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Identification of Human and Animal Fecal Contamination in Drinking Water Sources in the Kathmandu Valley, Nepal, Using Host-Associated *Bacteroidales* Quantitative PCR Assays

Bikash Malla [1], Rajani Ghaju Shrestha [1], Sarmila Tandukar [1], Dinesh Bhandari [2], Daisuke Inoue [3], Kazunari Sei [4], Yasuhiro Tanaka [5], Jeevan B. Sherchand [2] and Eiji Haramoto [6,*]

1 Department of Natural, Biotic and Social Environment Engineering, University of Yamanashi, 4-3-11 Takeda, Kofu, Yamanashi 400-8511, Japan; mallabikash@hotmail.com (B.M.); rajani_ghaju12@hotmail.com (R.G.S.); sar1234tan@gmail.com (S.T.)
2 Institute of Medicine, Tribhuvan University, Maharajgunj, Kathmandu P.O. Box 1524, Nepal; me.dinesh43@gmail.com (D.B.); jeevanbsherchand@gmail.com (J.B.S.)
3 Division of Sustainable Energy and Environmental Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan; d.inoue@see.eng.osaka-u.ac.jp
4 Department of Health Science, Kitasato University, 1-15-1 Kitasato, Minami-ku, Sagamihara, Kanagawa 252-0373, Japan; ksei@kitasato-u.ac.jp
5 Department of Environmental Sciences, University of Yamanashi, 4-4-37 Takeda, Kofu, Yamanashi 400-8510, Japan; yasuhiro@yamanashi.ac.jp
6 Interdisciplinary Center for River Basin Environment, University of Yamanashi, 4-3-11 Takeda, Kofu, Yamanashi 400-8511, Japan
* Correspondence: eharamoto@yamanashi.ac.jp; Tel: +81-552-208-725

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Abstract: This study identified the sources of fecal contamination in the groundwater of different land covers. A total of 300 groundwater samples were collected in the Kathmandu Valley, Nepal, in the dry (n = 152) and wet (n = 148) seasons of 2016. Fecal indicator bacteria were initially enumerated, and then fecal contamination sources were identified using human (BacHum), ruminant (BacR), and pig-associated (Pig2Bac) *Bacteroidales* quantitative polymerase chain reaction assays. Sixty-six percent (197/300) of the tested groundwater samples had *Escherichia coli* concentrations higher than the World Health Organization threshold for drinking (<1 most probable number/100 mL). The fecal contamination of the groundwater was of human (22%, 55/250), ruminant (11%, 28/250), and pig (3%, 8/250) origin. Deep tube wells were less likely to be positive for *E. coli* and fecal markers compared to shallow dug wells. The human fecal marker was more likely to be detected in sources from built-up as compared to agricultural areas (Adjusted odds ratio (AOR) = 3.60, p = 0.002). Likewise, the ruminant fecal marker was more likely to be detected in sources from agricultural as compared to built-up areas (AOR = 2.90, p = 0.018). These findings suggest the preparation of mitigation strategies for controlling fecal pollution based on land cover and well types.

Keywords: drinking water source; fecal contamination; land cover; microbial source tracking; water quality

1. Introduction

Fecal pollution in water sources is a major concern for public health. Drinking water sources with fecal contamination may cause outbreaks of illness and economic degradation [1]. Groundwater

is a major source for domestic water use, and fecal contamination has been widely reported in many developing countries [2–6], and appears to be a persistent problem [7]. High risks of diarrhea result from drinking enteropathogen-contaminated groundwater [8] and from consuming vegetables washed in contaminated groundwater [9]. Therefore, there is a need for better understanding of fecal contamination sources to prepare mitigation strategies for controlling fecal pollution.

Land use practices, particularly urbanization, likely elevate the fecal contamination of shallow well water, increasing the risk of diarrheal diseases [10]. Urbanization and agriculture can also substantially degrade water quality, causing eutrophication and fecal contamination [11,12]. The primary source of the fecal pollution of water sources may be the improper management of human waste, which is often coupled with leakage in sewer pipes, the improper construction of septic tanks, and the use of pit latrines, all of which dispose of human waste in the subsurface [13]. In some agricultural areas in Cambodia, animals have been found to be primary sources of the fecal pollution of water sources [3] and significant association of livestock ownership with drinking water contamination in Ghana and Bangladesh has been reported [14].

In developing countries, animal farming is less intensive compared to that in developed countries; waste management is mostly unpracticed, and non-point sources of fecal pollution appear to be predominant [3]. In addition, the use of raw animal waste as fertilizer on agricultural farms is a common practice [15]. Fecal contamination from animals was found to be more prevalent than human contamination in the domestic environment, including source and stored drinking water and soil in rural India and Bangladesh [16–18].

Fecal indicator bacteria (FIB), such as *Escherichia coli* and total coliforms, have been used as indicators for fecal contamination [19]. However, FIB do not provide information on the fecal pollution sources, such as human, ruminant, or pig feces [20]. The application of a microbial source tracking (MST) technique using host-associated *Bacteroidales* genetic markers can resolve this limitation by identifying and quantifying the sources of fecal pollution in environmental water samples [21–25]. The correct identification of the origin of fecal contamination is useful when preparing efficient management strategies for controlling fecal pollution, particularly in low-income countries where resources are limited.

Potential fecal sources in the Kathmandu Valley include the human and animal densities, farm animal waste, manure application and livestock grazing, and the poor management of wastewater treatment plants. However, information regarding the identification of sources and the distribution of human and animal fecal contamination for different land covers in the Kathmandu Valley is lacking. The land cover factor can be used as proxy for determining which fecal sources should constitute the focus on remediation, and it could be helpful for policy makers to formulate strategies for land cover management in order to improve the microbial quality of the groundwater sources.

In this study, we applied host-associated *Bacteroidales* quantitative polymerase chain reaction (qPCR) assays recently validated in Nepal [26] to assess human-, ruminant-, and pig-associated fecal contamination in drinking water sources. The validation of such assays is essential prior to their application because some assays are found to cross-react with the feces of non-target hosts, resulting in variations in the sensitivity and specificity of these assays in different geographical locations [27]. BacHum (human-associated) [28], BacR (ruminant-associated) [29], and Pig2Bac (pig-associated) [30] assays were validated as the best assays to track fecal sources in each host category, human, ruminant, and pig, respectively, in the valley [26]. However, in the Malla et al. (2018) study [26], a limited number of groundwater samples (n = 74) were tested to identify the fecal-source contamination, which did not allow a clear picture to emerge of the distributions of human and animal fecal contamination based on different land cover types.

The aims of this study are: (i) to determine the distributions and concentrations of FIB in drinking water sources; (ii) to identify and quantify host-associated fecal markers using the validated *Bacteroidales* qPCR assays; and (iii) to determine the potential distribution of fecal contamination in different groundwater sources and for different land covers in the valley. These results could help

policy makers improve the microbial quality of the groundwater sources by implementing proper land cover management strategies. This study adds value to the limited use of MST in less developed countries by highlighting different dominant fecal contamination sources depending on land cover.

2. Materials and Methods

2.1. Study Site Description

The Kathmandu Valley, with an area of 664 km^2 and a population of 2.51 million [31], primarily consists of three major land covers: built-up (116 km^2), agriculture/open space (265 km^2), and forest/shrub (215 km^2) [32,33]. The land cover used in this study has been adapted from Uddin et al. (2015) [32]. The broad leaved open and closed forest, needle leaved open and closed forest, and shrub land are considered as forest; grassland and agriculture as agricultural areas; and built-up as built-up areas. A "built-up area" in this study is defined as area comprising densely populated city, whereas an "agricultural area" is defined as area comprising land with crop and vegetable farming activities [34]. Kathmandu Upatyaka Khanepani Limited is the only organization that supplies piped water in the valley, tapping water from the surface and groundwater [35]. However, there is a high supply deficit, with a current average supply of 107 million liters per day (MLD) serving a demand of 415 MLD [35]. Because of this, for drinking and domestic purposes, 52% of households depend on groundwater sources [36] in which the contamination of pathogens has been reported [5,8,37,38]. In the valley, the populations of animals such as cattle (ruminant) and pigs are 157,900 and 21,000, respectively [39]. The sanitation coverage in urban Nepal was 91% in 2011 and the percentage of households with toilets was 98% in 2012 in the valley [40].

2.2. Collection of Water Samples

In this study, four different groundwater source types were accessed for random sampling to distribute the sampling sites uniformly in the Kathmandu Valley (Figure 1). In the dry season (February and March 2016), a total of 152 groundwater sources were collected: shallow dug wells (n = 94), shallow tube wells (n = 23), deep tube wells (n = 25), and stone spouts (n = 10). Out of 152 groundwater sources collected in dry season, 127 could be accessed in wet season (August, 2016), and 21 new groundwater sources were accessed additionally: shallow dug wells (n = 87), shallow tube wells (n = 22), deep tube wells (n = 25), and stone spouts (n = 14). The water sources mostly used by the community people were selected to cover a large number of population using them. The depth of shallow wells was below 50 m, and those of deep wells was above 50 m from the ground surface. Altogether, 300 groundwater samples (200 mL each) were collected in this study. According to land cover, 207 samples ($n_{dry\ season}$ = 106; $n_{wet\ season}$ = 101) fell in built-up and 93 ($n_{dry\ season}$ = 46; $n_{wet\ season}$ = 47) in agricultural areas. The water samples were collected in two autoclaved 100-mL plastic bottles, which had been thoroughly washed and rinsed with ultrapure water prior to autoclaving, stored in an icebox, and transported to the laboratory as soon as possible. Before collecting the sample, the bottles were rinsed several times with water from the source. The tube wells were flushed by pumping five times before collecting the water samples.

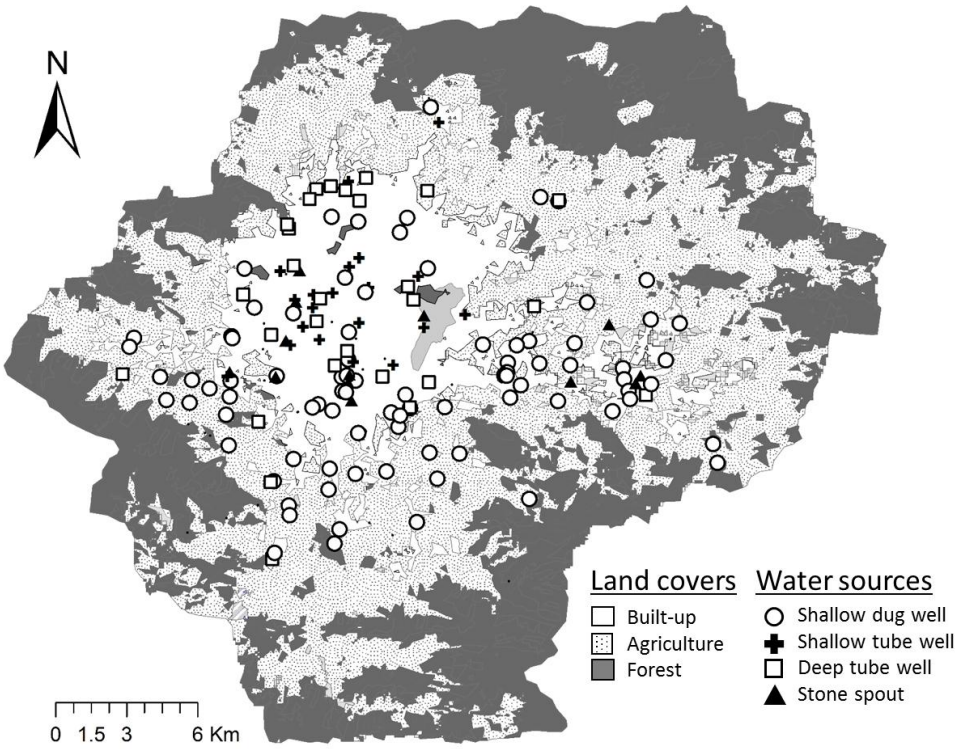

Figure 1. Map showing the locations of the sampling sites.

2.3. Detection of E. coli and Total Coliforms

The concentrations of *E. coli* and total coliforms in the water samples were quantified using the Colilert method (IDEXX Laboratories, Westbrook, ME, USA) following the manufacturer's protocol, as described previously [37].

2.4. Bacterial DNA Extraction

A disposable filter unit preset with a nitrocellulose membrane (diameter, 47 mm; pore size, 0.22 µm; Nalgene, Tokyo, Japan) was used to filter 100 mL of each water sample. A solution-based extraction using a CicaGeneus DNA Extraction Reagent (Kanto Chemical, Tokyo, Japan) was used to extract bacterial DNA from the water sample, as described previously [37], with a slight modification. The membrane filter was transferred into a 50-mL tube, and 5 mL of Tris-EDTA buffer (pH 7.4) was added, followed by shaking and vortex mixing steps at 50 °C with a speed of 300 rpm to resuspend the sample. In this study, 160 µL of the resuspended sample, 20 µL of Buffer A, and 200 µL of Buffer B were used to obtain a final volume of extracted DNA of 300 µL.

2.5. Application of the Selected Assays to the Fecal-Source Identification

The BacHum, BacR, and Pig2Bac assays were used as human-, ruminant-, and pig-associated fecal markers to identify the sources of fecal contamination in the water samples (n = 250). The applicability of these assays to the Kathmandu Valley was validated in our previous study using target and non-target fecal-source samples [26]. All three assays had a sensitivity of 100%, while specificities were 77%, 88%, and 75% for BacHum, BacR, and Pig2Bac assays, respectively. For the purpose of identification of possible fecal contamination sources in water samples, 250 water samples were selected out of 300 samples based on the *E. coli* concentration, location, season, and source type. When two water samples of the same source type in the same season and similar location had comparable *E. coli* concentrations, only one sample was selected. For qPCR, the primer and probe concentrations and temperature protocol were changed from the original publications [28–30]. A qPCR mixture and the thermal condition were similar to those of a previous validation study [26]. A final qPCR

mixture of 25 µL was prepared by mixing 12.5 µL of the Probe qPCR Mix (Takara Bio, Kusatsu, Japan), 10.0 pmol each of the forward and reverse primers, 5.0 pmol of the TaqMan (MGB) probe, 7.0 µL of PCR-grade water, and 2.5 µL of the template DNA. Marker amplifications were performed using a Thermal Cycler Dice Real Time System TP800 (Takara Bio) with thermal cycle conditions as follows: 95 °C for 30 s, followed by 45 cycles at 95 °C for 5 s, and 60 °C for 30 s. Six 10-fold serial dilutions of artificially-synthesized plasmid DNA were used as standard samples. A negative control was included to check if any contamination occurred during each run. All qPCR runs were performed in duplicate. The lowest concentration of the standard plasmid DNA that was amplified was considered as an assay's limit of quantification (LOQ). A cycle threshold (Ct) value of 40 was set as a cutoff point, and the sample with a Ct value of <40 was judged positive. The sample between the LOQ and a Ct value of 40 was considered as detected but not quantifiable (DNQ). The average efficiencies of standard curves of BacHum, Pig2Bac, and BacR assays were 98 ± 15%, 105 ± 8%, and 106 ± 7%, respectively. The LOQ values of BacHum, Pig2Bac, and BacR assays were 5.01, 4.28, and 3.82 log copies/100 mL of water sample, respectively.

2.6. Statistical Analysis

For comparison of the concentrations of *E. coli* between water source types, a one-way analysis of variance (ANOVA) was used for the statistical analysis. The samples below the detection limit were imputed with a one-tenth value of limit of detection (1 most probable number (MPN) per 100 mL for *E. coli*), as mentioned previously [41,42]. The associations of the water source types and land cover with the *E. coli* concentrations were examined by the multiple linear regression model Equation (1). Similarly, the multiple logistic regression models Equation (2) were used to examine the associations of the water source types and land cover with the detection of fecal markers, separately. The detection ratios of pig fecal markers between two land covers were compared using Fisher exact test due to limited number of samples. In order to identify association of the water source types and land cover with the *E. coli* concentrations and the detection of the fecal markers, generalized estimation equation (GEE) models were used which will control the dependency of observation for samples that were repeatedly collected in dry and wet seasons. Since shallow dug wells were the prevalent source, the associations of land cover with the *E. coli* concentrations and with the detection of the fecal markers were examined using multiple linear and logistic regression models, respectively. $p < 0.05$ was considered significant in all analyses. SPSS version 23 (IBM Corporation, Armonk, USA) was used for the statistical analyses.

$$Y^* = b_0 + b_1X_1 + b_2X_2 + \ldots \ldots + b_pX_p \quad (1)$$

where, Y^* is the predicted value of the dependent variable, X_1 through X_p are p distinct independent or predictor variables, b_0 through b_p are the estimated regression coefficients.

$$\hat{p} = exp\,(b_0 + b_1X_1 + b_2X_2 + \ldots \ldots + b_pX_p)/1 + exp\,(b_0 + b_1X_1 + b_2X_2 + \ldots \ldots + b_pX_p) \quad (2)$$

where, $\hat{p}$ is the expected probability that the outcome is present, X_1 through X_p are p distinct independent variables, b_0 through b_p are the regression coefficients.

3. Results

3.1. Detection of FIB and Host-Associated Bacteroidales Genetic Markers

The results of the detection of *E. coli* and total coliforms in the groundwater samples are shown in Table 1. *E. coli* and total coliforms were detected in 66% (197/300) and 94% (282/300) of the groundwater samples, respectively. The concentrations of *E. coli* and total coliforms had value ranges of <0.00–5.38 log MPN/100 mL and <0.00–7.08 log MPN/100 mL, respectively.

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 (log MPN/100 mL)		No. of Positive Samples (% Positive)	Concentration (log MPN/100 mL)	
			Mean ± SD [a]	Max		Mean ± SD [a]	Max
Shallow dug well	181	149 (82)	1.61 ± 1.59	5.38	178 (98)	3.60 ± 1.27	7.08
Shallow tube well	45	13 (29)	−0.22 ± 1.40	4.46	36 (80)	1.64 ± 1.77	5.38
Deep tube well	50	17 (34)	−0.17 ± 1.35	4.46	44 (88)	1.79 ± 1.51	4.74
Stone spout	24	18 (75)	1.34 ± 1.80	4.51	24 (100)	2.69 ± 1.25	4.74
Total	300	197 (66)			282 (94)		

[a] SD, standard deviation.

Table 2 shows the results of the detection of fecal markers in the groundwater samples. At least one of the three *Bacteroidales* genetic markers tested was detected in every source type (shallow dug and tube wells, deep tube well, and stone spout): the detection ratios of human, ruminant, and pig fecal markers were 22% (55/250), 11% (28/250), and 3% (8/250), respectively. Of the 250 water samples tested, total coliforms, *E. coli*, and at least one of the three fecal markers were detected in 96%, 78%, and 33% of the samples, respectively. Human and ruminant mixed fecal markers were detected in four, human and pig mixed fecal markers in two, and ruminant and pig mixed fecal markers in two water samples. The concentrations of ruminant-, human-, and pig-associated fecal markers were in the ranges of 4.25–7.81, 5.01–7.17, and 4.39–5.77 log copies/100 mL, respectively.

Table 2. Detection of host-associated *Bacteroidales* genetic markers in water samples.

Source	No. of Tested Samples	BacHum (Human)		BacR (Ruminant)		Pig2Bac (Pig)		No. of Samples Positive for at Least One Fecal Marker (% Positive)
		No. of Positive Samples (% Positive)	Conc. [a] (log Copies/100 mL)	No. of Positive Samples (% Positive)	Conc. [a] (log Copies/100 mL)	No. of Positive Samples (% Positive)	Conc. [a] (log Copies/100 mL)	
Shallow dug well	166	40 (24)	5.04–7.17	21 (13)	4.25–7.81	5 (3)	4.39–4.68	59 (36)
Shallow tube well	33	6 (18)	5.01–6.91	1 (3)	4.47	2 (6)	5.77	8 (24)
Deep tube well	30	2 (7)	DNQ [b]	3 (10)	4.52–5.18	1 (3)	DNQ [b]	5 (17)
Stone spout	21	7 (33)	5.50–6.88	3 (14)	4.67–5.57	0 (0)	ND [c]	10 (48)
Total	250	55 (22)		28 (11)		8 (3)		82 (33)

[a] Range of concentrations of samples detected with concentrations higher than LOQ values; [b] DNQ, detected but not quantifiable; [c] ND, not detected.

Water samples from 127 sampling sites were collected consecutively in both seasons for the analysis of FIB. Microbial source tracking using host-associated fecal markers was performed in 86 sites of the total 127 repeated sampling sites. Of these samples, 114 (90%), 66 (52%), and 18 (21%) sites were contaminated by total coliforms, *E. coli*, and at least one fecal marker in both seasons, respectively, indicating consistent fecal contamination. A total of 12% (8/68) of the shallow dug well sites were contaminated consistently by human fecal markers, and 6% (4/68) were consistently contaminated by ruminant fecal markers. None of the groundwater samples were contaminated with all three of the fecal markers.

3.2. Comparison of FIB and Fecal Markers between Water Source Types

E. coli was detected in every water source type (shallow dug well, shallow tube well, deep tube well, and stone spout) tested in this study, with the detection ratios of 82% (149/181), 75% (18/24), 34% (17/50), and 29% (13/45) in shallow dug wells, stone spouts, deep tube wells, and shallow tube wells, respectively (Table 1).

Table 2 shows the detection ratio of human, ruminant, and pig fecal markers in different source types. All three fecal markers were detected in each source type, except for stone spouts, in which only human and ruminant fecal markers were detected. Human fecal markers were detected in 33% (7/21) of stone spouts, 24% (40/166) of shallow dug wells, 18% (6/33) of shallow tube wells, and 7% (2/30) of deep tube wells (Table 2). Likewise, 14% (3/21) of stone spouts, 13% (21/166) of shallow dug wells, 10% (3/30) of deep tube wells, and 3% (1/33) of shallow tube wells were contaminated with ruminant fecal markers. Pig fecal markers were detected in 6% (2/33) of shallow tube wells, 3% (5/166) of shallow dug wells, and 3% (1/30) of deep tube wells but not in stone spouts. The detection ratios of at least one fecal marker were 48% (10/21), 36% (59/166), 24% (8/33), and 17% (5/30) in stone spouts, shallow dug wells, shallow tube wells, and deep tube wells, respectively (Table 2).

In order to compare the level of contamination among the different source types, ANOVA was carried out to compare the mean concentration of *E. coli*. Shallow dug wells (1.61 ± 1.59 log MPN/100 mL) had significantly higher concentration compared to that in shallow tube wells (−0.22 ± 1.40 log MPN/100 mL) (ANOVA, $p < 0.001$) and deep tube wells (−0.17 ± 1.35 log MPN/100 mL) (ANOVA, $p < 0.001$). Likewise, stone spouts (1.34 ± 1.80 log MPN/100 mL) had significantly higher concentration than that in shallow tube wells (ANOVA, $p < 0.001$) and deep tube wells (ANOVA, $p = 0.001$). On the other hand, there was no significant difference in the concentrations between shallow dug wells and stone spouts (ANOVA, $p = 0.855$) and between shallow tube wells and deep tube wells (ANOVA, $p = 0.999$) (Table 1). These results suggest lower contamination in both types of tube wells compared to shallow dug wells and stone spouts.

For further analysis, a multiple linear regression analysis was used to examine the association between source types and *E. coli* concentration and three separate multiple logistic regression models were used to examine the association between source types and human, ruminant, and at least one fecal marker while controlling for land cover and season (Table 3). Considering shallow dug wells as prevalent and highly polluted among different sources, they have been kept as a reference source in multiple logistic regression analysis. Shallow tube wells (unstandardized beta coefficient (β) = −1.44, 95% confidence interval (CI) = −2.16 – (−0.73), $p < 0.001$) and deep tube wells (β = −1.43, 95% CI = −2.13 – (−0.73), $p = 0.001$) were negatively associated with *E. coli* concentration, but not shallow dug wells. Both shallow tube wells and stone spouts did not show significant difference from shallow dug wells in terms of detection of markers. When analyzing repeated samples using GEE, shallow tube wells (β = −1.81, 95% CI = −2.74 – 0.89, $p < 0.001$) and deep tube wells (β = −1.64, 95% CI = −2.58 – (−0.71), $p = 0.001$) were negatively associated with *E. coli* concentration but not shallow dug wells. Similar to the previous results of multiple logistic regression analysis, deep tube wells were significantly less likely to be *E. coli*, human fecal marker, and at least one fecal marker positive compared to shallow dug wells (Table 3). These results from several analyses suggest that the tube wells could be a safer source type compared to shallow dug wells and stone spouts.

Table 3. Association of land cover and source types with FIB and fecal markers detection.

Factors	*E. coli* Concentration				BacHum Detection				BacR Detection				At Least One Marker Detection			
	β [a]	95% CI [b]		*p*	AOR [c]	95% CI [b]		*p*	AOR [c]	95% CI [b]		*p*	AOR [c]	95% CI [b]		*p*
		Lower	Upper			Lower	Upper			Lower	Upper			Lower	Upper	
	Multiple Linear Regression				**Multiple Logistic Regression**											
Land cover																
Built-up	−0.18	−0.57	0.20	0.345	3.60	1.63	7.92	**0.002**	**Ref.**				1.48	0.79	2.75	0.219
Agricultural area					**Ref.**				2.90	1.20	7.02	**0.018**	**Ref.**			
Source types																
Shallow dug well	0.32	−0.31	0.95	0.315	**Ref.**				**Ref.**				**Ref.**			
Shallow tube well	−1.44	−2.16	−0.73	**<0.001**	0.41	0.15	1.10	0.076	0.34	0.04	2.81	0.317	0.44	0.18	1.09	0.076
Deep tube well	−1.43	−2.13	−0.73	**<0.001**	0.14	0.03	0.63	**0.010**	0.86	0.23	3.27	0.828	0.26	0.09	0.75	**0.012**
Stone spout	NA				0.94	0.34	2.61	0.911	1.79	0.44	7.39	0.418	1.29	0.49	3.37	0.602
Season																
Dry					**Ref.**				**Ref.**				**Ref.**			
Wet	1.18	0.85	1.51	**<0.001**	1.88	0.97	3.66	0.061	1.69	0.71	4.01	0.236	2.10	1.18	3.74	**0.012**
Generalized Estimation Equation in Repeated Samples																
Land cover																
Built up	−0.02	−0.43	0.39	0.914	**Ref.**				**Ref.**				**Ref.**			
Agricultural area					0.32	0.12	0.83	**0.019**	2.01	0.81	4.97	0.130	0.66	0.32	1.34	0.251
Source types																
Shallow dug well	0.16	−0.70	1.02	0.715	**Ref.**				**Ref.**				**Ref.**			
Shallow tube well	−1.81	−2.74	0.89	**<0.001**	0.37	0.13	1.08	0.068	0.39	0.06	2.73	0.342	0.44	0.17	1.13	0.088
Deep tube well	−1.64	−2.58	−0.71	**0.001**	0.18	0.04	0.85	**0.030**	0.42	0.05	3.53	0.424	0.20	0.05	0.74	**0.016**
Stone spout	NA				0.71	0.18	2.89	0.634	2.03	0.53	7.80	0.304	1.11	0.33	3.74	0.860
Season																
Dry					**Ref.**				**Ref.**				**Ref.**			
Wet	1.18	0.83	1.53	**<0.001**	2.17	1.15	4.10	**0.016**	1.28	0.59	2.75	0.532	2.23	1.31	3.79	**0.003**

[a] Unstandardized beta coefficient; [b] CI, confidence interval; [c] AOR, adjusted odds ratio; *p*: *p*-value; Ref.: Reference group; NA: not applicable.

3.3. Comparison of FIB and Fecal Markers between Land Covers

Built-up and agricultural areas were the two land cover types considered in this study based on the dominance of either human or agricultural activities. The detection ratios of *E. coli* in agricultural and built-up areas were 82% (76/93) and 58% (121/207), with mean concentrations of 1.57 ± 1.60 log MPN/100 mL and 0.77 ± 1.77 log MPN/100 mL, respectively. The distribution of fecal markers in different land covers is shown in Figure 2. At least one fecal marker was detected in 35% (57/165) and 29% (25/85) of samples in built-up and agricultural areas, respectively.

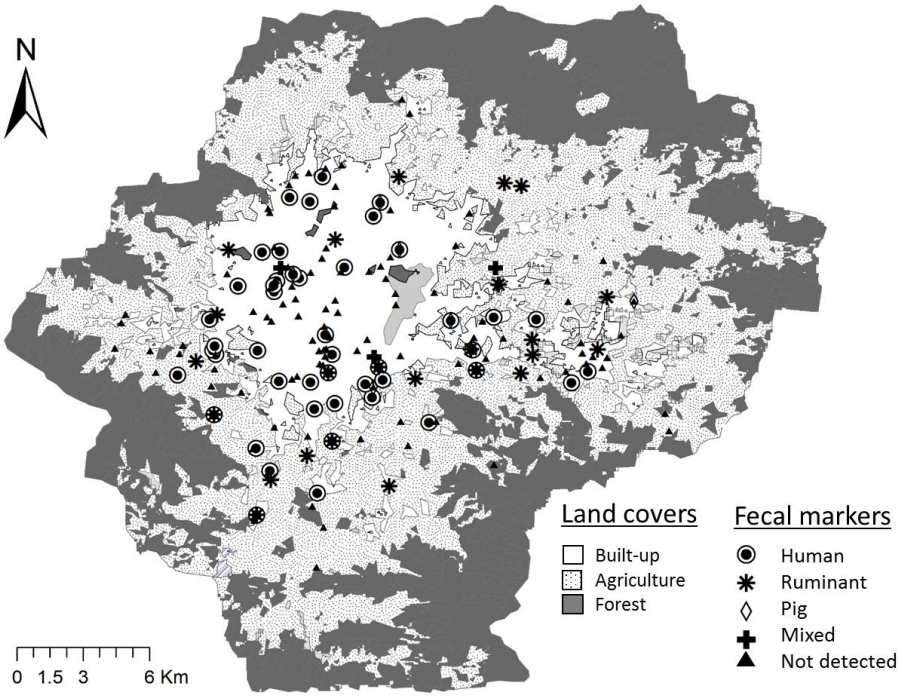

Figure 2. Spatial distribution of the fecal markers.

The results of four separate multiple regression models, as mentioned in earlier section, were further used for explaining the association between land cover and detection of *E. coli*, human, ruminant, and at least one fecal marker while controlling for source types and season (Table 3). Human fecal marker detection was significantly higher in built-up (27%, 45/165) compared to agricultural areas (12%, 10/85) (AOR = 3.60, 95% CI = 1.63–7.92, $p = 0.002$), whereas ruminant fecal marker detection was significantly higher in agricultural areas (19%, 16/85) compared to that in built-up areas (7%, 12/165) (AOR = 2.90, 95% CI = 1.20–7.02, $p = 0.018$). However, the detection ratio of pig fecal markers in built-up areas (4%, 45/165) was not significantly different from that in agricultural areas (2%, 2/85) (Fisher exact test, $p = 0.720$). While performing four separate GEE models on repeated samples, agricultural areas were significantly less likely to be positive for the human fecal marker compared to built-up areas (AOR = 0.32, 95% CI = 0.12–0.83, $p = 0.019$).

Shallow dug wells being the predominant source type in both land covers, multiple linear and logistic regression models were used to analyze the association between land cover and concentration of *E. coli* and the detection of fecal markers, respectively, while controlling for season. Land cover was not significantly associated with *E coli* concentration ($\beta = -0.20$, 95% CI = −0.60 – 0.21, $p = 0.340$); however, built-up areas were significantly more likely to be human marker positive (AOR = 3.63, 95% CI = 1.51–7.50, $p = 0.003$), whereas agricultural areas were significantly more likely to be ruminant marker positive (AOR = 2.70, 95% CI = 1.02–7.14, $p = 0.045$).

3.4. Relationship between E. coli and Fecal Markers

Percentile values (0.25, 0.50, and 0.75) were used to identify three cut points in the *E. coli* concentration to divide the entire dataset equally into four categories to observe the general trend between *E. coli* concentration and the detection ratios of fecal markers (Table 4). The result showed that the detection ratios of human and ruminant fecal markers seemed to be increased as the concentration of *E. coli* increased. In the category with the highest *E. coli* concentration (>2.7 log MPN/100 mL), 61% (38/62) of the samples tested positive for at least one type of fecal marker. However, fecal markers were detected even in *E. coli*-negative samples (23%, 13/56).

Table 4. Detection of the fecal markers at different concentration ranges of *E. coli*.

Concentration of *E. coli* (log MPN/100 mL)	No. of Samples	No. of Positive Samples (% positive)		
		Human	Ruminant	At Least One Fecal Marker
<0.00	56	10 (18)	2 (4)	13 (23)
0.00–1.50	64	5 (8)	5 (8)	10 (16)
1.51–2.70	68	12 (18)	9 (13)	21 (31)
>2.70	62	28 (45)	12 (19)	38 (61)

4. Discussion

Protecting groundwater sources from fecal contamination is essential, because they are important sources of water for households for drinking and domestic purposes [36,43]. However, it is very challenging to identify the exact source and primary mechanism that introduced the human and animal fecal contamination into the water sources when there are multiple sources, which makes it difficult to predict the health risk to humans and to control or improve the water quality. In this study, we examined the abundance and distribution of FIB and host-associated *Bacteroidales* genetic markers to evaluate the distribution of human and animal fecal contamination in groundwater sources of different land covers in the Kathmandu Valley.

The *E. coli* levels exceeded the World Health Organization guideline value for drinking (<1 MPN/100 mL) in 66% of the tested groundwater samples, which indicates the unsuitability of the groundwater for drinking purposes [44]. However, FIB do not provide information on the contamination sources. In this study, we used human-, ruminant-, and pig-associated *Bacteroidales* genetic markers to identify the fecal pollution sources. The detection ratio of human fecal markers was the highest of the host-associated markers tested in spite of high sanitation coverage (91%) and high percentage of households with toilets (98%) in the valley [40]. However, water sources contaminated with animal feces also pose health risk because animal feces carry microorganisms infectious to human, such as pathogenic *E. coli*, *Campylobacter*, and *Salmonella* [45]. In a developing country such as Nepal, mixed crop-livestock farming is practiced [14] and the use of animal feces, primarily ruminant and chicken, as fertilizer is common, which may explain the detection of ruminant fecal markers in the groundwater sources in this study. A study done in Bangladesh [46] has reported an association of animal and animal feces with domestic fecal contamination. Pig farms were mainly spotted near the banks of the rivers at the time of fecal sample collection for the validation study [26], and the practice of using pig feces as fertilizer is not very common in the valley. In addition to this, the population of pigs is 7.5 times less than that of cattle [39]. This could explain the relatively low prevalence of pig fecal markers in the tested groundwater sources. Haramoto (2018) [24] has also reported the detection of human, ruminant, and pig fecal markers in the shallow groundwater sources in the valley. Detections of animal fecal markers in the water sources indicate high risks of exposure to animal feces and zoonotic pathogens, which can be detrimental to human health [47]. Diarrhea has been reported to be associated with human and animal fecal markers in the domestic environment in rural India [18]. In the current study, the concentrations of human-associated fecal marker (BacHum) in the groundwater samples ranged from 5.01 to 7.17 log copies/100 mL, which exceeded the human marker concentration thresholds for gastrointestinal illness established by Boehm et al. (2015) (3.45 log copies/100 mL for

HumM2 and 3.62 log copies/100 mL for HF183) [48] and Ahmed et al. (2018) (3.51 log copies/100 mL for HF183) [49]. This indicated the possibility of higher human health risk when using the groundwater sources in the study area. The detections of at least one fecal marker in 21% of the sampling sites and of the human fecal marker in 12% of the sampling sites in both seasons indicate consistent contamination, which could be minimized if a detailed investigation is conducted.

Comparing the water source types, the probabilities of the detection of *E. coli* and fecal markers were found to be significantly higher in shallow dug wells and stone spouts compared to tube wells. These findings indicated that tube well sources are safer than shallow dug wells and stone spouts. An Indian study has also reported less frequent detections of the same human fecal marker in deep tube wells [16]. Their construction and well depths might make shallow dug wells and stone spouts more vulnerable to contamination compared to both types of tube wells. Deep tube wells are expensive to construct; therefore, inexpensive shallow wells are widely used, which results in large populations being vulnerable to potential hazards [13].

In this study, the dominance of host-associated *Bacteroidales* genetic markers revealed a pattern which was in accordance with the land cover type. While applying several statistical approaches in this study, human fecal markers were significantly more likely to be detected in built-up areas compared to agricultural areas. This could be due to relatively higher population density in built-up areas than in agricultural areas. The population densities in areas within built-up land cover varied from 6000–118,000 persons/km^2, while in areas of agriculture land cover, they varied from 2000–5000 persons/km^2 in the study area [31]. High population density coupled with poor sanitation infrastructures such as improperly designed septic tanks [50], leaky sewerage systems [51], partially functional or completely non-functional wastewater treatment plants [52] could have resulted in the dominance of human fecal markers in built-up areas. Improper solid waste management in the built-up area in the valley [53] could be another possible attribute, because when solid waste is disposed of openly into the environment, which may include child feces and other sources of fecal waste [54], it may contaminate groundwater sources [55]. Although not as a dominant fecal marker in agricultural areas, the human fecal marker was detected in a similar ratio to the ruminant fecal marker in this study. This might be due in part to the limited connection of households to the sewerage system [56], poorly constructed septic tanks, temporary pit latrines used by farm workers, and even open defecation [57] in the agricultural areas in Nepal. Use of sewage and river water for irrigation of agricultural lands is also a common practice in the Kathmandu Valley [58]. In this study, ruminant fecal markers were significantly more likely to be detected in agricultural areas compared to built-up areas, while using different statistical analytical approaches. The application of raw livestock manure as a fertilizer is a common agricultural practice in Nepal [15], and the applied manure can contaminate groundwater with pathogenic microorganisms [59]. In addition, livestock farming is less intensive in developing countries, and thus, animals defecate in pasture lands while grazing, and animal waste is usually improperly managed within farms as well. These features of livestock farming in developing countries are the potential causes of non-point sources of fecal contamination in the environment [60]. A previous study has suggested that livestock ownership is a significant risk factor for the contamination of drinking water sources [14]. Surprisingly, in this study, animal fecal markers were also detected in densely populated built-up areas. This result was in alignment with the result of a study in Dhaka, Bangladesh, which found ruminant fecal contamination of water in densely populated urban areas [17].

In this study, in spite of assays' sensitivity of 100% each, and relatively high target gene concentrations of BacHum (2.3), BacR (4.7), and Pig2Bac (3.5 log copies per MPN *E. coli*) in the fecal source samples [26], none of the tested source-associated fecal markers were detected in 125 *E. coli*-positive samples. This might be due to the fecal contamination of these samples by other animals, such as dogs or chickens, whose markers were not tested, the high limit of detections of tested assays, low concentration of target genes in the tested water samples, and/or the difference in methods of measurement of fecal concentration [61]. This could be explained by the use of a large water sample

volume (100 mL) for a culture method, while only 2.5 µL of the extracted DNA, which was equivalent to 0.02 mL of the original water sample, was used for qPCR. Higher detection ratios of the human and animal fecal marker might have been obtained if a larger volume of water had been filtered. More importantly, at least one fecal marker was detected in 23% (13/56) of the culture-based *E. coli*-negative samples. One of the reasons could be because culture method detects live cells only, whereas the qPCR method detects DNA from both live and dead cells. However, a previous study [17] also reported the detection of source-associated fecal markers in *E. coli*-negative samples in other study areas and other sample types. This finding may indicate that *E. coli* is not suitable to confirm the absence of fecal markers in the groundwater samples tested in this study. *E. coli* has also been found to not be suitable to confirm the absence of waterborne pathogens in groundwater samples in the Kathmandu Valley [24].

Our results showed a seasonal trend in *E. coli* concentration, as the concentration increased in wet season compared to dry season (Table 3). However, as water samples were collected only once in each season (the dry and wet seasons), long-term monitoring of the water quality using host-associated markers is recommended to identify the seasonal patterns of fecal contamination sources in a complex watershed [62]. Further work could include studies using greater volumes of groundwater, thereby enhancing the detection of markers. The validation of host-associated bacterial markers targeting possible fecal sources such as chickens [63] and dogs [64], which were not tested in this study, and their application in the MST of water sources, would be an important next step to obtain a clear picture of the fecal contamination scenarios for different land covers in the valley.

5. Conclusions

This study provided insights into the distribution and abundance of FIB in the groundwater sources throughout the entire Kathmandu Valley, and their detection in groundwater indicated unsuitability for drinking purposes. The shallow aquifer sources, mainly shallow dug wells and stone spouts, pose a potentially higher risk to humans compared to tube wells due to the potential presence of pathogens in fecal sources, as indicated by the high prevalence of FIB and fecal markers in these sources. This study also highlighted the use of host-associated *Bacteroidales* genetic markers, which can be useful in environmental water monitoring to identify fecal pollution sources in the groundwater of different land covers. Human and ruminant fecal contaminations were found to be predominant in built-up and agricultural areas, which indicates that both human and ruminant feces management strategies need to be implemented in both land cover areas to control fecal pollution. The findings also highlight the risk of fecal contamination from both human and animal origins, suggesting that prioritizing only human fecal pollution control without considering animal fecal contamination will not be sufficient to prevent groundwater contamination. Further studies should focus more on the distribution and genetic analyses of zoonotic pathogens to assess the risk of passing infections from animals to humans.

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