Title:

Microbiological and physicochemical analysis of water from natural springs in the Bhaderwah region of Jammu and Kashmir, India

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Summary

Natural springs are the main source of water supply for domestic and agricultural use for humans living in the mountainous regions of Asia. Increasing anthropogenic activities with associated waste load, coupled with inadequate sanitation, and contamination of natural water resources and the environment are emerging as important public health issues. We performed a prospective microbiological and physicochemical investigation of water samples from seven distinct natural springs situated at an altitude of 1615 m in the Bhaderwah region of Jammu and Kashmir, India. Bacterial groups belonging to opportunistic pathogens such as members of *Moraxellaceae* (*Acinetobacter*), *Arcobacteraceae* (*Pseudoarcobacter*), *Pseudomonadaceae* (*Pseudomonas*),*Oxalobacteraceae* (*Massilia*), and *Flavobacteriaceae* (*Flavobacterium*) were observed. The total coliform test indicated an intermediate level of risk of fecal contamination of the springs, except for one site. Through a questionnaire-based survey of the local population, we discovered that around 40% of participants had suffered from waterborne diseases including typhoid (~14%) and diarrhea (~11%). Our data suggests that increased surveillance of fecal contamination and heterotrophic opportunistic pathogens is needed to enhance water quality and reduce health

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Introduction

Water from springs is considered critical for rural families in mountainous regions of Asia since it is essential for domestic consumption, agriculture and animal husbandry.[1] Out of five million springs across India, nearly 3 million are in the Indian Himalayan Region (IHR), serving an estimated 50 million people.[2] Despite this crucial role in sustenance of human settlements, springs do not get the political and scientific attention required for monitoring and maintaining the water quality.

Water from the natural sources is of varying quality since it contains various microorganisms and contaminants due to weathering, erosion, and leaching processes that are a part of the biogeochemical cycles of aquatic systems.[3] In the recent years, rise in the issue of degraded spring water quality has been of major concern as it is directly related to the health index of people.[4] Both geogenic and anthropogenic sources cause deterioration of the spring water quality.[2] Spring waters are highly vulnerable to contamination from anthropogenic activities which arise from increased sewage and waste disposal, agricultural run-off, poor sanitation and animal husbandry practices.[3,5] Contaminated water and poor sanitation are directly linked to transmission of various gastrointestinal diseases such as dysentery, cholera, diarrhea, hepatitis A, typhoid, etc. According to UNICEF-India report 2019, nearly 37.7 million Indians are affected by water-borne diseases, however approximately 1.5 million children die due to diarrhea with posing huge economic burden of nearly USD 600 million per year in India. Therefore, access to safe and readily available water with improve hygienic practices along with proper treatment is essential for public health and to prevent water-borne diseases for developing nations.

It is well known that during rainy seasons, surface and groundwater can be very rapidly influenced by microbes originating from human or animal faecal materials due to improper sanitation and sewage practices.[4,6] As a result, diarrhea has become fourth leading cause of death worldwide. Conventional methods for assessing water quality and its potability is based on evaluation of *Escherichia coli,* total coliform and *Enterococcus* in the drinking water using WHO guidelines.[7] With advances in molecular microbial ecology, techniques such as next generation sequencing, provide opportunities for more robust assessment of bacterial load and capturing the unculturable microbial populations.[8-10] Recently, we investigated water quality index of the seven distinct springs by measuring various physico-chemical properties for assessing its potability in this region.[1] However, due to rise in water-borne diseases and continued dependence of local residents on spring water for their consumption and livelihoods, an in-depth microbiological assessment of spring water in this region is required to help develop comprehensive plans for its restoration, management, and improve water quality. Recent advent of high-throughput sequencing technology now allows more comprehensive assessment of contamination of water with a range of bacteria in a more robust manner. Apart from identification of potential pathogenic and opportunistic waterborne microbes it allows for study of association between physicochemical properties and microbes. To investigate this relationship, we performed a study on water samples collected from seven distinct natural springs in Bhaderwah, Jammu and Kashmir, situated at an altitude of 1615 m, using 16S rRNA gene-based targeted sequencing.

Materials and Methods

Sampling sites, sample collection, and measurement of physicochemical parameters

This study involved natural springs located in the hilly town of Bhaderwah in Doda district, Jammu and Kashmir, India (**Figure 1**). Detailed description of the springs is presented in the appendix p 2. These perennial springs serve as a source of potable water for thousands of inhabitants and is also used for irrigation purpose. Out of seven springs, one is Limnocrene spring (Sp1) and rest are Rheocrene (Sp2-Sp7). Limnocrene springs emerge as groundwater originating from a large, deep pool of water. Due to their relatively uniform temperature and chemistry, the sources of these springs may support aquatic species that are different from surrounding habitat influenced by surface water. Rheocrene springs flow from a defined opening flowing spring, emerges into one or more stream channels. Multiple water samples from the springs were collected in sterile containers for microbiological and physicochemical investigation.

Microbiological investigation of total coliforms and thermotolerant coliforms was assessed as per APHA 2017 protocol.[11] The risk level/categories for the spring samples were based on the total coliform and total faecal coliform concentration as per WHO guidelines.[12]

Acid wash containers were used for collection of water samples for physicochemical analysis and transported to the laboratory in an ice box to maintain a temperature of less than 6 °C. Detailed sample collection and measurement of various environmental parameters such as carbonate, bicarbonate, total alkalinity, calcium, magnesium, total hardness, sodium, potassium, chloride, nitrate, sulfate, phosphate, free CO_2, were performed as described previously.[1]

Questionnaires for the survey to determine the prevalence of waterborne diseases such as typhoid, diarrhea, dysentery, skin disease, gastroenteritis, and hepatitis were developed and are provided in the appendix p 4-5. Two hundred local residents were selected for the survey.

DNA extraction and amplicon sequencing

To assess the microbial diversity in the spring samples, representative water samples (1000 ml) from each spring were subjected to total DNA extraction. Water samples were filtered using 0.22 µm filter paper, further these filtered papers were used for community DNA extraction using DNeasy PowerSoil Pro kit as per manufacturer protocol. The extracted DNA from each sample was subjected to quality checking using nanodrop and Qubit 4.0 (Thermo fisher Scientific, USA). The V4 region of the 16S rRNA gene was selected for amplification using universal primers (515F and 806R)[13]. Negative control without DNA template was used as PCR control. The amplified PCR product was purified using AMPure XP beads (Beckman Coulter) followed by reduced cycle (8 cycles) PCR for indexing. The purified indexed libraries were pooled at equimolar concentration (4nM) and was sequenced on the in-house Illumina MiSeq platform using MiSeq reagent v2 kit-Illumina Inc. (2 ×250 bp chemistry).

Data analysis and statistics

The paired end raw reads were quality checked using FastQC[14] followed by analysis using DADA2 package v1.6.0[15] in R 3.6.0. The low-quality reads and primers were removed. Chimaeras were removed using the removeBimeraDenovo function of DADA2. Silva Database (silva_nr99_v138.1_train_set.fa.gz) was used for the taxonomic assignment of the amplicon sequence variants. Phyloseq v3.4.2 R package[16] was used to calculate the alpha diversity parameters. Various R packages were used for data visualizations such as ggplot2[17], Reshape2[18], vegan[19], and RColorBrewer[20], in RStudio version 1.2.1335. The fraction of ASVs shared among samples was visualized using the UpSet plot[21] .

In order to understand the correlation between the microbial genera and environmental variables, Spearman correlation analysis was performed. Heatmap based clustering of samples and major families was performed based on Bray-Curtis dissimilarity matrix.

Heatmap cum clustering was performed using pheatmap package[22]. Bray-Curtis dissimilarity matrix was used for clustering the samples and bacterial families. Spearman Correlation was calculated between environmental variables and bacterial taxa and displayed in the form of matrix using corrplot package[23] .

Results

Physicochemical parameters and water quality

Detailed description of the sample's properties and physicochemical characteristics are presented in the appendix p 3. Briefly physicochemical analysis showed that water samples from the seven springs were near-neutral with a pH range of 6.32-7.14. The temperature of the water ranged from 12.21-15.58 ^{o}C with dissolved oxygen (DO) varied from 2.18-8.28 mg/L. The total hardness (TH) and total alkalinity (TA) varied between 54.36 to 202.71 and 42.42 to 206.17 mg of $CaCO_3$ equivalent per litre, respectively. Spring water less disturbed by human activity had lower alkalinity and hardness compared to moderately and highly disturbed springs. Similarly, the total dissolved solid (TDS) and electrical conductivity (EC) were also found to be low in the less disturbed springs (Sp3 and Sp1) and varied from 48.92 to 241.5 mg/L and 97.75 to 484.92 µS/cm, respectively across the samples. Interestingly, most of these parameters were found to be significantly correlated to each other (figure 2). Spearman correlation-based analysis showed the negative association between pH and DO ($p < 0.05$). In contrast, TDS, EC, TH and TA were significantly positively correlated with each other at $p < 0.05$. However, these parameters were not found to be significantly correlated with pH, temperature, DO, etc. Water chemistry showed that anionic composition was dominated with HCO_3^- (66.32 -247 mg/L) ions followed by Cl^- (10.94-27.45 mg/L), SO_4^{2-} (2.35-12.39 mg/L), NO_3^-

(0.22-5.0 mg/L), and PO_4^{3-} (0.01-0.57 mg/L). HCO_3^- was found to be positively correlated with TH and TA ($p < 0.05$). However, SO_4^{2-} was found to be positively correlated with Cl^- and NO_3^- at $p < 0.05$ and negatively correlated with DO at $p < 0.05$. Water samples were high in Ca^{2+} (12.29-66.23 mg/L) than Mg^{2+} (2.86-12.31 mg/L) and both were found to be positively correlated ($p<0.05$) with each other along with TH, TA, and HCO_3^- ($p < 0.05$).

Total coliform and faecal coliform in the spring samples and indication of risk level

The total coliform/faecal coliform counts varied across the samples. It was interesting to note that sample Sp3 which was less disturbed with human activities had least total coliform count (1.05±1.72 MPN/100mL) and could be categorized under low risk level while other samples (Sp1: 28.67±5.42 MPN/100mL, Sp2: 31.67±9.46 MPN/100mL, Sp4: 27±8.95 MPN/100mL, Sp5: 31.09+7.83 MPN/100mL, Sp6: 22.72±5.41 MPN/100mL, Sp7: 23.50±9.0 MPN/100mL) were at intermediate risk level. Thermotolerant faecal coliform counts followed the similar trend and varied among the sites (Sp1: 8.85±9.48 MPN/100mL, Sp2: 12.89±8.76 MPN/100mL, Sp4: 3.93±4.66 MPN/100mL, Sp5: 17.05+11.8 MPN/100mL, Sp6: 6.84±6.61 MPN/100mL, Sp7: 5.72±5.37 MPN/100mL) with no detection in sample Sp3 (less disturbed with human activities). In order to assess the correlation between the coliform counts and geochemical/physicochemical parameters, it was found that total coliform/faecal coliform counts were not significantly ($p>0.05$) influenced with any geochemical/physicochemical parameters.

Microbiological investigation of spring samples with targeted amplicon sequencing

16S rRNA gene based targeted sequencing revealed that alpha diversity metrics were varied across the samples (figure 3a). In total 559 Amplicon sequence variants (ASVs) were detected across the samples. Interestingly, among the Rheocrene type spring, the least number of ASVs were detected in the Sp3 while Sp7 had highest ASVs counts. Remaining four samples from the Rheocrene (Sp2, Sp4, Sp5, and Sp6) had intermediate ASVs counts. Moreover, one water sample (Sp1) from limnocrene spring showed second highest ASVs count among all the seven spring water samples collected for the study. Shannon and Simpson diversity indices values showed that Sp7 had high microbial diversity followed by Sp2, Sp1, Sp3, Sp6, Sp4 and Sp5. Spearman correlation-based analysis revealed that number of observed ASVs was found to be positively correlated with TH, TA, Ca^{2+}, and HCO_3^- at $p < 0.05$. In contrast other diversity indices did not show any significant correlation with environmental variables. However, all the diversity indices metrics calculated were found to be positively correlated with each other at $p < 0.05$. In order to understand the share and unique microbiome of the spring samples, UpSet plot showed that 11 ASVs constituted the part of core microbiome and contributed nearly 8-97% of the total reads across the spring samples (figure 3b).

Our data further revealed that bacterial populations were dominated by *Proteobacteria* followed by *Campylobacterota* and *Bacteroidota* in water samples from rheocrene spring (figure 4a). In contrast, limnocrene spring water sample (Sp1) was predominated by *Bacteroidota*, followed by *Proteobacteria, Campylobacterota*, *Cyanobacteria*, *Patescibacteria*, etc. (figure 4a). Moreover, based on the distribution and abundance pattern of the top 20 bacterial families', samples were clustered in to two distinct clades (figure 4b). Sp1 and Sp7 clustered together from rest of the samples.

Rheocrene water samples were mostly represented by five bacterial families affiliated to *Moraxellaceae*, *Oxalobacteraceae*, *Pseudomonadaceae*, *Arcobacteraceae*, and *Flavobacteriaecae* (figure 4b). However, water sample (Sp7) from rheocrene spring showed abundance of few other taxa affiliated to *Chromobacteriaceae*, *Comamonadaceae*, *Rhodocyclaceae*, *Halomicrobiaceae*, *Haloferraceae*, Contrastingly, water sample (Sp1) from limnocrene spring constituted of *Spirosomaceae*, *Comamonadaceae*, and *Arcobacteraceae* along with *Moraxellaceae*, *Oxalobacteraceae*, *Pseudomonadaceae*, and *Flavobacteriaecae* (figure 4b)*.* Interestingly, microbial diversity was profoundly noticed at genera level distribution (figure 5). *Acinetobacter* (11-86%) and *Pseudomonas* (2-24%) were prevalent in rheocrene spring water samples (figure 5). However, several bacterial genera were found to be detected in few of the rheocrene samples in substantial abundance. *Pseudoarcobacter* was detected in Sp2, Sp5 and Sp7 while *Falvobacterium* was mostly noticed in Sp2, Sp3, Sp4 and Sp7. Additionally, *Duganella, Rhodoferax, and Massilia* were observed in Sp2, Sp3, and Sp7. Interestingly, *Vogesella and Dechloromonas* were only detected in substantial abundance in Sp7*. Undibacterium* was mostly a part of Sp2, Sp3, Sp6 and Sp7 samples. Limnocrene water sample was predominated with *Flectobacillus (51%), Pseudoarcobacter (17%) Rhodoferax (8.5%), Limnohabitans (8.4%), Flavobacterium (3%), Pseudomonas (3%), Perlucidibaca (2%), Roseateles (1%).*

Correlation between environmental variables and bacterial genera

In order to understand the correlation between the microbial genera and environmental variables, Spearman correlation analysis was performed (figure 6). The results showed that 14 genera (*Vogesella, Dechloromonas, Janthinobacterium, Ferribacterium, Alkanindiges, Halolamina, Halobacillus, Halomarina, Saccharopolyspora, Curvibacter, Alteromonas, Salimicrobium, Natronomonas, and Halogranuma*) were found to be positively correlated with EC, TDS, TH, TA, HCO_3^- and Ca^{2+} at $p<0.05$. However, *Flectobacillus, Limnohabitans, Arcicella, Sphaerotilus, Azospirillium, Caulobacter, Rhizobi*um, *Perlucidibaca, Cellvibrio,* and *Gemmobacter* were negatively correlated with Temperature and $SO_4^{2-.}$ *Acinetobacter* was found to be negatively correlated (p <0.05) with pH while Rhodoferax, *Limnohabitans, Arcicella, Sphaerotilus, Azospirillium, Caulobacter, Rhizobi*um, *Perlucidibaca, Cellvibrio, Fluviicola, Pseudoarthobacter, paucibacter, Roseateles,* and *Gemmobacter* were positively correlated with pH at p<0.05. *Flectobacillus, Limnohabitans, Arcicella, Azospirillium, Cellvibrio,* and Gemmobacter were positively correlated with DO at p <0.05.

Potential pathogenic microbes and incidence of waterborne diseases reported during course of study period

We further, identified several genera which generally contain common water borne/opportunistic pathogenic species from our microbiome datasets; however, 16S gene sequence did not allow species- or strain-level resolution. The abundance of these bacterial genera varied across the samples. *Acinetobacter, Pseudomonas, Legionella, Klebsiella, Pseudoarcobacter, Massilia, Clostridium, Yersinia,* and *Flavobacterium* are the known taxa for causing disease in human and animals. Interestingly, the *Escherichia, Shigella, Vibrio,* and *Salmonella* were not detected in our amplicon datasets might be due to its low abundance in the spring water. Additionally, we had surveyed approximately 200 people who were consuming this spring water for drinking purpose to understand the incidence of waterborne diseases such as typhoid, diarrhea, dysentery, skin disease, gastroenteritis, and hepatitis during the course of study. Detailed summary response of the survey is presented in appendix p 6-7. Nearly 40% of the people (80 out of 200) were infected with any one of the abovementioned diseases. Among all, ~14 % of people were infected with typhoid followed by diarrhea (~11%) (Table 1).

Discussion

In the present study, we have explored the bacterial communities in seven natural springs from hilly region and their impact on water quality which could be used in developing the strategies for its restoration, management, and monitoring. Earlier, we reported the detailed physicochemical properties of these springs and it was found that the water quality index of the less disturbed spring with human/animal activities was excellent, indicating the role of anthropogenic and geogenic activities in maintaining the water quality.[1]. However, our aim was to understand the microbiological distribution as well as their impact on the water quality. The total coliform test showed the presence of coliform bacteria across the seven spring with varied proportions, indicating the faecal contamination in the water body, which ultimately increased risk of human health and unsuitability of water for drinking. Our survey further confirmed the incidence of water-borne diseases such as typhoid and diarrhoea in the people who are regular consumer of this spring for drinking purpose which are well corroborated with our total coliform data. Open defecation, animal husbandry, and improver sewage disposal are the major sources faecal matter contamination in the spring.[3] This clearly indicates that these spring need special attention to monitor the microbiological indicators before its use for various domestic or irrigation purpose.

Our amplicon data showed a broader and in-depth picture about the indigenous microbial community of the spring water. Interestingly, the microbiome profile varied substantially across the springs, indicating the role of environmental factors in its assemblages, which is well evident from our correlation-based analysis of microbes and physicochemical parameters. The results suggested that spring water chemistry, including pH, temperature, DO, and nutrients (nitrate, sulfate, and phosphate) along with alkalinity and TDS favoured the emergence of spring water microbiome, which in turn determine the chemical characteristic of the spring as well. Our aim was to identify the potential pathogenic microbes in the spring from the next generation sequencing technology which help us to assess the water quality and improve our monitoring system. We identified various opportunistic pathogenic microbes in the spring waters from the microbiome profiling. *Moraxellceae*, is one of the major dominated taxa present in most of the springs and it harbors potential pathogenic microbial members such as *Acinetobacter*, which are known to cause various diseases in humans.[24] Higher abundance of *Acinetobacter* has been previously reported from various drinking water distribution system as well as in municipal tap water and drinking bottled water.[25,26] Similarly, *Arcobacteracae*, which is prevalent in the spring water is of great concern. Members of *Arcobacteraceae* are found in diverse habitats including underground water, surface water, sewage and sea water etc. and are one of the agents for foodborne or waterborne transmitted diseases.[27] Hence, presence of *Arcobacteraceae* species in water samples can be useful to determine their role as a vehicle for the transmission of infectious agents and the potential zoonotic risk of these samples.[27]

Thus, considering the presence of *Arcobacteracea*e in the spring samples and its potential risk to human health, the present study suggests for proper monitoring and surveillance of spring water towards its quality and potability. *Pseudomonas* is highly versatile genera, distributed across every environment and its presence in the spring sample is not surprising. *Pseudomonas aeruginosa* is reported as a pathogenic bacterium[28] and has been reported from drinking water, surface water, tap water system.[26,29,30] Hence, it is indeed necessary to identify the *Pseudomonas* species in the water system for assessing its quality and reduce the health risk to human. *Massilia* is another bacterial genus which has been previously reported from the drinking water distribution system and its presence in the spring samples is well corroborated.[31] Despite of its inability to cause any disease, recently it has been reported from the patient suffering from endophthalmitis[32], indicating its potential to cause human disease. *Legionella* is an opportunistic pathogen of public health concern for water-based bacterial infections and potable water is the main source of its transmission to human.[33] Hence, presence of *Legionella*, even in low abundance in the spring samples is alarming and of great health concern. Other than these known human-associated pathogenic bacteria, another bacterial taxon; *Flavobacterium* which are mostly harmless but recently few species are described as opportunistic or true pathogens for fish and are causative agent of columnaris disease and bacterial gill disease in fishes, has been detected in our spring system.[34] Overall, we successfully identified several heterotrophic opportunistic pathogens from spring water samples and this data could be useful for monitoring and surveillance the water quality of springs.

Conclusions

Water is considered as the valuable natural resource on earth and plays an important role in the development of different sectors of the economy worldwide.[35,36] However, the demand of high category potable freshwater is increasing day by day worldwide due to rise in human population. Due to the improper restoration and management, poor sanitary practices, inappropriate waste/sewage disposal system, and ultimately climate change and human development, the sustainability of the freshwater supply is compromised.[3,37] In order to maintain the quality and quantity of these water resources, monitoring and conserving of the natural resources are the two ultimate strategies for improving the health index of people as well as supply of safe drinking water. Combination of both routine assessment of microbiological assay for water quality and targeted amplicon sequencing can reveal the presence of opportunistic pathogens in the water samples. Hence, continuous assessment and monitoring of faecal contamination and heterotrophic opportunistic pathogens should be prioritised for improved water quality and reduce health risk. Use of 16S rRNA gene-based targeting is useful in microbiological examination of spring water and can be applied for continuous assessment and monitoring before it is declared safe for human consumption. It is of utmost important to develop policies on conserving natural water sources and reduce the risk of various diseases via proper sanitation, sewage disposal system and treatment of the water before use.

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Declarations

The authors declare no conflict of interest

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Table 1: Incidence of water borne diseases reported during course of study period

S. No.	Waterborne diseases	Total number of participants	Number of respondents	Percentage (%)
1	Typhoid	202	29	14.36
2	Diarrhea	202	21	10.40
3	Skin Diseases	202	10	4.95
4	Gastroenteritis	202	10	4.95
5	Dysentery	202	9	4.46
6	Hepatitis E	202	1	0.5
	Total respondents		80 out of 202	39.60

Figures Legends

Figure 1 Map of the study area, a portion of the Neeru watershed, indicating the locations of water sample stations

Figure2 Heatmap based Spearman correlation between physicochemical parameters and alpha diversity indices. * depicts significant p value ($p < 0.05$)

Figure 3 Alpha diversity parameters of spring samples (a). Shared and core microbiome across the spring samples (b).

Figure 4 Relative abundance of major taxa across the springs. Relative distribution of major phyla across the springs (a). Heatmap based clustering of samples and major families. Clustering was performed based on Bray-Curtis dissimilarity matrix.

Figure 5 Bubble plot of major genera detected across the spring samples.

Figure 6 Heatmap based Spearman correlation between physicochemical parameters major bacterial genera. * depicts significant p value ($p < 0.05$).