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*
Institute of Environmental Science & Research Ltd (ESR), New Zealand

ABSTRACT

Our aim was to determine whether human-pathogenic viruses are present in surface water used as the source of drinking-water. We regularly sampled two rivers over two years. Enteric viruses were concentrated using ultrafiltration and detected using PCR for adenovirus, norovirus, enterovirus, rotavirus, and hepatitis E virus (HEV).

Target viruses were detected in 97% (106/109) of samples, with 67% of samples positive for three or more viral types at one time. Adenovirus, norovirus and rotavirus were detected the most frequently, while HEV was detected the least but at least once in both rivers. There was an increase in the frequency of animal-norovirus and rotavirus over the lambing and calving period (July-October).

Our results suggest that New Zealand would benefit from assessing the ability of drinking-water treatment plants to remove a certain viral concentration from source water, and this assessment could be based on the viral concentration of adenovirus, norovirus and rotavirus.

KEYWORDS

Microbial risk assessment and management; water pollution and diseases; enteric viruses; drinking water

1 INTRODUCTION

The overall purpose of our project is to generate sufficient data for a quantitative microbial risk assessment (QMRA) that can be used to estimate the level of drinking-water treatment required to reduce the risk of viral-related waterborne disease from the consumption of drinking water to an acceptable level. To do this, it is necessary to determine the viral presence in rivers used as sources of drinking water. The results from a QMRA can be used to inform water suppliers of the risks from viral contamination of water sources and the need for appropriate treatment. Our aims were to determine the waterborne human enteric viruses relevant to New Zealand, viral present in water that is to be treated for purposes of human consumption, and the appropriate human viral target/indicators for assessing the challenge to water treatment processes. This paper reports the testing of river water for a suite of enteric viruses and suggests at least three as potential target/indicators.

The waterborne viruses that were identified as significant to New Zealand are norovirus (NoV genogroup I-III), adenovirus (AdV), enterovirus (EnV), rotavirus (RoV) and hepatitis E virus (HEV) (Pulford et al., 2005a; Wolf et al., 2007; Wolf et al., 2009). These enteric viruses are often shed in high numbers in faeces and are transmitted by the faecal-oral route. The reasons for selecting these viruses include their impact on human health, that international experience has suggested they are of waterborne significance, and the potential to inform about viral contamination likely to challenge drinking-water treatment.

Noroviruses infect mammals including humans, cattle and pigs, and are transmitted to new hosts. Norovirus have been associated with outbreaks of acute gastroenteritis in various setting including hospitals, cruise ships and after consumption of shellfish (Gallimore et al., 2005; Boxman et al., 2006; Costantini et al., 2006; Le Guyader et al., 2006). Although the infection tends to be self-limiting to 48–72 hours, shedding of infectious viruses continues for 1–2 weeks post-infection (Maunula, 2007). The ease with which NoV spreads amongst

closely associating people of all ages and its presence in shellfish, strongly suggests that this pathogenic virus is likely to be spread by water contaminated with faecal matter (Hewitt et al., 2007), making it an appropriate target virus for this project. The three NoV GI-GIII targeted in this study cover most human (NoV-GI and NoV-GII), porcine (NoV-GII), and bovine/sheep (NoV-GIII) NoV strains (Wolf et al., 2007; Wolf et al., 2009).

Enteroviruses are found in mammals including humans, cattle and pigs, and are present in many different environmental settings including water, where they can be used to indicate faecal contamination. Despite their presence in faeces and waterborne potential, EnVs do not typically cause gastroenteritis in humans, however they have been associated with other diseases including diabetes (Klemola et al., 2008). Their ubiquitous environmental reporting may be due to their relative ease of detection by culture methods (Pulford et al., 2005a), which led to them be a widely used indicator of environmental contamination by human pathogenic viruses. Therefore, the inclusion of EnV as a target in this project is consistent with international methods and a previous study of New Zealand river water indicated EnV was present in about a third of the samples (Till et al., 2008).

Indicator organisms ideally need to fulfil several criteria, including being present when the pathogen is present, not multiplying in the environment, having a constant ratio between itself and the pathogen(s) or the risk of waterborne infection, being present at greater concentrations than the pathogen, having the same environmental persistence as the pathogen, being non-pathogenic, and being easy to quantify/detect (Moe, 2002). Enteroviruses have been used as an indicator or 'index' virus (Murray et al., 2000); some types are relatively easy to grow from environmental samples (e.g. some coxsackie B viruses and poliovirus). Since 1998, it has been suggested that AdV would make a good molecular index for the assessment of human viral contamination in the environment (Pina et al., 1998; Lewis and Greening, 2000; Pulford et al., 2005a; Pulford et al., 2005b). Other indicator organisms proposed include faecal bacteria (e.g. *E. coli* and enterococci) and bacteriophage. However, the correlation between bacteria and phage indicators and human pathogenic viruses is not necessarily strong, with occasions where water considered potable by the absence of bacterial indicators contained high levels of pathogenic viruses (Pulford et al., 2005a).

Adenoviruses are an alternative an indicator (to EnV) of faecal contamination; they are double-stranded DNA, species specific, environmentally stable and are suitable for source tracking of faecal contamination (Pulford et al., 2005a). However, many AdVs are difficult to culture, making molecular-based assays excellent for their ready detection in environmental samples. Like EnV, most strains of AdV do not commonly cause gastroenteritis in people, although two principal strains (AdV 40 and 41) are commonly associated with gastroenteritis and maybe shed in faeces for several months post-infection (Pulford et al., 2005a). The limitations associated with culturing AdV may underlie the difficulty in establishing a strong causative association between waterborne disease and AdV. Internationally, AdV are strong candidates as viral indicators or indices and are thus a necessary component for surveying environmental faecal contamination and the quality of surface and drinking water (Pina et al., 1998; Lewis and Greening, 2000). The importance of AdV is illustrated by their inclusion (along with only three other viruses: echoviruses, coxsackieviruses (both of which are EnV) and NoV) on the USA Environment Protection Agency's (EPA) "*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.

Rotavirus has been identified as a major causative agent of severe gastroenteritis in children, and while infections do occur in older people, these infections are typically asymptomatic or mild compared with the primary infection (Pang et al., 2004; Zhao et al., 2005). Consequently, waterborne outbreaks are not common. There are a number of reports of reassortant RoVs; viruses that contain gene segments from both animal and human RoV strains. Reassortant RoV suggest zoonotic episodes where co-infection with both animal and human RoV must have occurred in a human or animal host (Maes et al., 2003; Khamrin et al., 2006). If RoV were zoonotic, there is ample opportunity for RoV to cross the species barrier due to its long shedding phase and constant seasonal transmission *via* renewable susceptible hosts (lambs and calves). Rotavirus infections are a serious problem in calf rearing in the dairy and beef industries (about 40% prevalence in USA (Maes et al., 2003)), and suggest that RoV may be a viral pathogen of significance to New Zealand

Hepatitis E virus is endemic in pigs and humans in many developing and tropical countries, and large waterborne outbreaks have occurred due to failure of drinking-water treatment or poor sanitation (Centre for

Emerging Issues, 2003). Hepatitis E virus is considered an emerging human disease in temperate countries, and is widespread among domestic pig populations in New Zealand (Garkavenko et al., 2001). New Zealand isolates cluster with human and swine isolates from the USA and Europe (Garkavenko et al., 2001), suggesting that there is nothing particularly unusual about the New Zealand strains. Reports of HEV antibodies in deer, cats, dogs, cattle and sheep, taken together with the close genetic similarity between human and swine isolates, suggest that HEV is a zoonotic virus (Chobe et al., 2006). However, direct epidemiological evidence is lacking (Centre for Emerging Issues, 2003). Unlike hepatitis A, B, and C, HEV is not a notifiable disease in New Zealand and routine clinical procedures have not been established, both of which may result in under-reporting. Hence, as an example of an emerging disease, HEV is an appropriate virus to survey in New Zealand environments.

2 MATERIAL AND METHODS

2.1 WATER SAMPLES

Two rivers were sampled. The Oreti River in Southland was sampled at Branxholme water treatment plant, which serves the local communities and Invercargill, and the Waikato River in Waikato was sampled at the Huntly water treatment plant, which serves the community of Huntly. These rivers were selected because of high virus prevalence in a previous study (Till et al., 2008). The Oreti River represented an agriculturally-impacted river and the Waikato River represented a river with significant urban interactions. From February 2007 to June 2009, 10 L river water samples were collected approximately two-weekly and transported to the laboratories for immediate processing within 48 h.

The water samples were taken at the drinking water treatment-plant's normal sampling point for assessing other microbiological determinants of the drinking-water intake. Hence the sample represents a true abstraction sample (raw/untreated river water that will be treated for to be drinking water).

2.2 CONCENTRATION OF 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 pre-treatment of the filter with 0.01% (w/v) sodium polyphosphate ($NaPO_3$) solution for 15 min, the sample was pumped through the filter using a peristaltic pump at a permeate rate of 120-150 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) $NaPO_3$. The retentate was centrifuged (10,000 × g 20 min) to pellet solid material, and the supernatant stored (SN1). An elution solution containing 3 % (w/v) beef extract and 0.05 M glycine was added at a ratio of 5:1 (v/w) to the pellet and then adjusted to pH 9. Following shaking (120 rpm) for 1 h at room temperature, the material was centrifuged (10,000 × g 20 min), supernatant was added to the stored SN1 and the pellet was then discarded. Viruses were further concentrated using polyethylene glycol 6000 (PEG, 10 % w/v) and sodium chloride (NaCl, 1.75 % w/v) and adjusting to pH 7.2. Following shaking (120 rpm) overnight at 4 °C, the material was centrifuged (10,000 × g 30 min) and pellet was resuspended in 5 mL phosphate buffered saline (PBS, pH 7.2). The suspension was sonicated in an ultrasonic wave bath for 2 min and eluted for 30-60 min on a shaker (120 rpm) at room temperature. Following sonicating for another 2 min, the suspended solids were removed by centrifugation (13,000 × g 15 min) and the concentrated sample was stored at −80 °C until use.

2.3 VIRAL DNA/RNA EXTRACTION

Viral DNA/RNA was extracted from water concentrates (2 × 200 µL) by 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 Armored RNA®-Norwalk Virus GI (aRNA; Asuragen, USA) was added prior to extraction as an inhibition reference control to each 200 µL aliquot of sample or dH_2O. Inhibition was considered present when the cycle threshold (Ct) value obtained from the sample was more than one cycle higher than the mean Ct-value that was obtained from the negative control in the aRNA PCR.

2.4 REAL-TIME PCR/RT-PCR

Published primer sets and probes were used for the detection of EnV, AdV, NoV-GI, NoV-GII, NoV-GIII, RoV, and HEV (Table 1). Primers and probes were synthesised by Invitrogen (Carlsbad, USA) and Applied Biosystems (Carlsbad, USA) or Biosearch Technologies (Navato, USA).

For real-time PCR for NoVs and AdV, each 25 ul reaction contained 5 ul of cDNA, 1x qPCR Supermix-UDG (Invitrogen) and primers/probes at different concentrations (Table X). Initial activation of the HotStar polymerase at 95°C for 5 min was followed by a two-step cycling protocol for NoV, comprising denaturation at 95°C for 15s and annealing/extension at 57°C for 60s over 45 cycles, and a three step protocol for AdV, comprising 95°C for 20s, annealing at 55°C for 15s and extension at 62°C for 60s over 45 cycles . All real-time assays were carried out in a Rotor-GeneTM 3000 or 6000 real-time rotary analyzer (Corbett Life Science, Sydney, Australia).

Reverse transcription for EnV, RoV and HEV was carried out in 40 µL reaction volumes by using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions and random hexamers were used as primers. The TaqMan real-time PCR reactions were set up in 25 µL reaction volumes by using Platinum qPCR Supermix-UDG (Invitrogen) for EnV, AdV, NoV and RoV or Universal TaqMan Reagent for HEV (Applied Biosystems) with the concentration for each primer and probe as indicated in Table 1. The fluorescent signal emitted during PCR was detected using the ABI Prism 7700 Sequence Detection System (Applied Biosystems) with cycling conditions as described in Table 1. Post-PCR data analysis was performed using the Sequence Detector Software (Applied Biosystems). The baselines were determined between cycles 3 and 15. The threshold of EnV, RoV or HEV PCR was set at 0.1 for all runs, which was about the halfway point in the linear part of the semi-log scale amplification plot. Each DNA/RNA extract was tested in duplicate to give 4 results for each water sample for each target virus.

A specific primers and probe set (Table 1) to detect aRNA was used to detect RT-PCR inhibition. The criterion we used to define a sample as positive is any replicate PCR reaction that had a Ct-value equal to or less than where we could detect the target on a PCR run of 45 cycles[1], which we determined to be the limit of detection. This value is between 39 and 43 Ct-units depending on the virus; NoV-GI 43, NoV-GII 41, NoV GIII 43, AdV 43, EnV 40, RoV 43, and HEV 43. While in most cases when high Ct values were found (i.e. low numbers of target present), they were often associated with only one replicate being positive, however this was not necessarily the case.

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). Humans and pigs can be infected by NoV-GII strains, therefore for environmental samples it is possible that a NoV-GII-positive sample could be from either or both species. However, because the most human cases of NoV in New Zealand have been associated with NoV GII and there is little information on NoV GII in New Zealand pigs, we assumed that NoV GII detected is likely to be of human origin. However, we acknowledge that a sample may return a false human-positive (actually be a animal NoV) by this definition

[1] The higher the Ct value the less target is present, thus a Ct value of 40 has less target present in a sample than a Ct value of 38, which has less than a sample with a Ct value of 30.

Table 1 Real-time PCR/RT-PCRs primers, probes and cycling conditions.

Target virus	Primer and probe sequences (concentration)	PCR cycling conditions	Amplicon size and region	Reference
Enterovirus	Fw: 5'-ggcccctgaatgcggctaat-3' (0.6 μM) Rv: 5'-caccggatggccaatccaa-3' (0.6 μM) 5'dFAM-ggacacccaaagtagtcggttccg-BHQ-1,3' (0.25 μM)	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	192-bp 5' untranscribed region	Donaldson et al. (2002)
Adenovirus	Fw: gcc acg gtg ggg ttt cta aac tt (0.6 μM) Rv: gcc cca gtg gtc tta cat gca cat c (0.6 μM) 6-FAMd-tgc acc aga ccc ggg ctc agg tac tcc ga-BHQ-1 (0.25 μM)	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	132-bp hexon gene	Heim et al. (2003)
Norovirus	NoV GI/IIa Fw: 5'-atgttyagrtggatgagrttyt-3' (0.4 μM) NoV GI/IIb Fw: 5'-atgttccgytggatgcgvtt-3' (0.4 μM) NoV GII Rv: 5'-tmgaygccatcwtcattcac-3' (0.4 μM) NoV-GII: 5'dFAM-cacrtgggagggcgatcgcaatc-BHQ-1,3' (0.2 μM)	hold at 95°C for 5 min; 45 cycles of 95°C for 15 sec and 57°C for 1 min	NoV GII is 97-bp, junction between ORF-1 and ORF-2 regions	Wolf *et al*. (2007)
Rotavirus	Fw: 5'-accatctacacatgaccctc-3' (0.3 μM) Rv: 5'-ggtcacataacgcccc-3' (0.3 μM) FAM-atgagcacaatagttaaaagctaacactgtcaa-TAMRA (0.2 μM)	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	87-bp non-structural protein 3	Pang et al. (2004)
Hepatitis E virus	Fw: 5′-ggtggtttctggggtgac-3' (0.4 μM) Rv: 5′-aggggttggttggatgaa-3′ (0.4 μM) FAM-tgattctcagcccttcgc-MGBNFQ (0.35 μM)	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	69-bp ORF3 region	Jothikumar et al. (2006)

3 RESULTS AND DISCUSSION

A total of 109 samples were taken from the two rivers; 106 were positive for at least one of the target viruses and no viruses were detected in three samples. For the Oreti River, 54/56 samples were positive for at least one target virus and 39 samples were positive for three or more target viruses (from the same individual sample). For the Waikato River, 52/53 were positive for at least one target virus and 28 samples were positive for three or more target viruses.

Adenovirus, combined-NoV and RoV were the most frequently found target viruses, with 71 and 70 samples positive of the 109 samples, respectively. Norovirus-GII and NoV-GIII were less frequently detected, with 66 and 56 samples positive, respectively (Table 2). In most cases, for each target virus the Oreti River had more samples positive than the Waikato River. However, caution should be used when interpreting these data, and it is incorrect to interpret this finding as "Oreti River has more viral contamination than the Waikato River". This is because we found that the Waikato River water was interfering with at least some methods along the concentration, extraction and detection pathway. These methods will not detect all the targets all the time, suggesting that the number of positive samples is a conservative estimation of viral presence.

To increase the probability of detecting a target virus if it were present, several replicates from the same sample are analysed, and of these, usually several determinations were also carried out. The Oreti River had more samples where all replicates were positive than seen for the Waikato River (Figure 1). This suggested that care should be taken when drawing a conclusion about whether one river is more or less contaminated by faecal material or by a viral type; assay inhibition may be underlying differences between sites or virus types. The results clearly show the benefit of taking replicate extractions from one river water concentrate when testing environmental samples. Detection assays do not detect all viruses equally and concentration procedures do not concentrate all viruses equally within a sample or between samples. Therefore, in most cases less than 1-in-4 of the target viruses in samples are being detected, and replication will increase the ability to estimate risk. The detection of viruses by PCR methods does not indicate whether the viruses were infective nor how recent the faecal contamination occurred.

Table 2 Presence-absence data from the Oreti and Waikato Rivers.

	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%
				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%
				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%

* NoV = norovirus; AdV = adenovirus; EnV = enterovirus; RoV = rotavirus; HEV = hepatitis E virus

The detection of viruses in the Oreti and Waikato Rivers was often close to the limit of detection. It is hypothesised that water quality and behaviour of individual viruses (e.g. clumping by RoV) will significantly impact on the accuracy of detection. This means for any one sample there are usually four PCR reactions per viral PCR (n=4 replicates; for some repeats only duplication was carried out). The considerable information from the replicates can be captured by including the percentage of replicates that were positive for individual samples for an individual virus, within the number of samples that were positive for a target virus (Figure 1).

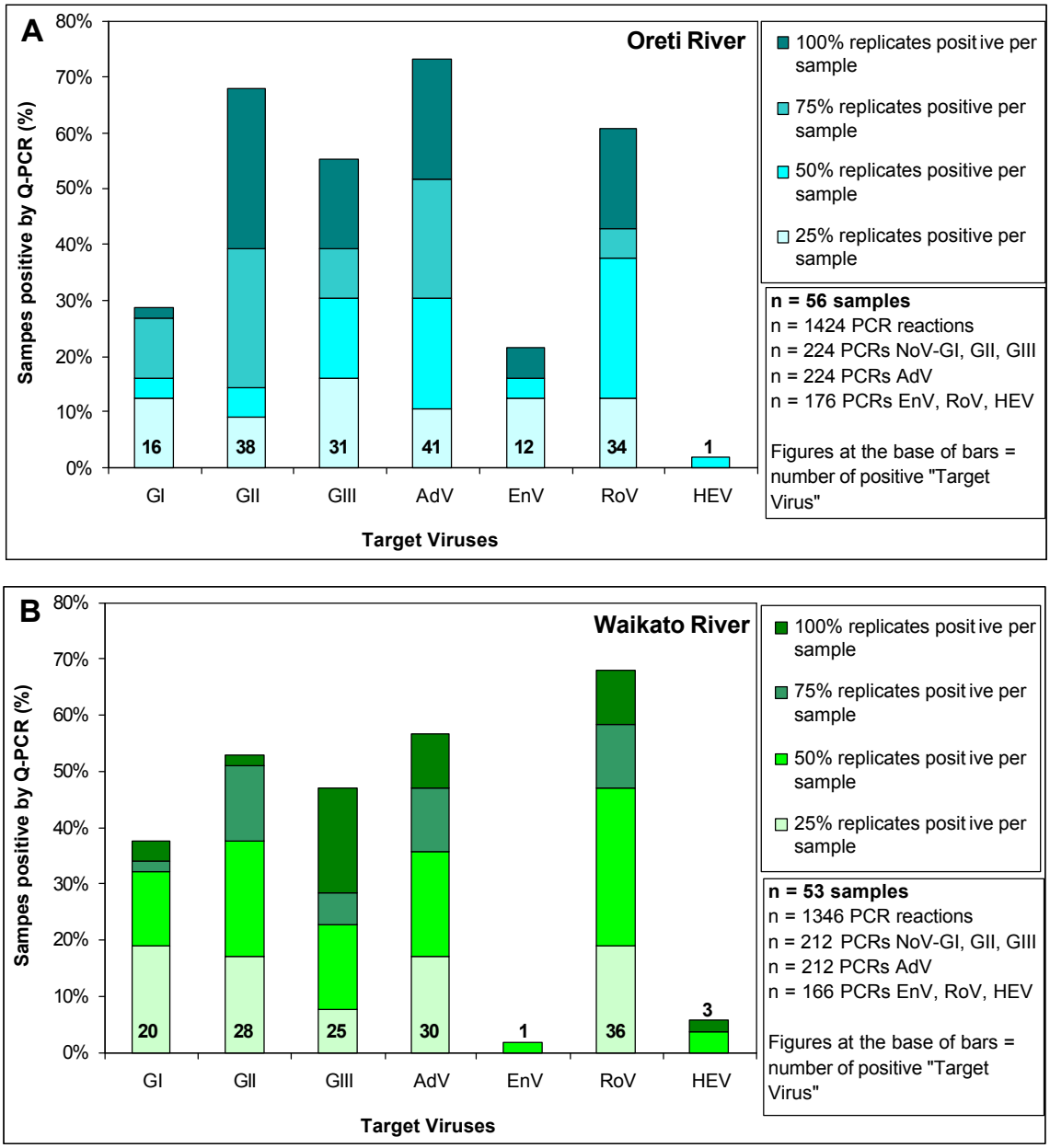

Figure 1 Percentage of samples positive for target viruses, with the number of replicates for each individual sample for each individual virus shown, where 100 % replicates positive is equivalent to 2/2 or 4/4 of the replicates positive, while 50 % replicates positive is equivalent to 1/2 or 2/4 of the replicates positive, for (A) the Oreti River and (B) the Waikato River.

4 CONCLUSIONS

This paper brings together a data set for the presence-absence of seven enteric viruses that were identified as being of importance in New Zealand surface waters used as drinking-water sources. These viruses were NoV (GI-GII), AdV, EnV, RoV and HEV and all are shed in faeces and therefore have the potential to enter waterways. From 109 river water samples taken over two years from the Oreti River and the Waikato River, 106/109 were positive for at least one of the target viruses and 67/109 samples had three or more of the targets present at any one time. We suggest the target viruses AdV, NoV and RoV as indicators of enteric virus contamination because they were present in most samples.

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