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An Investigation into the Microbiological Quality of Ground water in an Industrial Hub in Mid-Hills of Northern India

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Abstract

Frequent water-borne disease outbreaks affecting human health have been reported in district Solan, which is a rapidly industrialing region of Himachal Pradesh, a northern state of India. For ascertaining the cause, microbiological assessment of drinking groundwater sources of the region was undertaken. For this, thermotolerant coliform study and genome typing were undertaken. Multiple tube fermentation technique was employed in selected five sources to detect thermotolerant coliforms during the seasons of monsoon and post-monsoon in the years 2018-19. Thermotolerant coliforms varied in Most Probable Number from 2-34 per 100 ml water indicating contaminated water sources, densities being significantly more in monsoon months (p< 0.05). Four different coliform morphotypes were obtained from all the sources on Eosin Methylene Blue plates. Of these, the only strain, designated as Nalagarh Water Strain (NGW), was recovered from all sources, suggesting its prevalence. Based upon biochemical and morphological attributes, the strain was recognized as *Escherichia coli.* Conversely, molecular characterization (16S rRNA ribotyping) identified it as *Raoultella planticola* (GenBank accession No. MK318824). The strain was deposited in a National Culture Collection Centre, National Centre for Microbial Resources, Pune, Maharashtra (India) with deposition accession number MCC 4064 for future utility in public research activities. Microbiological assessment evinced contaminated drinking water sources, probably causing diarrhoeal diseases. 16S rRNA ribotyping assisted in correct identification of the microorganism.

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Introduction

Globally, various anthropogenic activities related with industrialization, urbanization and chemical based farming have affected the physical, chemical and biological properties of drinking water.[1,2] Polluted water transmits various infectious diseases such as shigelosis, dysentery, diarrhoea, cholera, hepatitis etc.[3,4] Poor water sanitation and hygiene is responsible for about 88% of the diarrhoeal disease across the world.[5]

In India, anthropogenic activities lead to about two million tons of human waste into water courses daily.[6] District Solan, a rapidly industrializing region in Himachal Pradesh, a state in northern India, has also witnessed rapid growth in urbanization and industrialization which has led to unabated pollution of water,[7] necessitating water quality assessment. The utility of the thermotolerant coliform group, specifically *Escherichia coli*, as an indicator of microbiological water quality, is definitive evidence of faecal contamination.[8]

Sometimes, the sole identification of indicator organism can be misleading. Therefore, many advanced microbiological analytical (DNA-based typing methods) tests have been used recently.[9] The use of rRNA molecules was developed for comparisons based on phylogenetic traits.[10] The16S rRNA gene (1550 bp) has been the most used marker.[11] Henceforth, the study was undertaken with the objective of the microbiological assessment of region's drinking water sources by looking for the presence of indicator organisms, the thermotolerant coliforms and further identification of the organism by genome typing.

Materials and Methods

In order to construct the water borne disease burden strata of the district, the standard stratification method was employed to the five-year diarrhoeal disease incidence secondary data.[12] This was collected from the district health department for the 49 PHIs (Public Health Institutes) of the district for the years 2012-16.[13] Approximation statistics were applied and disease burdened areas were divided in four strata namely Very low, Low, High, and Very

high. The Public Health Institute (PHI) Nalagarh of industrial hub located in Shivalik foothills of North Western Himalayas of district Solan, India, having high disease burden, was selected for assessing the microbiological attributes of the sources of drinking water.

Hydrogeology of Study Region

In the study region, groundwater occurs in unconsolidated alluvial formation comprising sand, silt, gravel, cobbles/pebbles. In Nalagarh, wells and tube wells are the main groundwater obstruction structures. Nalagarh has dendrite drainage pattern having sandstaone-clay alteration. The uppermost horizon consists of conglomerate with well-rounded clast of grey quartizite possibly derived from shale.

Study Design and Data Sampling For Micro-biological Assessment

A cross-sectional survey was conducted in the industrial region being served by PHI Nalagarh. KAP attributes (Knowledge, Attitude and Practice) of 180 adult residents was assessed for water, sanitation and hygiene. A structured pre-tested standardized questionnaire was used. Five commonly used sources of water were inferred from survey. These sources (three bore-wells and two unprotected springs) were selected purposively for the further study (Fig.1). The groundwater samples were drawn by the grab water sampling technique. The sampling was undertaken during the monsoon months of June, July and August and during the post monsoon months of September, October and November in 2018-19.[14]

Detection of Thermotolerant Coliforms

For determining the presence of thermotolerant coliforms, the technique of multiple tube fermentation was used.[14] Appropriate decimal dilutions of water sample were inoculated in a series of tubes. Three stage procedure was employed for identifying aerobic and facultative anaerobic rod shaped bacteria which were rod shaped, non spore forming and gram negative. These bacteria (coliforms) fermented lactose with acid and gas formation within 48 hours at $35 \pm 2^{o}C$,. Most Probable Number (MPN) per 100ml water was used to represent the results.
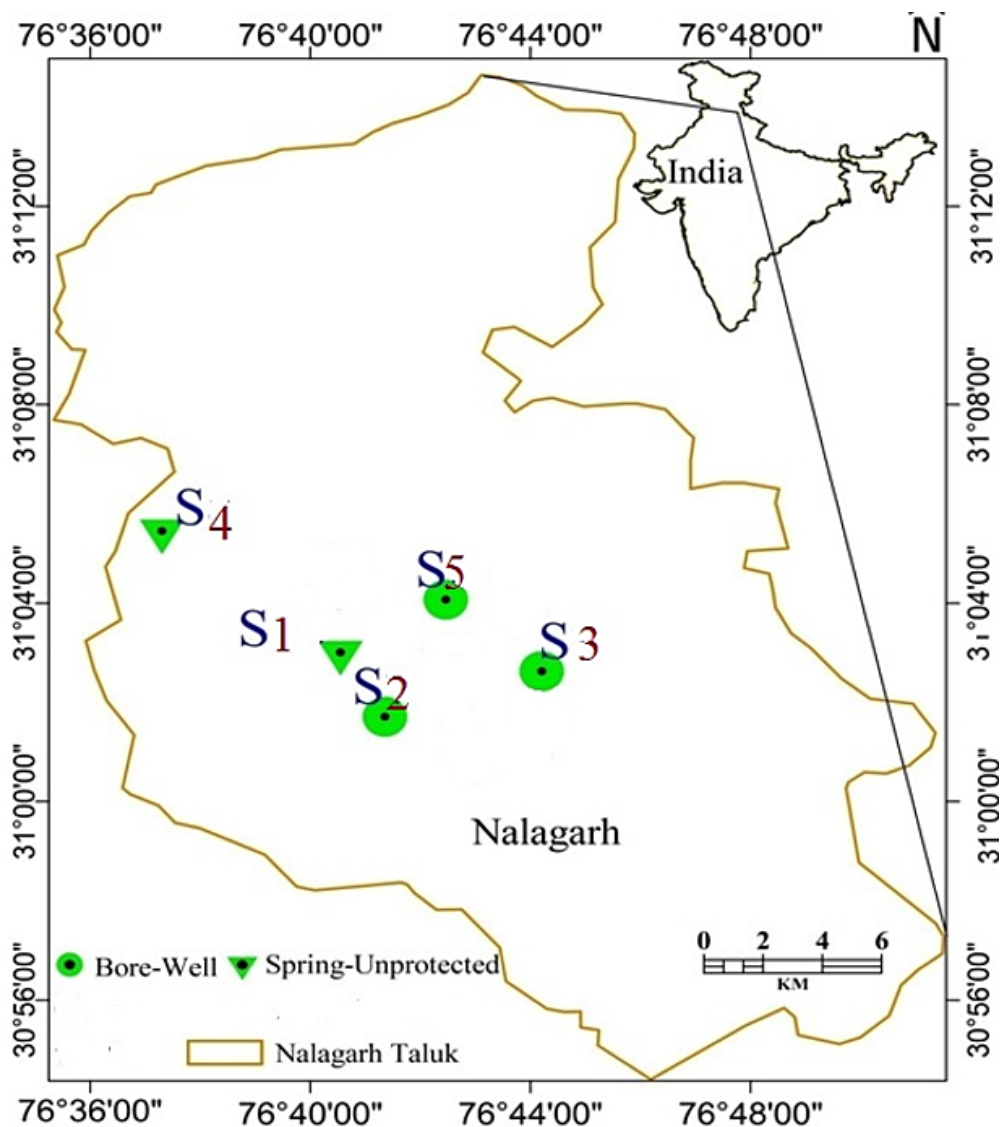
Fig. 1: Drinking water sources of Nalagarh

Morphological and Biochemical Characterization
Different morphotypes of coliform bacteria obtained on Eosin Methylene Blue (EMB) agar plates in the present study were identified based on their morphological and biochemical characteristics according to the standard methods described in Bergey, Manual of Systematic Bacteriology.[15]
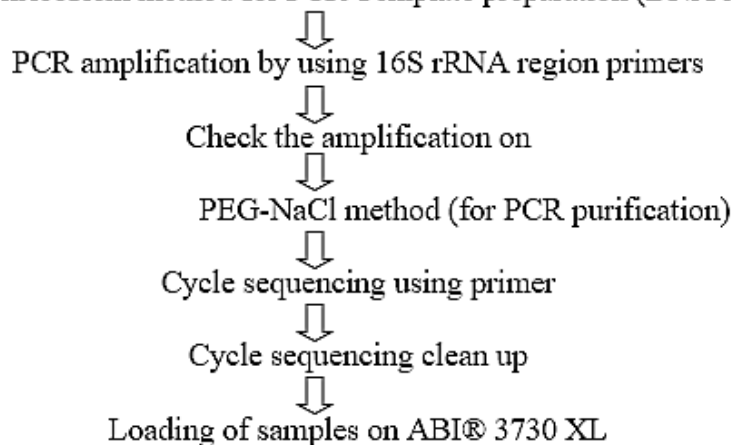
Molecular Characterization
Molecular characterization of the most prevalent indicator organism was done using 16S rRNA gene sequencing as per the standard protocol (Fig.2).

The isolate was identified at National Centre for Cell Science Pune, Maharashtra, India. Genomic DNA was isolated by phenol/chloroform extraction method. Thereafter, 16S rRNA gene amplification by PCR was achieved by employing the universal primers such as 16F27 [5'-CCA GAG TTT GAT CMT GGC TCA G-3'] and 16R1492 [5'-TAC GGY TAC CTT GTT ACG ACT T-3']. For purifying the amplified 16S rRNA gene PCR product the method of PEG-NaCl precipitation was adopted. Thereafter, it was directly sequenced on an ABI® 3730XL automated DNA sequencer. For sequencing, additional internal

primers were utilized from both the ends ensuring that each position was read at least twice. Thereafter, Lasergene package was used for assembly. EzBioCloud database (http://ezbiocloud.net/eztaxon) was then employed for identification. MEGA 7 software programme was used for phylogenetic analyses. The gene sequences of 16S rRNA were deposited in the Gene Bank of NCBI (National Centre for Biotechnology Information), USA.

Phenol-Chloroform method for PCR Template preparation (DNA isolation)
⬇
PCR amplification by using 16S rRNA region primers
⬇
Check the amplification on
⬇
PEG-NaCl method (for PCR purification)
⬇
Cycle sequencing using primer
⬇
Cycle sequencing clean up
⬇
Loading of samples on ABI® 3730 XL

Fig. 2: General Sequencing Pool

Deposition of Bacterial Strain NGW
The bacterial strain *Raoultella planticola* NGW, identified in the present study was deposited in a national culture collection centre/repository namely the National Centre for Microbial Resource and the National Centre for Cell Science at Pune in Maharashtra state of India, for making its accessibility easy as reference strain by the research community.

Analysis
Randomized Block Design (Factorial) suggested by Gomez and Gomez was used for data analysis16. Five water sources were presumed to be the replications for the study. The years were considered as factor one with two levels i.e. 2018 and 2019. Seasons were factor two having monsoon and post-monsoon as two levels. Least Significant Difference (LSD) at 5 % level of probability was utilized for comparing the treatment means.

Results and Discussion
Table 1 inferred MPN values of thermotolerant coliforms. It varied from 2-34 per 100 ml water across all the water sources in both seasons. This indicated varying contamination levels in drinking water of the region. the seasons (monsoon and post-monsoon) significantly influenced the concentration of thermotolerant coliforms in water. These bacteria were detected more in concentrations during the monsoon season ($p = 0.02$, 95% confidence interval). However, the concentration of the coliforms was not influenced by the year of the study. Also, no effect on the concentration of the coliforms occurred due to any interaction between the two factors i.e. the year and the season. The average highest total coliforms (MPN- 27 per 100 mlwater)were detected in the water sources during monsoon, 2019. This was followed by statistically equivalent values noticed during the monsoon, 2018.

Morphological Characterization of Coiliform Morphotypes
A total of four different morphotypes of coliform bacteria were obtained from the five different drinking water sources of industial region Nalagarh on EMB agar plates. All morphotypes were further purified on EMB agar plates. Of these, only one isolate, designated as NGW (Nalagarh Water) strain showed green metallic sheen on EMB plates (Fig. 3 and Table 2). During the monsoon season, NGW

strain was detected in all the water sources. This suggested the prevalence/dominance of NGW strain among other strains, in the selected water sources. On the contrary, NGW strainwas observed in only two water sources during post monsoon

Table 1: Temporo-spatial distribution of thermotolerant coliforms in drinking water sources of Nalagarh

Year	Season	Water source[a] (MPN per 100 ml water)					Mean mean	Yearly
		A	B	C	D	E		
2018	M[b]	17	34	33	33	17	26.80	19.00
	PM[c]	17	17	9	9	4	11.20	
2019	M	17	34	34	33	17	27.00	18.50
	PM	12	17	2	2	17	10.00	

Least Significant Difference (0.05)
Year: Not significant; Season: 7.48; Year x Season: Not significant

[a]Drinking water sources of : A-Ward 1; B-Ward 3; C- Near bus stand; D- Ward 7; E- Raj Mahal area, [b]Monsoon, [c]Post monsoon
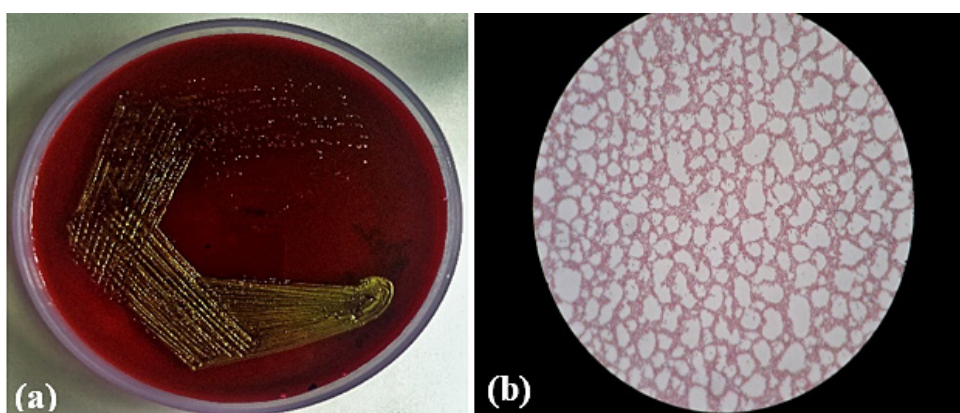
Fig. 3(a): Morphology of strain NGW on EMB agar plate after 24 h of incubation at 37 °C. (b) Gram reaction of strain NGW

**Table 2: Phenotypic characterization of Nalagarh water strain (NGW) strain**

Phenotypic characterization

1. Morphological characteristics (on Eosin Methylene Blue agar plate)

Colour	Margin	Shape	Texture	Elevation	Gram reaction	Metallic sheen-
Greenish	Entire	Circular	Smooth	Flat	Negative	Green

2. Biochemical characteristics

Positive reaction for -Methyl Red, oxidase, catalase, oxidative, triple iron sugar tests	Negative reaction for -Voges- Proskauer, indole, citrate and gelatinase tests	Positive reaction for carbohydrate ferme -ntation (Acid/Gas production) -Arabinose, Mannose, Mannitol, Xyl -ose, Rhamnose, Dextr -ose, Sorbitol, Lactose, Adonitol, Melibiose, Cellobiose and Sucrose	Probable identi -fication- *Escherichia coli*

Table 3: Molecular characterization of Nalagarh water strain (NGW) strain

Molecular characterization			
NCBI GenBank Accession number	**Closest neighbour**	**Percent Similarity**	**Sequence length (bp)**
MK318824	*Raoultellaplanticola* ATCC 33531 (T)	99.85	1200
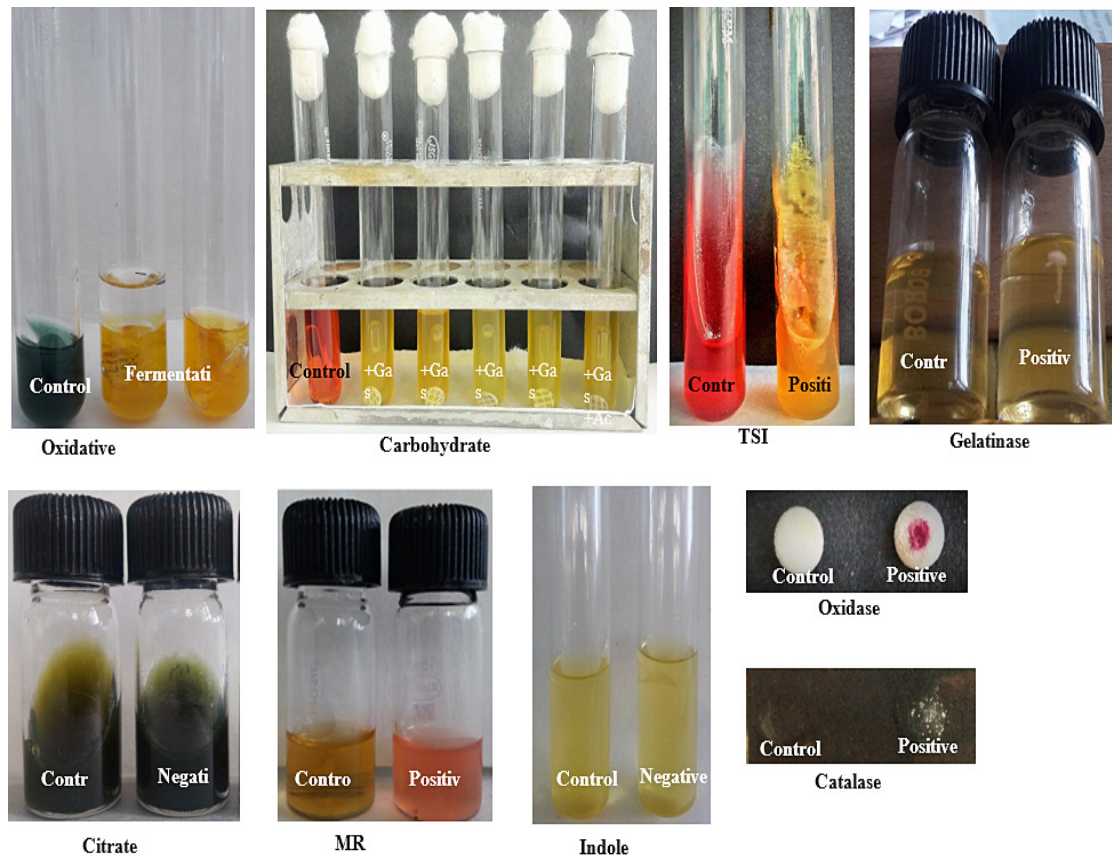
Fig. 4: Biochemical characteristics of NGW strain

Biochemical characterization

Based on biochemical characteristics, strain NGW was positive for MR, catalase, oxidase, O/F and TSI tests whereas, it showed negative reactions for VP, Indole and citrate tests (Fig. 4 and Table 2). All morphological characterization results, especially green metallic sheen formation on EMB agar plates and biochemical tests except indole tests, prompted the possible identification of this strain as *Escherichia coli*.

Molecular Characterization

16S rRNA ribotyping of the NGW strain revealed that it belonged to the genus *Raoultella* Your sentence may be unclear or hard to follow. Consider rephrasing.

and species *planticola*, GenBank accession no: MK318824 (Table 3). The sequence comparison with 16S rRNA gene sequences deposited in the GenBank indicated that nearest neighbors of strain NGW were *Raoultella planticola* ATCC 33531 (T), *Raoultella* ornithinolytica JCM 6096 (T), *Raoultella* electrica1GB (T) (AB762091), Enterobacter aerogenes KCTC 2190 (T) (CP002824) and *Klebsiella* grimontii 06D021 (T) (FZTC01000044). Phylogenetic tree constructed with Neighbor-Joining method interpreted that the strain NGW clustered among species of genus *Raoultella*. Henceforth, it was confirmed by the observations that the strain NGW belonged to the genus *Raoultella* (Fig. 5).
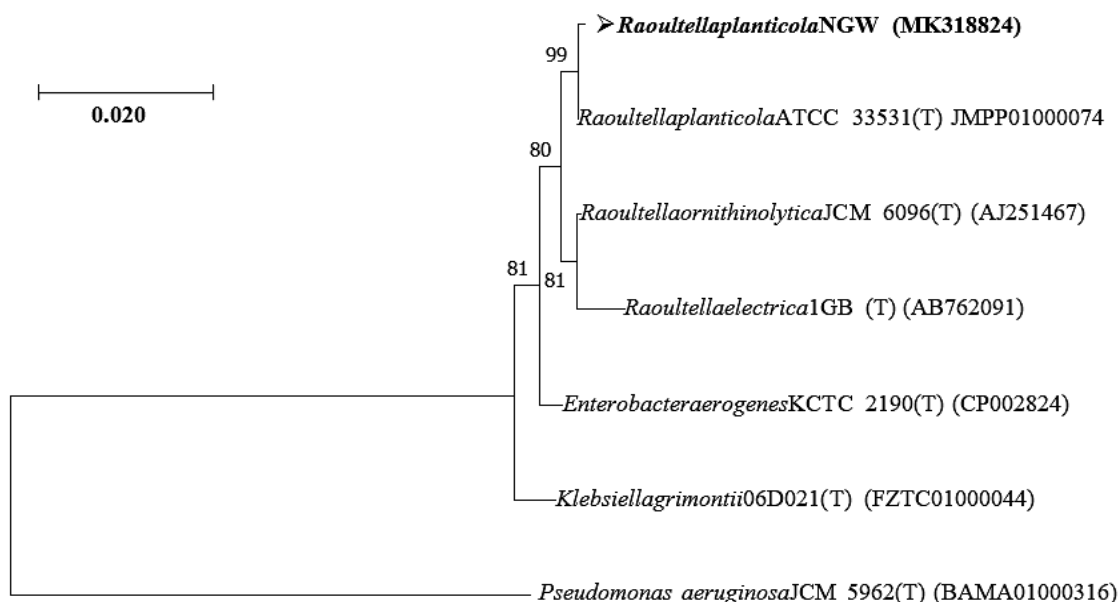
Fig. 5: 16SrRNA gene sequencing based Neighbor-Joining method Phlogenetic tree

It was observed in the present study that the concentrations of thermotolerant coliforms in the drinking water sources was high. Similar findings had been also ascertained by a KAP study undertaken in these regions by Singh and Bhardwaj.[2] Bain *et al.* had demonstrated that the chance of faecal contamination were more in the unprotected water bodies and that the odds of contamination in protected sources were lower (OR = 0.15) when compared[17] Nalagarh region had predominantly bore wells and unprotected springs as the source of drinking water. The present study has inferred high contamination levels in these sources.

In Baddi region, which was adjoining the study area, another study had documented high coliform concentrations in bore-well water.[18] Poor water quality due to adverse bacteriological profile has been observed in water samples of Shimla, the town in the vicinity of our study area.[19,20] The present study documented that drinking water sources were unprotected and prone to runoff and faecal contamination. A study in West coast of India had similarly documented high densities of coliforms in monsoon seasons.[21] Another similar study had inferred that the concentration of total coliforms and *E. coli* was more in the wet season as compared to

the dry season.[22] The water bodies of industrially dominated regions had shown *E. coli*, suggesting lack of sanitation services. This was leading to faecal contamination of these water sources of the region. The migratory populations of various industries practiced open defecation. This feature had also been observed during the KAP survey carried out in the present study. A similar finding wass observed in a study conducted Taiwan which demonstrated that the domestic sewage of areas having a large number of industries, was responsible for high concentrations of faecal coliforms[23] The study elicited low prevalence of *Raoultella planticola* in post monsoon as compared to monsoon season. In another similar study, conducted in the northeast state of Odisha, India, seasonal variation of *E.coli* fecal indicator organism was documented and low prevalence was observed in post monsoon season.[24]

It was demonstrated in the study that the thermotolerant coliforms are the indicator organisms which can be utilized for the sanitary quality assessment of drinking water sources. The presence of thermotolerant coliforms pointed out to the probable fecal contamination of water. Similarly, Pandey and Soupir (2013) had also demonstrated water having varying levels of *E. coli* and studied utility of *E. coli* for water quality management.[25]

The present study has concluded that the indentification of indicator organism i.e. *E. coli*, based upon phenotypic identification methods is not sufficient. An exact indentification of the micorganism involved in the contaminated water may be inferred only after detailed genotypic tests, as was the strain *Raoultella planticola* identified in our study. Primarily R. *planticola* is found in water and soil. This rod shaped bacteria is gram-negative, aerobic, encapsulated and nonmotile in nature. The bacteria is a causative agent for urinary tract infections, cellulitis, necrotizing fasciitis, bacteremia, soft tissue infections, conjunctivitis, peritonitis and pneumonia. It also causes conjunctivitis, pneumonia, cholangitis, urinary tract infection, peritonitis, cellulitis, and soft tissue infection.[26] R. *planticola* was initially included in the genus *Klebsiella*. In 1981, it was first described as *Klebsiella planticola*[27] and as *Klebsiella* trevisanii in 1983.[28] Comparative analysis of 16S ribosomal RNA and rpoB gene sequences identified *Raoultella* which according to current taxonomy,

has been described as a new genus of the family *Enterobacteriaceae*.[28]

Furthermore, phenotypic identification results revealed that the NGW strain may belong to *E.coli* while genotypic identification confirmed its identification as *Raoultella planticola* (formerly *Klebsiella planticola*). The strain identified has been documented elsewhere in recent studies as a new pathogen afflicting human health.[30] This discrepancy in results could be ascribed to the fact that identification systems based upon biochemical methods may sometimes be unreliable for environmental isolates as the computerized databases do not have sufficient information about these environmental bacteria. Similarly a study on the eggs of loggerhead sea turtle, biochemical (API and Microgen) and molecular methods (16S rRNA analysis) employed in the identification of bacterial isolates inferred a 74 percent discrepancy in the identification results.[31]

This type of discrepancy was also observed in a study which reported that physiological tests have certain uncertainty in the identification of *E. coli*.[32] *Escherichia coli* has been confirmed by the characteristics such as gas and indole formation at 44 and 44.5 ℃ respectively. This feature also makes it distinct from *Klebsiella* species. Similarly another study underlined the use of alternative microbial techniques for real identification of contaminant microorganism.[33]

The study indicated that the presence of thermotolerant organisms preliminarily evinced contaminated water sources. Genotypic molecular studies elucidated that the water sources used for drinking purposes, of the industrial region of district Solan, were contaminated by the organism *Raoultella planticola*. Henceforth, the bore well and the unprotected spring water sources need to be managed to check the contamination by this rare human pathogen. The town administration was appraised of the findings of the study and remedial actions thereafter had been put in place for the treatment of water sources.

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Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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