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Next-generation sequencing identification of pathogenic bacterial genes and their relationship with fecal indicator bacteria in different water sources in the Kathmandu Valley, Nepal

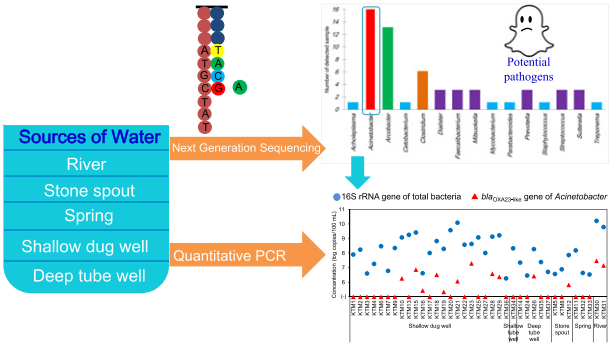
Rajani Ghaju Shrestha [a], Yasuhiro Tanaka [b], Bikash Malla [a], Dinesh Bhandari [c], Sarmila Tandukar [a,c], Daisuke Inoue [d,e], Kazunari Sei [d], Jeevan B. Sherchand [c], Eiji Haramoto [b,*]

[a] Integrated Graduate School of Medicine, Engineering, and Agricultural Sciences, University of Yamanashi, 4-3-11 Takeda, Kofu, Yamanashi 400-8511, Japan
[b] Graduate Faculty of Interdisciplinary Research, University of Yamanashi, 4-3-11 Takeda, Kofu, Yamanashi 400-8511, Japan
[c] Institute of Medicine, Tribhuvan University, Maharajgunj, Kathmandu, Nepal
[d] Department of Health Science, Kitasato University, 1-15-1 Kitasato, Minami-ku, Sagamihara, Kanagawa 252-0373, Japan
[e] Division of Sustainable Energy and Environmental Engineering, Osaka University, Suita, Osaka 565-0871, Japan

HIGHLIGHTS

- Bacterial communities in water sources in Kathmandu Valley were characterized by NGS.
- Eighty-one out of 525 genera identified were considered potential pathogens.
- *Acinetobacter*, *Arcobacter*, and *Clostridium* were highly abundant.
- $bla_{OXA23\text{-like}}$ genes of *Acinetobacter* were detected by qPCR in 35% of water samples.
- No sufficient correlation was found between fecal indicator and pathogenic bacteria.

GRAPHICAL ABSTRACT



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ABSTRACT

Bacteriological analysis of drinking water leads to detection of only conventional fecal indicator bacteria. This study aimed to explore and characterize bacterial diversity, to understand the extent of pathogenic bacterial contamination, and to examine the relationship between pathogenic bacteria and fecal indicator bacteria in different water sources in the Kathmandu Valley, Nepal. Sixteen water samples were collected from shallow dug wells ($n = 12$), a deep tube well ($n = 1$), a spring ($n = 1$), and rivers ($n = 2$) in September 2014 for 16S rRNA gene next-generation sequencing. A total of 525 genera were identified, of which 81 genera were classified as possible pathogenic bacteria. *Acinetobacter*, *Arcobacter*, and *Clostridium* were detected with a relatively higher abundance (>0.1% of total bacterial genes) in 16, 13, and 5 of the 16 samples, respectively, and the highest abundance ratio of *Acinetobacter* (85.14%) was obtained in the deep tube well sample. Furthermore, the $bla_{OXA23\text{-like}}$ genes of *Acinetobacter* were detected using SYBR Green-based quantitative PCR in 13 (35%) of 37 water samples, including the 16 samples that were analyzed for next-generation sequencing, with concentrations ranging 5.3–7.5 log copies/100 mL. There was no sufficient correlation found between fecal indicator bacteria, such as *Escherichia coli* and

* Corresponding author at: Interdisciplinary Center for River Basin Environment, Graduate Faculty of Interdisciplinary Research, University of Yamanashi, 4-3-11 Takeda, Kofu, Yamanashi 400-8511, Japan.
E-mail address: eharamoto@yamanashi.ac.jp (E. Haramoto).

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total coliforms, and potential pathogenic bacteria, as well as the $bla_{OXA23\text{-}like}$ gene of *Acinetobacter*. These results suggest the limitation of using conventional fecal indicator bacteria in evaluating the pathogenic bacteria contamination of different water sources in the Kathmandu Valley.

1. Introduction

The Kathmandu Valley consists of three districts, namely, Kathmandu, Bhaktapur, and Lalitpur, covering an area of 664 km^2 with higher population density (estimated to be >2500 persons per km^2) (Asian Development Bank, 2011). Kathmandu Upatyaka Khanepani Limited (KUKL) is the only potable water supplying agent in the valley, which covers 45% of the total area with 105 million L per day (MLD) and 76 MLD in wet and dry seasons, respectively, whereas the demand is excessively high (i.e., 320 MLD) in 2009 (KUKL, 2009) and has been increased to 370 MLD in 2015 (KUKL, 2015; Thapa et al., 2015). Thus, the percentage of households using groundwater in the dry season is 52 (Shrestha et al., 2017). In Nepal, 5.5 million people have no access to safe drinking water; subsequently, 10,500 children die every year (Shrestha, 2012). In the valley, diarrhea accounts for 30% of total hospital cases and 69% suffer from waterborne diseases (Ministry of Health and Population, 2010).

Human and animal pathogenic and potentially pathogenic bacteria are released with wastewater into aquatic environments, causing water pollution; therefore, its recognition and understanding is essential. Several groundwater sources used in the valley, such as shallow dug wells as well as deep and shallow tube wells, have been reported to contain bacterial pollution, including fecal bacteria (Haramoto et al., 2011; Inoue et al., 2014; Tanaka et al., 2012). According to this, groundwater in the Kathmandu Valley has exceeded the drinking water quality standard specified in the World Health Organization (WHO) guidelines (Prasai et al., 2007; Warner et al., 2008), which indicates poor management of the water source. Microbial contamination of water in this valley has been evaluated by measuring fecal indicator bacteria, such as *Escherichia coli* and total coliforms. However, there was no significant correlation found between these fecal indicator bacteria and other pathogens (Bradshaw et al., 2016; Haramoto et al., 2012; Inoue et al., 2014), indicating that the detection of the indicator bacteria is not enough to predict pathogen contamination.

Diversity and dynamics of bacterial communities, including pathogens and their impact on pollution in water have been characterized through different molecular methods (Inkinen et al., 2016; Maheux et al., 2014). Within these, intensive application of next-generation sequencing (NGS) helps to explore the diversity of microorganisms, including enhanced sensitivity and increased dynamic range (Staley et al., 2013). Based on this background, the present study aimed to analyze *E. coli* and total coliforms by culture method along with bacterial communities in various water sources in the Kathmandu Valley by NGS and to determine the relationship between fecal indicator bacteria and potential pathogenic bacteria.

2. Materials and methods

2.1. Collection of water samples

In August 2014, a total of 37 samples were collected from shallow dug wells ($n = 23$), deep tube wells ($n = 5$), a shallow tube well ($n = 1$), stone spouts ($n = 3$), springs ($n = 3$), and rivers ($n = 2$) in the Kathmandu Valley. The depth of shallow wells and deep tube wells was below 50 m and above 50 m from the ground surface, respectively (data not shown). The samples were collected in sterile 100 mL plastic bottles, kept in bags containing ice packs, and brought to the laboratory as soon as possible.

2.2. Detection of total coliforms and E. coli

Total coliforms and *E. coli* were enumerated by the most probable number (MPN) method using a Colilert test kit (IDEXX Laboratories, Westbrook, USA), following the manufacturer's procedure. Briefly, one pack of Colilert powder was added to a 100 mL water sample, where the undiluted samples and dilutions of 10^{-2}, 10^{-4}, and 10^{-6} were prepared in a sterile vessel and shaken gently until dissolved. Then, the mixture was poured into a Quanti-Tray 2000 and sealed in an IDEXX Quanti-Tray sealer. The sealed tray was placed in an incubator at 37 °C for 24 h. The result was interpreted as follows: yellow-colored large and small wells were counted as total coliforms, whereas for *E. coli*, the tray was observed under UV light and blue-fluorescent large and small wells were counted. Finally, the MPN value was calculated using an MPN generating software (IDEXX Laboratories), considering the dilution ratio.

2.3. Bacterial DNA extraction

One hundred milliliters of the water sample was filtered using a sterilized disposable filter unit preset with a nitrocellulose membrane (diameter, 47 mm; pore size, 0.22 μm; Nalgene, Tokyo, Japan). The membrane filter was transferred into a 50 mL plastic tube, and microorganisms trapped on the filter were resuspended in 5 mL of Tris-EDTA buffer (pH 7.4). Following shaking and vortex mixing steps at 50 °C with a speed of 300 rpm, total DNA was extracted from the resuspended sample using the CicaGeneus DNA extraction kit (Kanto Chemical, Tokyo, Japan) to obtain a final volume of 100 μL of purified DNA.

2.4. NGS analysis for characterization of bacterial community

Among the 37 samples collected, 16 samples (12 shallow dug wells, one spring, one deep tube well, and two river water samples) were selected for Illumina MiSeq 16S rRNA gene sequencing using the MiSeq platform (Illumina, Atsugi, Japan), considering the results obtained from the detection of *E. coli* and total coliforms, as well as covering area of the Kathmandu Valley. The universal primers U515F and U806R (Baker et al., 2003; Takai and Horikoshi, 2000) linked with barcode sequences (5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3′ for U515F and 5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3′ for U806R) were used for the first 16S rRNA gene amplifications. The reaction was performed as follows: at 95 °C for 30 s, followed by 23–29 cycles (to minimize the PCR bias, the number of cycles was decreased as follows: 23 cycles for 3 samples (KTM21, -30, and -31); 26 cycles for 5 samples (KTM15, -18, -20, -25, and -28); 28 cycles for 1 sample (KTM26) and 29 cycles for 7 samples (KTM6, -10, -11, -13, -22, -23, and -29), at 95 °C for 10 s, 50 °C for 30 s, and at 72 °C for 30 s, followed by 72 °C for 5 min.

The amplicons were sent to FASMAC Co. Ltd. (Atsugi, Japan) for amplification of the second PCR using the above barcode sequences as primers and sequencing by the Illumina MiSeq gene sequencer. A compiled set of sequences was derived in the form of operational taxonomic units (OTUs) where the OTU data obtained were further analyzed and classified based on bacterium domain, phylum, family, and genus from the FASMAC Co. Ltd. Bio-safety level 2 or 3 pathogens were distinguished, where the pathogenic species percentage within the genus was first calculated by the number of species considered bio-safety level 2 or 3 (from American Biological Safety Association, https://my.