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FULL PAPER

Occurrence of two Norovirus outbreaks in the same cafeteria in one week

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SUMMARY

In October 2017, two outbreaks of gastroenteritis (GE) occurred among patrons of a cafeteria in Italy in one week. Virological and bacteria investigations on stool samples, environment and food were conducted to identify the infectious agents and the possible source of infection.
Forty-five cases occurred in the two outbreaks, including 13 laboratory-confirmed cases of norovirus GI. Nine staff members were interviewed, six were confirmed positive for NoV GI and 3 experienced GE symptoms. Bacteria faecal indicators and other bacteria pathogens were not detected in either environmental swab samples or food. A low level of NoV GII was detected in two environmental swab samples. The same GI.6 strain was identified in cases related to both outbreaks, suggesting a common source of infection. Since the two outbreaks occurred in one week, the NoV contamination could have persisted in the cafeteria. Furthermore, virological investigation revealed confirmed cases among food handlers who had worked at the cafeteria between and during the two outbreaks. Several studies highlighted the importance of excluding symptomatic food handlers to prevent contamination of foods and environment.

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INTRODUCTION

Noroviruses (NoVs; genus norovirus, family *Caliciviridae*) are the main causative agents of viral acute gastroenteritis (AGE) in humans worldwide (van Beek *et al.*, 2013), frequently associated with the occurrence of outbreaks. NoVs are small (26-35 nm), non-enveloped icosahedral viruses with a single-stranded positive sense RNA. The viral genome (7.5 to 7.7 kbp) consists of three open reading frames (ORFs). ORF1 encodes a large polyprotein that is cleaved into six non-structural proteins involved in viral replication. The ORF2 encodes the major structural capsid protein (VP1) and the ORF3 encodes a minor structural protein (VP2). Currently, NoVs are classified into six genogroups (Zheng *et al.*, 2006; Martella *et al.*, 2009) and genogroup 7 has been proposed (Vinje 2015). Strains belonging to GI, GII and GIV genogroups infect humans. Based on the amino acid sequence of the full-length capsid region, genogroups are further divided into approximately 40 genotypes (Kroneman *et al.*, 2013).
Genogroup II genotype 4 (GII.4) is the predominant genotype across the globe, identified in 62% of outbreaks (Siebenga *et al.*, 2009; Ahmed *et al.*, 2014; Verhoef *et al.*, 2015). A new antigenic variant of the GII.4 emerges every 2-3 years to escape human herd immunity, replacing the previous variant (Siebenga *et al.*, 2007; Lindesmith *et al.*, 2012).
In countries with active surveillance of NoVs, the virus is responsible for a high percentage of GE outbreaks, 47% as reported in Spain and up to 96% in the US (Atmar and Estes 2006; Patel *et al.*, 2008). The symptoms of norovirus infection are diarrhoea and vomiting and, less frequently, nausea, abdominal cramps and headaches.
Due to the low infection dose of NoV, the virus is easily transmitted and the occurrence of secondary cases is frequent. Infection occurs primarily via person-to-person transmission through the faecal-oral route (Tung-Thompson *et al.*, 2015), but foodborne and waterborne are also common routes of transmission (Maunula *et al.*, 2005; Di Bartolo *et al.*, 2011). Food can be contaminated by human faeces through irrigation water (e.g., soft fruits or vegetables) or by harvesting shellfish from contaminated water without appropriate depuration or indirectly by unhygienic food handling.
NoVs belonging to the GI are more frequently detected in environmental water settings (Fuentes *et al.*, 2014) and in outbreaks linked to consumption of contaminated water or shellfish (Mathijs *et al.*, 2012; Kokkinos *et al.*, 2017), while the NoV GII are frequently detected in patients in all age classes (Nguyen *et al.*, 2017), causing the majority of the outbreaks reported.

Key words:
Norovirus, outbreak, environmental contamination, gastroenteritis, foodborne.

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The Public Health Unit of the Autonomous Province of Bolzano (Northern Italy) received notification of gastrointestinal illness in customers who had attended a private birthday meal at a cafeteria on the 6 and 14 October 2017. The outbreak investigation was initiated to identify the infectious agent and possible source of infection.

MATERIALS AND METHODS

A probable case was defined as a person who displayed at least two of the following symptoms: vomiting, diarrhoea, nausea or abdominal pain, 12-48 hours after lunch at the cafeteria on 6 October 2017 or dinner on 14 October 2017. Confirmed cases were probable cases that also had NoV RNA detected in stools by real time RT-PCR. A total of 24 and 47 guests attended the lunch and the dinner respectively, of which only 33 were interviewed to outline the epidemiological analysis. Nine food handlers who worked at the restaurant during the two outbreaks were also interviewed. A questionnaire was administered to collect information on demographic data, symptoms and food consumed during the aforementioned events. Informed consent was obtained from all individual participants in the study.

The Local Health Authority conducted the environmental inspection on 9 October 2017 after the first outbreak notification. Good hygienic practices were examined at the restaurant. Seven environmental swabs on surfaces (handles, kitchen surfaces), 6 foods stored in the fridge or at room temperature in the cafeteria (raw julienne vegetables, partially boiled rice used for stuffing, mayonnaise, meat, burrata-cheese) and 1 leftover (tartar sauce) were collected (*Table 1*). Food and environmental samples were tested at the Biological Laboratory of the Agency for Environment and Climate Protection of the Autonomous Province of Bolzano. Foods were tested for total mesophilic count (ISO 4833-1:2013), mesophilic lactic acid bacteria (ISO 15214:1998), *E. coli* (ISO 16649-2:2001), coagulase positive Staphylococcus (ISO 6888-2:1999/Amd 1:2003), *Bacillus cereus* (by ISO 7932:2004), *Clostridium perfringens* (ISO 7937:2004), *Salmonella* (AFNOR BRD 07/06-07/04), *Listeria monocytogenes* (AFNOR BIO 12/2-06/94), staphylococcal enterotoxins (ANSES-EULR-VIDAS Staph enterotoxin II SET 2 Version 5:2010). Norovirus GI and GII were tested in partially boiled rice (used for stuffing) and julienne vegetables according to ISO/TS 15216-2:2013 because the method was considered unsuitable for the other food (*Table 1*).

Extraction of viral RNA was performed from 25 gr of food according to ISO /TS 15216-2:2013; treatment with pectinase was not performed.

Following the same procedures, total bacterial count, *E. coli*, *Enterobacteriaceae* (ISO 21528-2:2017), coagulase positive *Staphylococcus*, *Bacillus cereus*, *Clostridium per-*

Table 1 - *Sampling scheme and performed analysis.*

Sampling Date	*Type*	*Source*	*Analysis*	*Outcome*
9 October	ES	Flush toilet device (toilet used by food handlers)	TMC, BCER, ENT, ECO, STF+, CPER, LST, SLM	Negative
	ES	Working surface (kitchen)	TMC, BCER, ENT, ECO, STF+, CPER, LST, SLM, NoV	Negative
	ES	Blast chiller handle (kitchen)	TMC, BCER, ENT, ECO, STF+, CPER, LST, SLM, NoV	Negative
	ES	Door handle (dining room)	NoV	Negative
	ES	Fridge handle (larder)	NoV	Positive NoV GII
	F	Julienne vegetables	TMC, MLAB, ECO, BCER, STF+, CPER, LST, SLM, TOX, NoV	Positive NoV GII
	F	Tartare sauce[c]	TMC, MLAB, ECO, BCER, STF+, CPER, LST, SLM, TOX,	Negative
	F	Rice partially boiled (for stuffing)	TMC, MLAB, ECO, BCER, STF+, CPER, LST, SLM, TOX, NoV	Positive NoV GII
	F	Mayonnaise	TMC, MLAB, ECO, BCER, STF+, CPER, LST, SLM, TOX,	Negative
	F	Burrata-cheese	ECO, LST, SLM, TOX, STF+, BCER, CPER, STEC	Negative
	F	Bovine raw meat	ECO, CPER, LST, SLM, CAMP, STEC	Negative
18 October	ES	Working surface (kitchen)	NoV	Negative
	ES	Blast chiller handle (kitchen)	NoV	Negative
	ES	Flush toilet device (toilet used by food handlers)	NoV	Negative
	ES	Fridge handle (larder)	NoV	Negative
	ES	Wash handle	NoV	Negative
	ES	Oven handle	NoV	Negative
	ES	Door handle (Dining room)	NoV	Negative
	ES	Door handle (Bar)	NoV	Negative
	ES	Locker surface	NoV	Negative

ES: Environmental swab, F: Food, TMC: total mesophilic count, BCER: *Bacillus cereus*, ENT: *Enterobacteriaceae*, ECO: *E. coli*, STF+: coagulase positive *Staphylococcus*, CPER: *Clostridium perfringens*, LST: *Listeria monocytogenes*, SLM: *Salmonella*, CAMP: *Campylobacter* spp, STEC: shiga-toxin producing *E. coli*, NoV: norovirus GI-GII, MLAB: mesophilic lactic acid bacteria, TOX: staphylococcal enterotoxins; [c]leftover food consumed during the dinner on 6 October 2017.

fringens, *Salmonella* and *Listeria monocytogenes*; NoV GI and GII were also tested in environmental swab samples collected after the first outbreak to identify possible faecal contamination and presence of gastrointestinal pathogens (*Table 1*).

A second environmental inspection was organized on 18 of October 2017, after the second outbreak, to review food preparation practices; ten additional environmental swabs (handles and work surfaces) were sampled and analysed for NoV (*Table 1*).

Stool specimens were collected from 21 guests and 9 food handlers.

Nucleic acid extraction was performed from 10% faecal suspension (w/v) using the STARMag 96x4 Universal Cartridge Kit on the MicroLab Nimbus System (Seegene, Korea) following manufacturer's instructions.

A multiplex real-time reverse transcription PCR (RT-PCR) assay for detection of 6 gastrointestinal viruses (Norovirus GI, Norovirus GII, Rotavirus group A, Astrovirus, Sapovirus and Adenovirus) was performed using the kit Allplex™ Gastrointestinal Panel 1 Assay (Seegene, Korea) on the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Italy).

RNA from samples positive for norovirus by real-time RT-PCR was also analysed by end-point RT-PCR, with primer sets G1SKF/G1SKR and G2SKF/G2SKR annealing to ORF2 and specific for GI and GII genogroups, respectively (Kojima *et al.,* 2002). Furthermore, a one-step RT-PCR using primers JV12 and G1SKR which amplify a region overlapping the ORF1/ORF2 junction (814 bp of the RdRp, C-terminal ORF1 and 330 bp of the capsid gene N-terminal ORF2) was performed on three samples, followed by a semi-nested PCR, using JV12 and JV13 primers set annealing to the ORF1 region (Vinje and Koopmans 1996). DNA amplicons were purified by the QIAquick PCR Purification Kit (Qiagen, Milan, Italy) or the ExoSAP (Affimetrix, USA) enzyme, and sequences were performed by Eurofins (Italy). Sequencing data were edited and aligned using MEGA6. The genotypes were assigned using the public database Noronet typing tool (http://www.rivm.nl/mpf/norovirus/typingtool). Nucleotide sequences have been submitted to GenBank database under accession numbers: MH569698-99; MH569690-97.

Faecal samples were also tested for bacteria (Salmonella, Shigella and Campylobacter).

RESULTS

The first group of 19 cases occurred 12-24 hours after a private lunch (24 participants) on 6 October 2017 at the cafeteria. One week later, on 14 October, a second epidemic event occurred after a private dinner at the same cafeteria. In the second outbreak 25 out of 47 (53.2%) experienced GE symptoms; all cases were recorded on 16 October (incubation period ~24 hours).

Nine food handlers were interviewed after the first outbreak; three reported experiencing GE 12-24 hours after the private lunch on 6 October and did not work at the cafeteria during the week. The six employees who did not experience symptoms worked during and between the two events linked to the outbreaks (6 and 14 October).

Among the 33 interviewed cases, 20 (60.6%) were female and 13 (39.4%) were male. The median age of cases was 25.5 years, ranging from 1 to 84 years.

Virological investigation was conducted on 11 cases (out of 19) and 2 asymptomatic people from the first outbreak, and 8 cases (out of 25) from the second. Stool samples were also collected from food handlers enrolled after the first outbreak.

Overall, 9 out of 11 and 4 out of 8 stools from probable cases from the first and the second outbreaks, respectively, were found positive for NoV GI as revealed by Real time RT-PCR. Results from the two stools from the asymptomatic subjects gave the same positive NoV GI results. No other viruses were detected. Among the food handlers, six subjects were found positive for NoV GI, of whom only three experienced GE. Investigated bacterial pathogens (Salmonella, Shigella and Campylobacter) were not detected in stool samples.

A similar menu was served at both events (Tartare Salmon and steak Tartare, prawn tempura, herb risotto, cake, calamari salad, fried vegetables). All the guests, including the food handlers, consumed the same meal, except for one subject who did not experience GE symptoms and did not consume any food from the cafeteria except the cake. The cake was not prepared at the cafeteria but bought outside.

To ascertain if any food was contaminated, during the Local Authority inspection after the first outbreak, food was sampled and analysed (*Table 1*). No leftovers were available except for the tartare sauce, which was tested only for bacteria and gave a negative result. Among the food items sampled, only julienne vegetables and partially boiled rice (rice used for stuffing) were tested for norovirus and both samples were found positive for NoV GII (Ct value >39).

The other food items were found negative for all other pathogens tested (*Table 1*).

No additional food was collected after the second outbreak.

After the first outbreak, swabs were collected from the kitchen (handles and working surfaces) and the toilet used by the food handlers. Only one swab from the fridge handle was positive for NoV GII with a Ct value =39 (*Table 1*). The results of other analyses were negative or in compliance with hygienic and storage parameters. After the second outbreak, the cafeteria was closed. Thorough disinfection was performed, following instructions from the Local Authority and education on Good Practices was conducted among food handlers. After that, environmental swabs were collected (18 October 2017) from the kitchen and tested for NoV. None of the environmental samples were positive for norovirus.

NoV genotype identification was based on the sequencing of 330 bp in the ORF2 gene and of 326 bp in the RdRp gene, conducted on 9 guests from both outbreaks and on 5 food handlers.

Sequences of the capsid fragments were assigned to GI.6 by matching with the Noronet typing tool. For three samples, the RdRp fragments were also sequenced and matched with GI.Pb, confirming occurrence of a recombinant strain, GI.Pb_GI.6.

Sequence comparisons showed 100% nucleotide identity of the NoVs detected during the two outbreaks. Unfortunately, no sequences from the environmental samples could be obtained.

DISCUSSION

The two outbreaks of GE described in the study occurred in the same setting (a small cafeteria) in one week and

were linked to norovirus infection as confirmed by epidemiological (high attack rate; short incubation period and symptoms) and virological (detection of Norovirus RNA in stool specimens) evidence. Although stool samples were available from a limited number of subjects from the two outbreaks, laboratory investigation confirmed the involvement of a single identical norovirus strain typed as GI.Pb_GI.6 in both outbreaks. This data confirmed a persistent source of norovirus contamination that unfortunately we could not identify. We could not conduct a case control study because no controls were interviewed. However, we can exclude contamination in the kitchen environment or in food because samples investigated for norovirus resulted negative for NoV GI. Nevertheless, poor hygienic conditions were confirmed by revealing NoV GII on the handle of the fridge and in two food ingredients sampled after the first outbreak that, although not consumed in the meals, were stored in the cafeteria kitchen. The detection of different genogroups of NoV in the confirmed cases and in the environment was surprising. We ruled out possible cross-contamination during the virological test in humans, food and environmental samples by re-testing all samples. Unfortunately, due to the low level of contamination in food and environment it was not possible to sequence the NoV GII samples. The presence of double infections was also excluded because all samples were tested with Real-Time RT-PCR that distinguishes GI and GII. Likewise, none of the human samples was positive for GII and none of the environmental samples was positive for GI. The hypothesis is that since GII noroviruses circulate very commonly in people, the environment in the kitchen was contaminated by the GII NoV and therefore could have contaminated the environment and food items. The GI causing the outbreak was not derived from the kitchen or from food, which were negative for NoV GI.

The occurrence of two epidemic events in the same cafeteria in one week generates the hypothesis that either the source of contamination persisted in the environment and caused the second outbreak or that it was linked to cafeteria employees. Six food handlers, three of whom declared GE symptoms after the first epidemic event, were found positive for NoV GI.Pb_GI.6 and were working in the cafeteria during both events. Shedding of NoV may continue several days after recovery from symptoms and can also be shed in asymptomatic patients. Prolonged shedding of norovirus makes contamination probable (Sabria *et al.*, 2016), although we are uncertain if foods or the environment were contaminated by workers since NoV GI was not detected. Furthermore, cafeteria employees had not declared symptoms before the first outbreak.

The source of contamination causing the two outbreaks was not identified; however, food business operators should exclude employees with a foodborne transmissible infection from work for 48 hours after symptoms stop naturally (Food Standard Agency, 2009). As revealed by our study and widely described in the literature, the shedding of the virus in faeces can also occur in the absence of symptoms (Teunis *et al.*, 2015). NoVs are highly resistant in the environment, can remain infectious for two weeks on surfaces, and are also resistant to common decontamination practices; a <2log reduction was measured by washing the hands with regular soap (Cook *et al.*, 2016). Therefore, a bleach of 1000 p.p.m. sodium hypochlorite is recommended for disinfection. Environmental contamination with NoV positive fomites has been indicated frequently as a transmission route of infection, with the potential to maintain a sustained series of outbreaks aboard a cruise ship, hotel and football team (Towers *et al.*, 2018). Something similar could have occurred at the cafeteria; no other cases were reported after appropriate cleaning procedures. In Italy, NoV surveillance is still limited. The disease is now recognized worldwide as a public health concern causing a high number of cases with great economic loss and serious health consequences in children and the elderly (de Graaf *et al.*, 2016). An appropriate communication of NoV impact on public health and on how to manage NoV outbreaks or NoVs infection among food handlers is continuously needed to prevent the spread of the virus and the occurrence of outbreaks.

Conflict of interest

The authors declare no conflict of interest.

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