Investigation and control of a Norovirus outbreak of probable waterborne transmission through a municipal groundwater system

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ABSTRACT

During March 2011 an outbreak of gastroenteritis occurred in Santo Stefano di Quisquina, Agrigento, Sicily, Italy. Within two weeks 156 cases were identified among the 4,965 people living in the municipality. An epidemiological investigation was conducted to characterize the outbreak and target the control measures. A case was defined as a person developing diarrhea or vomiting during February 27–March 13, 2011. Stool specimens were collected from 12 cases. Norovirus (NoV) genotype GII.4 variant New Orleans 2009 was identified in stool samples from 11 of 12 cases tested (91.7%). Epidemiological investigations suggested a possible association with municipal drinking water consumption. Water samples from the public water system were tested for NoV and a variety of genotypes were detected during the first 3 months of surveillance, including GII.4 strains belonging to different variants from that involved in the gastroenteritis outbreak. Contamination of the well and springs supplying the public water network was eventually thought to be the source of the NoV contamination.

Key words | drinking water, Italy, Norovirus, outbreak, waterborne

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INTRODUCTION

Noroviruses (NoVs) are considered as the major cause of acute gastroenteritis (AGE) in both children and adults, being responsible for sporadic cases and outbreaks worldwide (Patel *et al.* 2008; Matthews *et al.* 2012). NoVs are transmitted primarily through the fecal–oral route either by direct person-to-person spread or by consumption of contaminated food or water (Patel *et al.* 2009). It has been estimated that 10–100 virions of NoV are capable of causing human infection (Lindesmith *et al.* 2003). NoVs have been detected in both raw and treated sewage waters (van den Berg *et al.* 2005; Bosch *et al.* 2008; Calgua *et al.* 2013). Treated sewage waters are frequently discharged into surface waters and may contaminate them if they still contain viruses (Laverick *et al.* 2004; Bosch *et al.* 2008). Viruses dispersed into the aquatic environment can also reach the groundwater resources (Locas *et al.* 2007; Gabrieli *et al.* 2009). Use of these contaminated waters for drinking water production, shellfish culture, and recreational purposes may pose a risk to public health (Hafliger *et al.* 2000; van den Berg *et al.* 2005; Matthews *et al.* 2012). Waterborne outbreaks of NoV have been linked to contaminated drinking water in North America, Europe, and Asia (Hafliger *et al.* 2000; Boccia *et al.* 2002; Parshionikar *et al.* 2003; Hoebe *et al.* 2004; Nygard *et al.* 2004; Kim *et al.* 2005; Yoder *et al.* 2008; Larsson *et al.* 2014). Recovery of NoVs from contaminated water can be challenging as large volumes of water need to be collected and concentrated to detect the virus (Calgua *et al.* 2013). Waterborne outbreaks can be prevented through water disinfection, providing chlorine levels which are adequate to inactivate NoVs (Shin & Sobsey 2008). NoVs can be classified into five genogroups, GI to GV (Green 2007). More than 30 genotypes within genogroups GI, GII, and GIV may infect humans (Kroneman *et al.* 2013), but a single genotype, GII.4, has been associated with the vast majority of NoV-related outbreaks and sporadic cases of AGE worldwide (Bok *et al.* 2009). Within the GII.4 genotype novel strains are periodically generated via accumulation of point mutations or recombination and this process of continuous genetic/antigenic diversification leads to the emergence of new GII.4 variants every two to three years (Siebenga *et al.* 2007). Members of the Italian Study Group for Enteric Viruses (ISGEV; http://isgev.net) have monitored the circulation of NoV and other enteric viruses in Italian children over the past decade. During this period the periodic introduction of new GII.4 NoV variants has been documented in Sicily, up to the most recent Sydney 2012 variant (Ramirez *et al.* 2006, 2008; Giammanco *et al.* 2013).

Here we report the results of an investigation carried out during an outbreak of NoV gastroenteritis associated with drinking water from a municipal supply network fed by groundwater in Santo Stefano di Quisquina, Agrigento, Sicily, Italy.

We investigated the outbreak in order to identify the source of infection and implement appropriate control measures.

METHODS

Outbreak description

On March 4, 2011, the Public Health Agency of Agrigento, Sicily, Italy, was alerted about an unexpectedly high number of individuals who had fallen sick with gastrointestinal symptoms during the previous days in Santo Stefano di Quisquina, a small municipality with an area of 85.94 km^2 and a population of 4,965 people located in the southwestern part of Sicily.

Seventy-nine outbreak cases were reported in the municipality area in 2 days, March 3 and 4. Shared events involving meals were ruled out by interviewing the cases, but the water supply of Santo Stefano di Quisquina was reported to be regularly used for drinking by the local population. Heavy rain was reported in the area just before the outbreak, suggesting that the risk of infiltration of contaminated superficial waters into the well and springs of the municipal service should be considered. As the initial information pointed towards drinking water as the most likely source of the outbreak, the inhabitants were informed that a suspected waterborne outbreak was involving the municipality. On March 4, 2011, the use of municipal drinking water was restricted (consumption was allowed only to

wash and cook after boiling) and alternative water supplies were provided to the population for drinking (bottled water). Chlorine concentration of the water supply was also increased from the original 0.2 mg/L, corresponding to the concentration currently recommended for waters intended for human consumption in Italy, to 0.4 mg/L, assuming this level could be recommended as safe during the outbreak. This increased level of chlorine was maintained through the whole surveillance period.

Investigations were started to identify the causative agents, the extent of the outbreak and the source of infection.

Epidemiological investigation

In this study we defined a probable outbreak case as a person who fell sick with vomiting (>1 episode/day), diarrhea (>3 stools/day), abdominal pain or fever ($T > 38\ ^{\circ}C$) after February 27, 2011 and who stayed in Santo Stefano di Quisquina prior to disease onset. A confirmed outbreak case was defined as a person who met the criteria of a probable case and whose stool sample was laboratory-confirmed for NoV. A system for enhanced surveillance of gastroenteritis was started on March 4: a dedicated phone line was set up to contact cases and to actively collect information concerning the disease, i.e., symptoms, date of onset, and geographical location of the cases. Simultaneously, the outbreak investigation team gathered information on case-patients presenting at the emergency unit of the local care continuity service and collected stool samples, when possible. A questionnaire was administered to all cases to collect information about the date of onset of symptoms, the number of household members suffering from AGE during the outbreak period, foods consumed in the previous 48 hours, and whether the household members regularly used the municipal water supply as drinking water or preferred commercial bottled water. Data were analyzed using EpiInfo version 6.3 software (CDC, USA).

Microbiological samples from cases

Fecal samples were obtained from 12 cases with dates of onset of illness between March 2 and 10, 2011, who presented to healthcare facilities (Table 1), generally on the day of onset of symptoms or a few days after. Samples were sent to the Department of Sciences for Health Promotion and Mother and Child Care (DSHP-MCC), University of Palermo, for microbiologic investigation. Feces were examined for pathogenic bacteria such as *Salmonella* and *Shigella* by standard bacteriological methods and were also assayed for rotavirus and adenovirus using the Diarlex Rota-Adeno latex agglutination kit (Orion Diagnostica, Espoo, Finland). For all samples, NoV detection was performed by a real-time reverse transcription (RT)-PCR assay that allows differentiation between GI and GII NoVs, using genogroup-specific primer sets targeting the open reading frame 1 (ORF1)–ORF2 junction region (Kageyama *et al.* 2003). Viral RNA was extracted from 140 μL of 10% fecal suspensions using the QIAamp Viral RNA Mini Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Samples were considered full-positive for NoV when the cycle threshold (ct) was passed within the 30th amplification cycle (ct < 31.00) and low-positive when the ct was >30.99 but <35.00. After real-time RT-PCR amplification screening, NoV-positive samples were confirmed by conventional RT-PCR with the specific primer pair JV12 and JV13 targeting the RNA-dependent RNA polymerase (RdRp) gene (Vinje & Koopmans 1996) and the amplification products were sequenced directly and analyzed using CLUSTALW and MEGA 5.0 (Tamura *et al.* 2011). The sequence of the epidemic strain obtained from fecal samples is available at NCBI with the following accession number: KF857575.

Environmental investigation

In Santo Stefano di Quisquina, the municipal water originates from a well and two springs (named Prisa well and Prisa springs No. 1 and 2, respectively) located at close proximity to each other and near the town. Before being distributed to the town, water is collected into a main reservoir (Reservoir No. 1) and is treated with hypochlorite to a final concentration of 0.2 mg/L. Two smaller reservoirs, named No. 2 and 3, are also used to store treated waters along the branches of the system. A third spring (named Capo Favara spring No. 3) is also close to the Prisa water sources but is not used by the municipality. In order to investigate the role of the municipal water supply in the outbreak, starting from March 4, 2011, the local health authorities initiated the collection of water samples from the public

Table 1 | Main clinical features and laboratory testing results on fecal samples of 12 cases presenting with AGE at the emergency unit of Santo Stefano di Quisquina (Agrigento, Italy) between March 4 and 9, 2011

					Symptoms								Norovirus		
Sample	Sex	Age	Onset of symptoms	Sampling date	Diarrhea (>3 stools/day)	Vomiting (>1 episode/day)	Abdominal pain	Fever ($T \geq 38$ °C)	Headache	*Salmonella Shigella*	Rotavirus	Adenovirus	Real-time RT-PCR	Viral load (ct)	Genotype
1	F	60	04 March	04 March	Yes	No	Yes	Yes	No	Neg	Neg	Neg	Pos	18.27	GII.4 vNO
2	F	21	02 March	06 March	Yes	Yes	Yes	Yes	No	Neg	Neg	Neg	Low pos	34.77	N.A.
3	M	56	05 March	05 March	Yes	Yes	Yes	No	No	Neg	Neg	Neg	Pos	18.04	GII.4 vNO
4	F	18	05 March	05 March	Yes	Yes	Yes	No	No	Neg	Neg	Neg	Neg	–	–
5	M	44	05 March	05 March	No	Yes	Yes	Yes	No	Neg	Neg	Neg	Pos	18.13	GII.4 vNO
6	F	74	04 March	04 March	Yes	Yes	Yes	No	No	Neg	Neg	Neg	Pos	15.54	GII.4 vNO
7	F	50	03 March	04 March	Yes	Yes	Yes	No	No	Neg	Neg	Neg	Pos	17.41	GII.4 vNO
8	F	41	05 March	05 March	Yes	No	Yes	No	No	Neg	Neg	Neg	Low pos	34	N.A.
9	M	51	06 March	06 March	Yes	No	Yes	Yes	No	Neg	Neg	Neg	Pos	18.19	GII.4 vNO
10	F	84	08 March	08 March	N.A.	N.A.	N.A.	N.A.	N.A.	Neg	Neg	Neg	Pos	17.34	GII.4 vNO
11	F	73	09 March	09 March	Yes	Yes	Yes	No	No	Neg	Neg	Neg	Pos	19.88	N.A.
12	M	14	09 March	09 March	Yes	Yes	Yes	No	No	Neg	Neg	Neg	Pos	24.24	N.A.

N.A., not available; vNO, variant New Orleans 2009.

well and springs, water reservoirs, public fountains and taps along the distribution system. The samples were sent to the local health authority laboratory of Agrigento for bacteriological analysis and to the Experimental Zooprophylactic Institute of Sicily, the DSHP-MCC in Palermo, and the Istituto Superiore di Sanità, Rome, for molecular analyses of NoVs. The municipal water supply of Santo Stefano is routinely tested for coliforms and enterococci by standard bacteriological methods once every 2 weeks, at fixed sampling points. Drinking water sample volumes were increased from the initial 310–400 mL to 10 L when the viral etiology of the enteritis outbreak was defined.

Places, times, and volumes of the water samplings are shown in Table 2.

For all water samples, NoV detection was performed by real-time RT-PCR and by two RT-PCR, amplifying both the ORF1 and ORF2 regions. Water samples were also tested for adenovirus (AdV) by a nested PCR (Formiga-Cruz *et al.* 2005). Water samples (volumes ranging from 0.31 to 10 L) were concentrated through an ultra-filtration system (Sartoflow® Slice 200 Benchtop Crossflow System, Sartorius AG, Goettingen, Germany), using appropriate membranes (SG Hydrosart 10 kDa) treated with 3% Beef Extract, pH 7 and pH 9.5 sequentially. Viral RNA was extracted from up to 560 μL of water concentrates, whose volumes varied from 9 to 25 mL, using the QIAamp Viral RNA Mini Kit (Qiagen), and was subjected to RT-PCR amplification of a 315-bp fragment in the ORF2 capsid protein region using G1SKF/G1SKR and G2SKF/G2SKR primer sets specific for NoV genogroups I and II, respectively (Kojima *et al.* 2002). To confirm diagnosis and identify possible multiple NoV genotypes, samples were also examined by a second RT-PCR assay targeting the ORF1 (RdRp) region (327-bp fragment) using degenerate primers JV12-JV13 (Vinje & Koopmans 1996).

Genotype characterization was accomplished by nucleotide sequencing of the amplified ORF1 or ORF2 region and comparison of sequences with both GenBank (www.ncbi.nlm.nih.gov/Genbank/) and the Foodborne Viruses in Europe databases (http://www.rivm.nl/mpf/norovirus/typingtool). PCR amplicons were sequenced using the PCR primers with the BigDye Terminator Cycle Sequencing Ready Reaction kit version 3.1 (Perkin Elmer, Applied Biosystems, USA) in an automated sequencer (ABI Prism 310 DNA sequencer, Applied Biosystems).

Sequences obtained in this study from water samples are available at NCBI with the following accession numbers: KF850522 and KF846529 (GI.4 and GII.7 sample 14951-7); KF850523 (GI.1 sample 14762-4); and KF850524 (GII.4 v2006b sample 14766-2). Three shorter sequence fragments were used only to identify the genotype (GII.21 sample 29765-3, GII.4 v2004 sample 14899-2, and GI.4 sample 15351-2).

RESULTS

In total, 156 inhabitants of Santo Stefano di Quisquina were defined as probable cases of the AGE outbreak described in this investigation according to the criteria for outbreak case definition. Figure 1 shows the epidemic curve with probable cases by date of onset of symptoms. The attack rate for the inhabitants was 3.14% (156/4,965). Age group-specific attack rates (Figure 2) ranged from 2.26% in the age group 25–44 years (28/1,238) to 4.78% (23/481) for the 5–14-year-old group. There was no fatality. Based on the available medical records, symptoms included vomiting (>1 episode/day; 78.4%), diarrhea (>3 stools/day; 73.9%), abdominal pain (75.9%), fever ($T \geq 38\,^{\circ}$C; 38.7%), and headache (15.7%). Stool samples were obtained from 12 probable cases that presented at the emergency unit between March 4 and 9, and were examined for enteric pathogens. Of these, 11 tested positive for NoV, and all were negative for rotavirus, adenovirus, *Salmonella* or *Shigella* (Table 1). In seven of the positive samples the NoV genotype was characterized as GII.4 New Orleans 2009 variant, through sequence analysis of the RdRp gene.

Information collected through the questionnaire administered to all cases revealed that 146/156 (93.6%) of them regularly drank the water from the municipal supply system, while no association was found with exposure to any specific food. No connection was found between the location of the cases and the branching of the municipal water distribution system (Figure 3), since all distribution sections, inside and outside the urban area of Santo Stefano di Quisquina, were involved in the epidemic and the number of cases was higher in those areas that were most densely populated. The prospect that the water supply could be involved in the outbreak induced the authorities to increase chlorine concentration of the municipal drinking water and

Table 2 | Water sampling and results of molecular analysis for Norovirus detection and typing

Sampling date	Sample no.	Sampling site[a]	Sampling volume (L)	Elution volume (mL)	Real-time RT-PCR [earliest ct] (no. positive replicates)	ORF1/ ORF2 RT-PCR	Sequence analysis	Chlorinated sample[b]
04 March 2011	15351-1	Distribution system section E	0.31	20	Neg	Neg		Yes
04 March 2011	15351-2	Distribution system section E	0.32	20	Neg	GGI	GI.4	Yes
04 March 2011	15351-3	Public fountain section A	0.35	20	Neg	GGII	NA	Yes
04 March 2011	15351-4	Distribution system section B	0.35	20	Neg	Neg		Yes
04 March 2011	14951-1	Prisa well + Prisa No. 1 spring (mixed waters)[c]	0.38	20	GGII [40] (1)	GGII	NA	No
04 March 2011	14951-2	Main reservoir (No. 1)	0.38	20	Neg	Neg		Yes
04 March 2011	14951-3	Reservoir No. 2	0.38	20	Neg	Neg		Yes
04 March 2011	14951-4	Reservoir No. 3	0.36	20	Neg	Neg		Yes
04 March 2011	14951-5	Public fountain section C	0.38	20	GGII [40] (1)	Neg	NA	Yes
04 March 2011	14951-6	Public fountain section E	0.38	20	Neg	Neg		Yes
04 March 2011	14951-7	Distribution system section OUA	0.4	20	GGII [38] (1)	GGI + GGII	GI.4 + GII.7	Yes
04 March 2011	14951-8	Public fountain section E	0.4	20	Neg	Neg		Yes
09 March 2011	14762-1	Prisa well + Prisa No. 1 spring (mixed waters)[c]	0.5	10	GGII [39] (1)	GGII	NA	No
09 March 2011	14762-2	Main reservoir (No. 1)	0.5	10	GGII [40] (2)	Neg	NA	Yes
09 March 2011	14762-3	Public fountain section D	0.5	10	GGII [40] (1)	Neg	NA	Yes
09 March 2011	14762-4	Public fountain section E	0.5	10	Neg	GGI	GI.1	Yes
09 March 2011	14762-5	Public fountain section C	0.5	20	Neg	Neg		Yes
09 March 2011	14762-6	Public fountain section A	0.5	10	GGII [38] (3)	GGII	NA	Yes
10 March 2011	14766-1	Prisa well + Prisa No. 1 spring (mixed waters)[c]	10	10	Neg	Neg		No
10 March 2011	14766-2	Main reservoir (No. 1)	10	20	GGII [40] (1)	GGII	GII.4 v2006b	Yes
10 March 2011	14766-3	Public fountain section C	5	10	Neg	Neg		Yes
11 March 2011	14899-1	Prisa No. 2 spring	6	20	Neg	GGI	NA	No
11 March 2011	14899-2	Prisa well + Prisa No. 1 spring (mixed waters)[c]	4	15	Neg	GGII	GII.4 v2004	No
22 March 2011	16959-1	Prisa well	10	20	GGII [38] (1)	Neg	NA	No
22 March 2011	16959-2	Prisa No. 2 spring	10	15	GGII [39] (3)	GGII	NA	No
22 March 2011	16959-3	Public fountain section A	10	15	GGII [39] (2)	Neg	NA	Yes
22 March 2011	16959-4	Public fountain section C	10	20	GGII [40] (1)	Neg	NA	Yes
22 March 2011	16959-5	Public fountain section E	10	15	Neg	Neg		Yes
22 March 2011	16959-6	Public fountain section E	10	25	Neg	Neg		Yes
28 March 2011	18422-1	Prisa well	10	15	Neg	Neg		No
28 March 2011	18422-2	Prisa No. 2 spring	10	10	Neg	GGII	NA	No
28 March 2011	18426-3	Capo Favara No. 3 spring	10	12	Neg	GGII	NA	No

(*continued*)

Table 2 | continued

Sampling date	Sample no.	Sampling site[a]	Sampling volume (L)	Elution volume (mL)	Real-time RT-PCR [earliest ct] (no. positive replicates)	ORF1/ ORF2 RT-PCR	Sequence analysis	Chlorinated sample[b]
01 April 2011	19843-1	Capo Favara No. 3 spring	10	22	Neg	GGII	NA	No
01 April 2011	19835-2	Prisa well	10	17	Neg	Neg		No
01 April 2011	19835-3	Prisa No. 2 spring	10	18	GGII [39] (1)	GGII	NA	No
01 April 2011	19835-4	Prisa No. 1 spring	10	20	Neg	GGII	NA	No
08 April 2011	21568-1	Prisa well	10	20	Neg	Neg		No
08 April 2011	21568-2	Margimuto river upstream of the well	10	19	GGII [36] (2)	Neg	NA	No
08 April 2011	21568-3	Margimuto river downstream of the well	10	18	Neg	Neg		No
11 May 2011	29765-1	Prisa well	10	19	GGI [40] (1) + GGII [39] (1)	Neg	NA	No
11 May 2011	29765-2	Main reservoir (No. 1)	10	20	GGI [39] (1)	Neg	NA	Yes
11 May 2011	29765-3	Public fountain section E	10	19	GGI [25] (1) + GGII [37] (1)	GGI + GGII	GII.21	Yes
08 September 2011	58406-1	Prisa well	10	25	Neg	Neg		No
08 September 2011	58406-2	Prisa No. 1 spring	10	15	Neg	Neg		No
08 September 2011	58406-3	Prisa No. 2 spring	10	40	Neg	Neg		No
21 October 2011	71512-1	Prisa well	10	16	Neg	Neg		No
21 October 2011	71512-2	Main reservoir (No. 1)	10	17	Neg	Neg		Yes
21 October 2011	71512-3	Public fountain section C	10	15	Neg	Neg		Yes
10 January 2013	2330-1	Prisa well	10	9	Neg	Neg		No
24 June 2013	43770-1	Prisa well + Prisa No. 1 spring (mixed waters)[c]	10	9	Neg	Neg		No

[a]A–E: sections inside the urban area of Santo Stefano di Quisquina divided according to the branching of the municipal water system; OUA: outside the urban area of Santo Stefano di Quisquina.
[b]In treated waters chlorine concentration was 0.2 mg/L for samples collected on the March 4, 2011 and thereafter was increased to 0.4 mg/L.
[c]Waters from the two sources are mixed before reaching the main reservoir (No. 1).
NA: not available, due to low amount of DNA amplified by RT-PCR or insufficient length and quality of sequences for NoV genotype characterization.

to advise the population to drink bottled water or boil the municipal water before use.

Regular water sampling and testing was started to monitor the effectiveness of the control measures implemented. From March 4 to May 11 of 2011, 42 water samples were collected from the well, the springs, the reservoirs, and several sites of distribution of the municipal water system (Figure 3). None of the samples resulted to be contaminated with *Escherichia coli*, coliforms, enterococci or AdV, but 26 (61.9%) of them tested positive for NoV by at least one of the molecular methods used (Table 2). Ten NoV-positive water samples were collected from sites dispersed over the distribution pipelines and three were from the main reservoir, while twelve others were raw untreated waters from the well and the springs (Prisa No. 1 and 2) supplying the water system and from the Capo Favara spring (No. 3), which is not used by the municipality (Figure 3). A NoV-positive water sample was also obtained from the river flowing in the Margimuto valley that borders the well and springs supplying the municipal water system. For six NoV-positive water samples, the ORF1 and/or ORF2 amplification fragments obtained yielded nucleotide sequences of sufficient length and quality for NoV genotype characterization. NoV genotypes GI.1, GI.4, GII.4 (two samples), GII.7 and GII.21 were detected in water samples. Neither of the two GII.4 NoVs corresponded to the New Orleans

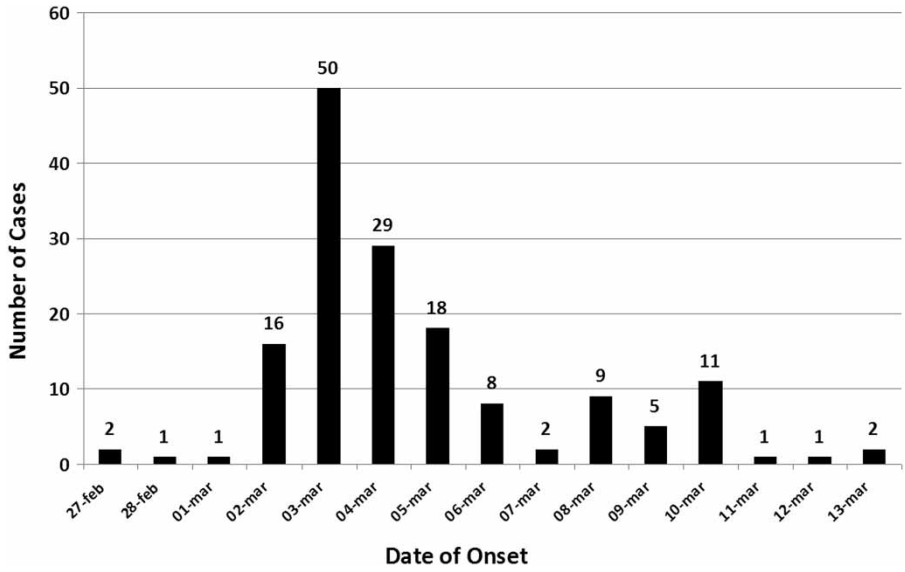

Figure 1 | Probable cases of NoV gastroenteritis ($n = 156$), by date of onset of symptoms in Santo Stefano di Quisquina (Agrigento, Italy), February 27–March 13, 2011.

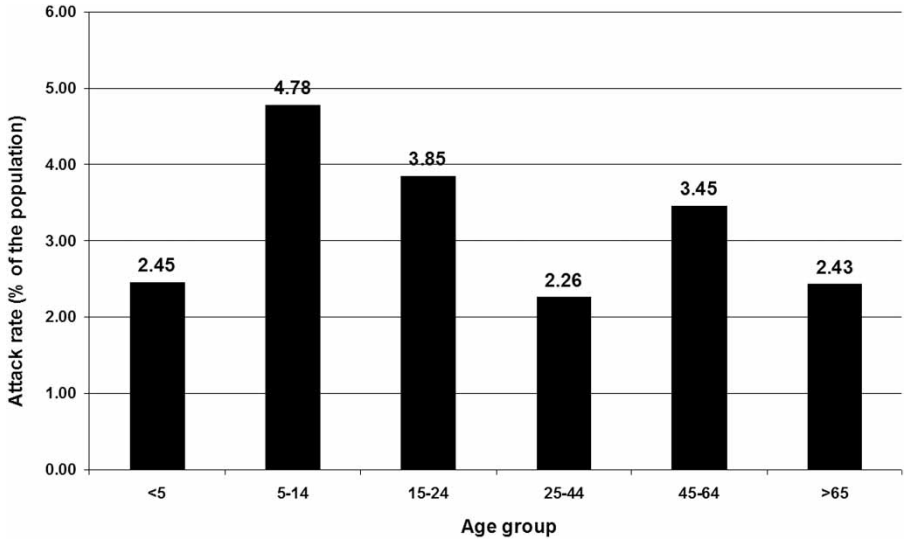

Figure 2 | Attack rate of NoV gastroenteritis ($n = 151$ cases) by age group. Outbreak in Santo Stefano di Quisquina (Agrigento, Italy), February 27–March 13, 2011. For five of the 156 total cases the age was not available.

2009 variant which had been detected in fecal samples, since their sequences clustered close to variant Hunter 2004 and Den Haag 2006b, respectively. The remaining NoV-positive samples could not be characterized due either to failure to amplify target genome fragments by RT-PCR and low amount of amplified DNA or to insufficient length and quality of the sequences obtained. Eight further water samples were taken and analyzed in September and October 2011 and January and June 2013, but none of them tested positive for NoV.

DISCUSSION

Between February 27 and March 13, 2011, an outbreak of gastroenteritis occurred in Santo Stefano di Quisquina,

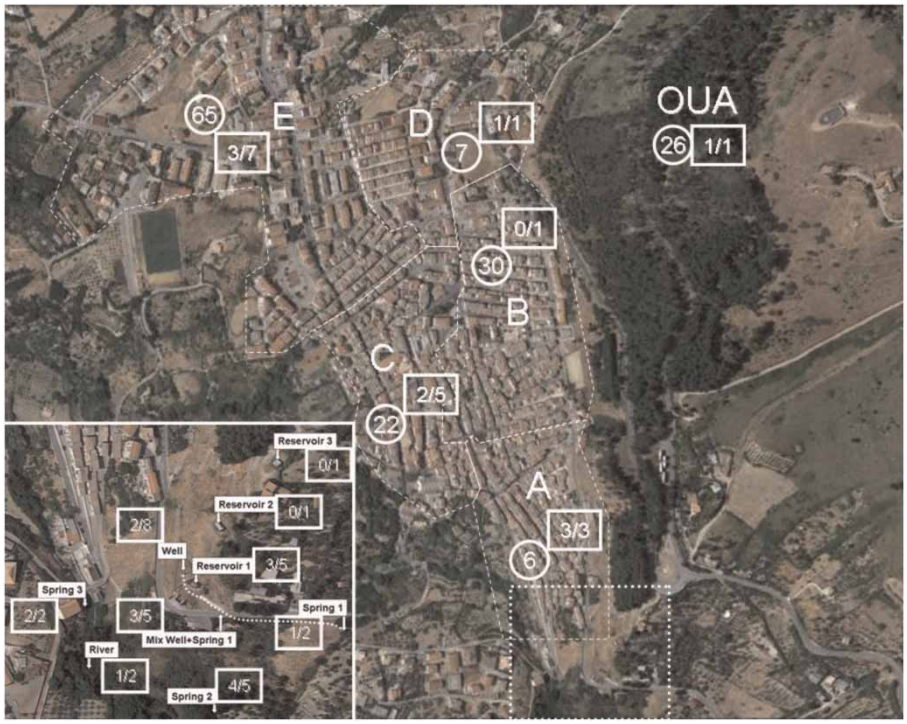

Figure 3 | Geographical location of gastroenteritis cases (circles: no. of cases) and water samplings (squares: no. of samples positive for NoV/no. of samples tested) along the municipal water distribution system of Santo Stefano di Quisquina (Agrigento, Italy). The urban area has been divided into sections (A–E, OUA: outside urban area) according to the branching of the municipal water system. The area in the dotted box is enlarged at the left-bottom corner of the figure to better show the location of the well and springs feeding the municipal water system, of the reservoirs used by the municipality, and of possible sources of contamination such as the river in the valley nearby. Figures inside the symbols indicate the no. of samples positive for NoV/no. of samples tested.

Agrigento, Italy. Cases were distributed uniformly throughout the municipality jurisdiction (Figure 3). All age groups were affected, with a slightly higher prevalence of cases among children older than 4 years, adolescents and young adults up to 24 years. The timing and geographical distribution of the cases suggested that the outbreak could be linked to the municipal drinking water (Figures 1 and 3).

In a recent systematic review of the epidemiological characteristics of NoV outbreaks, the median primary attack rate in waterborne outbreaks was estimated to be 38% (28–64%) (Matthews *et al.* 2012). Thus, the attack rate in the present outbreak appears to be unexpectedly low considering its likely waterborne transmission route. However, as mentioned previously, only a passive case search was performed, which may have led to a significant underestimation of cases (Matthews *et al.* 2012). Moreover, GII.4 outbreaks have been reported to be generally associated with lower attack rates when compared to other genotypes (Noda *et al.* 2008). The rather homogeneous attack rates observed throughout all age groups are consistent with the exposure of the entire population to a contaminated common source such as water from the municipal distribution system. Normally, exposure of a whole community to common pathogens is likely to produce more cases in the lower age groups, due to either lack of prior pathogen-specific immunity or to higher susceptibility of younger individuals to low infectious doses of the pathogen. In our survey children over 4 years, adolescents and young adults showed the highest attack rates. The younger children (0–4 years) were affected to a lesser extent (2.45% attack rate), which may be correlated with limited drinking of tap water by children of very small age, who are normally given bottled or boiled water to drink. An association of drinking water exposure with the occurrence and severity of gastroenteritis symptoms was impossible to evaluate since more than 90% of the cases reported drinking municipal water regularly. To confirm the origin of the epidemic with microbiological evidence, stool samples from patients and water samples, including raw

water and tap water from different parts of the distribution network, were collected and analyzed for NoV. Although limited, the number of fecal samples tested was sufficient to fulfill the recommendations for the selection of a representative sampling (Maunula *et al*. 2005). The finding of NoV genome in the feces of 11 out of 12 patients investigated, and the characterization of all strains typed as a unique sequence variant of the same genotype, suggests considering GII.4 NoV variant New Orleans 2009 as the causative agent of the outbreak. Water surveillance for NoVs showed that the network was contaminated by several different NoV genotypes, including GII.4, although none of them showed sequences identical to those recovered from the patients involved in the outbreak. A conclusive microbiological evidence for a waterborne NoV epidemic was lacking in our investigation due to the failure to identify an identical NoV genome sequence in both patients and water. This may depend on the small volume of water samples taken at the time of the outbreak, which likely reduced the sensitivity of the analyses. Furthermore, water contamination with the epidemic strain may simply have ended before the investigation started. However, the finding of at least five different NoV strains suggests that a large fecal pollution has been present in the municipal drinking water over a long period. NoV RNA was still detected in the water 2 months after (May 11) the end of the outbreak, disappearing only by September 2011. Bacteriological analyses carried out on the same water samples investigated for NoV showed the absence of indicators of fecal contamination such as *E. coli*, coliforms, and enterococci. Also, the routine water quality monitoring carried out for several months prior to the outbreak had not identified fecal contamination indicators. This is not surprising since previous reports showed that the presence of NoV may not correlate with bacterial indicators of fecal pollution (Kukkula *et al*. 1999; Lee *et al*. 2011). The occurrence of a NoV epidemic involving exclusively the GII.4 variant New Orleans 2009 strain despite a variety of NoVs was concurrently contaminating the municipal water source could have been facilitated by the lack of immunity to this specific strain in the population. In fact, New Orleans 2009 was the most recent GII.4 NoV antigenic variant known at the time of the outbreak. Despite the evidence of prolonged NoV contamination of the water supply, the control measures adopted were apparently effective in containing the outbreak since no cases were reported after March 13. In the fall of 2011, the NoV contamination finally disappeared from the water distribution system. Further evidence that the source of the epidemic was no longer effective came from the surveillance of gastroenteritis cases performed by the local health authorities, showing that no further cases of NoV gastroenteritis were reported to the care continuity service in the following months. However, the interruption of NoV transmission might also have been favored by a diffuse immunization of the local population through widespread circulation of the epidemic strain, also as subclinical or milder infections. It is also of interest that no NoV gastroenteritis cases were reported in summer 2011, when many natives of Santo Stefano di Quisquina who had migrated towards northern Italy or other European countries in the previous decades returned to spend their summer holidays, thus providing a naïve population for a possible re-emergence of the NoV epidemic.

Contamination with human feces of the well and springs supplying the public water network is thought to have caused the outbreak. We can assume that the contamination of underground water was due to illegal and uncontrolled sewage dumping. Meteorological data support this hypothesis since heavy rain was reported in the area just prior to the outbreak. The rainfall may have caused direct soil pollution by sewage overflowing from septic tanks and latrines. The well and springs contamination may have been caused by massive infiltration of contaminated superficial waters overcoming the natural capacity of soil to remove pollutants before reaching groundwater. The water sources in use by the municipality are located in the Margimuto river valley where water originating from rainfall usually flows into the river, particularly in low-flow periods. In times of continuous heavy rain, however, flowing water might infiltrate the aquifer. The finding that a water sample from the river flowing in the Margimuto valley and bordering the municipal well and springs was positive for NoV would support this idea.

Drinking water supply has been identified in the past as the source of water-borne infections in NoV outbreaks in Italy (Boccia *et al*. 2002; Rizzo *et al*. 2007; Scarcella *et al*. 2009). However, previous reports concerned small tourist resort water systems and a municipal supply fed by lake water. This is the first report of NoV contamination of a groundwater municipal supply in Italy.

CONCLUSIONS

Stringent natural groundwater protection and control programs are necessary to satisfy the demand for drinking water in Italy and globally. Despite the progress in water and wastewater treatment technology, waterborne diseases still have public health and socio-economic implications in both the developed and developing world. Many studies have shown that NoVs are present at high levels in wastewater, even after treatment processes (Laverick *et al.* 2004; van den Berg *et al.* 2005; Haramoto *et al.* 2006; da Silva *et al.* 2007; La Rosa *et al.* 2007; Katayama *et al.* 2008; Rodriguez *et al.* 2012). Moreover, commonly used bacterial indicators are unreliable in terms of viral contamination, and no correlation between levels of enteric bacteria and enteric viruses has been found (Gerba *et al.* 1979; Haramoto *et al.* 2006; Lucena *et al.* 2006). The risk associated with NoV contamination urges the definition of affordable and reliable indicators for the presence of such viral pathogens in drinking waters.

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