Detection of Waterborne Protozoa, Viruses, and Bacteria in Groundwater and Other Water Samples in the Kathmandu Valley, Nepal

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Abstract. In this study, the prevalence of various waterborne pathogens in water samples collected in the Kathmandu Valley, Nepal, and the applicability of *Escherichia coli* as an indicator of pathogen contamination in groundwater were assessed. Fifty-three water samples, including shallow groundwater and river water, were analyzed to examine the presence of protozoan (oo)cysts via fluorescence microscopy and that of viral and bacterial genomes via quantitative PCR. At least one of the seven types of pathogens tested (i.e., *Cryptosporidium*, *Giardia*, human adenoviruses, noroviruses of genogroups I and II, group A rotaviruses, and *Vibrio cholerae*) was detected in 68% (15/22) of the shallow dug well water samples; groundwater in the shallow dug wells was more contaminated compared with that in shallow tube wells (8/15, 53%). River water and sewage samples were contaminated with extremely high concentrations of multiple pathogens, whereas a tap water sample supplied by a water tanker tested positive for human adenoviruses and *V. cholerae*. The detection of host-specific *Bacteroidales* genetic markers revealed the effects of human and animal feces on groundwater contamination. The tested pathogens were sometimes detected even in *E. coli*-negative groundwater samples, indicative of the limitations of using *E. coli* as an indicator for waterborne pathogens in groundwater.

1. Introduction

High concentrations of waterborne pathogens, such as protozoa, viruses, and bacteria, are present in the feces of infected individuals; these pathogens are primarily transmitted via the fecal–oral route, including the consumption of contaminated food or water. An increasing number of studies have been reported on the prevalence of these pathogens in various water samples, mainly because of the rapid development of molecular biological methods; however, a limited number of studies have been conducted in developing countries.

Meanwhile, the Kathmandu Valley in Nepal has been plagued with waterborne diseases, which are considered as a serious public health concern; this issue is probably associated with the low coverage of drinking water and wastewater treatment systems. Because groundwater is the most important water source for drinking and domestic purposes in the valley [1], some recent studies have attempted to demonstrate the prevalence of waterborne pathogens, such as protozoa [2], [3], viruses [2], [4], and bacteria [5]–[7], in groundwater and other water samples. While these studies have contributed to reveal the prevalence of waterborne pathogens, most of them have examined one of the three major types of pathogens, limiting the assessment of the utility of conventional fecal indicator bacteria such as *Escherichia coli* for the management of microbiological safety of water. In addition, water sampling

is sometimes conducted only once, which may not be sufficient to support the generalization of the obtained conclusions. In reality, the author of this study reportedly examined two protozoa and three viruses; however, the number of analyzed samples was small (n = 10) [2].
On the basis of this background, this study aimed to determine the prevalence of various waterborne pathogens in water samples collected in the Kathmandu Valley during different seasons. Recently, the electronegative membrane-vortex (EMV) method [8] was utilized for the simultaneous concentration of protozoa and viruses from single water samples. In particular, it was employed to detect seven pathogens (two protozoa, four viruses, and one bacterium). *E. coli* and total coliforms were examined to evaluate their applicability as the indicators of pathogen contamination of groundwater. In addition, sources of fecal contamination of water were estimated on the basis of the detection of host-specific *Bacteroidales* genetic markers.

2. Materials and methods

2.1. Collection of water samples

Figure 1 shows the locations of the sampling sites in the Kathmandu Valley. As described previously [4], water sampling was conducted in August 2010 (wet season) and May 2011 (dry season) with a total of 43 samples comprising 16 groundwater samples from nine shallow dug wells (SG06, 17, 18, 26, 31, and 34–37); 12 groundwater samples from six shallow tube wells (SG07, 10, 12, 23, 25, and 33); 13 river water samples from eight sites along the Bagmati River and its tributaries (RW01–08); one sample of tap water supplied by a water tanker (TW01); and one sewage sample collected from a sewage pipe (SW01, not shown in Figure 1).

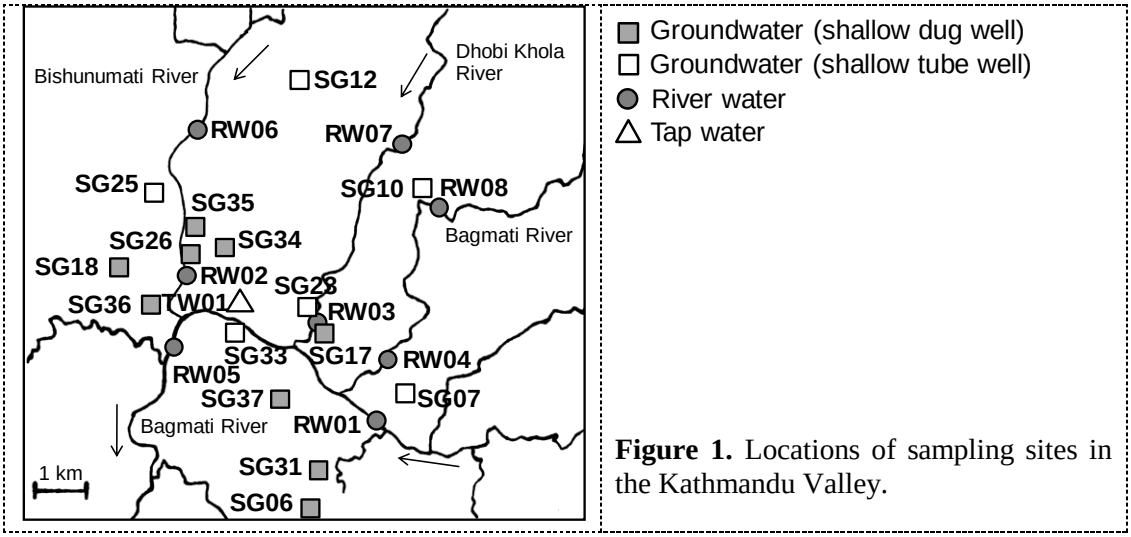

Figure 1. Locations of sampling sites in the Kathmandu Valley.

2.2. Detection of E. coli and total coliforms

The Colilert method (IDEXX Laboratories, Westbrook, ME, USA) was employed to quantify the concentrations of *E. coli* and total coliforms in the water samples according to the manufacturer's protocol, as described previously [2], [9]. Samples of groundwater from shallow dug wells have been tested in a previous study [9].

2.3. Detection of waterborne protozoa, viruses, and bacteria

2.3.1. Concentration. For all water samples excluding the sewage sample, the EMV method using a mixed cellulose ester membrane filter (pore size of 0.45 μm and diameter of 47 mm; Merck Millipore, Billerica, MA, USA) [8] was employed to simultaneously concentrate three types of waterborne pathogens (i.e., protozoa, viruses, and bacteria) in single water samples (50–3000 ml, depending on the sample type), as described previously [2], [4]. After the centrifugation of a 12-ml eluate at 2,000 ×

g for 10 min, a supernatant was obtained as a virus concentrate, whereas a pellet suspension was used to detect protozoa and bacteria. The sewage sample was directly subjected to centrifugation without any concentration step [4].

2.3.2. Detection of viruses. Viral DNA was extracted from virus concentrates using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), whereas viral RNA was extracted using a QIAamp Viral RNA Mini Kit (Qiagen), followed by reverse transcription using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA), as described previously [2, 4]. A Thermal Cycler Dice Real Time System TP800 (Takara Bio, Otsu, Japan) was used to perform quantitative PCR (qPCR) for human adenoviruses (HuAdVs) [10], noroviruses of genogroups I (NoVs-GI) and II (NoVs-GII) [11], and group A rotaviruses (RVAs) [12]. HuAdVs, NoVs-GI, and NoVs-GII were completely quantified using standard curves generated from 10-fold serial dilutions of standard samples, whereas semi-quantitative detection was conducted for RVAs based on threshold cycle values. All water samples, standard samples, and negative control were tested in duplicate.

2.3.3. Detection of protozoa. Eleven milliliters of the 12-ml pellet suspension was subjected to immunomagnetic separation (IMS) using a Dynabeads GC-Combo (Thermo Fisher Scientific). One half of the resulting IMS-purified sample (110 µl) was passed through a hydrophilic polytetrafluoroethylene membrane (pore size of 1.0 µm and diameter of 25 mm; Advantec, Tokyo, Japan), followed by direct fluorescent antibody staining using an EasyStain (BTF, North Ryde, Australia) according to the manufacturer's protocol. Fluorescence microscopy (BX60; Olympus, Tokyo, Japan) was employed for counting the number of *Cryptosporidium* oocysts and *Giardia* cysts.

2.3.4. Detection of bacteria. Bacterial DNA was extracted from the remaining portion of the pellet suspension using the QIAamp DNA Mini Kit, followed by qPCR for the *ctx*A toxic gene of *Vibrio cholerae* [13]. In addition, three host-specific *Bacteroidales* qPCR assays were performed to identify the sources of fecal contamination of the tested water samples: *gyr*B for human-specific assay [14], Pig2Bac for pig-specific assay [15], and BacR for ruminant-specific assay [16]. All qPCR runs were performed using a Thermal Cycler Dice Real Time System TP800.

3. Results and discussion

3.1. Detection of E. coli and total coliforms

Table 1 summarizes the results obtained from the detection of *E. coli* and total coliforms in the collected water samples. They were detected in almost all samples, except for a few shallow tube well water samples. The concentrations of *E. coli* and total coliforms in the groundwater samples from shallow dug wells were significantly greater than those from shallow tube wells (*t*-test, $P < 0.05$). This result is probably related to the more vulnerable structure of shallow dug wells than tube wells. According to the guidelines stipulated by the World Health Organization, *E. coli* should be absent in drinking water [17]; however, the results clearly indicated that shallow groundwater and water supplied by water tankers are not suitable for drinking purposes.

3.2. Detection of waterborne protozoa, viruses, and bacteria

Table 2 summarizes the results obtained from the detection of seven waterborne pathogens. In addition to the collected water samples, ten samples (six shallow dug well, three shallow tube well, and one river water samples) collected from the same locations in August–September 2009 were included in this table; the data for *Cryptosporidium* and *Giardia* have been reported previously [2], whereas those for viruses and *V. cholerae* has been analyzed herein.

At least one of the seven types of the tested pathogens was detected in 68% (15/22) of the shallow dug well water samples. Compared with the shallow dug wells, the shallow tube wells were less contaminated with pathogens (8/15, 53%); this result is in agreement with those obtained from the detection of fecal indicator bacteria (Table 1).

Table 1. Detection of *E. coli* and total coliforms in water samples.

Water sample	No. of tested samples	*E. coli*		Total coliforms	
		No. of positive samples (% positive)	Concentration[a] (log MPN[b]/100 ml)	No. of positive samples (% positive)	Concentration[a] (log MPN[b]/100 ml)
Shallow dug well water[c]	16	16 (100)	0.61–4.05	16 (100)	2.86–5.42
Shallow tube well water	12	7 (58)	0.00–1.43	11 (92)	0.93–3.96
River water	13	13 (100)	5.20–9.38	13 (100)	5.82–>9.38
Sewage	1	1 (100)	9.30	1 (100)	9.30
Tap water	1	1 (100)	2.04	1 (100)	3.31
Total	43	38 (88)		42 (98)	

[a] Range of concentrations among positive samples.
[b] MPN, most probable number.
[c] Reported previously [9].

Figure 2 shows the concentrations of the selected protozoa and viruses in the tested samples, clearly demonstrating that the river water samples contain a considerably higher number of pathogens compared with groundwater samples. The concentrations of pathogens at upstream sites (RW05–08) were sometimes lesser than those at densely polluted downstream sites (RW01–04) or even below the limit of detection.

Some shallow dug wells such as SG06 and SG37 were found to be highly contaminated with multiple pathogens, indicative of a serious risk of pathogen infections via contaminated groundwater. HuAdVs and *V. cholerae* were the only two pathogens found in the tap water supplied by a water tanker, which was also tested positive for *E. coli* and total coliforms. Tanker water is one of the major water sources in the Kathmandu Valley [1]; however, high levels of microbial contamination in water tanker samples were observed (unpublished data), indicative of the unsuitability of tanker water for drinking purposes.

Table 2. Detection of waterborne protozoa, viruses, and bacteria in water samples.

Pathogen	No. of positive samples (% positive)					
	Shallow dug well water (n = 22)	Shallow tube well water (n = 15)	River water (n = 14)	Sewage (n = 1)	Tap water (n = 1)	Total (n = 53)
Cryptosporidium	7 (32)[a]	0 (0)[a]	14 (100)[a]	1 (100)	0 (0)	22 (42)
Giardia	8 (36)[a]	1 (7)[a]	14 (100)[a]	1 (100)	0 (0)	24 (45)
HuAdVs	7 (32)	5 (33)	13 (93)	1 (100)	1 (100)	27 (51)
NoVs-GI	5 (23)	1 (7)	13 (93)	1 (100)	0 (0)	20 (38)
NoVs-GII	7 (32)	1 (7)	13 (93)	1 (100)	0 (0)	22 (42)
RVAs	1 (5)	0 (0)	10 (71)	1 (100)	0 (0)	12 (23)
V. cholerae	2 (9)	3 (20)	2 (14)	0 (0)	1 (100)	8 (15)
Total	15 (68)	8 (53)	14 (100)	1 (100)	1 (100)	39 (74)

[a] Including the data reported previously [2].

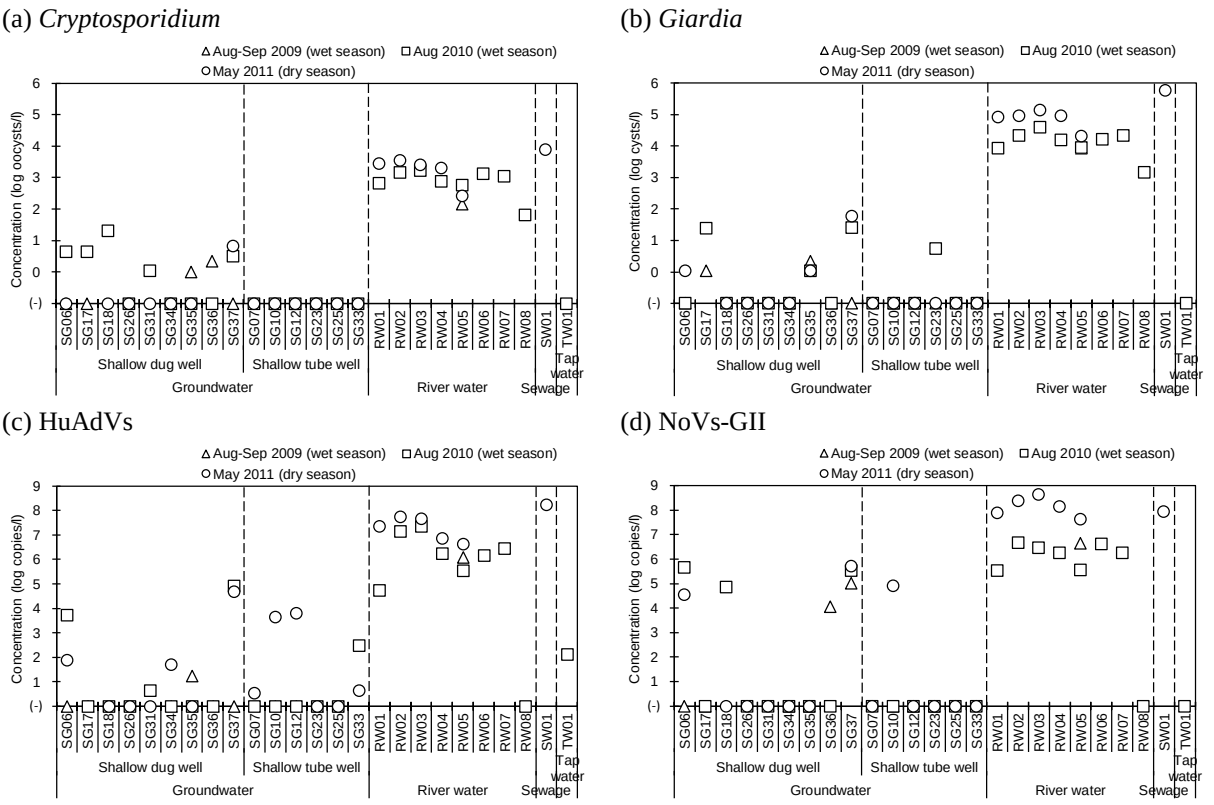

Figure 2. Concentrations of *Cryptosporidium*, *Giardia*, HuAdVs, and NoVs-GII in water samples.

3.3. Detection of host-specific Bacteroidales genetic markers

At least one of the three *Bacteroidales* genetic markers tested were detected in 18 (34%) of 53 samples, including the samples collected in August–September 2009; 14 (26%), 8 (15%), and 10 (19%) samples tested positive by human-, pig-, and ruminant-specific qPCR assays, respectively (Table 3). River water is clearly contaminated heavily with feces from humans and animals considering the low coverage of wastewater treatment plants and poor management of livestock wastewater.

Two shallow dug wells tested positive for the markers: the human-specific marker was detected in SG37 in August 2010, whereas both pig- and ruminant-specific markers were detected in SG35 in August–September 2009 and August 2010, respectively, indicative of the continuous animal fecal contamination of this well. The shallow tube well sample at SG10 in May 2011 tested positive for the ruminant-specific marker. These results emphasize the elimination of fecal waste from not only humans but also animals to improve the microbial quality of groundwater in the Kathmandu Valley. In addition, the EMV method is found to be effective for concentrating bacteria.

Table 3. Detection of host-specific *Bacteroidales* genetic markers in water samples.

Water sample	No. of tested samples	No. of positive samples (% positive)			
		*gyr*B (human)	Pig2Bac (pig)	BacR (ruminant)	Total
Shallow dug well water	22	1 (6)	2 (9)	2 (9)	3 (14)
Shallow tube well water	15	0 (0)	0 (0)	1 (7)	1 (7)
River water	14	12 (86)	6 (43)	6 (43)	12 (86)
Sewage	1	1 (100)	0 (0)	0 (0)	1 (100)
Tap water	1	0 (0)	0 (0)	1 (100)	1 (100)
Total	53	14 (26)	8 (15)	10 (19)	18 (34)

3.4. Relation between pathogens and E. coli

To assess the applicability of *E. coli* as an indicator of pathogen contamination of groundwater, shallow dug and tube well water samples were divided into four groups based on the *E. coli* concentrations. In general, the ratios of samples that tested positive for at least one of the seven types of pathogens tested and mean numbers of the types of pathogens detected in a single sample increased with increasing *E. coli* concentration, although the plots at a concentration of 1–10 most probable number (MPN)/100 ml did not follow this trend (Figure 3). In case a water sample contained *E. coli* at a concentration of >1000 MPN/100 mL, 73% of such samples tested positive for at least one type of pathogens, and on average, 2.9 types of pathogens were detected in a sample.

However, importantly, these pathogens were sometimes detected even in *E. coli*-negative samples with a positive ratio of 38%, indicating that *E. coli* is not suitable to confirm the absence of waterborne pathogens in the groundwater samples tested in this study. A similar result was obtained for total coliforms (data not shown).

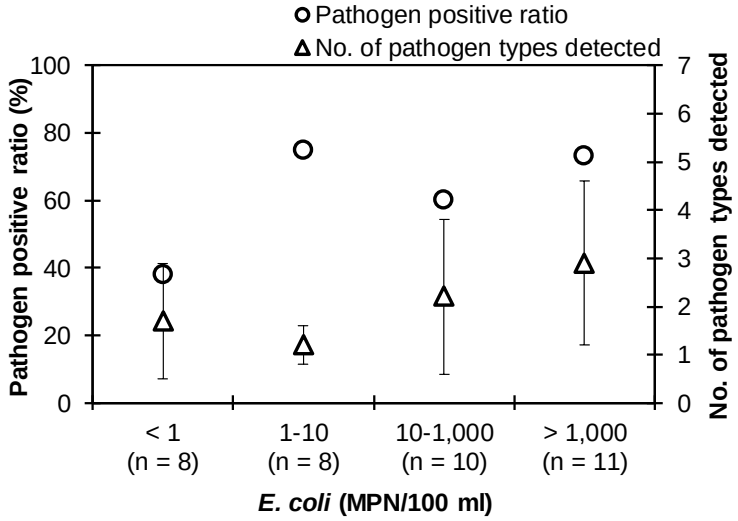

Figure 3. Relation between pathogens and *E. coli* in groundwater.

4. Conclusions

In summary, various types of waterborne pathogens, such as *Cryptosporidium*, *Giardia*, and HuAdVs, were successfully detected in the water samples in the Kathmandu Valley, Nepal. A higher prevalence of waterborne pathogens in the shallow dug wells compared with the shallow tube wells indicated a potentially higher risk of pathogen infections by drinking water from a shallow dug well. The limited applicability of *E. coli* as an indicator of the pathogen contamination of groundwater strongly emphasized the need for further studies related to the establishment of more suitable indicators. Furthermore, as not only human but also animal fecal contamination was identified in the tested water samples, future studies need to focus more on the prevalence and genetic analysis of zoonotic pathogens to evaluate the risk of infections from animals to humans.

5. References

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Acknowledgments
This study was supported by the Grant-in-Aid for the Global COE Program "Evolution of Research and Education on Integrated River Basin Management in Asian Region" from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The author thanks Prof. Kei Nishida and Dr. Takashi Nakamura (University of Yamanashi, Japan) for their help in water sampling.