Characterization of microbial communities distributed in the groundwater pumped from deep tube wells in the Kathmandu Valley of Nepal

Yasuhiro Tanaka, Kei Nishida, Takashi Nakamura, Saroj Kumar Chapagain, Daisuke Inoue, Kazunari Sei, Kazuhiro Mori, Yasushi Sakamoto and Futaba Kazama

ABSTRACT

Although groundwater is a major water supply source in the Kathmandu Valley of Nepal, it is known that the groundwater has significant microbial contamination exceeding the drinking water quality standard recommended by the World Health Organization (WHO), and that this has been implicated in causing a variety of diseases among people living in the valley. However, little is known about the distribution of pathogenic microbes in the groundwater. Here, we analysed the microbial communities of the six water samples from deep tube wells by using the 16S rRNA gene sequences based culture-independent method. The analysis showed that the groundwater has been contaminated with various types of opportunistic microbes in addition to fecal microbes. Particularly, the clonal sequences related to the opportunistic microbes within the genus *Acinetobacter* were detected in all samples. As many strains of *Acinetobacter* are known as multi-drug resistant microbes that are currently spreading in the world, we conducted a molecular-based survey for detection of the gene encoding carbapenem-hydrolysing β-lactamase ($bla_{\text{oxa-23-like}}$ gene), which is a key enzyme responsible for multi-drug resistance, in the groundwater samples. Nested polymerase chain reaction (PCR) using two specific primer sets for amplifying $bla_{\text{oxa-23-like}}$ gene indicated that two of six groundwater samples contain multi-drug resistant *Acinetobacter*.

Key words | *Acinetobacter*, deep tube well, groundwater, microbial community, multi-drug resistance

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INTRODUCTION

Nepal is an underdeveloped and small, land-locked Himalayan kingdom located in South Asia between China in the north and India in the east, south and west. This country is facing severe problems regarding drinking water quality and availability, similar to those occurring in many other developing countries (Warner *et al.* 2008).

The Kathmandu Valley, which is the main urban center of Nepal, is situated at an altitude of about 1,300 m above sea level and covers an area of roughly 500 km^2. Owing to rapid population growth in the area, the water demand in this valley has increased greatly. However, the quality of drinking water in the area is deteriorating because of the deficiency of treatment plants, direct discharge of sewage waste into surface water and inefficient management of the piped water distribution system (Asian Development Bank 2010). Currently, approximately 50% of the water supply (estimated to be 59.06 million litres a day) that is used for drinking and other domestic requirements in the

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Kathmandu Valley are derived from groundwater (Pandey *et al.* 2010).

Several groundwater sources including dug wells, shallow tube wells and deep tube wells are used in the valley (Warner *et al.* 2008; Asian Development Bank 2010), with water from the deep tube wells comprising about 32% (37.49 million litres a day) of the water used in the valley (Pandey *et al.* 2010). Although groundwater, particularly that pumped from deep tube wells, is generally thought to be advantageous when compared to the surface water with respect to quality (e.g. suspended solids and microbial contamination), recent studies have shown that the groundwater in the Kathmandu Valley has significant microbial contamination that exceeds the drinking water quality standard recommended by the World Health Organization (WHO) guideline (Prasai *et al.* 2007; Warner *et al.* 2008). Water-borne diseases such as diarrhoea and dysentery are very serious in Nepal. Indeed, Teku Hospital in Kathmandu reported that 16.5% of all deaths were due to complications related to water-borne diseases (Metcalf & Eddy 2000). As a result, microbial analysis and monitoring studies conducted in the region have focused on *Escherichia coli* and coliform bacteria that are commonly used as bacterial indicator for contamination of feces (Prasai *et al.* 2007; Warner *et al.* 2008). This has resulted in a lack of information regarding whether other microbes that could cause health problems have contaminated the groundwater. Based on 16S rRNA gene sequence analysis, our objective was to analyse the microbial communities distributed in the groundwater pumped from deep tube wells in the Kathmandu Valley to detect other suspected microbial pathogens.

METHODS

Water samples and DNA extraction

Groundwater samples were collected from six deep tube wells located in the areas Kamal Pokhari, Panipokhari, Kalimati, Balkumari, Sinamangal and Minbhawan (hereafter referred to as DG-KM, DG-PN, DG-KL, DG-BL, DG-SN and DG-MN, respectively) of the Kathmandu Valley during the monsoon season (August–September) in 2008. The depths of the wells were 270 m for DG-KM, 200 m for DG-PN, 280 m for DG-KL, 270 m for DG-BL, 280 m for DG-SN and 250 m for DG-MN. Four liters of water sample pumped from each well was filtered through the 0.45 μm-pore-sized Durapore membranes (47 mm-diameter; Millipore, Billerica, MA, USA). The filters were then stored at −20 °C in a sterilized 50 ml conical tube until subsequent analysis.

DNA extraction and purification from the filters was conducted using a QIAamp DNA Stool Mini kit (QIAGEN, Hilden, Germany) with a small modification at the initial step. Specifically, ASL buffer (20 ml) was added to the conical tube containing the membranes, and they were then mixed well by vortexing. After incubation at 70 °C for 30 min, the suspension was centrifuged (14,000 × *g*) for 1 min, and 1.2 ml of the supernatant was applied to the remaining steps according to the manufacturer's instructions.

Culture-independent microbial analysis

Polymerase chain reaction (PCR) amplification of the 16S rRNA genes from the water sample DNA was conducted using two bacterial universal primers, EUB8F (Weisburg *et al.* 1991) (5′-AGAGTTT GATC(A/C)TGGCTCAG-3′: corresponding to positions 8–27 of the *E. coli* 16S rRNA gene) and EUB1512R (Kane *et al.* 1993) (5′-ACGG(C/T) TACCTTGTTACGACTT-3′; corresponding to positions 1492–1512 of the *E. coli* 16S rRNA gene). The reactions were conducted as previously described (Matsuzawa *et al.* 2010), except for the numbers of cycles. To minimize the PCR bias, the number of cycles was decreased to 25–31 cycles (25, 25, 27, 31, 29 and 29 cycles for DG-KM, DG-PN, DG-KL, DG-BL, DG-SN and DG-MN, respectively). The amplified DNA fragments were purified using an illustra GFX PCR purification kit (GE Healthcare, Buckinghamshire, UK), after which they were cloned into *E. coli* strain DH5α using a pT7 Blue T-vector kit (TaKaRa, Shiga, Japan). The clonal DNA was subsequently amplified from randomly selected recombinants by colony direct PCR using the two primers, pT7-F (5′-GATCTACTAGTCATATG GAT-3′) and pT7-R (5′-TCGGTACCCGGGGATCCGAT-3′), which were specific to the vector sites flanking the insert (Matsuzawa *et al.* 2010). The DNA fragments obtained

were subjected to restriction fragment length polymorphism (RFLP) analysis by separate digestion with two types of restriction endonucleases, *Hha*I and *Hae*III (TaKaRa). Coverage (C) values for each of the clone libraries were calculated by the equation $C = [1–(n/N)] \times 100$ (Good 1953), where n is the number of unique clones and N is the total number of clones analysed. The PCR products from representative clones of each of the RFLP groups were purified using an Illustra GFX PCR DNA and gel purification kit (GE Healthcare), after which they were sequenced as previously described using primer EUB907R (Tamaki *et al.* 2005) (5′-CCG(C/T)CAATTC(A/G)TTT(A/G)AGTTT-3′). The obtained data were compared with those in the NCBI database by using the BLAST search program (http://www.ncbi.nlm.nih.gov/blast/).

Detection of carbapenem-hydrolysing β-lactamase gene

To determine if genes encoding carbapenem-hydrolysing β-lactamase ($bla_{\text{oxa-23-like}}$ gene) were present in the groundwater samples, nested PCR was conducted using two sets of nucleotide primers that were specific to the gene of *Acinetobacter*. The outer primers, OXA23-F1 (5′-AATATGTGCCAGCCTC TACA-3′) and OXA23-R1 (5′-TTAAATAATATTCAGCTGTT-3′), which amplify a 602-bp sequence of the $bla_{\text{oxa-23-like}}$ gene, were designed based on comparison with sequences of $bla_{\text{oxa-23-like}}$ genes from some bacterial strains belonging to the genus *Acinetobacter*. The primer set OXA23-F2 (5′-GATCGGATTGGAGAACCAGA-3′) and OXA23-R2 (5′-ATTTCTGACCGCATTTCCAT-3′) (Woodford *et al.* 2006), which was designed to amplify 501-bp sequence of the $bla_{\text{oxa-23-like}}$ gene, was used as the inner primer set. First step amplification was conducted using the primers OXA23-F1 and OXA23-R1. The reaction mixture for this PCR step was prepared using the same method used for amplification of the 16S rRNA gene. Thermal cycling was carried out as follows: 95 °C for 5 min; 35 cycles of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C; and final extension at 72 °C for 10 min. Next, 0.5 μl of the amplified product was applied to the second step PCR, which was conducted using the inner primers OXA23-F2 and OXA23-R2 in the same manner as described above. The PCR amplification products were analysed by agarose gel (2% w/v) electrophoresis followed by ethidium bromide staining.

To check whether the nested PCR amplified the correct target gene, cloning and sequencing of the amplified DNA fragments were performed using the same methods as analysis of 16S rRNA genes.

Nucleotide accession numbers

The nucleotide sequence data determined in this study have been deposited in the DDBJ/EMBL/GenBank databases under accession numbers AB635865 to AB636074 for the 16S rRNA gene clones and AB636075 to AB636104 for the $bla_{\text{oxa-23-like}}$ gene clones.

RESULTS AND DISCUSSION

Analysis of microbial communities

Investigation of the microbial communities distributed in the groundwater samples collected from the deep tube wells located in the Kathmandu Valley was performed by culture-independent method based on the 16S rRNA gene sequences. A total of 510 clones from six groundwater samples were analysed. The characterization of the communities was based on 85 clones from each 16S rRNA gene library. The clones were grouped by RFLP analysis, and the clones from the water samples of DG-KM, DG-PN, DG-KL, DG-BL, DG-SN and DG-MN were divided into 29, 28, 40, 41, 43 and 29 phylotypes, respectively. The coverage values were 74% for DG-KM, 78% for DG-PN, 69% for DG-KL, 74% for DG-BL, 65% for DG-SN and 81% for DG-MN.

Phylogenetic analysis showed that the sequences representing RFLP phylotypes from the clone libraries of DG-KM, DG-PN, DG-KL, DG-BL, DG-SN and DG-MN water samples, were affiliated with 6, 10, 7, 8, 6 and 9 bacterial taxonomic groups, respectively (Figure 1). *Betaproteobacteria* was the most abundant phylogenetic group in all groundwater samples.

Table 1 shows the genus level classification of 16S rRNA gene sequences obtained from the clones based on comparative analysis with the sequence obtained from a BLAST search. A homology greater than 95% of any known bacterial species was defined as the threshold for grouping at genus level. Interestingly, microbes that can grow on C_1 compounds

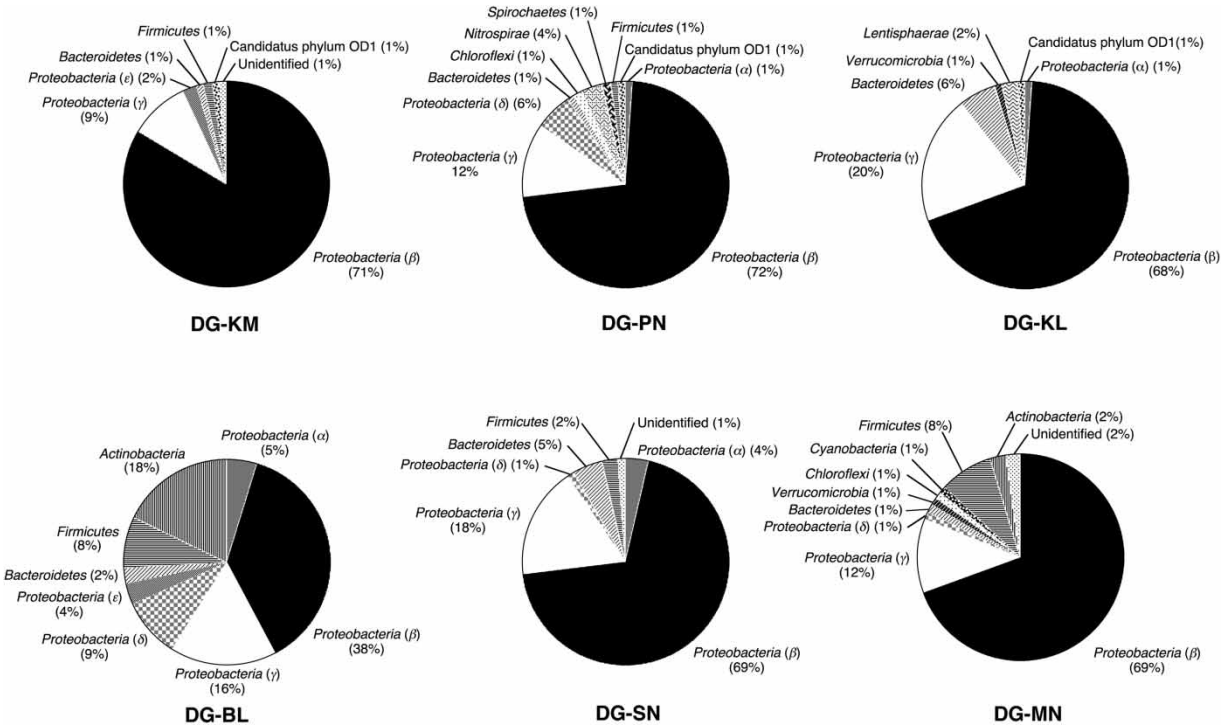

Figure 1 | Phylogenetic distribution of the 16S rRNA gene clones belonging to different bacterial taxa in the groundwater samples.

such as methane, methanol, formaldehyde and formate as the sole source of carbon and energy (*Methylobacterium*, *Methylophilus*, *Methylovorus*, *Methylotenera*, *Methylobacillus*, *Methylobacter* or *Methylomonas*) (Urakami & Komagata 1986; Jenkins *et al.* 1987; Govorukhina & Trotsenko 1991; Bowman *et al.* 1993; Van Aken *et al.* 2004; Kalyuzhnaya *et al.* 2006) were detected in all groundwater samples with relatively high yield; the yields of these microbial groups in the groundwater samples of DG-KM, DG-PN, DG-KL, DG-BL, DG-SN and DG-MN were 18, 28, 38, 5, 42 and 31%, respectively. These findings are consistent with the fact that high concentrations of methane gas have been dissolved into the groundwater in the Kathmandu Valley (JICA 1990). Although microbes that can directly utilize methane for growth have been reported in three of the aforementioned genera (*Methylobacterium*, *Methylobacter* and *Methylomonas*), other C_1 compounds-utilizers (*Methylophilus*, *Methylovorus*, *Methylotenera* and *Methylobacillus*) might use the metabolites, methanol, formaldehyde and formate, produced by methane-utilizing microbes in a groundwater ecosystem.

In general, obligate anaerobic sulfate- and metal-reducing microbes tend to be detected during the microbial analyses of deep groundwater samples due to their strong reducing (anoxic) conditions (Fry *et al.* 1997; Ishii *et al.* 2000; Junier *et al.* 2010). In this study, two clonal sequences closely related to the 16S rRNA gene sequences from the genera *Ferribacterium* and *Geobacter* indicating metal-reduction (Lovley *et al.* 1993; Cummings *et al.* 1999) were detected in the samples from DG-PN and DG-KL, respectively. However, many types of clones related to aerobic microbial groups (C_1 compounds-utilizers and the genera *Sphingomonas*, *Erythromonas*, *Acidovorax*, *Curvibacter*, *Acinetobacter*, etc.) were detected in all groundwater samples. Furthermore, *Escherichia* or microbes correlated with human and animal feces (*Lactobacillus*, *Leuconostoc*, *Weissella* and *Streptococcus*) (Godfree *et al.* 1997; Walter *et al.* 2001) were also observed in the water samples from the four deep tube wells, DG-KM, DG-PN, DG-SN and DG-MN. These data supported the information obtained in earlier studies that industrial and household wastewater

Table 1 | Number of clones found in each groundwater sample; the phylum and the genus detected

		Groundwater samples					
Phylum (Class)	**Genus**	**DG-KM**	**DG-PN**	**DG-KL**	**DG-BL**	**DG-SN**	**DG-MN**
Proteobacteria (α)	*Sphingomonas*				1		
	Erythromonas				3		
	Methylobacterium			1		1	
	Unidentified		1			2	
Proteobacteria (β)	*Acidovorax*	5		1			
	Aquabacterium	4		2			
	Curvibacter	4		4		3	7
	Ferribacterium			1			
	Gallionella		1		3		27
	Hydrogenophaga	1		1		1	
	Leptothrix			1			
	Limnohabitans	2		2			
	Massilia			1			
	Methylophilus	14	16	30		3	9
	Methylovorus		1			21	8
	Methylotenera		2				1
	Methylobacillus					2	
	Neisseria				3	2	
	Paucibacter			1			
	Perlucidibaca			1			
	Quatrionicoccus			2		1	
	Rhodoferax				3	1	
	Azospira		35				
	Pelomonas					2	
	Rubrivivax					3	
	Undibacterium					1	
	Vogesella			7			
	Dechloromonas						1
	Zoogloea						1
	Unidentified	41	6	4	23	19	5
Proteobacteria (γ)	*Acinetobacter*	4	3	8	8	4	2
	Comamonas			1			
	Escherichia				2	2	
	Pseudomonas			3			
	Rheinheimera	1		1			
	Methylobacter		3		4	6	
	Methylomonas	1	2	1		3	8
	Moraxella			1			

(*continued*)

Table 1 | continued

		Groundwater samples					
Phylum (Class)	**Genus**	**DG-KM**	**DG-PN**	**DG-KL**	**DG-BL**	**DG-SN**	**DG-MN**
	Unidentified	2	2	2			
Proteobacteria (δ)	*Geobacter*		1				
	Pelobacter		3				
	Unidentified		1		8	1	1
Proteobacteria (ε)	*Sulfuricurvum*				1		
	Unidentified	2			2		
Bacteroidetes	Unidentified		1	5	2	4	1
Verrucomicrobia	Unidentified			1			1
Chloroflexi	Unidentified		1				1
Nitrospirae	Unidentified		3				
Spirochaetes	Unidentified		1				
Lentisphaerae	Unidentified			2			
Cyanobacteria	Unidentified						1
Firmicutes	*Gemella*				2		
	Lactobacillus	1			2		
	Leuconostoc				2	1	3
	Weissella						1
	Streptococcus				1	1	3
	Unidentified	1	1				
Actinobacteria	*Propionibacterium*				14		
	Unidentified				1		2
Candidate division OD1	Unidentified	1	1	1			
Unidentified	Unidentified	1				1	2
Total		85	85	85	85	85	85

had been discharged into the deep groundwater in the Kathmandu Valley (Pandey *et al*. 2010).

The clonal sequences showing high similarities (>97%; which is the threshold for the same bacterial species defined by Stackebrandt & Goebel 1994) with 16S rRNA gene sequences from pathogenic or Biosafety level (BSL) 2 bacteria are summarized in Table 2. Various types of opportunistic pathogens were observed in the present study. There were no groundwater samples free from such microbes. Opportunistic microbes cause a wide variety of infections such as endocarditis, meningitis, pneumonia, osteomyelitis and septicemia in critically ill and immune-compromised patients. In Nepal, about 70% of all health problems and deaths are attributed to infectious diseases including meningitis, a typical symptom of opportunistic infection (Rai *et al*. 2001, 2002). Moreover, human immunodeficiency virus (HIV) infection has spread rapidly in Nepal (Pais 1996; Rodrigo & Rajapakse 2009). Taken together, these findings indicate that many opportunistic infections are likely to pose a great challenge to Nepal in the future.

Detection of the gene for carbapenem-hydrolysing β-lactamase

In this study, various opportunistic microbial species were detected as described above (Table 2). In particular, the clones related to any of four bacterial species belonging to the genus *Acinetobacter* were present in all groundwater samples. The genus *Acinetobacter* consists of more than 20 species, of which *A. baumannii*, *A. calcoaceticus*, *A. johnsonii*

Table 2 | Pathogenic or BSL2 microbes detected in this study

Bacterial species	Pathogenicity, etc.	BSL[a]	Detected groundwater sample[b]
Neisseria mucosa	Opportunistic infection (Endocarditis)	2	DG-BL (3)
Neisseria meningitidis	Opportunistic infection (Meningitis, Meningococcal disease)	2	DG-SN (2)
Acinetobacter calcoaceticus	Opportunistic infection (Meningitis, Endocarditis, Pneumonia, Endophthalmitis), Multi-drug resistance	2	DG-KM (1), DG-KL (2), DG-BL (4)
Acinetobacter baumannii	Opportunistic infection (Bacteremia, Meningitis, Pneumonia, Urinary tract infection, Wound infection), Multi-drug resistance	2	DG-KL (1)
Acinetobacter johnsonii	Opportunistic infection (Endocarditis), Multi-drug resistance	2	DG-KM (2), DG-KL (6), DG-SN (1)
Acinetobacter junii	Opportunistic infection (Blood stream infection, Bacteremia, Empyema, Peritonitis, Keratitis), Multi-drug resistance	2	DG-KM (1), DG-PN (2), DG-MN (2)
Escherichia coli	Haemolytic uraemic syndrome (Enterohemorrhagic *E. coli*)	2[c]	DG-BL (2), DG-SN (2)
Moraxella osloensis	Opportunistic infection (Osteomyelitis)	2	DG-KL (1)
Gemella haemolysans	Opportunistic infection (Meningitis, Endocarditis)	1	DG-BL (2)
Lactobacillus iners	Isolated from human clinical specimens (urine, vagina) and medical care products	2	DG-KM (1)
Streptococcus oralis	Opportunistic infection (Meningitis, Endocarditis)	2	DG-KL (1)
Streptococcus mitis	Opportunistic infection (Lung abscess, Meningitis, Endocarditis)	2	DG-SN (1)
Streptococcus parasanguinis	Opportunistic infection (Endocarditis), Asymptomatic Mastitis in sheep	2	DG-MN (2)
Streptococcus bovis	Opportunistic infection (Endocarditis, Septicemia, Meningitis), Correlative relationships with colon cancer and colonic neoplasm	2	DG-MN (1)
Propionibacterium acnes	Opportunistic infection (Spondylodiscitis, Endocarditis, Arthritis, Endophthalmitis, Pansinusitis)	2	DG-MN (5)

[a]The BSL was based on the criteria defined by the ATCC, DSMZ or NBRC.
[b]The clone number detected in each sample is provided in parentheses.
[c]Only a portion of the strains, e.g. Enterohemorrhagic *E. coli* strains, are BSL-2 pathogens.
Bacterial characters including pathogenicity were obtained from reports by Headings *et al*. (1978), Pal *et al*. (1981), Sugarman & Clarridge (1982), Carrascosa *et al*. (1994), Burnette-Curley *et al*. (1995), Jakab *et al*. (1996), Echeverría *et al*. (1998), Fernández-Garayzábal *et al*. (1998), Montejo & Aguirrebengoe (1998), Falsen *et al*. (1999), Filka *et al*. (2000), Grant *et al*. (2000), Hunt *et al*. (2000), Tronel *et al*. (2001), White *et al*. (2002), Gopal *et al*. (2003), Han & Tarrand (2004), Khan *et al*. (2004), Anil *et al*. (2007), Maragakis & Perl (2008), Renton *et al*. (2008), Dinani *et al*. (2009), Hung *et al*. (2009), Dessinioti & Katsambas (2010), Kim (2010), Pennington (2010), Turton *et al*. (2010), Uçkay *et al*. (2010) and Horii *et al*. (2011).

and *A. junii* detected in our study are usually associated with the clinical environment and nosocomial infections including meningitis, endophthalmitis, bacteremia, pneumonia, urinary tract infection, wound infection, endocarditis, blood stream infection, empyema, peritonitis and keratitis (Pal *et al*. 1981; Filka *et al*. 2000; Hunt *et al*. 2000; Gopal *et al*. 2003; Maragakis & Perl 2008; Hung *et al*. 2009; Turton *et al*. 2010; Horii *et al*. 2011). Members of this genus are also associated with multiple antibiotic resistances, and many widespread strains belonging to these species are resistant to almost all antibiotics currently in use.

Recent studies have indicated that the microbes having the genes for carbapenem-hydrolysing β-lactamases ($bla_{\text{oxa-23-like}}$ gene, $bla_{\text{oxa-24/40-like}}$ gene, $bla_{\text{oxa-51-like}}$ gene and $bla_{\text{oxa-58-like}}$ gene), which are key enzymes responsible for multi-drug resistance, have emerged globally, although the isolation sources of almost every strain were limited to hospital settings (e.g. clinical samples and hospital wastes) (Zarrilli *et al*. 2009). In Asian areas including Nepal, the main gene detected in the clinical isolates of *Acinetobacter* species was $bla_{\text{oxa-23-like}}$ (Gaur *et al*. 2007; Zarrilli *et al*. 2009). On the other hand, microbial strains producing extended-spectrum β-lactamases were very recently isolated from general environmental samples, river water in Paris (the first isolation of environmental multi-drug resistant *Acinetobacter*) and drinking water in New Delhi (Girlich

et al. 2010; Walsh et al. 2011). Therefore, we checked for the presence of the $bla_{\text{oxa-23-like}}$ gene in groundwater samples collected in this study by PCR. Firstly, single PCR using OXA23-F1 and OXA23-R1 primers were performed. But, no clear PCR products were observed in all samples (data not shown). So then we conducted nested PCR for detecting the $bla_{\text{oxa-23-like}}$ gene. As a result, positive amplifications, 501 bp of DNA fragments, were detected in groundwater samples from DG-KM and DG-KL (Figure 2). To check whether the amplified DNA fragments were derived from the $bla_{\text{oxa-23-like}}$ gene of *Acinetobacter*, we constructed the clone libraries from the two PCR products. The sequences of 15 clones from each library were analysed, and compared to the sequences in the NCBI database. All clonal sequences showed above 96% identities with $bla_{\text{oxa-23-like}}$ gene (Ho et al. 2010) from *A. baumannii*, which is the most commonly isolated multi-drug resistant nosocomial species, indicating that the PCR fragments in the DG-KM and DG-KL samples were derived from the correct targeted gene. Detections of $bla_{\text{oxa-23-like}}$ gene from two deep tube wells and *Acinetobacter* from all deep tube wells in this study suggest that water supplies in the Kathmandu Valley are potentially contaminated with multi-drug resistant *Acinetobacter*.

In recent years, outbreaks of carbapenem-resistant *Acinetobacter* species have been increasing in several parts of the world including developing countries, and these organisms have caused many serious diseases, especially in intensive-care-units (ICU) (Peleg et al. 2008). Since groundwater from deep tube wells accounts for 32% of water demand, and is used in various public places containing hospitals in the Kathmandu Valley (Pandey et al. 2010), further detailed research based on both culture-dependent and culture-independent methods for the multi-drug resistant microbes in the groundwater should be conducted to reveal their pathogenic properties and contamination sources. Moreover, not only in deep groundwater, but also in any other water sources such as shallow groundwater and river water, it would be desirable to measure the multi-drug resistant microbes as a new target in microbial surveys for understanding the water quality of Nepal, although the targets in the survey have so far been focused on faecal microbes.

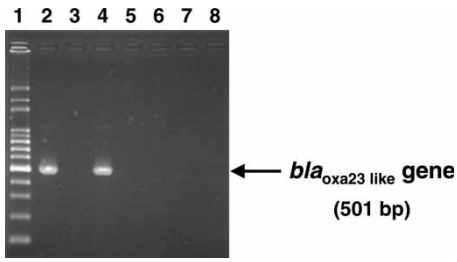

Figure 2 | Detection of the gene encoding carbapenem-hydrolysing β-lactamase ($bla_{\text{oxa-23-like}}$ gene) by nested PCR. Lane designations: 1, 100 bp DNA ladder marker (Bio Regenerations, Yokohama, Japan); 2, DG-KM; 3, DG-PN; 4, DG-KL; 5, DG-BL; 6, DG-SN; 7, DG-MN; 8, Negative control.

CONCLUSIONS

In the present study, we analysed the microbial communities distributed in the groundwater from the deep tube well in the Kathmandu Valley of Nepal based on samples collected from deep tube wells. The results revealed that the groundwater contained fecal microbes and a variety of aerobic microbes including C_1 compounds-utilizers, supporting the earlier reports that groundwater pollution due to anthropogenic activities had occurred in the valley. Furthermore, the analysis demonstrated the existence of various opportunistic microbes in the groundwater, and suggested they had the potential to emerge as infectious pathogens in Nepal.

This study also showed the occurrence of multi-drug resistant *Acinetobacter* species in the groundwater based on the detection of the carbapenem-hydrolysing β-lactamase gene ($bla_{\text{oxa-23-like}}$ gene). This is the first finding of multi-drug resistant microbes from the general environmental samples (non-clinical samples) in Nepal, and indicates an important clinical implication and the need to survey for such microbes in this country.

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