and leads to their destruction (or elimination) by an unknown mechanism. The latter process would be more efficient in the case of a GII.4 than of a GII.3 strain.

Shellfish species may also impact bioaccumulation as demonstrated comparing two oysters species (*Crassostrea ariakensis* and *C. virginica*). The GI.1 strain was more efficiently concentrated by *C. ariakensis* and persisted for a longer time compared to *C. virginica* [42••]. It will be interesting to compare the glycan ligand expression between these species.

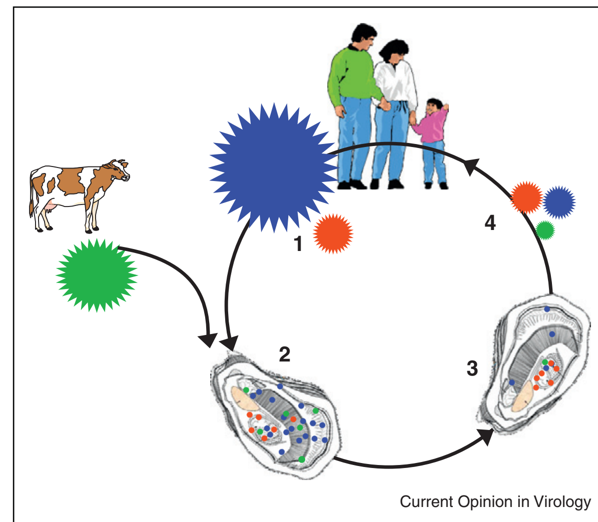
Since many environmental conditions may interfere with oyster's filter capacity and consequently with contamination, a field study was conducted to determine if the above observations performed in laboratory conditions are valid in the environment. Thus, concentrations of GI and GII NoVs in waters collected during a year were compared to concentrations in oyster digestive tissues. As expected, much higher concentrations of GII NoVs than of GI were detected in waters. GI NoVs were concentrated to a greater degree than GII strains, with GI viruses requiring 30 viral RNA copies/L water to bioaccumulate 1 viral RNA copy/g oyster tissue compared to GII viruses that required ~1200 viral copies/L of water to observe 1 viral copy per gram of oyster tissue. These data provide additional evidence for the specific selection and persistence of GI NoVs in oysters. This field study was conducted in an area with a large amount of cattle breeding. Bovine NoVs (GIII) were detected in 14% of water samples at high levels, but only one shellfish sample contained a GIII NoV strain [43]. The αGal HBGA epitope, identified as the virus-specific glycan ligand in bovine tissues [44], was absent from oyster tissues, potentially explaining the poor bioaccumulation efficiency observed for GIII NoV strains.

Conclusion

These data suggest a selective transmission of NoV strains via oysters through specific binding to carbohydrate ligands. Ligands that facilitate bioaccumulation (the A-like antigen) or that contribute to the elimination of the virus (the sialic acid-containing ligand) may both influence NoV accumulation and survival in oysters (Figure 2). For a long time, oysters were believed to act as filters or ionic traps, passively concentrating particles. However, this is clearly not the case for NoVs, especially for NoV GI.1 that is more actively and efficiently concentrated than GII strains. The differential accumulation efficiency provides a possible explanation for the unexpectedly high proportion of GI strains associated with shellfish-related outbreaks.

This new concept demonstrating a special relationship between oysters and NoV should be explored for other

Figure 2



Influence of oyster in the selection of NoV transmission. 1: Shedding in the environment of large amounts of GII NoVs (blue) and much lower amounts of GI strains (red) due to the overwhelming predominance of NoV GII in human outbreaks. Shedding of NoV GIII (green) in cattle is also shown. 2: Viruses present in seawater are ingested by oysters. GI NoVs particles are very rapidly directed to the gut, whereas GII particles are retained in mantle or gills possibly via a sialic acid containing ligand. GIII NoVs are probably randomly distributed. 3: NoV GI and GII are accumulated in the gut via an HBGA A-like ligand, most GII and GIII particles outside the gut are presumably destroyed. 4: Upon consumption of a NoV-contaminated oyster, infection caused by GI and GII strains occur with similar frequency because of the selective accumulation and retention of GI viral particles. GIII NoV transmission is unlikely to happen as few particles persist in oysters and humans do not express the glycan ligand.

enteric viruses, including Aichi virus and oysters, sapovirus and clams, and other foods such as NoV and berries or hepatitis A virus and tomatoes. Food trade may contribute to dispersal of a virus strain, as virus-contaminated imported shellfish have been responsible for outbreaks (Table 1) [66]. A better understanding of virus–food interactions may provide strategies to prevent contamination, to increase viral elimination, and thus to increase consumer safety.

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