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Detection of *Cryptosporidium*, *Giardia*, fecal indicator bacteria, and total bacteria in commercial jar water in the Kathmandu Valley, Nepal

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Abstract:

Introduction: Jar water is a convenient and common source of drinking water in the Kathmandu Valley. However, studies including detailed microbial analyses of this source of potable water are lacking. In this study, jar water samples were examined for the occurrence of *Cryptosporidium*, *Giardia*, fecal indicator bacteria, and total bacteria.

Methods: Thirty different brands of jars were collected in September 2014. *Escherichia coli* and total coliforms were determined using a Colilert reagent. Ten of the 30 brands were selected to test for *Cryptosporidium*, *Giardia*, and total bacteria. Bacterial DNA extraction from water samples was performed using the Cica Geneus DNA Extraction Kit, followed by quantitative polymerase chain reaction (qPCR) targeting the 16S rRNA gene of bacterial DNA. Protozoan detection was accomplished by concentrating the samples using the electronegative membrane vortex method, followed by immunomagnetic separation and fluorescent staining.

Results: *E. coli* was detected in 10% of the samples, with a maximum concentration of 2 most probable number (MPN)/100 mL, whereas total coliforms were detected in 97% of the samples, with a maximum and mean concentration of 7.3×10^2 and 3.8×10^1 MPN/100 mL, respectively. Total coliforms concentrations in 40% of the samples ranged from 10^2 to 10^3 MPN/100 mL. *Cryptosporidium* and *Giardia* were not detected in any of the tested samples. Concentrations of total bacteria in the samples ranged from 10^4 to 10^6 cells/100 mL.

Conclusions: Ninety-seven percent of the jar water brands were unsuitable for drinking without proper treatment based on the guideline values of the National Drinking Water Quality Standards (NDWQS) of Nepal. There is no guideline value for total bacteria in NDWQS however, high concentrations can be indicative of poor control on regrowth of bacteria and recontamination or inefficient water treatment methods.

Key words: *Cryptosporidium*, *Giardia*, Jar water, qPCR, Total bacteria

Introduction

In 2011, the Kathmandu Valley, comprising the capital city and the most urbanized area of Nepal, had a population of 2.51 million¹. Coupled with a rapid increase in the population, the valley is facing a severe water supply crisis. The demand for water is 320 million liters per day (MLD), whereas the supply is only 76 and 105 MLD in the dry and wet seasons, respectively². Although the people here depend on piped tap water,³ fecal contamination in tap water⁴ and the intermittent supply have compelled residents of the valley to search for another safe and easily available drinking water source. Jar water, in 20-L bottles, is being used by 35% of households and more than 60% of institutions as a source of drinking water³.

Jar water is affordable. Eighty percent of people perceive jar water to be a good source of drinking water⁵. People's perceptions of jar water as pure, safe, and of better taste may have popularized it in the market. According to the Department of Food Technology and Quality Control in Nepal, only 129 jar water companies have been officially registered⁶. Because of the increasing demand and consumption of jar water in the valley, this business is rapidly flourishing.

Jar water companies treat source water using different treatment methods, such as membrane filtration and reverse osmosis, and disinfect using methods such as ozonation, pre-chlorination, and ultraviolet (UV) disinfection. However, in the valley, 90% of bottled water brands were found to be unsafe for drinking⁷ and similarly 90% and 60% of jar water samples were found to be contaminated with total coliforms and fecal coliforms, respectively⁵. Microbial contamination of drinking water sources poses a risk for waterborne diseases, including diarrhea. In the valley, diarrhea accounts for 30% of total hospital cases and 69% of waterborne diseases⁸. The most prevalent bacteria among children and adult diarrheal patients have been enteropathogenic *Escherichia coli* and the most prevalent protozoa were *Giardia*, followed by *Cryptosporidium*^{9,10,11}. Hence, monitoring of microbial contamination of such an important water source should be conducted routinely. We can quantify the bacterial population using a rapid and accurate method to evaluate the bacterial growth in water.

Quantitative polymerase chain reaction (qPCR) is a culture-independent, highly sensitive, and specific technique in which nucleic acid is amplified using a primer. For conducting qPCR to quantify the total number of bacterial cells, 16S rRNA genes of bacteria are amplified using a universal eubacterial primer. Hence, qPCR provides an alternative molecular biological tool that can

be more sensitive, more accurate, and less labor intensive.

Limited research studies have reported microbial contamination in jar water^{5,7}. Hence, the objectives of this study were to examine protozoa, fecal indicator bacteria, and total bacteria targeting the 16S rRNA gene in jar water sold in the valley.

Methods

Collection of water samples

Thirty different brands of jar water (20 L each) were collected during September 15–20, 2014, in the Kathmandu Valley and brought to the laboratory of the Institute of Medicine, Tribhuvan University. Only 10 out of 30 samples were selected to enumerate the total bacterial cells and to detect *Cryptosporidium* and *Giardia*. This selection was based on the detection of *E. coli* and high and low concentrations of total coliforms in the water samples.

Detection of *E. coli* and total coliforms

E. coli and total coliforms were determined using a Colilert reagent (Idexx Laboratories). In brief, 100 mL of each sample was mixed with one tube of Colilert reagent in a bottle and poured into a Quanti-Tray and sealed. Next, the tray was incubated at 37°C for 24 hours and viewed under visible light conditions for the total coliforms count. Yellow lights were counted for total coliforms and for the *E. coli* count, the trays were viewed under a UV lamp and the blue lights were counted for *E. coli*. Both the small and large wells were counted. And, finally, the most probable number (MPN) value for the *E. coli* and total coliforms present in 100 mL of the water samples were determined by using MPN generating software (Idexx Laboratories).

Quantification of total bacterial cells

One hundred milliliters of each water sample was filtered using a sterilized disposable filter unit preset with a nitrocellulose membrane filter (diameter, 47 mm; pore size, 0.22 µm; Nalgene). The filters were stored at –25°C in a sterilized 50-mL conical tube until further analysis was performed. Next, the filter was suspended in 5 mL of Tris-EDTA (TE) buffer (pH 7.4) and incubated at 50°C for 30 min. Then, 70 µL of the sample was transferred to a 1.5-mL microtube, followed by the addition of 10 µL of TE buffer and a mixture of 10-µL Buffer A and 100-µL Buffer B of the Cica Geneus DNA Extraction Kit (Kanto Chemical). Later, the tube was incubated at 72°C for 20 min followed by 94°C for 3 min. Finally, the microtube was centrifuged at 20,400 × g for 5 min at room temperature. After centrifugation, 100 µL of supernatant was recovered as DNA extract in a new microtube.

SYBR Green-based qPCR targeting of 16S rRNA genes of bacterial DNA was performed using a Thermal Cycler Dice (Takara Bio). To make a standard sample, 10-fold serial dilutions (1.98×10^2 – 1.98×10^6 genome DNA/ μ L) of *E. coli* genome DNA (LA PCR Genome DNA kit; Takara Bio) were prepared. Since an *E. coli* genome DNA has seven copies of the 16S rRNA genes¹², the standard samples included 1.39×10^3 – 1.39×10^7 16S rRNA gene copies/ μ L. Two microliters of template DNA or *E. coli* genome DNA was mixed with 0.1 μ L of 50 pmol/ μ L Eub-V3-F primer (5'-CKGYCCAGACTCCTACGGG-3'; corresponding to positions 330–348 bp of *E. coli* 16S rRNA gene), 0.1 μ L of 50 pmol/ μ L Eub-V3-R primer (TTACCGCGGCTGCTGGCAC; corresponding to positions 515–533 bp of *E. coli* 16S rRNA gene)¹², 12.5 μ L of SYBR Premix Ex Taq II (Takara Bio), and 10.3 μ L of sterilized ultrapure water. The thermal conditions were as follows: initial activation at 95°C for 30 s; followed by 30 cycles of denaturing at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 20 s. To check the specificity of amplification, at the end of 30 cycles of PCR amplification, product melting curve analysis was performed at 95°C for 15 s, at 60°C for 30 s, and at 95°C for 15 s. To calculate the total bacterial cell counts, the data of 16S rRNA gene copy numbers obtained in qPCR were divided by the average of 16S rRNA gene copy numbers (4.2) in a genome DNA of Eubacteria¹³.

Detection of *Cryptosporidium* and *Giardia*

Cryptosporidium and *Giardia* in the water samples were concentrated using the electronegative membrane vortex (EMV) method as described previously¹⁴ with slight modifications. In summary, 5 L of jar water was mixed with 50 mL of 2.5 mol/L MgCl₂. This solution was then filtered through a sterilized mixed cellulose membrane filter (diameter, 47 mm; pore size, 0.8 μ m; Merck Millipore) using the same disposable filter unit utilized for total bacteria. After filtration, the membrane filter was removed and kept in a 50-mL plastic tube. Subsequently, 10 mL of elution buffer containing 0.2 g/L NaP₂O₇ 10H₂O, 0.3 g/L C₁₀H₁₃N₂O₈Na₃ 3H₂O, and 0.1 mL/L Tween 80 was added to the plastic tube, followed by vigorous vortexing and crushing of the filter. The solution was then transferred to a new plastic tube. The same process was repeated by adding 5 mL of the elution buffer. The resulting 15 mL solution was then centrifuged at 2000 \times g for 10 min at 4°C. Pellets in the tubes were used for protozoa analysis. Since the EMV method adopted in this study can simultaneously concentrate on protozoa and viruses,¹⁴ the supernatant was further processed and stored in a microtube at –20°C for future virus analysis.

Phosphate buffered saline i.e., saline (PBS (–)) was added to the pellet, followed by vortexing and centrifugation at 2000 \times g for 10 min at 4°C. Finally, a 10 mL solution was obtained, which was subjected to immunomagnetic separation using Dynabeads GC-Combo (Invitrogen) and fluorescent staining using DAPI and EasyStain™ (BTF). The numbers of *Cryptosporidium* oocysts (round-shaped, 4–6 μ m diameter) and *Giardia* cysts (oval-shaped, 5–8 μ m diameter and 8–12 μ m width) were counted using a fluorescent microscope (Olympus).

Results

Prevalence of *E. coli* and total coliforms

As shown in Table 1, *E. coli* was detected in 3 (10%) out of 30 samples, with a maximum concentration of 2 MPN/100 mL. In contrast, total coliforms were detected in 29 (97%) out of 30 samples. The concentrations of total coliforms in the positive jar water samples were in the range of 1–7.3 $\times 10^2$ MPN/100 mL. Overall, 12 samples (40%) had total coliforms values between 10¹ and 10² MPN/100 mL and 12 samples (40%) had values between 10² and 10³ MPN/100 mL (Table 2). Total coliform concentrations in the three *E. coli* positive samples ranged from 10² to 10³ MPN/100 mL and the mean concentration was $2.2 \pm 0.5 \log_{10}$ MPN/100 mL. However, the mean concentration in the *E. coli* negative samples was $1.5 \pm 0.8 \log_{10}$ MPN/100 mL, which was similar to that of the *E. coli* positive samples. Similarly, total bacterial cells concentration in the *E. coli* positive samples ranged from 10³ to 10⁴ cells/100 mL and the mean concentration was $5.7 \pm 0.6 \log_{10}$ MPN/100 mL. The mean concentration in *E. coli* negative samples was $5.5 \pm 0.3 \log_{10}$ MPN/100 mL, which was similar to that in the *E. coli* positive samples.

Table 1. Prevalence of total coliforms, *E. coli*, total bacteria, and *Cryptosporidium* and *Giardia* in jar water

	Total coliforms	<i>E. coli</i>	Total bacteria	<i>Cryptosporidium</i>	<i>Giardia</i>
Detection ratio, % (no. of positive samples/no. of tested samples)	97 (29/30)	10 (3/30)	100 (10/10)	0 (0/10)	0 (0/10)
Unit	MPN/100 mL	MPN/100 mL	cells/100 mL	oocyst/L	cyst/L
Minimum	1.0	1.0	9.1×10^4	NA	NA
Maximum	7.3×10^2	2.0	1.3×10^6	NA	NA
Geometric Mean	3.8×10^1	NA	4.9×10^5	NA	NA

NA: Not applicable (Only 3 samples out of 30 were *E. coli* positive and all selected samples were negative for *Cryptosporidium* and *Giardia*)

Prevalence of *Cryptosporidium* and *Giardia*

As shown in Table 1, none of the 10 jar water samples were positive for either *Cryptosporidium* or *Giardia*.

Prevalence of total bacteria

Total bacteria were quantified in all the tested samples ($n = 10$). The scores in jar water ranged from 9.1×10^4 to 1.3×10^6 cells/100 mL (Table 1) and 90% of the samples had a concentration above 10^5 cells/100 mL (Table 2).

Table 2. Frequency distribution of total coliforms, *E. coli*, and total bacteria in jar water

Total coliforms		<i>E. coli</i>		Total bacteria	
Concentration (\log_{10} MPN/100 mL)	No. of samples (%)	Concentration (\log_{10} MPN/100 mL)	No. of samples (%)	Concentration (\log_{10} cells/100 mL)	No. of samples (%)
<0	1 (3)	<0	27 (90)	<5.0	1 (10)
0–1	5 (17)	0–1	3 (10)	5.0–5.5	4 (40)
1–2	12 (40)			5.5–6.0	4 (40)
2–3	12 (40)			6.0–6.5	1 (10)

Discussion

In this study, jar water samples were analyzed for total coliforms, *E. coli*, *Cryptosporidium*, *Giardia*, and total bacteria. According to WHO guideline values for drinking water, *E. coli* should not be present in any of the samples tested. The guideline value for *E. coli* is the same in the National Drinking Water Quality Standards (NDWQS) of Nepal, which also dictates absence of total coliforms in 95% of the samples tested. In this study, 97% of the jar water brands were not suitable for drinking when compared to these water quality guideline values. The result for total coliforms in jar water in this study is in line with previous studies.^{4,6} However, the positive ratio for *E. coli* was slightly lower in our study (10%) compared to that of Subedi and Aryal (2010) (44%). While considering the distribution of the concentration of total coliforms, most of the samples had concentrations on the high end (\log_{10}

1–2 and \log_{10} 2–3 MPN/100 mL). This result indicates that both the detection ratio and the level of pollution were high in the jar water samples. The presence of total coliforms and *E. coli* in treated and processed jar water may indicate that there is either recontamination during storage or inefficiencies in the treatment and disinfection procedures followed by the production companies.

Protozoa analysis of the jar water in the valley is the uniqueness of this study although none of the tested samples were positive, neither for *Giardia* nor for *Cryptosporidium*. *Cryptosporidium* and *Giardia* were the most prevalent pathogens found in children and adult diarrheal patients in the valley,^{9,10,11} but they were absent in our samples. This result could be because of the small sample size or an effective water treatment method for protozoa. Another reason can be the small volume

(5 L) of experimental water that was used in the protozoa concentration step.

The concentrations of total bacteria in the jar water ranged from 9.1×10^2 to 1.3×10^4 cells/mL. The total bacteria in almost all the water samples were above 10^5 cells/100 mL. We also tested for heterotrophic bacteria in all 30 jar water samples using Sanita-kun sheet (Chisso Corporation) and the concentrations ranged from 1.2×10^2 to 8.4×10^3 CFU/mL (data not shown). In comparison, the concentrations of total bacteria in the water samples were higher than those of heterotrophic bacteria, except for in one sample. Furthermore, an increasing tendency of heterotrophic bacteria concentration was seen with an increase in total bacteria concentration. This comparison indicates that qPCR provides a wider range of microbial concentration in the water environment. Therefore, it may indicate that total bacteria detection by qPCR can be an alternative method for culturing heterotrophic bacteria.

The guideline value for total bacteria in drinking water was not available in NDWQS of Nepal. However, as mentioned by one study¹⁵ in the valley, WHO considered total bacteria <10 CFU/mL in drinking water as a guideline and they reported that 82.6% of the drinking water samples exceeded that guideline value. If we consider this to be the guideline value, then almost all the jar water brands would be unsuitable for drinking. Prasai et al. (2007) conducted total bacterial count of various drinking water sources using pour plate technology. Hence, this study is unique in two ways; first, it is the first study to enumerate the total bacterial count in jar water and second, none of the previous studies have performed qPCR to enumerate total bacteria in the Kathmandu Valley. Large number of total bacteria in treated and processed bottle water implies that there is poor control on regrowth of bacteria during storage and distribution. There is also the possibility of recontamination after production or poor treatment and disinfection procedures of the jar water.

There are several limitations to this study. First, only one jar from each company was tested. Microbial quality in jar water can be influenced by the quality of the jar being used. Hence, if water from each company had been tested repeatedly, we can be more certain of the quality of water being produced by that company. Second, we analyzed only 30 jar water, one each from 30 different companies. Therefore, the findings will not be helpful for making legislative decisions. Despite such limitations, our results have shown some important findings regarding the suitability of jar water for drinking as well as indicating the possibility of poor treatment or poor control and regrowth

of bacteria during storage and distribution.

This study has opened opportunities for future research concerning detailed microbial analysis in jar water, before and after treatment, and during storage, as well as assessing the chlorine level in processed water as a measure for controlling regrowth of bacteria.

Conclusion

In this study, *E. coli* was detected in 10% and total coliforms in 97% of the samples collected. Based on NDWQS, almost all jar water brands in the Kathmandu Valley have exceeded acceptable limits and are unsuitable for drinking without proper treatment. However, *Cryptosporidium* and *Giardia* were not detected in the samples. Total bacteria in the samples ranged from 10^5 to 10^6 cells/100 mL of DNA. Such high concentrations can be indicative of recontamination and poor control of the regrowth of bacteria. We recommend an increased surveillance of the jar water industry in Nepal. Comparative studies of microbial analysis between source water and treated and stored jar water in the market can provide a better understanding of the current status of jar water production and storage. Such studies can be helpful for lowering public health risks.

Conflict of interest: None declared.

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