Enteric viruses in New Zealand drinking-water sources
W. M. Williamson, A. Ball, S. Wolf, J. Hewitt, S. Lin, P. Scholes, V. Ambrose, B. Robson and G. E. Greening

ABSTRACT
This study determined whether human pathogenic viruses are present in two New Zealand surface waters that are used as drinking-water sources. Enteric viruses were concentrated using hollow-fibre ultrafiltration and detected using PCR for adenovirus (AdV), and reverse transcription PCR for norovirus (NoV) genogroups I-III, enterovirus, rotavirus (RoV) and hepatitis E virus (HEV). Target viruses were detected in 106/109 (97%) samples, with 67/109 (61%) samples positive for three or more viral types at any one time. AdV, NoV and RoV were detected the most frequently, and HEV the least frequently. Human NoV was not usually associated with animal NoV. Our results suggest that New Zealand would be well served by assessing the ability of drinking-water treatment plants to remove viruses from the source waters, and that this assessment could be based on the viral concentration of AdV-NoV-RoV. The long-term aim of our work is to use this information to estimate the risk of waterborne viral infection.

Key words | drinking-water, enteric viruses, microbial risk assessment and management, water pollution

INTRODUCTION
Viruses are commonly found in surface water sources (Hurst 1991; Till et al. 2008) and are thought to be responsible for an appreciable proportion of waterborne disease cases (Moore et al. 1994). While the drinking-water standards used in most developed countries contain compliance criteria for bacteria and protozoa, they do not include criteria for viruses. The bacteriological criteria in drinking-water standards are mostly based on indicator bacteria (Escherichia coli, coliforms or faecal (thermotolerant) coliforms), which are generally less resistant to disinfection than viruses (Hrudey & Hrudey 2004). Furthermore, waterborne outbreaks have occurred in supplies that tested negative for indicator bacteria (Craun et al. 1997; Kukkula et al. 1999; Brieseman et al. 2000).

Five groups (seven types) of waterborne viruses were selected as our target viruses: the norovirus genogroups I-III (NoV GI-GIII), adenovirus (AdV), enterovirus (EnV), rotavirus (RoV) and hepatitis E virus (HEV). These groups of waterborne viruses were selected due to their transmission by the faecal-oral route, impact on human health, international prevalence in waters and potential to inform about viral contamination likely to challenge drinking-water treatment (Ashbolt 2004; Albinana-Gimenez et al. 2006).

NoV are shed from mammals including humans, cattle and pigs. The ease with which NoV spreads amongst closely associating people and its presence in shellfish, strongly suggests that this pathogenic virus is likely to be spread by
water contaminated with faecal matter (Le Guyader et al. 2006), making it an appropriate target virus for this project. The three NoV genogroups targeted covered most human (NoV GI and NoV GII), porcine (NoV GII), and bovine/sheep (NoV GIII) NoV strains (Wolf et al. 2007, 2009).

EnV are found in mammals including humans, cattle and pigs, and are present in many different environmental matrices, including water. Their ubiquitous environmental reporting may be due to their relative ease of detection by culture methods, which has led to them becoming a widely used indicator of environmental contamination by human pathogenic viruses. EnV were detected in about one-third of samples in a previous study of New Zealand surface waters (Till et al. 2008).

AdV have considerable advantages as indicators of faecal contamination and appear to be species-specific. While AdV are environmentally stable, many are difficult to culture, making current molecular tools excellent for their ready detection in environmental samples. AdV are included on the USA Environmental Protection Agency’s “Drinking Water Microbial Candidate Contaminant List” (USA-EPA Federal Register 2006), which lists contaminants that may occur in public water systems and are research priorities in the treatment of water.

RoV circulation in the environment is anecdotally quite high as many young mammals, including sheep and cattle, are susceptible to RoV infections (Schroeder et al. 1985). RoV infections are a serious problem during calf rearing in the dairy and beef industries, where the prevalence is about 40% (Maes et al. 2003). RoV may cross the species barrier due to their long shedding phase and constant seasonal production of susceptible hosts (lams and calves). There are an increasing number of reports of atypical RoV that have features associated with more than one species, strongly suggesting that RoV may be zoonotic (Maes et al. 2003; Khamrin et al. 2006).

HEV is considered an emerging human disease in temperate countries, and waterborne outbreaks have occurred due to failures in drinking water treatment or poor sanitation (CEI 2003; Clemente-Casares et al. 2003). HEV antibodies have been reported in deer, cats, dogs, cattle, pigs and sheep, and taking this together with the close genetic similarity between human and swine isolates, it has been suggested that HEV is a zoonotic virus (Chobe et al. 2006). HEV is widespread among domestic pig populations in New Zealand, with isolates clustering with human and swine isolates from the USA and Europe (Garkavenko et al. 2001).

Our project was designed to investigate viral concentrations in source waters for drinking-water treatment plants as part of a programme to develop virus compliance criteria for New Zealand’s drinking-water standards. Our long-term aim is to conduct a worst-case quantitative microbial risk assessment using virus concentrations from source waters that are heavily impacted by human and animal faeces. This information could be used to estimate the amount of drinking-water treatment required to reduce the risk of viral-related waterborne disease to an acceptable level.

This paper reports the testing of river water for a suite of enteric viruses and suggests three as potential target/indicators, while the aims of our ongoing study are to determine: Which human pathogenic viruses are relevant to New Zealand? Are these viruses present in drinking-water sources (water that is to be treated for purposes of human consumption)? Finally, what appropriate human virus(es) can be used to assess the challenge viruses pose to water treatment processes?

**MATERIALS AND METHODS**

**Water samples**

The two river sites selected for this study had previously demonstrated high virus prevalence (Till et al. 2008). The Waikato River in the North Island (Figure 1) provides drinking-water to the Huntly community (~8,000 people) and is impacted by agriculture. The Oreti River in the South Island (Figure 1) provides drinking-water to Invercargill City (~51,000 people) and is impacted by agriculture. From February 2007–June 2009, 10 L water samples from intakes on the two rivers were collected approximately fortnightly and transported to laboratories for processing to recover the target viruses within 48 h of sampling.

The water samples were taken from the same drinking-water treatment plant sampling points routinely used for sampling for other microbiological determinants. Hence the samples represented true abstraction samples. Fifty-three and 56 samples were taken from the Waikato River and the Oreti River, respectively. The viruses targeted in this work were: AdV, NoV with three genogroups targeted (NoV GI, NoV GII, NoV GIII), EnV, RoV and HEV.

**Concentration of viruses in water samples**

Viruses were concentrated from each 10 L water sample by hollow fibre ultrafiltration using Hemoflow® HF80S dialysis filters (Fresenius Medical Care) as described by...
Hill et al. (2005) with modifications. Briefly, following pretreatment of the filter with 0.01% (w/v) sodium polyphosphate solution for 15 min, the sample was pumped through the filter using a peristaltic pump at a permeate rate of 100–120 mL/min to complete filtration within approximately 2 h. The filter was back-flushed with a solution of 0.5% (v/v) Tween 80 and 0.01% (w/v) sodium polyphosphate. The retentate was centrifuged (10,000 × g 20 min) to pellet solid material, and the supernatant stored at 4 ℃ (SN1). An elution solution containing 3% (w/v) beef extract and 0.05 M glycine (pH 9) was added to the pellet at a ratio of 5:1 (v/w). Following shaking (120 rpm) for 1 h at room temperature, the material was centrifuged (5,000 × g 10 min EnV, RoV, HEV; 10,000 × g 10 min AdV, NoV), the supernatant was added to the stored SN1 and the pellet discarded. Viruses were further concentrated using polyethylene glycol 6000 (10% w/v) and sodium chloride (1.75% w/v). After shaking (120 rpm) overnight at 4 ℃, the material was centrifuged (10,000 × g 30 min) and the pellet was resuspended in 5 mL phosphate buffered saline (pH 7.2). The suspension was sonicated in an ultrasonic wave bath for 2 min and eluted for 30 min on a shaker (120 rpm) at room temperature. Sonication for another 2 min was followed by removal of the suspended solids by centrifugation (13,000 × g 15 min). The concentrated sample was stored at −80 ℃ until nucleic acid extraction.

Recovery efficiencies were determined using Waikato River and Oreti River water samples spiked with AdV, NoV GI, NoV GII, EnV and RoV. Norovirus GIII and HEV were not available for spiking experiments.

**Assay controls**

To evaluate reverse transcription (RT)-PCR inhibition, Armored RNA®-Norwalk Virus GI (aRNA; Asuragen, USA) was used. If assay inhibition were detected, the sample was diluted to minimise its effect on RT and PCR; a relatively low dilution will reduce the effect of inhibitors while, because the PCR is a very sensitive method, the dilution will not significantly reduce the ability to detect the virus (Kopecka et al. 1993). The net result of dilution is to reduce inhibitor effects and so increase the ability to detect target viruses in a sample.

**Viral DNA/RNA extraction**

Viral DNA/RNA was extracted from river water sample concentrates (2 × 200 μL) using the High Pure Viral Nucleic Acid kit (Roche Molecular Diagnostics Ltd, Mannheim, Germany) according to the manufacturer’s instructions. Viral DNA/RNA was eluted in 50 μL elution buffer. To evaluate RT-PCR inhibition, 10 μL of 10-fold diluted aRNA was added to each 200 μL aliquot of sample (or control) prior to extraction.

**Real-time PCR/RT-PCR**

Published primer sets and probes were applied for the detection of EnV (human enterovirus, poliovirus, coxsackievirus, echovirus), AdV (all human types), NoV GI (human), NoV GII (human and swine), NoV GIII (animal – bovine and sheep), RoV (mammalian – human, swine, canine, bovine), HEV (human, swine) and aRNA (Table 1). Armored RNA primers and probes were as previously described by Greening & Hewitt (2008). Primers and probes were synthesised by Invitrogen (Carlsbad, USA) and Applied Biosystems (Carlsbad, USA) or Biosearch Technologies (Navato, USA).
Reverse transcription for NoV, EnV, RoV and HEV was carried out with random hexamers (EnV, RoV and HEV) or specific reverse primers (NoV GI-III) using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. Real-time PCRs were set up in 25 μL reaction volumes by using the Platinum qPCR Supermix-UDG (Invitrogen) for EnV, AdV, NoV and RoV or the Universal TaqMan Reagent for HEV (Applied Biosystems). The cycling conditions with primer and probe concentrations used for each PCR, are shown in Table 1. The real-time assays were carried out in a ABI Prism 7700 Sequence Detection System (Applied Biosystems) with post-PCR data analysis using Sequence Detector Software (Applied Biosystems) for EnV, RoV and HEV and in a Rotor-GeneTM 3000 or 6000 real-time rotary analyzer (Corbett Life Science, Sydney, Australia) for NoV GI-III and AdV. Each viral DNA/RNA extract was tested in duplicate to give four results for each water sample for each target virus. Samples that were inhibitory to the PCR, as shown by the aRNA results, were diluted 1:5 and the (RT) PCR assay repeated.

For the EnV, RoV and HEV PCR assay, after the first 24 sample we found interference from aRNA as a multiplex assay. Therefore, samples 1–24 were repeated for EnV, RoV

<table>
<thead>
<tr>
<th>Target virus (ref.)</th>
<th>Primer and probe sequences (concentration)</th>
<th>PCR cycling conditions</th>
<th>Amplicon size and region</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus (Heim et al. 2003)</td>
<td>Primers at 0.6 μM, Probe at 0.25 μM</td>
<td>Hold at 50°C for 2 min; hold at 95°C for 3 min; 45 cycles of 95°C for 20 sec and 55°C for 15 sec and 62°C for 1 min</td>
<td>132-bp hexon gene</td>
</tr>
<tr>
<td>Norovirus (Wolf et al. 2007)</td>
<td>Primers at 0.4 μM, Probes at 0.2 μM</td>
<td>Hold at 50°C for 2 min; hold at 95°C for 5 min; 45 cycles of 95°C for 15 sec and 57°C for 1 min</td>
<td>NoV GII is 97-bp, junction between ORF-1 and ORF-2 regions</td>
</tr>
<tr>
<td>Enterovirus (Donaldson et al. 2002)</td>
<td>Primers at 0.6 μM, Probe at 0.25 μM</td>
<td>Hold at 50°C for 2 min; hold at 95°C for 5 min; 45 cycles of 95°C for 20 sec and 60°C for 1 min</td>
<td>192-bp 5’ untranscribed region</td>
</tr>
<tr>
<td>Rotavirus (Pang et al. 2004)</td>
<td>Primers at 0.3 μM, Probe at 0.2 μM</td>
<td>Hold at 50°C for 2 min; hold at 95°C for 5 min; 45 cycles of 94°C for 20 sec and 60°C for 1 min</td>
<td>87-bp non-structural protein 3</td>
</tr>
<tr>
<td>Hepatitis E virus (Jothikumar et al. 2006)</td>
<td>Primers at 0.4 μM, Probe at 0.35 μM</td>
<td>Hold at 50°C for 2 min; hold at 95°C for 10 min; 45 cycles of 95°C for 15 sec and 60°C for 1 min</td>
<td>69-bp ORF3 region</td>
</tr>
<tr>
<td>NV-GI aRNA (Greening and Hewitt 2008)</td>
<td>Primers at 0.1 μM, Probe at 0.2 μM</td>
<td>As per individual virus assay</td>
<td></td>
</tr>
</tbody>
</table>
and HEV for both rivers with the aRNA as a separate assay. However, due to resource limitation samples 1–24 were repeated in duplicate extractions with no additional replication. From sample 25 onwards for EnV, RoV and HEV, each DNA/RNA extract was extracted in duplicate and each extract tested in duplicate to effectively give four replicates for each water sample for each target virus, with the aRNA run as separate assays to assess inhibition. Consequently, positive replicates are referred to as “a percentage of replicates positive” to account for the variation in replication for EnV, RoV and HEV. Hence, if 4/4 or 2/2 of the replicates were positive, this is referred to as “100% replicates positives”, and if 2/4 or 1/2 were positive this is referred to as “50% replicates positives”.

RESULTS AND DISCUSSION

A total of 109 samples were taken from the Waikato River and the Oreti River (Table 2). Of these, 106/109 samples were positive for at least one of the target viruses and no viruses were detected in three samples. For the Waikato River, 52/53 samples were positive for at least one target virus and 28/33 were positive for three or more target viruses. For the Oreti River, 54/56 samples were positive for at least one target virus and 39/56 were positive for three or more target viruses (from the same sample). These results indicate that not only do the drinking-water sources contain enteric viruses they usually contain more than one virus type.

AdV and RoV were the most frequently found target viruses, with 71/109 (65%) and 70/109 (64%) samples positive, respectively, and NoV GII and NoV GIII the next most frequently found target viruses with 66/109 (61%) and 56/109 (51%) samples positive, respectively (Table 2).

Our spiking data and aRNA control data indicated variable recovery efficiencies within and between virus groups, and that the Waikato River water assays were prone to interference from components of the river water. Percentage recoveries from Waikato River water (mean ± standard deviation; n = 5) were: AdV 3.8 ± 2.3, NoV GI 20.9 ± 21.7, NoV GII 33.8 ± 22.1, EnV 7.8 ± 3.9 and RoV 15.9 ± 13.4. Percentage recoveries from Oreti River water (mean ± standard deviation; n = 5) were: AdV 13.7 ± 10.7, NoV GI 22.6 ± 19.4, NoV GII 27.4 ± 17.5, EnV 27.9 ± 19.7 and RoV 36.3 ± 48.8. NoV GIII and HEV were not available for spiking experiments. Samples from the Waikato

| Table 2 | Presence-absence of norovirus genogroups I-III (NoV GI-III), adenovirus (AdV), enterovirus (EnV), rotavirus (RoV) and hepatitis E virus (HEV) from the Waikato River and Oreti River |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | NoV GI | NoV GII | NoV GIII | AdV | EnV | RoV | HEV |
| Overall Totals |        |         |         |     |     |     |     |
| Total number of samples | 109   | 109     | 109     | 109 | 109 | 109 | 109 |
| Samples positive    | 36    | 66      | 56      | 71  | 13  | 70  | 4   |
| Samples negative    | 73    | 43      | 53      | 38  | 96  | 39  | 105 |
| Positive (%)        | 33%   | 61%     | 51%     | 65% | 12% | 64% | 4%  |

| Waikato           |        |         |         |     |     |     |     |
| Total number of samples | 53   | 53      | 53      | 53  | 53  | 53  | 53  |
| Samples positive   | 20    | 28      | 25      | 30  | 1   | 36  | 3   |
| Samples negative   | 33    | 25      | 28      | 23  | 52  | 17  | 50  |
| Positive (%)       | 38%   | 53%     | 47%     | 57% | 2%  | 68% | 5.7%|

| Oreti              |        |         |         |     |     |     |     |
| Total number of samples | 56   | 56      | 56      | 56  | 56  | 56  | 56  |
| Samples positive    | 16    | 38      | 31      | 41  | 12  | 34  | 1   |
| Samples negative    | 40    | 18      | 25      | 15  | 44  | 22  | 55  |
| Positive (%)        | 29%   | 68%     | 55%     | 73% | 21% | 61% | 1.8%|
River were more prone to RT-PCR inhibition than those from the Oreti River (data not shown).

A negative (non-detect) result may mean that viruses are absent or below the detection limit, or that the assay failed to detect target viruses due to interference from some component of the sample concentrate (Kopecka et al. 1993). Analysing a larger portion of the sample would overcome the first problem, but may lead to greater interference (RT-PCR inhibition). So we optimised the recovery by analysing multiple small volume replicates.

Three genogroups of NoV were assayed for (NoV GI-GIII): NoV GI and NoV GII genogroups were defined as human-associated for the purpose of this project, while NoV GIII was defined as an animal associated NoV genogroup (Wolf et al. 2007, 2009). Humans and pigs can be infected by NoV GII types, therefore for environmental samples it is possible that a NoV GII-positive sample could be from either or both species. However, because most human cases of NoV in New Zealand have been associated with NoV GI and there is little information on NoV GII in New Zealand pigs, we made the assumption that the NoV GII detected was most likely to be of human origin. We acknowledge that a sample may return a false human-positive (and actually be animal NoV) by this definition.

To aid interpretation and use all the information in the replicates we have presented the presence-absence data as a cumulative proportion of samples where viruses were detected, with this broken down into the proportion of replicates positive (Figure 2). The Oreti River had more samples where all replicates were positive than the Waikato River (Figure 2). However, as mentioned above, our control assays indicated that there were likely to be more RT-PCR inhibitory substances present in the Waikato River samples, which may reduce our ability to detect our target nucleic acids. Our results indicate that caution should be used when interpreting whether there are true differences in viral presence in the Waikato River and the Oreti River (environmental samples). Since the concentration method did not recover viruses equally and recoveries were usually less than 30% (based on spiking studies; see Material and Methods), our results suggest that the number of positive samples may be a conservative estimate of viral presence in these two rivers.

**CONCLUSIONS**

This paper presents a dataset for the presence-absence of seven enteric viruses from 109 river water samples taken over two years from the Waikato River in the North Island and the Oreti River in the South Island of New Zealand. Of the 109 water samples, 106 were positive for at least one of the target viruses and 67 samples had three or more of the target viruses present at any one time. Therefore, there is ample evidence that all the target viruses are present in both rivers and that some of the target viruses such as AdV, NoV and RoV are present most of the time. The results reaffirm the need for water treatment to protect public health from potentially pathogenic waterborne viruses and suggest that the ability of drinking-water treatment plants to remove viruses from the source waters could be estimated from the combined viral concentration of AdV-NoV-RoV.

The detection of target viruses by molecular biology techniques does not, currently, provide information about the potential infectivity of the detected viruses. Viral particles
can be inactivated by environmental conditions, but still be detected by PCR. Kopecka et al. (1993) estimated that for EnV the ratio of infectious units to physical viral particles may be lower than 0.01, with this ratio decreasing even further when viruses originated from treated wastewater. It is unknown whether the virus recovery methods, such as ultrafiltration used in this work, preferentially recover infective viruses or non-infective viruses, or whether the infectivity status is neutral to recovery efficiencies.

The current data will be used to estimate the risk of viral infection from drinking untreated river water and then to determine the degree of water treatment required to reduce the risk of viral infection to an acceptable level. In doing so, we will use a precautionary approach to public health risk by assuming that the detected viruses are potentially infectious, even given the caveats of Kopecka et al. (1993). We will take this approach because our recovery data indicate that we may be detecting only a low percentage of the viruses present in samples and we reason that these viruses must have originated from faecal contamination and represent structurally intact viral particles because naked nucleic acid has a short half-life in most environmental contexts (Tsai et al. 1995). Further work is needed to determine the infectivity of viruses detected by molecular biology tools, whether infectivity status alters recover potential, and the effectiveness of the water treatment plants in removing/inactivating human pathogenic viruses.

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