Comprehensive Detection of Pathogenic Bacteria in Jar Water, Community Well Groundwater, and Environmental Water in the Kathmandu Valley, Nepal

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Abstract

Microbial contamination of water sources remains a serious problem in the Kathmandu Valley, Nepal. This study was conducted to comprehensively investigate the occurrence of pathogenic bacteria in water samples collected from the valley, most of which were used for drinking and other domestic purposes. The water samples consisted of 10 samples of commercial jar water, 10 of groundwater from shallow dug wells, 3 of groundwater from deep tube wells, 1 of stone spout water, 2 of spring water, and 2 of river water. DNA microarray analysis targeting 941 different pathogenic bacterial species/groups showed the prevalence of Acinetobacter, Arthrobacter, Brevibacterium, Pseudomonas, and Legionella and the presence of biosafety level 3 pathogens in some samples, which were partially confirmed by genus-specific PCR quantification for Legionella and Brucella. Additionally, the occurrence of most pathogens detected via DNA microarray analysis was not correlated with that of fecal indicator bacteria (total coliforms and Escherichia coli). These results will aid in understanding the overall picture of pathogenic bacterial contamination and in screening important pathogens that should be investigated in detail.

Keywords: DNA microarray analysis, Kathmandu valley, pathogenic bacteria, water samples

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INTRODUCTION

The Kathmandu Valley includes the capital city and most urbanized areas of Nepal. Along with the recent spike in population growth, water demands have significantly increased in the Kathmandu Valley. However, due to severe shortage and low quality of piped tap water in the valley, jar water, groundwater, and stone spouts represent the major water sources. Jar water is used by 35% of households and >60% of institutions as a drinking water source\(^1\). Additionally, groundwater from shallow dug, shallow tube, and deep tube wells as well as stone spouts are generally preferred over surface water, the quality of these water sources has also deteriorated due to the direct discharge of untreated sewage into surface water and inadequate treatment facilities.

Microbial contamination of water sources in the Kathmandu Valley has been an important cause of waterborne diseases, with diarrhea accounting for 69% of waterborne diseases in the valley\(^3\). Therefore, microbial contamination of important water sources in the valley has been investigated during the previous decade. Previous studies have demonstrated the presence of fecal indicators and selected pathogens, including protozoa, viruses, and pathogenic bacteria, in jar water\(^4,5\) and groundwater in the valley\(^6-9\), confirming that the water sources were unsafe. Additionally, very recently, we investigated total bacterial communities in groundwater and spring and river water samples collected from the valley using next-generation sequencing (NGS), and revealed the occurrence of diverse potential pathogenic bacteria, relatively abundant occurrence of several potential pathogenic bacteria such as \textit{Acinetobacter}, \textit{Arcobacter} and \textit{Clostridium}, and no sufficient correlation between fecal indicator bacteria and potential pathogenic bacteria\(^10\). The evidence emphasized the importance of further investigation of the occurrence of pathogenic bacteria in water sources in the valley. In particular, comprehensive analysis of diverse pathogenic bacteria, including both abundant and scarce, is of great importance, given the serious health risk posed by some pathogens even at low abundance.

Therefore, this study aimed at comprehensively investigating the occurrence of pathogenic bacteria in water samples collected from the Kathmandu Valley, which were mostly used for drinking and other domestic purposes. DNA microarray analysis targeting >900 different pathogenic bacterial species/groups\(^9\) was performed, and genus-specific PCR was used to confirm the occurrence and quantify selected pathogenic bacteria. DNA microarray analysis which targets only pathogenic bacteria would be useful especially for clarifying the occurrence of low abundance pathogenic bacteria that might be overlooked in NGS targeting whole bacterial communities that was conducted in our previous study\(^10\).

MATERIALS AND METHODS

Water samples A total of 28 water samples including 10 from commercial jar water, 14 from community well groundwater, and 4 from environmental water, all of which were collected from the Kathmandu Valley from August to September 2014\(^5,10\), were used in this study. Ten jar water samples investigated here were selected from 30 different brands of jar water investigated previously\(^5\) based on the detection of \textit{Escherichia coli} and high and low concentrations of total coliforms. Community well groundwater samples were sourced from 10 shallow dug wells, 3 deep tube wells, and 1 stone spout. Environmental water samples included 2 spring water and 2 river water samples. Community well groundwater and environmental water samples investigated here were also selected from 37 samples investigated previously\(^10\) in the light of the occurrence and abundance of \textit{E. coli} and total coliforms and geographical locations.

Bacterial DNA extraction Bacterial DNA in water samples was extracted with CicaGeneus DNA extraction reagent ST (Kanto Chemical, Tokyo, Japan) as previously described\(^10\).

DNA microarray analysis Pathogenic bacteria were comprehensively detected using
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DNA microarray analysis\(^9\), in which 16S rRNA oligonucleotide probes were used for targeting 941 pathogenic bacterial species/groups, including all biosafety level (BSL) 2 and 3 pathogens according to the Japanese Society for Bacteriology and other opportunistic ones (BSL 1) that are infectious to humans, animals, plants, fish, and shellfish. The DNA microarray employed in this study was designed using the eArray software (Agilent Technologies, Santa Clara, CA, USA) on the 8 × 15 K format.

PCR amplification and Cy3-labeling of partial 16S rRNA genes were conducted as described by Inoue \(\text{et al.}\)^9\(^,\) with the modification that the PCR amplification cycle was set at 22 cycles. After purification with NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel, Düren, Germany), Cy3-labeled DNA (600 ng) was hybridized with the aforementioned microarray slides at 55°C for 16 h in the hybridization oven set at 10 rpm, after which the microarray slides were washed, according to the manufacturer’s instructions. Hybridized microarray slides were then scanned with a GenePix 4000B microarray scanner (Molecular Devices, Sunnyvale, CA, USA), after which the scanned images were processed with the GenePix Pro 7 software (Molecular Devices). The signal to noise (S/N) ratio of test probes was calculated, and test probes with a mean S/N ratio \((n = 4)\) greater than the maximum S/N ratio of negative probes were considered positive.

**Most probable number–PCR** (MPN–PCR)

As representative pathogenic bacterial groups, *Legionella* spp. and *Brucella* spp. were quantitatively detected using MPN–PCR\(^10\), with five replicated PCR amplifications of each 10-fold dilution series. Detection of partial 16S rRNA gene sequences of *Legionella* spp. was performed using semi-nested PCR with the genus-specific primers LEG225, LEG448, and LEG858 (LEG225 and LEG858 for the first PCR and LEG448 and LEG858 for the second PCR) according to the protocol described by Miyamoto \(\text{et al.}\)^13. Partial sequences of 16S rRNA genes from *Brucella* spp. were PCR amplified using conventional PCR with the genus-specific primers F4 and R2 according to the protocol described by Romero \(\text{et al.}\)^13.

**Statistical analysis**  To clarify the correlations between the occurrence of each pathogenic bacterial species/groups and fecal contamination level (total coliforms and *E. coli*), regression analysis was performed using GraphPad Prism 5J (GraphPad software, La Jolla, CA, USA). The S/N ratio obtained from DNA microarray analysis was applied as the indicator of relative abundance of pathogenic bacteria. A correlation was considered significant if the correlation coefficient \((r^2)\) and variant \((p)\) values were \(\geq 0.8\) and \(< 0.05\), respectively.

**RESULTS AND DISCUSSION**

**Contamination by total coliforms and *E. coli***

In the 28 samples investigated in this study, total coliforms and *E. coli* were detected \((\geq 1.0 \text{ MPN/100 mL})\) in 24 (86%) and 16 (67%) samples, respectively (Table 1, rearranged from Malla \(\text{et al.}\)^5 and Ghaju Shrestha \(\text{et al.}\)^10). Total coliforms and *E. coli* were detected in all the water sources. As discussed in detail in our previous studies, most water sources investigated were not suitable for drinking\(^5,\)\(^10\).

**Occurrence of pathogenic bacteria**

| Water source type | Total coliforms | *E. coli* |
|-------------------|----------------|----------|
| Jar water         | \(8/10 (5.0 \times 10^1 - 7.3 \times 10^2)\) | \(2/10 (1.0 \times 10^0 - 2.0 \times 10^0)\) |
| Shallow dug well  | \(9/10 (2.2 \times 10^1 - 1.3 \times 10^7)\) | \(8/10 (4.1 \times 10^0 - 1.2 \times 10^5)\) |
| Deep tube well    | \(2/3 (1.0 \times 10^3 - 2.0 \times 10^3)\) | \(1/3 (8.4 \times 10^1)\) |
| Stone spout       | \(1/1 (1.6 \times 10^4)\) | \(1/1 (9.1 \times 10^3)\) |
| Spring            | \(2/2 (7.7 \times 10^2 - 6.9 \times 10^4)\) | \(2/2 (2.0 \times 10^1 - 5.1 \times 10^3)\) |
| River             | \(2/2 (1.7 \times 10^1 - 2.4 \times 10^5)\) | \(2/2 (4.4 \times 10^6 - 8.2 \times 10^6)\) |

\(^a\)Values are represented as positive/total samples (concentration range, MPN/100 mL). A single value in the parenthesis indicates one positive sample.
According to DNA microarray analysis, 8–226 species/groups of pathogenic bacteria were detected in 28 samples under study (Fig. 1). The total pathogenic bacterial species/groups detected in at least 1 sample was 411, > 60% of which were classified as BSL 2 or 3. More diverse pathogenic bacteria were present in jar water and groundwater samples from shallow dug wells than in other water source types. The diversity of pathogenic bacteria was higher in groundwater from shallow dug wells in cultivated land than in those in built-up areas. Among 12 potential (BSL 2 and 3) pathogenic bacterial genera detected with relatively high abundance (> 0.1% at least in a sample) in community well groundwater and environmental water samples by previous NGS analysis, Acinetobacter, Arcobacter and Staphylococcus were also detected in some of the same samples in this study. This would verify the validity of the results in DNA microarray analysis.

In the DNA microarray analysis, 10 species were detected at high frequencies of > 80% (Table 2). A classification at the genus level also revealed that 5 genera (Acinetobacter, Arthrobacter, Brevibacterium, Pseudomonas, and Staphylococcus) were detected in some of the same samples. This would verify the validity of the results in DNA microarray analysis.

![Fig. 1 Numbers of pathogenic bacterial species/groups detected in different water sources by DNA microarray analysis. Samples are listed in descending order of the number of pathogenic bacterial species/groups within each water source. Symbols: black bar, BSL 3; gray bar, BSL 2; white bar, others.](image)

**Table 2** Most frequently detected pathogenic bacterial species/groups according to DNA microarray analysis

| Pathogenic bacteria                  | BSL | Jar water | Shallow dug well | Deep tube well | Stone spout | Spring | River | Total |
|--------------------------------------|-----|-----------|------------------|----------------|-------------|--------|-------|-------|
| Arthrobacter globiformis             | 1   | 9/10      | 10/10            | 3/3            | 1/1         | 2/2    | 1/2   | 26/28 |
| Brevibacterium oitidis               | 1   | 9/10      | 8/10             | 2/3            | 1/1         | 2/2    | 2/2   | 24/28 |
| Legionella longbeachae               | 2   | 9/10      | 9/10             | 3/3            | 1/1         | 2/2    | 2/2   | 26/28 |
| Legionella pneumophila               | 2   | 9/10      | 10/10            | 2/3            | 1/1         | 2/2    | 1/2   | 25/28 |
| Pseudomonas aeruginosa               | 2   | 10/10     | 8/10             | 1/3            | 1/1         | 2/2    | 1/2   | 23/28 |
| Pseudomonas chichiori                | 2   | 10/10     | 10/10            | 3/3            | 1/1         | 2/2    | 2/2   | 28/28 |
| Pseudomonas fluorescens              | 1   | 10/10     | 9/10             | 3/3            | 1/1         | 2/2    | 2/2   | 27/28 |
| Pseudomonas marginalis               | 1   | 10/10     | 10/10            | 3/3            | 1/1         | 2/2    | 1/2   | 27/28 |
| Pseudomonas syringe                  | 1   | 10/10     | 9/10             | 3/3            | 1/1         | 2/2    | 1/2   | 26/28 |
| Pseudomonas viridiflava              | 1   | 10/10     | 9/10             | 3/3            | 1/1         | 2/2    | 2/2   | 26/28 |
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Among the 214 genera that were targeted in DNA microarray analysis, a number of feces-related pathogens, such as *Klebsiella*, *Enterobacter*, and *Serratia*, were detected in > 60% of the samples, including most jar water samples, which was consistent with the aforementioned results. *Legionella* spp. were detected in all but one jar water sample, and *Legionella longbeachae* and *Legionella pneumophila* were most prevalent species (Table 2). Both species can cause legionellosis. The occurrence of *Legionella* spp. in shallow well groundwater was also reported in our previous study. The occurrence of *Legionella* in groundwater has also been reported in North America, Portugal, and the Netherlands. To confirm their prevalence and estimate their contamination levels in the samples, MPN-PCR employing genus-specific PCR primers was performed. Consequently, *Legionella* spp. were detected in 1 jar water, 9 shallow well groundwater, 1 spring, and 2 river water samples at $2.7 \times 10^3$–$4.5 \times 10^5$ MPN-copies/100 mL (Fig. 2). The detection of *Legionella* at high frequencies and concentrations in shallow well groundwater and river water samples along with the presence of high numbers of total coliforms and *E. coli* suggested a relationship between the occurrence of *Legionella* and fecal contamination, although, in general, *Legionella* is not a feces-related bacterium. Contrastingly, lack of detection of *Legionella* spp. in the samples, where they were detected in DNA microarray, by MPN-PCR may be attributed to the relatively high detection limit of MPN-PCR method ($2.7 \times 10^3$ MPN-copies/100 mL) and to the possibility of false-positive detections in DNA microarray analysis.

*Acinetobacter* spp. was also detected prevalently in the investigated samples. Previous studies employing clone library methods and NGS found that *Acinetobacter* is a dominant genus in various water sources in the Kathmandu Valley. *Acinetobacter* is an opportunistic bacterium capable of causing pneumonia in immunocompromised individuals. In addition, some members of *Acinetobacter* are associated with multiple antibiotic resistances, enabling them to spread the resistance genes to other bacteria. In our previous study, the blaOXA23-like genes of *Acinetobacter*, which were associated with the production of carbapenem-hydrolyzing $\beta$-lactamases, were detected in 35% of the 37 water samples, including those from shallow dug wells, deep tube wells, stone spouts, and rivers, with concentrations of $5.3$–$7.5 \log$.

![Fig. 2 Concentrations of 16S rRNA genes of *Legionella* and *Brucella* as determined by genus-specific PCR methods in different water sources (detection limit: $2.7 \times 10^3$ MPN-copies/100 mL). Samples are listed in the same order as in Fig. 1. Error bar indicates the 95% confidence interval.](image-url)
copies/100 mL\(^{10}\)). Taken together, *Acinetobacter* is a potential pathogen group whose sources and fates should be further investigated.

Among the BSL 3 pathogens, 7 species/groups were detected in at least 1 sample by DNA microarray analysis, and the detection frequency was highest (50%; 14/28 samples) for *Anaplasma marginale/centrale* group, followed by 21% (6/28 samples) for *Brucella melitensis* and *Salmonella enterica*. Incidence of infectious diseases caused by *Brucella* (brucellosis) in animals and humans of Nepal, including Kathmandu, has been reported recently\(^{118}\). Also, the infection of *Anaplasma* sp. in hyperthermic dogs in Kathmandu\(^{19}\) and of *A. marginale* in dairy cattle in mid western Nepal\(^{20}\) has been found. Due to methodological limitations, genus-specific quantitative detection by MPN-PCR was performed only for *Brucella*. As shown in Fig. 2, *Brucella* spp. were detected in 5 of the 28 samples at 2.7 × 10\(^3\) –3.3 × 10\(^5\) MPN–copies/100 mL; these samples were associated with relatively low fecal contamination. Livestock animals are representative carriers of *Brucella*, and stray cows are widely distributed in the Kathmandu Valley. Therefore, the distribution of stray cow may be associated with the occurrence of *Brucella*; however, this association needs to be further studied.

In our previous investigations applying NGS, 12 potential pathogenic bacterial genera (*Acholeplasma*, *Acinetobacter*, *Arcobacter*, *Cetobacterium*, *Clostridium*, *Dialister*, *Faecalibacterium*, *Mitsuokella*, *Prevotella*, *Staphylococcus*, *Streptococcus* and *Sutterella*) were found with relatively high abundance in community well groundwater and environmental water samples\(^{10}\). By contrast, the present study could reveal the occurrence of other BSL 2 and 3 pathogenic bacteria in the same water samples by applying DNA microarray analysis. This demonstrated the usefulness of DNA microarray analysis as a comprehensive screening tool of a variety of pathogenic bacteria including low abundance ones.

**Correlations between the occurrence of pathogenic bacteria and fecal indicators**

Correlation analysis was performed between the relative abundance of pathogenic bacteria detected using DNA microarray analysis and the concentrations of total coliforms or *E. coli* (Table 1) was performed, targeting a total of 182 pathogenic bacterial species/groups detected in at least 6 samples in DNA microarray analysis. Only 8 (4%) and 15 (8%) pathogens exhibited a significant positive correlation with total coliforms and *E. coli*, respectively (Table 3). In addition, more than half of them were feces-related (*Enterobacteriaceae* and related species). The remaining 162 pathogenic bacterial species/groups did not show a significant positive correlation with any fecal indicators tested here. They included both fecal related and non-fecal-related pathogens; however, the number of the latter ones was higher than that of the former ones, although it might be influenced by the differences in the total number of target species/groups. It was thus suggested that conventional fecal indicators were insufficient to assess the occurrence of most pathogenic bacterial species/groups, especially non-fecal-related ones that are prevalent in water sources in the Kathmandu Valley. These findings were in accordance with a previous study that assessed the correlation between selected potential pathogenic bacterial genera and fecal indicators\(^{10}\).

**CONCLUSIONS**

This study revealed the occurrence of a variety of pathogenic bacteria, including BSL 3 ones, in water sources that are important for drinking and other domestic uses in the Kathmandu Valley, and identified some prevalently distributed pathogens. We also found that conventional fecal indicators (total coliforms and *E. coli*) were ineffective at assessing the occurrence of most of pathogenic bacteria, especially non-fecal-related ones, in the valley. Overall, the results of this study will be helpful for understanding pathogenic bacterial contamination in the valley and for screening important pathogens that should be investigated in detail.

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Table 3  Pathogenic bacterial species/groups showing significant positive correlation with fecal indicators

| Pathogenic bacteria                          | BSL | Total coliforms | E. coli |
|---------------------------------------------|-----|----------------|---------|
|                                            |     | P value | r²  | P value | r²  |
| Acinetobacter calcoaceticus                | 2   | 0.0015  | 0.94 | 0.0404  | 0.92 |
| Arcobacter genus                           | 2   | 0.0084  | 0.98 |
| Brenneria rubrifaciens                     | 1   | 0.0022  | 0.87 |
| Citrobacter braakii                        | 1   | 0.0352  | 0.93 |
| Citrobacter freundii                       | 2   | 0.0081  | 0.86 |
| Enterobacter cancerogenus                  | 2   | <0.0001 | 0.95 |
| Enterobacter cloacae                       | 2   | 0.0231  | 0.86 |
| Enterobacter dissolvens                    | 1   | 0.0002  | 0.80 |
| Legionella birminghamensis                 | 2   | 0.0057  | 0.88 |
| Legionella sainthelesi                     | 2   | 0.0054  | 0.81 | 0.0014  | 0.89 |
| Pectobacterium cacticida                   | 1   | 0.0032  | 0.91 |
| Pectobacterium carotovorum subsp. brasiensi| NA  |        |      |
| Pectobacterium carotovorum subsp. wasabiae | 1   | 0.0062  | 0.99 |
| Pectobacterium chrysanthemi                | 1   | 0.024   | 0.95 |
| Pseudomonas atlantica group                | 1   | <0.0001 | 0.86 |
| Pseudobutyricibrio ruminis                | 1   | <0.0001 | 0.99 |
| Rathayibacter toxicus                      | 1   | <0.0001 | 1    | 0.0137  | 1    |
| Ruminococcus torques                      | 1   | 0.0139  | 0.81 |
| Serratia rubidaea                         | 2   | 0.0012  | 0.85 |
| Staphylococcus lugdunensis                | 2   | 0.0001  | 1    |

aBlank indicates without significant positive correlation.
bNA, not assigned.
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