
Aichi Virus, Norovirus, Astrovirus, Enterovirus, and Rotavirus Involved in Clinical Cases from a French Oyster-Related Gastroenteritis Outbreak

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Abstract:

Following a flooding event close to a shellfish production lagoon, 205 cases of gastroenteritis were linked to oyster consumption. Twelve stool samples from different individuals were collected. Analysis showed that eight samples were positive for multiple enteric viruses, and one stool sample had seven different enteric viruses. Analysis of shellfish implicated in the outbreak allowed detection of the same diversity of enteric viruses, with some viral genomic sequences being identical to those obtained from stool sample analysis. Shellfish were contaminated by as many as five different enteric viruses. For the first time in Europe, Aichi virus was identified in oyster samples. Shellfish samples collected over 3 weeks following the outbreak showed a progressive decline in the level of virus contamination as measured by the virus diversity detected and by quantitative reverse transcription-PCR.

1 INTRODUCTION

2 Foods play an important role in the transmission of enteric viruses. For example, for
3 noroviruses (NoVs), the predominant agents of non bacterial gastroenteritis in humans, as many
4 as 40% of infections are estimated to be linked to contaminated food consumption (3, 16, 28).
5 Other enteric viruses, including rotavirus (RV), astrovirus (AV) and Aichi virus (AiV), also all
6 cause gastroenteritis with symptoms more or less similar to that caused by NoV, but their
7 etiologic importance in outbreaks is not really known. However, these viruses have occasionally
8 been shown to be transmitted by foods (21). Food may be contaminated at different stages of
9 production, such as fecal contamination of shellfish growing waters, use of nightsoil to fertilize
10 crops, fecal contamination of water to wash fruits after harvest, or poor hand hygiene by an
11 infected food handler (16, 21). While a single strain is usually responsible for clinical cases
12 associated with direct contamination by food handlers, multiple strains may be detected when a
13 poorly functioning sewage treatment plant is responsible for contamination of foods such as
14 oysters.

15 Viruses that cause gastroenteritis multiply in the intestines and are excreted in large
16 quantities in human feces. Human waste is processed in sewage treatment plants, but the
17 treatment procedures do not completely remove enteric viruses from the water effluents leaving
18 the plant (8, 10, 12). Strains that cause severe symptomatic infection as well as those that cause
19 subclinical infection are excreted into sewage. Thus, wastewaters reflect a wide range of strains
20 circulating in the population (8, 13). When the sewage treatment process is overwhelmed, as can
21 occur with some flooding event, accidental contamination of shellfish-growing beds may provide
22 an opportunity for enteric viruses other than NoV to infect people and cause disease.

1 This study reports a recent event that occurred in France following oyster consumption.
2 Stool analyses identified up to seven different strains in one stool. The two major points of this
3 study are firstly the analysis of shellfish related to the outbreak allowing the detection of the same
4 human enteric viruses and secondly, for the first time in Europe the presence of Aichi virus in
5 oyster samples. Environmental investigations identified the leading cause of the lagoon oyster
6 production area contamination, but prevention measures such as prolonged depuration time
7 implemented to satisfy European regulation (*E.coli* counts) for oysters were unable to avoid
8 human contamination.

9

10 **Materials and Methods**

11 **Epidemiological data.** All data concerning clusters of gastroenteritis in the affected area during
12 February 2006 were collected either from medical doctors or directly from sanitary services
13 (DDASS, DDSV). A standardized questionnaire covering foods consumed, symptoms, and
14 timing of illness was completed for each participant in the study. Association between food
15 consumption and illness was estimated by calculation of the relative risk (RR) and its 95%
16 confidence interval (CI 95%) using Epi Info Version 6.

17 **Clinical sample analysis.** Twelve fecal samples collected from twelve patients from three
18 clusters were analyzed. Group A rotaviruses were detected by EIA with group-specific
19 monoclonal antibodies as previously described (1). Astroviruses and adenoviruses types 40 and
20 41 were detected with EIA kits, IDEIA Astrovirus (Dako Diagnostics Ltd.) and Adenoclone type
21 40/41 EIA (Meridian Diagnostics Inc.) respectively. Results were then confirmed by RT-PCR
22 (1). For typing and other enteric virus detection, nucleic acids (NA) were extracted and purified
23 using a QIAmp Viral RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's

1 instructions. NoVs and sapoviruses were detected by several RT-PCRs that amplified regions of
2 the RNA dependent, RNA polymerase and the capsid genes (1, 19), HAV by amplification of a
3 VP1 gene fragment (1), and enteroviruses by amplification of the 5'UTR (1). Typing of AV and
4 G and P typing of RV-A positive samples were performed as reported (1). Aichi virus was
5 detected by amplification of a 519 bp fragment (1).

6 **Shellfish sample analysis.** Sixty-six samples were collected. Four oyster samples (*Crassostrea*
7 *gigas*) directly linked to human consumption (two leftovers from refrigerators in a private home
8 and in a restaurant and two from exactly same batch in producers) were analyzed, as were 62
9 oyster samples collected from the same producing area. Shellfish, kept at 4°C during shipment,
10 were washed, shucked, and the total weight recorded. The stomach and digestive diverticula were
11 removed by dissection, and divided into 1.5 g portions. For analysis, digestive tissues were
12 homogenized, extracted with chloroform-butanol, and precipitated with Cat-floc (Calgon,
13 Ellwood City, PA, USA), followed by polyethylene glycol 6000 (Sigma, St Quentin, France)
14 precipitation (2). Viral nucleic acids (NA) were extracted and purified using proteinase K,
15 phenol-chloroform and CTAB as previously described (19). The NA was suspended in 100µl of
16 RNase-free water with 20 units of RNase inhibitor (Invitrogen) and analyzed immediately or
17 kept frozen (-80°C).

18 *Real-time RT-PCR:* All shellfish NA extracts were first screened by real-time RT-PCR
19 using previously published primers and probe for NoV (8), HAV (7), and EV (9). *rRT-PCR* was
20 performed on a MX3000 (Stratagene, France) or an ABI Prism 7000 SDS detector (Applied
21 Biosystem, France), using the Platinum[®] Quantitative RT-PCR ThermoScript[™] One-Step System
22 (Invitrogen, France). Briefly, 5 µl of undiluted or ten-fold diluted RNA extracts were added in
23 duplicate to 20 µl of a mixture containing 1X of ThermoScript reaction buffer, 200 nM of the

1 probe and primers, 0.5 μ M of Rox reference dye 50 X, 0.5 μ l of Thermoscript Plus/Platinum *Taq*
2 enzyme mix and 2 U of RNase Inhibitor (Applied Biosystems, France). Reverse transcription
3 was performed for 30 min at 50 °C, and denaturation for 5 min at 95 °C, followed by 45 cycles of
4 PCR amplification (denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min).
5 The cycle threshold (Ct) was defined as the cycle at which a significant increase in fluorescence
6 occurred (i.e. when fluorescence became distinguishable from background) (8). Precautions such
7 as isolated rooms for various steps and the usage of filter tips were taken to prevent false positive
8 results. Two negative amplification controls (water) were included in each amplification series
9 and no more than six samples were analyzed in a RT-PCR assay.

10 The number of NoV RNA copies present in positive samples was estimated using standard
11 curves generated from RNA transcripts. In brief, the two first open reading frames of the GI.1
12 Norwalk virus (nt 146-6935) and the sequence between nucleotides 4191 and 5863 of the GII.4
13 Houston virus (Hu/Houston/TCH186/2002/US, Genbank EU310927) were each cloned in the
14 pCRII TOPO (Invitrogen) vector. *In vitro* transcription was performed on linearized plasmid
15 samples using the Promega riboprobe system. After DNase treatment, RNA was purified and
16 quantified by optical density at 260 nm (OD₂₆₀) (10). A standard curve was generated using from
17 5.3 to 530,000 and 7.5 to 750,000 copies of transcript for GI and GII respectively, and the
18 genomic copy number was determined by interpolation using the Ct values generated from the
19 shellfish extracts. To be evaluable, all wells had to yield a Ct value \leq 41. The final concentration
20 was then adjusted based on the NA volume analyzed (5 μ l of 100 μ l of NA extract) and reported
21 per g of DT (1.5g analyzed).

22 The presence of RT-PCR inhibitors was evaluated by co-amplification of 2.5 μ l of each NA
23 extract with 2.5 μ l containing 100 copies of GI or GII RNA internal controls in separate

1 experiments (7). Amplification of RNA indicated that no more than partial inhibition was
2 present; no adjustments to quantitative estimates were made for samples with partial inhibition.

3 *Standard RT-PCR:* The viruses that were detected in samples by real-time RT-PCR were
4 typed by sequencing after amplification with a standard, two-step RT-PCR format using 40 cycles
5 of amplification (17, 19). For NoV, 6 primer sets targeting the polymerase gene and 3 targeting
6 the capsid gene were used (17, 19). In some cases, a semi-nested PCR was performed using the
7 same amplification conditions, taking precautions to avoid cross contamination (each sample was
8 amplified alone and with negative controls) (19).

9 RV detection was performed by amplification of a portion of the VP6 gene and confirmed
10 by hybridization (27). AV was detected by amplification of a small fragment in the 3' non coding
11 region, and positive samples were typed using same primers as for stool samples (1). AiV was
12 detected using primers that amplify the polymerase gene (24, 29). Virus-specific amplicons were
13 identified by liquid hybridization. Nested amplification was performed to generate an amplicon of
14 179 bp which could then be sequenced.

15 **Sequence analysis.** Amplicons from virus-positive samples were purified and sequenced with the
16 BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequences were analyzed
17 through the European Foodborne Viruses Database (<https://hypocrates.rivm.nl/bnwww>; FBVE
18 QLK1-CT-1999-00594) for identification of the NoV genotypes. For other enteric viruses
19 sequence homologies obtained from all samples were evaluated using the BLAST-search program
20 in GenBank.

21 **Environmental investigations.** Oysters implicated in the outbreaks were produced in several
22 sites, all of which were located in one lagoon in southern France. Data from climate events
23 (Meteo France), and epidemiological status of the population (Sentiweb,

1 <http://www.sentiweb.org>) were obtained. At the same time, Ifremer surveillance network (REMI,
2 <http://www.ifremer.fr>) data for shellfish quality (*E.coli* concentrations) were collected.

3

4 **RESULTS**

5 **Epidemiological investigation.** A total of 38 clusters of cases with acute gastroenteritis were
6 traced to oyster consumption between February 2 to 27 (Figure 1). Thirty-one clusters were
7 reported by ill people directly to sanitary services, and seven were reported by physicians. A total
8 of 205 cases with acute gastroenteritis were identified. Vomiting was reported in 96% of cases,
9 diarrhea in 92%, abdominal pain in 92% and fever in 50%. Two persons were hospitalized for
10 one day. Median incubation periods were between 12 and 54 hours. Twenty-two (58%) of the
11 clusters were associated with oysters purchased at market and consumed in private homes.
12 Thirteen (35%) of clusters were associated with oysters consumed in a restaurant. One event
13 occurred after oyster consumption in a school and one in a banquet during a scientific meeting.

14 The largest cluster of cases was on February 3 at the scientific meeting with 77 attendees.
15 Twenty-seven attendees were interviewed by phone on February 10. Participants were adults
16 (between 26 and 59 year old) with a sex ratio of 1.6 (M/F). Eleven attendees had symptoms of
17 gastroenteritis with diarrhea (73%), abdominal pain (64%), vomiting (55%), nausea (82%) and
18 fever (64%). The median incubation period was 39 hours (range, 27 to 60 hours) (Figure 2). The
19 retrospective cohort study showed that people who ate oysters had a 4.5 greater risk of illness
20 compared to people who did not eat oysters (RR= 4.5, IC at 95%: 1.6-13 ;3, p=0.003). Other
21 foods served (fish soup, chicken, vegetables, chocolate mousse) were not associated with disease.
22 Twenty-six of 27 Italian persons who also participated in the meeting were later contacted.

1 Seventeen ate oysters and were ill. Of the nine who did not consume oysters, only one reported
2 gastroenteritis symptoms.

3
4 **Stool analysis results.** A total of 12 stool samples collected from different sick patients were
5 analyzed: two from people who ate oysters during a family dinner (cluster #1), four from people
6 who attended the scientific meeting (cluster #2) and six from people who bought their oysters to
7 the same producer on a market (cluster #3). All stools were negative for adenovirus, sapovirus
8 and HAV. One stool (E1201) had no viral pathogens detected, and the other 11 were positive for
9 at least one virus (Table 1). Nine (75%) samples were positive for NoV, six for AiV (50%), six
10 for EV (50%), three for AV (25%) and two for RV-A (17%). Eight samples were positive for
11 multiple enteric viruses, and one stool had seven different viruses (AiV, AV type 8, EV, NoV
12 GI.1, NoV GII.17, RV G1P[8] and RV G9P[8]). One stool was contaminated only with AiV and
13 two only with NoV. Five stools were contaminated by two different NoV strains. One stool
14 (E1203) had two rotavirus strains G1P[8] and a G9P[8].

15 A large number of different virus strains were identified by sequence analysis. There were
16 seven NoV genotypes (GI.1, GI.2, GII.2, GII.4, GII.7, GII.17, GIIb), two AV types (4 and 8) and
17 three AiV strains.

18
19 **Shellfish analysis results.** Shellfish samples that could be linked to consumers based on
20 consumer, producer, and environmental data were identified (Table 1). Inhibitor removal was
21 evaluated for all samples and only samples showing less than 50% inhibition were considered for
22 further analysis. Two samples left over from consumers (#1739, #140) were positive for AiV,
23 AV, NoV (one having both GI and GII strains) and RV (Table 1). Two other samples (#138,

1 #139) from producers were also virus-positive, but only contained AV (not included in the table
2 as no stool specimens linked to these samples were collected). Sample #1739 had a low level of
3 virus contamination near the limit of detection as estimated by rRT-PCR, but the same
4 approximate value was consistently obtained on repeated testing (about 72-130 RNA copies/g of
5 DT) (Table 2). Repeat analyses of samples from the same lot (except sample #140 for which only
6 a limited number of oysters were available such that only one extraction could be performed)
7 yielded quantitative estimates within 4-fold (2 Ct) of each other, except for that obtained for
8 sample 109 (Table 2).

9 A total of 62 samples were collected from the same production area in the lagoon during a
10 period of 30 days (February 6 to March 8, 2006). Among the 62 samples analyzed within the four
11 weeks period of time (the two last days were added to week 4), seven (11%) samples were virus-
12 negative, 16 (26%) were contaminated by one enteric virus, and 39 (63%) were contaminated by
13 at least two different enteric viruses. Sixteen of the 17 samples collected during the first week,
14 were contaminated by two or more different enteric viruses. Five different viruses were detected
15 in one sample and four viruses in four samples (Figure 3). Of the 15 samples collected during the
16 second week, one was contaminated with 5 different viruses and six with at least two viruses. In
17 weeks three and four, 31% of 13 samples and 40% of 17 samples, respectively, were
18 contaminated by more than one (all with only two) virus strain. AV was detected in a large
19 number of samples (41), primarily during the two first weeks (94% and 78% positive the first and
20 second week, respectively) (Table 3). RV was detected in 15 samples throughout most of the
21 study period, except during the third week (Table 3). NoV was detected in 33 samples with 76%
22 of samples collected the first week being positive, then 33, 30 and 59% for the following weeks.
23 The first week, 12 samples (among 17) were positive for GII NoVs, and GI NoVs were detected

1 in 10 samples, nine samples being contaminated by both genogroups (Table 3). At the end of the
2 study, GII NoVs were detected in 3 samples and GI NoVs in 7 samples (among 17) (Table 3) and
3 no sample was contaminated by both genogroups. The first week, the mean concentration was
4 about 7×10^3 RNA copies/g for GI NoV and 1.3×10^3 RNA copies/g for GII NoV (Table 4). After
5 three weeks the concentrations decreased and reached an average of 2×10^2 RNA copies/ g for
6 each genogroup (Table 4).

7 AiV was detected in five samples (two samples collected the first week, two during the
8 second week, one during the third week and none during the last week) (Table 3). Only one
9 sample was found contaminated by EV and no samples were contaminated by HAV.

10
11 **Sequence comparisons.** Only one sequence (GII.4) could be obtained from the sample #140
12 directly linked to the clinical cases, and none from the sample #1739. The GII.4 sequence
13 obtained by standard RT-PCR targeting capsid region was identical to the sequences obtained
14 from two stool samples (E1204, E1205). For AiV, the same sequence was detected in two
15 shellfish samples (# 115 and 131) and three stool samples (Figure 2). Two other stool samples
16 contained a virus sequence that differed at a single nucleotide (nt), and one shellfish sample
17 collected during the third week contained a virus with three nucleotide changes over the 140 nts
18 sequenced. The same type 8 AV sequence was obtained in one shellfish sample (# 115) and two
19 stool samples (E1203, E1208). Two other AV sequences were identified: a type 4 strain in stool
20 sample E1197 and a type 1 strain in a shellfish sample 107. One additional shellfish sample,
21 collected on February 10, was also positive for AV type 1. For NoV, three genotypes with
22 identical sequences were detected in shellfish and stool samples: two genogroup I (GI.1 and GI.2)
23 and one genogroup II (GII.4). Other genotypes were also detected either in stool or shellfish

1 samples (Table 1). No sequence data could be obtained for shellfish samples positive for EV or
2 RV.

3

4 **Environmental data and sanitary measures.** In 2006, winter gastroenteritis outbreaks reached
5 a peak in the population (up to cases 742 cases/100 000 inhabitants, with an outbreak threshold of
6 182 cases/100 000) in this area of France from January 7 to 15
7 (<http://websenti.b3e.jussieu.fr/sentiweb/>). One week later, heavy rain (138.2 mm) occurred in this
8 area during one week (January 23-29) with 76 mm on one day (Meteo France data). This amount
9 of water within one week or even one day is much more that the monthly average of 65.18 mm
10 shown by data collected over the previous 43 years (since 1963). On January 30, Ifremer shellfish
11 surveillance network (REMI) increased shellfish sampling in this area and set up an alert
12 informing producers. Samples collected on January 30 showed an increase of *E.coli*
13 contamination in the area (concentrations between 700 to 4800 *E.coli* organisms/100g for 9
14 samples). Based on *E. coli* results on January 31, the administration advised producers about the
15 risk and asked them to adopt depuration times to improve shellfish quality based on *E. coli* counts
16 for a period of two weeks. On February 06, all shellfish collected in this area met European
17 regulation requirements (class B, i.e. less than 3600 *E.coli* organisms/100g of shellfish meat). On
18 February 07, the first information on outbreaks was received and thus, on February 10, producers
19 were requested to withdraw shellfish collected between January 30 and February 5 from the
20 market. On February 13, *E. coli* controls showed no evidence of bacterial contamination. On
21 February 20, as human cases were still reported, shellfish collected before February 17 were
22 withdrawn from the market. On March 1, one month after the contaminating event, the area was
23 closed and all marketing of shellfish collected from this lagoon was prohibited for three weeks.

1 On March 20, three shellfish samples were analysed. One was positive for NoV GI (near the
2 limit of detection). The area was reopened and no additional outbreak related to shellfish
3 consumption was reported.

4

5 **Discussion.**

6 Shellfish are known to be vulnerable to contamination with sewage due to their filter
7 feeding activities, and contamination of shellfish growing waters has been a cause of
8 gastroenteritis outbreaks. Food contamination with sewage has often been suspected when
9 multiple strains are detected in food or patient stools, as previously reported with shellfish (4, 5,
10 15, 18, 19). Only noroviruses were detected or reported in prior reports of gastroenteritis
11 outbreaks caused by multiple strains. For example Boxman et al. (5) described a mixed infection
12 in four patients, with one person infected by three genotypes. Two of these strains were detected
13 in implicated shellfish samples. Kageyama et al. (15) reported that the majority of mixed
14 norovirus infections were associated with shellfish consumption. Previously, based on
15 epidemiological investigation and shellfish analysis implicated in the outbreak, we detected
16 multiple norovirus contamination (up to five different NoV strains) involved in the same area of
17 production in Southern France (19).

18 The outbreak reported here is remarkable for the diversity of human enteric viruses
19 detected both in clinical and oyster samples. Up to seven different viruses were detected together
20 in environmental or clinical samples. Few studies have reported mixed infections with several
21 different enteric viruses. In a hepatitis A outbreak linked to frozen clams imported from Peru,
22 rotavirus and enterovirus were also detected in shellfish samples, but no data linked to human
23 illness were reported (4). NoVs and human enteroviruses were detected in both oyster and stool

1 samples in one other report (6). The enteroviruses were suspected to be responsible for secondary
2 symptoms such as myalgias and arthralgias. In the present study, clinical signs could not be used
3 to discriminate the role of the different viruses detected in fecal samples of symptomatic persons.
4 We are also not able to determine whether co-infection with multiple viruses contributed to the
5 severe symptoms reported by some affected persons. However, the large number of enteric
6 viruses involved in the outbreaks and symptom severity may explain the unusually higher number
7 of direct reports to sanitary authorities.

8 To our knowledge AiV was not reported yet in a shellfish outbreak in Europe. AiV was
9 first recognized in 1989 as the cause of oyster-associated nonbacterial gastroenteritis in Japan
10 (29). There is a single previous report of an AiV-associated outbreak in Europe, but no details of
11 the outbreak, which occurred in Germany, were provided (24). AiVs have not been found in
12 other reports of outbreak investigations in Finland (14) and The Netherlands (25). In a
13 retrospective study performed in France, 0.9% of stool samples collected from children between
14 2001 and 2004 were positive for AiV (1). Our previous attempts to detect AiV in shellfish
15 implicated in outbreaks failed (data not shown). The detection of AiV in shellfish from the
16 contaminated harvesting area and the identification of this virus as the sole pathogen found in at
17 least one person's stool sample suggest that AiV contributed to the illness burden seen in these
18 outbreaks.

19 There is still relatively little quantitative information on the levels of NoV contamination in
20 shellfish implicated in outbreaks. Quantification of noroviruses in shellfish is a complex
21 procedure. It is subject to problems with inhibition of the RT-PCR reaction by shellfish tissue
22 components, which can cause false negatives. Several precautions were taken in this study to
23 avoid such false negative (persistence of RT or PCR inhibitors was evaluated) or false positive

1 (separate room, filter tips, several negative controls). When sufficient amounts of shellfish tissues
2 were available, two extractions were performed. All but one sample had quantitative estimates
3 that were within four-fold (two Ct values) of each other. Sample shellfish lot 109 had a larger
4 variation, which may have been due to greater variability in levels of virus contamination among
5 the shellfish collected for this lot. The quantitative estimates reported here are likely to be
6 minimal values, as several factors may have led to underestimating the actual level of virus
7 contamination. For example, nucleic extraction efficiencies using an external added virus, as
8 proposed by Costafreda et al. (7), were not used in this study. In addition, we made no
9 adjustments for PCR amplification efficiency, which may have been adversely affected by partial
10 sample inhibition or by sequence variation in different norovirus genotypes leading to
11 mismatches with the primers used in the real-time assay. The latter concern is offset by the
12 results of previous studies that have demonstrated the broad reactivity of the NoV-specific
13 primers and probes used in the current study (8, 15, 25).

14 The level of NoV contamination in the present report is similar to levels of viral shellfish
15 contamination described for NoV outbreaks (18, 19), for an HAV outbreak (7) and for a field
16 production area in Japan (23). More oyster samples were found to be contaminated with GII
17 NoVs than GI strains in the first week after the contamination event, but after four weeks a
18 greater percentage of samples contained GI NoV strains. Previous studies have shown that GI
19 NoVs are more resistant to sewage treatment (8) and are more often implicated in food related
20 outbreaks than are GII NoV strains (5, 11, 15, 19). The findings in the present study support the
21 hypothesis that GI NoVs are more stable in the environment and may explain the relatively higher
22 frequency in which strains from this genogroup are associated with foodborne outbreaks.

1 Shellfish are regularly consumed in France and it is important to avoid such outbreaks to
2 protect consumer health. The harvesting area, classified as a B area, is known to be sensitive to
3 rainfall events and sewage contamination (19, 22). When heavy rains were observed during the
4 winter gastroenteritis outbreaks, conditions previously responsible for a large shellfish outbreak
5 four years ago, an advisory alert was set up with increased evaluation of bacteriological controls
6 and producer information. Ifremer and sanitary service (DDASS) recommended closure of the
7 production area on January 30, but this advice was not followed by the regional authority. Instead
8 producers were instructed to increase depuration so that shellfish conformed to bacteria
9 contamination regulatory requirements in accordance with European Regulation. This approach
10 was not sufficient and the outbreaks were not prevented, as shown by data presented here. Some
11 shellfish samples, kept for several days in depuration tanks before being sent to the laboratory for
12 analysis, were still contaminated by different types of enteric viruses (data not shown). This is
13 additional evidence that short-term depuration to meet recommended bacterial regulatory
14 requirement is not efficient at removing contaminating viral pathogens. Specific binding of
15 noroviruses to shellfish tissues may contribute to delayed depuration of these viruses (20, 26), but
16 the importance of this mechanism for other enteric viruses is not known at this time.

17 In summary, this study is informative for several points: (1) it presents additional evidence
18 that coliform indicators are not reliable for viral elimination and that depuration process as done
19 is not efficient for enteric viruses; (2) the closure of the harvesting area just after the flooding
20 event as proposed by Ifremer likely would have prevented some of the outbreaks; (3) low levels
21 of NoV were infectious in consumers and the number of virus-contaminated samples decreased
22 slowly over a one month period; (4) many different enteric viruses were found both in stool and

1 shellfish samples showing the potential impact of sewage contamination for emerging strains; and
2 (5) it presents the first documentation of Aichi virus transmission by food in Europe.

3

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- 1 Aichi virus, a new member of the picornavirus family associated with gastroenteritis in
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1 Legends for figures:

2 Figure 1: Clusters occurring during February

3
4 Clusters are reported for each day of February (x axis) and number of clusters reported per day (Y
5 axis). Each cluster is represented by a square and the number of cases per cluster is written in
6 each square.

7
8 Figure 2: Scientific meeting cluster: onset of symptoms

9 Oysters were consumed on February 03 for lunch (arrow). Each box represents one clinical case.

10

11

12 Figure 3: Multiple contaminations observed in shellfish samples over time.

13

14 Black: two or more different enteric viruses detected per sample, grey: one virus detected per
15 sample, white: no virus detected. X axis % of positive samples, y axis: week.

16

17

18 Figure 4: Alignment of Aichi virus sequences obtained from stool and shellfish samples.

19

20 Sequence aligned here are between nucleotide 6298 to 6438 based on the reference strain
21 A846/88 (AB010145)(16), five sequences obtained from stool samples (numbered E1197 to
22 E1207) and three from shellfish samples (131, 115, 152). Conserved nucleotide are represented
23 by a dash (-) and nucleotide differences are shown by base changes.

1 Table 1: Results obtained from stool sample and related shellfish samples.

Cluster	Stool	Shellfish	AiV	AV	EV	RV	NoV GI*	NoV GII*
1-Feb.02	73		-	-	+	-	+ GI.2	-
	74		+	-	+	-	+ GI.1	+ GII.2
		109	+	+	-	-	+ GI.4	-
		1739	+	+	-	+	+ GI	-
2-Feb.03	E1196		-	-	+	-	-	+ GII.7, GIIb
	E1197		+	+	+	-	-	-
	E1201		-	-	-	-	-	-
	E1202		+	-	-	-	-	-
		93	-	+	-	-	-	+ GII.4
		107	+	+	+	-	+ GI.1	+ GII
	110	-	+	-	+	+ GI.4	+ GII	
3-Feb.04	E1203		+	+	+	+	+ GI.1	+ GII.17
	E1204		-	-	-	-	-	+ GII.4
	E1205		-	-	-	-	+ GI.1	+ GII.4
	E1206		-	-	+	-	+ GI.1	-
	E1207		+	-	-	-	+ GI.2	+ GII.7
	E1208		+	+	-	+	-	+ GIIb,
		140	+	+	-	+	+ GI	+ GII.4
		115	+	+	-	-	-	-
	130	-	+	-	-	+ GI.2		

131 + - - + + GI + GII.4

1 *: genotype when available

2

1 Table 2: NoV estimated concentrations found in shellfish related to human cases.

Cluster	Shellfish	NoV GI		NoV GII	
		Genotype	# RNA copies/g DT*	Genotype	# RNA copies/g DT*
1	109	+ GI.4	150-3700	-	
	1739	+ GI	72-130	-	
2	93	-		+ GII.4	1600-2500
	107	+ GI.1	5000-16000	+ GII	DL
	110	+ GI.4	DL	+ GII	DL
3	140	+ GI	2300	+ GII.4	1100
	130	+ GI.2	610-2300	-	
	131	+ GI	260-880	+ GII.4	<DL-79

2 *Numbers represent copy numbers observed from two separate extractions

3 DL: sample too close to the limit of detection for quantification. – : no virus detected

4

1 Table 3: Viral contamination in shellfish over four weeks.

Week	# samples	AiV	AV	RV	NoV	NoV	NoV	Neg.
					Both GI+GII	GI only	GII only	
1	17	2	16	6	9	1	3	0
2	15	2	12	4	2	3	1	0
3	13	1	6	0	1	2	1	6
4	17	0	7	5	0	7	3	1

2

3

1 Table 4: Detection and quantification of NoV in shellfish samples.

Week	# samples	Genogroup I		Genogroup II	
		Nb pos. (%)	Mean conc*	Nb pos. (%)	Mean conc*.
1	17	10 (59)	6900	12 (70)	1300
2	15	5 (33)	3100	3 (20)	120
3	13	3 (23)	120	2 (15)	DL
4	17	7 (41)	220	3 (17)	200

2

3 *: concentration expressed in RNA copies / g of digestive tissues.

4

5

6

7

8

Figure 1:

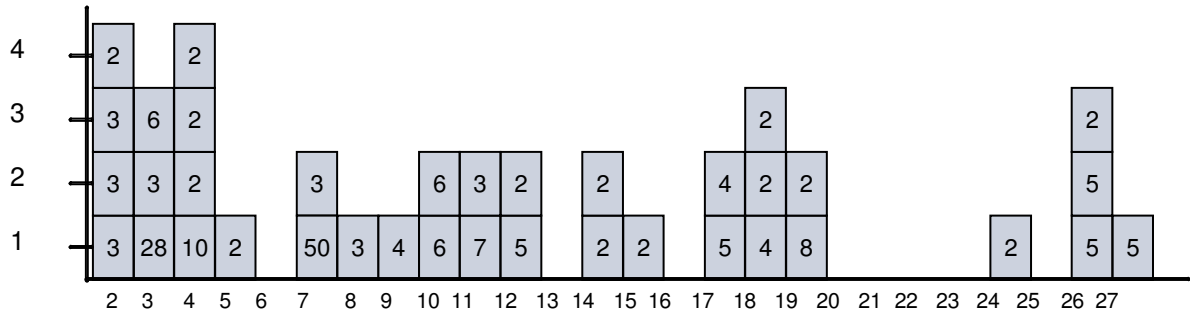


Figure 2:

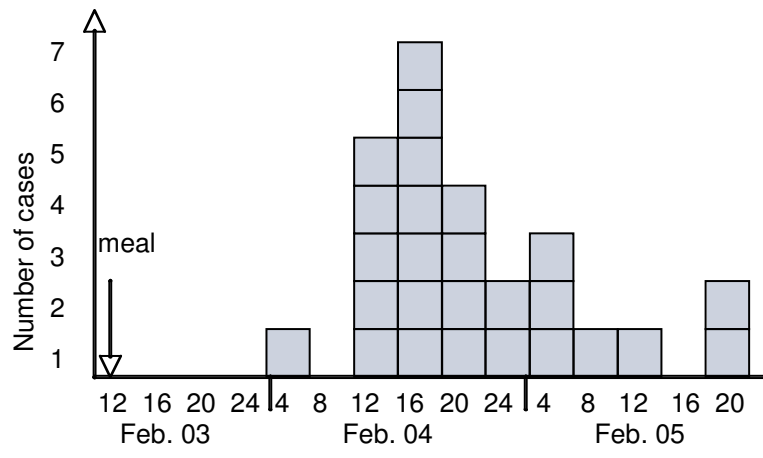


Figure 3:

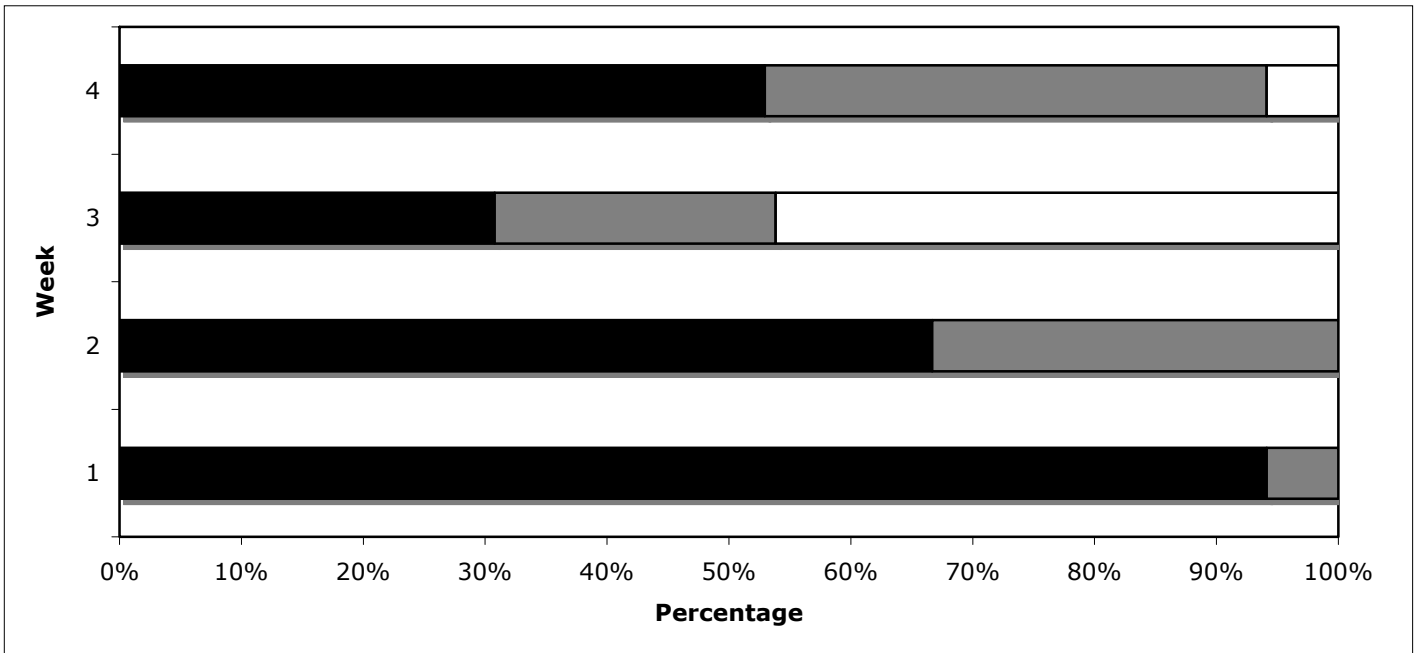


Figure 4

```

A846/88 cttcatcgggcgcccatccccaccccactggactccttgtttcccaattcaaagctgcccc
E1197 -----t-----
E1202 -----t-----
E1203 -----t-----g-----
E1208 -----t-----
E1207 -----t-----g-----
115 -----t-----
131 -----t-----
152 -----
  
```

A846/88 cctactatgtccgcatctccgacaaccggatcctggaccttgacttccccggagtcgtcg
E1197 t-----c-----
E1202 t-----c-----
E1203 t-----c-----
E1208 t-----c-----
E1207 t-----c-----
115 t-----c-----
131 t-----c-----
152 t-----c-----

A846/88 tctgtaagcagggcgtacgga
E1197 ----c--a-----
E1202 ----c--a-----
E1203 ----c--a-----
E1208 ----c--a-----
E1207 ----c--a-----
115 ----c--a-----
131 ----c--a-----
152 ----c-----