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Gastroenteritis Outbreak Caused by Waterborne Norovirus at a New Zealand Ski Resort▽

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In July 2006, public health services investigated an outbreak of acute gastroenteritis among staff and visitors of a popular ski resort in southern New Zealand. The source of the outbreak was a drinking water supply contaminated by human sewage. The virological component of the investigation played a major role in confirming the source of the outbreak. Drinking water, source stream water, and 31 fecal specimens from gastroenteritis outbreak cases were analyzed for the presence of norovirus (NoV). Water samples were concentrated by ultrafiltration, and real-time reverse transcription-PCR (RT-PCR) was used for rapid detection of NoV from both water and fecal samples. The implicated NoV strain was further characterized by DNA sequencing. NoV genogroup GI/5 was identified in water samples and linked case fecal specimens, providing clear evidence of the predominant pathogen and route of exposure. A retrospective cohort study demonstrated that staff who consumed drinking water from the resort supply were twice as likely to have gastroenteritis than those who did not. This is the first time that an outbreak of gastroenteritis in New Zealand has been conclusively linked to NoV detected in a community water supply. To our knowledge, this is the first report of the use of ultrafiltration combined with quantitative real-time RT-PCR and DNA sequencing for investigation of a waterborne NoV outbreak.

Noroviruses (NoVs), belonging to the family *Caliciviridae*, are a common cause of outbreaks of viral gastroenteritis worldwide. In New Zealand in 2006, there were approximately 200 recorded NoV outbreaks with almost 4,000 associated cases (14). Waterborne NoV outbreaks caused by contaminated drinking water are well documented (1, 2, 4, 9, 19, 24). The low infectious dose and environmental persistence of NoV allow for its spread by water, as well as by food and direct person-to-person transmission (6, 16, 18).

Although enteric viruses have been detected in a range of waters since the 1970s, identification of the virus in the implicated water sample is often difficult because virus levels are often low, requiring concentration from large volumes of water. In addition, reverse transcription-PCR (RT-PCR) inhibitory factors may be coextracted and purified with viruses, potentially causing false-negative results (32). In 1997, the first identification of NoV in both water and fecal samples from epidemiologically linked cases in an outbreak investigation occurred (2). Since then, due to improved concentration and detection methods, many investigators have shown identical NoV in both contaminated drinking water and related fecal samples from cases (1, 4, 9, 19, 22, 24). Viral concentration methods from water include adsorption-elution methods using negatively (10, 28) and positively charged membrane filters (1, 2, 25, 29, 30). More recently, ultrafiltration (UF) methods have been used to detect enteric viruses in water for either surveillance or method validation (11, 28, 33).

Following the concentration and viral nucleic acid extraction steps, detection of NoV in water samples is generally achieved by conventional RT-PCR, followed by nested PCR (1, 2, 4, 17, 22, 24, 25). Recently, real-time RT-PCR assays have been used to detect NoV in water and sewage samples (10, 20, 21, 28, 30, 34) but to our knowledge have not been applied to a real-time investigation of a NoV waterborne outbreak. Identification of the implicated NoV is usually performed by DNA sequencing which can confirm links between cases and the suspect contaminated water (4, 9, 25).

In this report, we describe the virological investigation of an outbreak of NoV gastroenteritis at a New Zealand ski resort. The epidemiological and environmental investigations are briefly described to obtain a wider picture of the outbreak.

MATERIALS AND METHODS

Gastroenteritis outbreak setting. On 27 July 2006, public health authorities were informed about a possible outbreak of gastroenteritis among staff and visitors at a popular ski resort. As well as skiing facilities, the resort provided two restaurants, two early child care services, a health care/trauma center, and 15 apartments. Approximately 480 full and part-time staff members were employed, and the resort catered for up to 3,800 visitors per day.

Epidemiological investigation. On 25 and 26 July 2006, there were 48 and 83 resort staff members absent, respectively, with acute gastrointestinal illness. Absenteeism from illness was normally three or four staff members daily. Also, three small outbreaks of gastroenteritis among groups who had recently visited the resort were reported to the public health authorities on 26 and 27 July. A retrospective cohort investigation using a standardized questionnaire and analysis based on Epi Info v 3.3.2 (7) was conducted among ski resort staff to investigate the source of illness. Exposure between 22 and 25 July 2006 was assessed. The incidence of illness among skiers at the resort was assessed from complaints to the resort management, notifications from general practitioners,

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and self-reporting following media publicity of the outbreak. A case was defined as a resort staff member or visitor who developed diarrhea or vomiting on or after 21 July 2006.

Environmental investigation. Information was sought from the ski resort management about the design and management of the water supply and the sewerage system. The restaurant, child care, and health care facilities were also inspected.

Microbiological investigation of fecal specimens. Following notification of the outbreak, fecal samples were collected from 31 staff, visitors, and apartment dwellers with recent symptoms of acute gastroenteritis. A further 42 fecal samples were collected from the community via family physicians from patients with gastroenteritis apparently unrelated to the resort outbreak. Fecal specimens from outbreak cases were referred for microbiological and virological analyses. For NoV analysis, a 10% (wt/vol) fecal suspension clarified with chloroform and centrifugation was prepared as previously described (8).

Microbiological investigation of water samples. Prior to the outbreak, the presence of total coliforms and *Escherichia coli* in the water supply was monitored weekly by resort management using a Colilert substrate assay (IDEXX Laboratories, Westbrook, ME). On 27 July 2006, routine testing produced a positive result for total coliforms, but not *E. coli*. On the same day, public health officials collected water samples from four points along the water supply for additional coliform and *E. coli* testing (IDEXX Quanti-Tray) and for NoV analysis, one 20-liter water sample was collected from a tap in the main building. Water (20 liters) was also sampled from a suspected contaminated source stream later in the investigation (3 August 2006).

For NoV detection, water samples were concentrated by hollow-fiber UF using Hemoflow HF80S dialysis filters (Fresenius Medical Care, Bad Homberg, Germany) as described by Hill et al. (11) with modifications. Briefly, following pretreatment of the filter with 0.01% sodium polyphosphate ($NaPO_3$) solution for 15 min, $NaPO_3$ was added to each sample to give a final concentration of 0.01% (wt/vol) and pumped through the filter using a peristaltic pump at a permeate rate of 120 to 150 ml/min to complete filtration within approximately 2 h. Filters were back flushed with a solution of 0.5% (vol/vol) Tween 80 and 0.01% (wt/vol) $NaPO_3$, and the retentate was further concentrated using polyethylene glycol 6000 (10% [wt/vol]) and sodium chloride (1.75% [wt/vol]). Following incubation on a horizontal shaker (120 rpm) overnight at 5°C ± 3°C, the material was centrifuged (10,000 × *g* for 25 min), and each pellet was resuspended in 5 ml phosphate-buffered saline (pH 7.5).

RNA extraction. Viral RNA was extracted from fecal suspensions (200 μl) and water concentrates (two 200-μl and two 500-μl samples) using the High Pure Viral Nucleic Acid kit (Roche Molecular Biochemicals Ltd., Mannheim, Germany) per the manufacturer's instructions.

To monitor for RT-PCR inhibition, 10 μl diluted Armored RNA-Norwalk virus genogroup I (NV GI aRNA; Asuragen Diagnostics, TX), RNA packaged in a bacteriophage coat protein (27) (1,000 RT-PCR units [RTPCRU]), was added prior to extraction as an internal control to two 500-μl aliquots of sample and two 500-μl aliquots of nuclease-free water. The extent of RT-PCR inhibition in the concentrated water sample was determined by the difference in the cycle threshold (C_T) values obtained between the sample and nuclease-free water.

NoV detection from fecal specimens by real-time RT-PCR. For fecal specimens, monoplex one-step NoV GI and GII real-time RT-PCR assays were performed using the Platinum Quantitative RT-PCR Thermoscript One-Step System (Invitrogen, CA). Rotor-Gene 3000 and 6000 rotary analyzers (Corbett Life Science, Sydney, Australia) were used in all assays. Viral RNA (2.5 μl) was added to 12.5 μl of 2× Mastermix, 0.5 μl reverse transcriptase-*Taq* polymerase mix, and 20 U RNase inhibitor (RNaseOUT; Invitrogen), using primers (0.4 μM) and probes (0.2 μM) as previously described (15) in a final volume of 25 μl.

The RT-PCR comprised an initial 30-min RT step at 60°C, 95°C for 5 min, and PCR cycling protocol of 95°C for 20 s and 56°C for 1 min over 45 cycles. Raw data were analyzed using the Rotor-Gene software to calculate the C_T values.

NoV detection from water samples by real-time RT-PCR. RT-PCR assays were performed as described above for the fecal specimens, but using monoplex two-step real-time assays. Briefly, 10 μl cDNA was produced using 5 μl viral RNA, 100 U SuperScript III reverse transcriptase (Invitrogen), 10 U RNase inhibitor (RNaseOUT; Invitrogen), 0.2 μM of each NoV GI and GII reverse primer (COG1/2R) (15), 1 mM of each deoxynucleoside triphosphate, and 1× First Strand RT buffer (Invitrogen). The RT reaction was carried out at 50°C for 30 min, followed by 95°C for 4 min. Each PCR mixture contained 5 μl cDNA, 12.5 μl of 2× Platinum *Taq* Quantitative PCR Supermix-UDG (Invitrogen), 0.2 μM of either GI or GII probe, and 0.4 μM of each GI or GII forward and reverse primer (COG1F/R or COG2F/R) (15) in a final volume of 25 μl. Following 95°C for 5 min, the same PCR cycling protocol used for fecal samples was performed. Each RNA extract was tested in duplicate to give four results for each 200- or 500-μl sample volume. A specific primer and probe set to detect NV GI aRNA was used to detect RT-PCR inhibition.

Anticontamination procedures were followed for all RNA and DNA procedures. Controls (water and 1,000, 100, and 10 RTPCRU of GI/3 and GII/4 NoVs) were included in each RNA extraction and RT-PCR assay.

For NoV quantitation purposes, a fragment of NoV from a GI/1 NoV-positive fecal sample was cloned into a DNA plasmid. Once the plasmid concentration was determined, dilutions were made to produce a NoV GI standard curve. Briefly, NoV inserts were cloned into TOPO vectors with transformation into One Shot TOP10 *E. coli* cells (Invitrogen). The plasmid was purified by using a PureLink Quick Plasmid Miniprep kit (Invitrogen), and the concentration of plasmid was determined with the Quant-iT double-stranded DNA high-sensitivity assay kit and Qubit fluorometer (Invitrogen).

NoV genotyping. Samples that were NoV positive by real-time RT-PCR were further characterized (genotyped) by DNA sequencing. For the fecal specimens, samples were amplified using Mon 431, 432, 433, and 434 primers in NoV RNA polymerase "region B" to give a 213-bp product (31). The one-step RT-PCR consisted of an initial 10-min RT step at 42°C, 3-min denaturation at 94°C, followed by 40 cycles of 94°C for 30 s, 50°C for 90 s, and 60°C for 30 s, with a final extension of 72°C for 7 min.

For the water samples, a seminested PCR in "region B " was performed. Briefly, cDNA was prepared as for the monoplex two-step real-time RT-PCR assay but using SWGIrev/SWGIIrev primers (34) instead of COG1/2R primers (15). The primary PCR comprised 5 μl of cDNA, 12.5 μl Qiagen *Taq* PCR Master Mix reagent (Qiagen Ltd., Germany), 0.4 μM each primer (Mon 431 and SWGIIrev or Mon 432 and SWGIrev), with a final concentration of 2 mM $MgCl_2$ in a 25-μl reaction mixture. An initial 2-min denaturation at 94°C was followed by 40 cycles of 95°C for 30 s, 53°C for 40 s, and 72°C for 60 s, with a final extension of 72°C for 5 min to produce a 284-bp product. Primary PCR product (1 μl) was then subjected to 30 cycles of seminested PCR using 12.5 μl Qiagen *Taq* PCR Master Mix reagent (Qiagen), 0.4 μM each primer (Mon 431/433 or 432/434), 2 mM $MgCl_2$ (final concentration) in a 25-μl reaction mixture using the same parameters as in the primary PCR. The seminested PCR produced a 213-bp product in "region B" as for the fecal samples, allowing for direct comparison of NoVs from water and fecal samples.

All PCR products were purified using the QIA quick PCR purification kit (Qiagen) followed by DNA sequencing using the Big Dye-Terminator Ready Reaction mix (Applied Biosystems Corp., CA) on an ABI PRISM 3130xl DNA analyzer. Sequences were compared with known NoV sequences and with each other using BioNumerics analysis software v 4.6 (Applied Maths BVBA, Sint-Martens-Latem, Belgium).

Nucleotide sequence accession number. The sequence of the NoV GI/5 reported here has been deposited in GenBank under the accession number EF527258.

RESULTS

Epidemiological investigation. A total of 218 cases were identified (115 ski resort staff and 103 visitors) in this outbreak. The cohort study recruited 214 resort staff members (45% of staff) with 111 identified as having gastroenteritis (Fig. 1). Symptoms included nausea (71%), vomiting (79%), or diarrhea (67%). Four more cases among staff members were identified through family physician notification and laboratory results. Staff who drank water at the staff cafeteria on 24 or 25 July 2006 were twice as likely to develop acute gastroenteritis than those who did not (relative risk, 2.0; 95% confidence interval, 1.5 to 2.8). However, the questionnaire did not differentiate between consumption of boiled water (from a hot water dispenser) and cold tap water. This may have lowered the relative risk due to the protective effect of boiled water, which many staff would have used for hot drinks. No other exposures were associated with a statistically significant increased risk of illness (data not shown). Comprehensive control measures were implemented starting 27 July 2006; these included work restrictions for sick staff, hand hygiene signage for staff and visitors, emergency chlorination of the water sup-

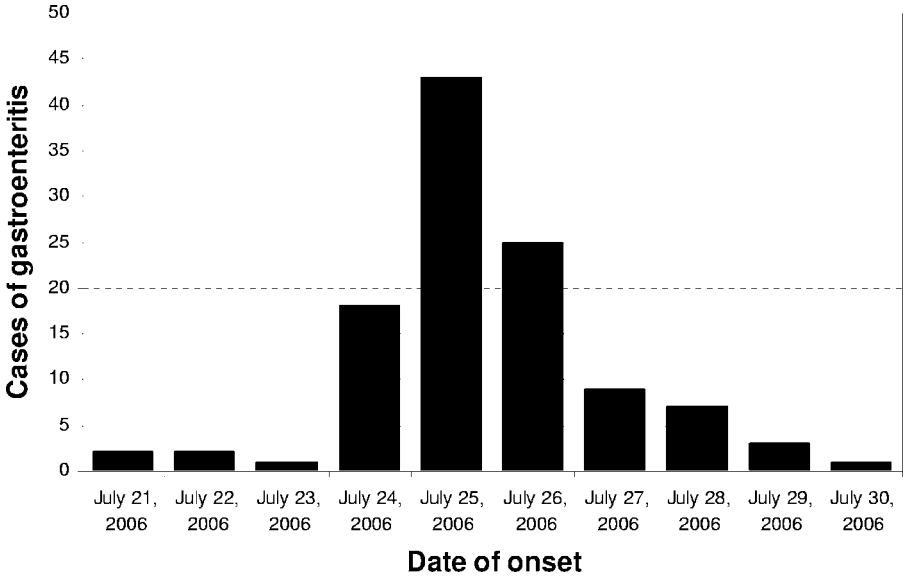

FIG. 1. Epidemic curve showing the number of cases of gastroenteritis in staff members that fit the case definition ($n = 111$) from date of onset (21 to 30 July 2006).

ply, and comprehensive environmental cleaning of the resort's facilities.

Environmental investigation. Water for the ski field was supplied from a natural spring which fed a small lake uphill of the base resort. Water was gravity fed to a holding tank and then pumped to six storage tanks above the base facility and on to the buildings at the resort. Water treatment at the base facility consisted of filtration and UV treatment. Water was dispensed for drinking purposes at numerous outlets throughout the restaurants and base facility. Initial reports were that no recent problems had been observed with the treatment system and that there had not been any recent maintenance work carried out on the sewerage system. However, important new information was divulged during the week following notification of the outbreak and as microbiological results became available. On 22 to 23 July 2006, a septic tank had become blocked and had overflowed at the resort. A drainage contractor had been employed to clear the blockage. Another important disclosure was that water had been pumped from a mountain stream to the lake to augment the main water supply during peak demand. The point of the sewage overflow was above the stream, and sewage was likely to have reached the water extraction point in the stream. Water from this stream continued to be used for 3 days after the outbreak was first recognized.

Microbiological investigation of fecal and water samples. The water samples taken on 27 July 2006 showed significant levels of *E. coli* (range, 7.4 to 220 per 100 ml) and total coliforms (range, 72 to 1,100 per 100 ml) from the four points along the water supply, including the lake, storage tanks, and the main building, which housed the restaurants, and child care and health care services.

NoV GI was detected in fecal specimens from 11 of the 31 cases who provided specimens. In addition, cases of rotavirus (one case), cryptosporidium (four cases), and campylobacter (two cases) were also identified. A small number (3/42) of community cases of acute gastroenteritis who had not visited the ski resort were positive for NoV GI. Six community cases were positive for NoV GII. One specimen from the community was positive for both NoV GI and GII. Water samples taken from the tap in the main building on 27 July 2006 and from the source stream on 3 August 2006 were positive for NoV GI.

PCR inhibition. Inhibition of the real-time RT-PCR was detected in the 500-μl sample extractions of main building tap water concentrate. This was shown by an increase of more than four C_Ts in the amplification of the internal NV GI aRNA control seeded into 500-μl samples of tap water (mean C_T, 33.0 ± 2.4) compared with the nonmatrix sample (mean C_T, 28.4 ± 0.8). Furthermore, this inhibition was also reflected in the NoV GI RT-PCR assay where only two of four replicates were NoV GI positive (C_T values of 38.1 and 38.8) in 500-μl sample extractions compared to all four replicates positive in the 200-μl samples extracted (mean C_T, 34.9).

NoV quantification. Mean NoV GI concentrations (± SD) in the source and tap water were 1,080 ± 569 and 856 ± 335 NoV copies/liter, respectively. These figures are based on mean C_T values (± SD) of 34.9 (±1.1) and 34.2 (±0.9) from the four test replicates for the source and tap water, respectively, and were calculated from the NoV GI plasmid standard curve. Due to the probable loss of NoV during the processing and RT steps, these figures are only estimated levels but can be used to compare NoV quantities in samples.

NoV genotyping. The NoV GIs detected in the fecal specimens from the outbreak cases and the community cases were typed as GI/5, as were the NoV isolated from the main building tap and source stream water. The NoVs were identified as GI/5 (most similar to GI/5 Apalachicola Bay GenBank accession number AF414406) and were indistinguishable by DNA sequencing with 100% DNA sequence homology for the 172-nucleotide "region B" sequence of the NoV RNA polymerase gene.

DISCUSSION

We report the largest waterborne NoV outbreak documented in New Zealand thus far. The water supply for the ski resort was contaminated with human sewage. Virological investigations identified NoV GI/5 in both fecal specimens from cases of gastroenteritis and the water supply, thereby establishing a linkage between cases and the source water. Epidemiological investigations supported the association between consumption of the resort drinking water and illness. Environmental investigations eventually revealed a likely event by which sewage contamination of the resort water supply had occurred.

Early environmental investigations did not support a common source of infection, but identification of *E. coli* contamination of the water supply and the subsequent identification of NoV in the feces of individuals with gastroenteritis suggested that the water supply had been contaminated by human sewage. The finding of gastroenteritis cases due to rotavirus and *Cryptosporidium* infection also supported sewage contamination.

The UF method with polyethylene glycol precipitation was used successfully to concentrate NoV. To our knowledge, this is the first time that this approach has been used to investigate a NoV outbreak in a real-time situation. The UF method was found to be rapid and simple to use. The disposable cartridges are low cost, and the method has been shown to give good recovery of NoV, enterovirus, and adenovirus from tap water (up to 100 liters) and river water (up to 20 liters) as determined by both PCR and cell culture (data not shown).

As described elsewhere (26), the use of an internal control allowed detection of RT-PCR inhibition. In this study, through the use of Armored RNA, RT-PCR inhibition was detected in RNA extracted from 500-μl volumes of water concentrate. Successful analysis was achieved through the extraction of lesser volumes (200 μl). Wyn-Jones et al. (35) also identified this issue demonstrating RT-PCR inhibition when larger volumes were extracted.

The rapid detection and genogrouping of NoV by real-time RT-PCR from fecal and water samples provided timely virological evidence of a possible link between cases of gastroenteritis and the suspected contaminated water to support the epidemiological investigation. In addition, further evidence was obtained through DNA sequencing when the NoV strains identified in water and fecal samples were genotyped and found to be indistinguishable. Genotyping viruses from environmental samples can be difficult because sequencing PCRs may lack the sensitivity required and are generally less sensitive than the real-time PCR assays which are used for their initial detection. Trujillo et al. (30) found that although 8/33 water samples were NoV positive by real-time RT-PCR (mean C_T, 37.9), only the sample with the lowest C_T value (C_T, 33.4) was successfully sequenced. Our approach to use a seminested PCR for the genotyping step was successful and enabled a direct comparison of NoV sequences from the fecal and water samples.

It is interesting that NoV GI was the causative agent rather than the more commonly found NoV GII. In New Zealand in 2006, GII/4 was responsible for 73% of identified NoV outbreaks, while NoV GI accounted for only 8% of outbreaks. Furthermore, GI/5 was responsible for less than 2% of identified outbreaks in 2006 (G. E. Greening, unpublished data). In the only other reported waterborne NoV outbreak in New Zealand, which occurred at a ski resort in 1996 (3), NoV GI/2 was identified in the fecal samples associated with cases of gastroenteritis, but no virological analysis of water was carried out (8). Other researchers have described the involvement of GI strains in waterborne outbreaks (9, 12, 17, 22, 25, 26). Maunula et al. (22) found that while 13% of NoV outbreaks in Norway between 1998 and 2003 were caused by NoV GI, approximately half the waterborne outbreaks were attributable to NoV GI strains (either GI/3 or GI/6) and postulated that there may be differences in virus stability between genotypes. Interestingly, fecal samples containing NoV GI have been shown to have 100 times less viral load compared with the viral load in NoV GII-positive samples. This difference suggests that transmissibility via the fecal-oral route may be lower for GI than GII (5) and may explain why secondary cases in the community were not a major feature of this outbreak. It does not, however, explain why NoV GI appears to be predominant in waterborne outbreaks, and in our view, this observation warrants further investigation.

The outbreak described in this paper highlights problems with the current regulatory approach to privately operated community drinking water supplies and provides supporting evidence for current efforts to improve drinking water legislation in New Zealand (23). The impact of this outbreak on the ski resort has lead to wider recognition of the vulnerability of water supplies at all ski resorts and alpine venues. Government agencies in New Zealand are reviewing their approach to water supply management with a particular focus on improved training for supply managers and the development of risk management plans.

For this outbreak, the virological data were critical to confirming the source of the outbreak. The development and use of the methods described provided an additional tool for outbreak investigation which could potentially have a significant impact on the recognition of waterborne infection and could subsequently lead to improvements in drinking water management and quality.

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