

Waterborne norovirus outbreak during a summer excursion in Northern Italy

Ilaria Di Bartolo¹, Enrico Pavoni², Silvia Tofani¹, Marta Consoli², Elisa Galuppini², Marina Nadia Losio², Franco Maria Ruggeri¹, Giorgio Varisco²

¹Istituto Superiore di Sanità, Department of Veterinary Public Health and Food Safety, Rome Italy;

²Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, "B. Ubertini", Department of Food Safety, Brescia Italy

SUMMARY

In September 2011, an acute gastroenteritis outbreak affected 33 children in Northern Italy. Patients had drunk river water during an excursion. Identical GI.4 norovirus genomes were detected from one patient's stools and from the river water. Improper discharge of human sewage into the river may have caused this waterborne outbreak.

KEY WORDS: Norovirus, Outbreak, Waterborne, Genogroup I, GI.4.

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Norovirus (NoV) is a leading cause of acute non-bacterial gastroenteritis worldwide. The virion contains a single-stranded RNA genome of 7.2kb, which comprises three open reading frames (ORF1-3). ORF1 codes for a large polyprotein including the viral RNA-dependent RNA polymerase (RdRp) and other non-structural proteins, ORF2 encodes the major capsid protein VP1, and ORF3 a protein (VP2) of unknown function (Atmar and Estes, 2006).

NoVs can be assigned to five distinct genogroups based on their nucleotide sequences. Three genogroups (GI, GII, and GIV) infect humans (Zheng *et al.*, 2006), whereas GIII and GV infect bovines and mice, respectively. The high genetic diversity among NoVs is also demonstrated by the existence of more than 30 different genotypes within the known genogroups (Kroneman *et al.*, 2013). Besides, NoVs also exhibit high environmental stability, low infectious dose, and high attack rates in the course of outbreaks (Atmar and Estes, 2006).

The main transmission route of NoVs is per-

son to person, but foodborne or waterborne transmission has also been frequently associated with norovirus infection (Kroneman *et al.*, 2008). In particular, NoVs are recognized as the third most frequent pathogens involved in waterborne disease (Seitz *et al.*, 2011). Several large foodborne and waterborne NoV outbreaks have been described, although epidemiological and environmental data have been confirmed by molecular analyses only in few reports. Inadequate water treatment is the most frequent cause of waterborne outbreaks (Seitz *et al.*, 2011), as recently described also in Italy (Di Bartolo *et al.*, 2011; Giammanco *et al.*, 2014).

This study describes a gastroenteritis outbreak in September 2011 involving 33 guests of a hotel in Northern Italy who developed acute symptoms after a daily excursion in the countryside near a small river.

Patients were aged between 8 and 18 years, and developed diarrhoea and vomiting within 12-36 hours after the excursion. The primary case was defined by the presence of vomiting and acute diarrhoea (>2 episodes) within 3 days after exposure. The symptoms of the first case began in the afternoon of September 1, and the last case occurred the day after during the night. Nine subjects were examined in a local hospital, being dismissed after six hours when symptoms had regressed.

Corresponding author

Ilaria Di Bartolo

Istituto Superiore di Sanità

Department of Veterinary Public Health and Food Safety

Viale Regina Elena, 299 - 00161 Rome, Italy

E-mail: ilaria.dibartolo@iss.it

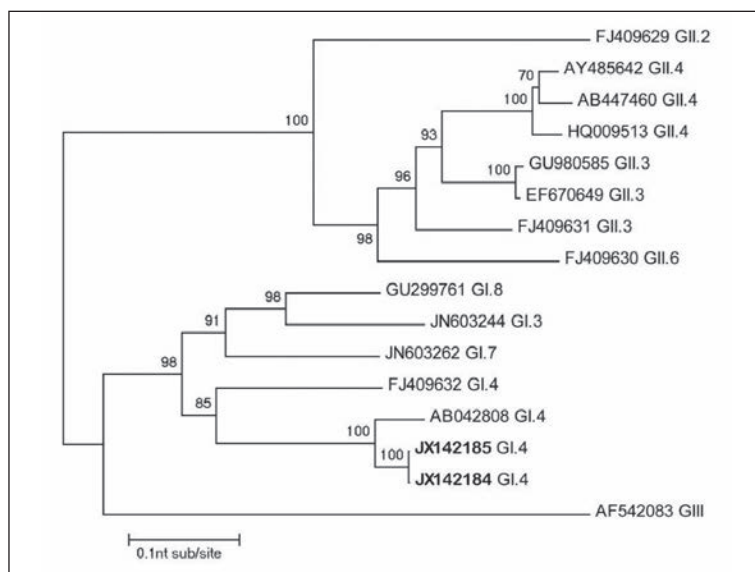


FIGURE 1 - Neighbor-joining tree, based on a 900nt genomic fragment, including 700nt of the RdRp (ORF1) and 200nt of the capsid region (ORF2). Relevant bootstrap values (100 replicates) are indicated. Reference human norovirus strains with the accession numbers are reported: GII.2, GII.4, GII.3, GII.6, GI.8, GI.3, GI.7, GI.4, GIII (bovine norovirus). Strains detected in this study are indicated in bold.

An 8-year-old girl had severe symptoms, and was kept in the hospital for a longer time. Only one faecal sample and a rectal swab were made available for virological analysis. Twenty-four hours after the index case, both parents of this patient also developed gastrointestinal symptoms. Although they had not participated in the excursion, they had taken care of their sick child. An interview was conducted to collect information on the patients and on the possible source of infection. During the tour, all cases had drunk the river water flushing out of a collecting pipe. None of the food items consumed in the 72 hours before symptoms onset was associated with an increased risk of infection.

Water samples (500 ml) were collected from both the pipe (1 sample) and the river (2 samples), and filtered through glass wool filter (Lambertini *et al.*, 2008). The flow-through was treated with $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ (0.005M), adjusted to pH 3.5, and filtered again through a positively charged 0.45- μm nitrocellulose membrane (Zetapore, Millipore S.p.A., Italy). Virions were eluted from the membrane with a solution containing 3% beef extract in water, pH 9.5, and used for RNA extraction (1 ml).

The rectal swab sample was soaked and vortexed in 1 ml of water, and the suspension was treated for RNA extraction. The faecal sample (0.5 g) was suspended in 0.5 ml of water at 4°C, vortexed for 2 min, and centrifuged at 10.000xg for 5 min. For all samples, viral RNA was ex-

tracted using QIAamp viral RNA kit (Qiagen S.r.l., Germany) following the manufacturers' instructions.

Six microliters of viral RNA were retro-transcribed using random hexamers and the commercial kit GeneAmp RNA PCR (Lifetechnologies, USA). Briefly, the MuLV Reverse Transcriptase (2.5 U/ μl) was added to the sample in the presence of RNase Inhibitor (1 U/ μl), random hexamers (2.5 μM), dNTPs (4 mM), and 1X specific buffer. Reverse transcription was performed at 45°C for 60 minutes.

The resulting cDNA was amplified using two distinct Real-Time PCR protocols, respectively specific for GI and GII NoV genogroups (da Silva *et al.*, 2007). To further confirm the presence of NoV, a conventional PCR was also conducted using a booster PCR approach (De Medici *et al.*, 2007). To perform sequence analysis of a longer DNA stretch the positive samples were further tested by a conventional end-point RT-PCR using the Superscript III One-step RT-PCR System with Platinum Taq DNA polymerase (Lifetechnologies, USA), with primers JV12 (Vinje and Koopmans, 1996) and G1SKR or G2SKR (Kojima *et al.*, 2002), to amplify a 1144nt fragment that includes 814nt of the RdRp (C-terminal ORF1) and 330nt of the capsid gene (N-terminal ORF2). Reactions were performed following the manufacturer's instructions, with annealing temperatures of 50°C and an extension time of 90 s. DNA amplicons were purified by the QIAquick

PCR Purification Kit (Qiagen, S.r.l., Germany), and sequences were performed by Genechron Laboratory (Ylichron S.r.l. - ENEA - Rome, Italy). Only one out of three water samples and both faecal and swab samples resulted positive by both Real-Time PCR and conventional PCRs. The faeces, the rectal swab and water samples analysed were positive for NoV GI by Real-Time PCR, with Ct values of 26.7, 25, and 29.3, respectively. A quantitative Real-Time was not conducted, the Ct values were determined on the machine default threshold. Two DNA amplicons obtained using GI specific primers, amplifying a 1144nt genome fragment that overlaps ORF1 and ORF2 genes, were subjected to nucleotide sequencing (Acc. no. JX142184 and JX142185) and analysed against the NCBI GenBank database, confirming the involvement of a GI.4 norovirus strain (93% nucleotide identity with the prototype strain Chiba Acc. no. AB042808). Sequence comparisons showed 100% nucleotide identity among the NoVs detected in river water and in the clinical samples from the patient investigated, corroborating the epidemiological data and suggesting that NoV contaminated water was the source of the outbreak.

Although all subjects involved in the outbreak were housed in the same hotel, no risk factor was identified other than participation in the river-side excursion. In particular, no correlation was found with consumption of any food. No cases of acute gastroenteritis were described among the population of the nearby village in the same period, although possible cases might have not been reported to the local health authority.

Norovirus transmission by contaminated river water was supported by the observation that disease occurred only in subjects who had consumed water from the river pipe, whereas the sick parents assisting a NoV-positive daughter most certainly represent intra-household secondary cases. An environmental investigation was conducted in the area to identify the possible origin of faecal pollution. An on-site inspection revealed a small village in the upstream proximity of the river, suggesting that the river might have been contaminated with human sewage probably caused by an improper spill over. Although the main mode of transmission of norovirus is person-to-person, foodborne and to a

lesser extent waterborne outbreaks altogether amount to more than 10% of cases in Europe (Kroneman *et al.*, 2008). Often, person-to-person transmission also participates in food and waterborne outbreaks, supporting secondary cases and rapid spreading of the infection, and detection of NoVs in water and food is hampered by the low levels of virus and by its high genetic variability. These reasons sometimes make it hard to identify the primary source of infection with certainty (Kroneman *et al.*, 2008; Verhoef *et al.*, 2011).

It is interesting to note that the NoVs causing the outbreak belonged to the GI.4 genotype, which has been frequently associated with waterborne outbreaks in several countries (Nenonen *et al.*, 2012), including a norovirus outbreak due to consumption of contaminated water recently reported in Northern Italy (Di Bartolo *et al.*, 2011). This might be in line with the hypothesis that GI norovirus genotypes exhibit a higher resistance and longer persistence in the environment than GII (Perez-Sautu *et al.*, 2012). Unfortunately, it was not possible to collect and analyse faecal samples from other subjects taking part in the excursion or from the parents of the only patient hospitalized. Nonetheless, the abrupt onset, the kind and short duration of symptoms within the tourist group, as well as the immediate occurrence of secondary cases, make it reasonable to conclude that at least one NoV strain of human origin caused the outbreak. In Italy, norovirus remains neglected as an important cause of acute gastroenteritis outbreaks. Nevertheless, several waterborne outbreaks have been described, associated with exposure to surface or drinking water in tourist resorts or municipal communities (Boccia *et al.*, 2002; Rizzo *et al.*, 2007; Migliorati *et al.*, 2008; Di Bartolo *et al.*, 2011; Giammanco *et al.*, 2014). The identification of the source of infection guided the implementation of proper hygienic measures to control the spread of infections. Further efforts should be undertaken to promptly investigate epidemic cases like the present one, extending early microbiological testing from only the classical bacterial enteropathogens also to norovirus, regardless of the possibly rapid cessation of the episode.

Sequences Acc. no.: JX142184- JX142185

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