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MOLECULAR CHARACTERIZATION OF SELECTED VIRULENT AND MULTIDRUG-RESISTANT GROUNDWATER BACTERIA FROM KOZHIKODE, KERALA

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ABSTRACT

Background: Groundwater serves as a major source of domestic water in Kerala; however, routine microbiological monitoring is often limited to indicator organisms and may overlook bacterial populations having clinically significant virulence traits and multidrug resistance.

Objective: This study aims to check the molecular characterization selected virulent and multidrug-resistant bacterial isolates recovered from groundwater sources in Kozhikode, Kerala**.**

Methods: A total of 392 groundwater samples were analysed using culture based microbiological methods, yielding 256 bacterial isolates. A comprehensive screening approach incorporating phenotypic identification, string test, biofilm formation assay, and antimicrobial susceptibility testing was employed to find high-risk isolates. Six representative isolates showing pronounced virulence-associated phenotypes and multidrug resistance were selected for genomic DNA extraction, 16S rRNA gene amplification, and molecular characterization.

Results: Initial screening revealed the predominance of *Escherichia coli* and *Klebsiella spp*. among the recovered isolates. *Klebsiella spp*. showed a strong hypermucoviscosity-associated phenotype, while *Klebsiella* and *Pseudomonas* groups showed greater biofilm-forming capacity compared with other taxa. Six representative multidrug-resistant isolates, comprising GW70 and GW141 (*Escherichia coli*), GW109 and GW155 (*Klebsiella pneumoniae*), GW188 (*Klebsiella oxytoca*), and GW132 (*Pseudomonas aeruginosa*), were selected for molecular characterization based on their resistance to ceftazidime, cefotaxime–clavulanic acid, meropenem, gentamicin, and ciprofloxacin. Genomic DNA extracted from these isolates yielded concentrations ranging from 85.3 to 110.2 ng/µL, with A260/A280 purity ratios of 1.79–1.88, indicating DNA of suitable quality for downstream analysis. Subsequent 16S rRNA gene amplification produced PCR products with concentrations ranging from 37.9 to 50.1 ng/µL and A260/A280 ratios of 1.82–1.90. Molecular analysis subsequently confirmed the taxonomic identity of all six selected isolates.

Conclusion: The findings suggest that groundwater sources in Kozhikode may serve as reservoirs of virulent and multidrug-resistant bacteria with potential public health significance. These results highlight the need for enhanced groundwater surveillance strategies incorporating molecular characterization alongside conventional indicator-based microbiological assessments.

KEYWORDS: groundwater microbiology; 16S rRNA gene; multidrug-resistant bacteria; molecular characterization; biofilm formation; hypermucoviscosity; groundwater quality; Kozhikode, Kerala.

INTRODUCTION

Groundwater constitutes a primary source of domestic water for a considerable proportion of the population in India, particularly in Kerala, where dependence on wells and localized aquifer systems is still widespread. Although groundwater is regarded as microbiologically safer than surface water, its quality can be compromised by faecal contamination, surface runoff, inadequate sanitation infrastructure, and other anthropogenic activities that help the introduction and persistence of pathogenic microorganisms (Girija et al., 2020). Conventional microbiological surveillance of groundwater primarily focuses on indicator organisms, such as coliform bacteria, which may not adequately reflect the presence of environmentally persistent or clinically significant bacterial populations showing virulence-associated traits and antimicrobial resistance (Nath et al., 2023).

The public health implications of groundwater contamination extend beyond the detection of indicator bacteria. Certain environmental bacterial species have adaptive characteristics that enhance their survival, persistence, and pathogenic potential. Among these, biofilm formation is particularly important, as it helps microbial attachment to abiotic surfaces, protects cells from environmental stress, and contributes to resistance against disinfectants and antimicrobial agents. Similarly, hypermucoviscosity-associated phenotypes, commonly seen in *Klebsiella* species, are linked to increased capsule production and may contribute to enhanced environmental fitness and virulence. The coexistence of such traits with multidrug resistance (MDR) further increases the public health significance of groundwater-associated bacterial populations by limiting treatment options in the event of human infection (Kamatham et al., 2020; Yousuf et al., 2026).

Recent studies have highlighted the emergence of antimicrobial-resistant bacteria in environmental water sources, emphasizing the role of aquatic ecosystems as reservoirs and dissemination pathways for resistance determinants. Groundwater systems used for domestic purposes may therefore serve as important but underrecognized reservoirs of bacteria capable of persistence, environmental adaptation, and transmission of clinically relevant resistance traits. Molecular characterization of selected high-risk isolates can provide exact taxonomic identification and strengthen the interpretation of phenotypic observations obtained through conventional microbiological methods. In a broader groundwater surveillance investigation conducted in Kozhikode, Kerala, 392 groundwater samples yielded 256 bacterial isolates. Phenotypic screening based on bacterial identification, biofilm-forming ability, hypermucoviscosity-associated phenotype, and antimicrobial susceptibility profiles showed a subset of isolates exhibiting characteristics indicative of increased environmental persistence and multidrug resistance. To further confirm their identity and assess their significance, representative high-risk isolates were selected for molecular analysis.

Therefore, the present study aimed to molecularly characterize selected phenotypically virulent and multidrug-resistant bacterial isolates recovered from groundwater sources in Kozhikode, Kerala, through 16S rRNA gene-based analysis. The findings contribute to a more comprehensive understanding of the microbial quality of groundwater and highlight the importance of incorporating molecular approaches into routine groundwater surveillance programs.

2. MATERIALS AND METHODS

2.1 Study Design and Sample Collection

This is laboratory based cross sectional study was conducted using groundwater samples collected from multiple locations across Kozhikode district, Kerala, India. A total of 392 groundwater samples were collected from wells and other groundwater sources following standard aseptic procedures and transported to the laboratory for microbiological analysis. Samples were processed at once or stored under proper conditions until analysis.

2.2 Isolation and Preliminary Identification of Bacterial Isolates

Groundwater samples were cultured on Blood Agar and MacConkey Agar using standard microbiological techniques. Following incubation, bacterial isolates were examined for colony morphology, pigmentation, texture, haemolytic activity, and lactose fermentation characteristics. Preliminary identification was based on colony appearance, Gram-staining reaction, and growth characteristics on selective and differential media. Blood Agar was used to evaluate colony morphology and haemolytic patterns, while MacConkey Agar helped differentiation between lactose-fermenting and non-lactose-fermenting organisms.

2.3 Biochemical Characterization

Representative isolates were further characterized using conventional biochemical tests, including indole production, methyl red (MR), Voges–Proskauer (VP), citrate utilization, urease activity, triple sugar iron (TSI) reaction, oxidase test, and catalase test. Based on phenotypic and biochemical characteristics, the predominant bacterial groups recovered from groundwater samples were identified as *Escherichia coli*, *Klebsiella* spp., *Pseudomonas* spp., and *Enterococcus faecalis*.

2.4 Phenotypic Screening and Selection of High-Risk Isolates

All recovered isolates were screened for virulence-associated phenotypes and antimicrobial resistance. Hyper mucoid phenotype was assessed using the string test, while biofilm-forming ability was evaluated by the crystal violet tube assay. Antimicrobial susceptibility testing was performed using standard procedures against ceftazidime, cefotaxime-clavulanic acid, meropenem, gentamicin, and ciprofloxacin. Based on the combined assessment of multidrug resistance, biofilm-forming capacity, and hypermucoviscosity-associated phenotype, six representative isolates showing high-risk characteristics were selected for molecular characterization. The selected isolates included representatives of *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, and *Pseudomonas aeruginosa*.

2.5 Genomic DNA Extraction and 16S rRNA Gene Amplification

Genomic DNA was extracted from the selected bacterial isolates using standard bacterial DNA extraction protocols. DNA quantity and purity were figured out spectrophotometrically by measuring absorbance at 260 and 280 nm and calculating the A260/A280 ratio. DNA samples exhibiting acceptable purity were used for amplification of the 16S rRNA gene using universal bacterial primers.

Polymerase chain reaction (PCR) amplification was performed under optimized conditions. Amplified products were analysed by agarose gel electrophoresis to verify the presence of the expected amplicons. The concentration and purity of PCR products were later found spectrophotometrically prior to molecular identification and sequence-based confirmation. Molecular identification of the selected isolates was performed based on 16S rRNA gene amplification and sequence confirmation. The quality of genomic DNA and PCR products was evaluated using concentration measurements and A260/A280 absorbance ratios.

2.6 Data Analysis

Descriptive statistical methods were used to summarize the findings. As this study represents a molecular follow-up investigation of a larger groundwater surveillance program, the Results section primarily focuses on the characterization and molecular confirmation of selected virulent and multidrug-resistant isolates, including DNA quality parameters and 16S rRNA gene amplification outcomes.

3. RESULTS

3.1 Screening overview and selection of isolates for molecular study

A total of 392 groundwater samples were processed, yielding 256 bacterial isolates in the broader screening dataset. The screening workflow identified *Escherichia coli* and *Klebsiella spp.* as the dominant groups and highlighted a smaller subset of phenotypically high-risk isolates for molecular follow-up. Six representative isolates were ultimately forwarded for 16S rRNA-based molecular characterization: GW70 and GW141 (*E. coli*), GW109 and GW155 (*Klebsiella pneumoniae*), GW188 (*Klebsiella oxytoca*), and GW132 (*Pseudomonas aeruginosa*).

Parameter	Observation
Groundwater samples processed	392
Total isolates recovered in broader screening dataset	256
Principal taxa identified during initial screening	*Escherichia coli, Klebsiella spp., Pseudomonas spp., Enterococcus faecalis*
Basis for selecting isolates for molecular study	Combined phenotypic virulence screening and multidrug resistance
Number of isolates forwarded to molecular work-up	6
Selected isolate IDs	GW70, GW141, GW109, GW155, GW188, GW132

Table 1. Screening overview and isolate selection summary.]

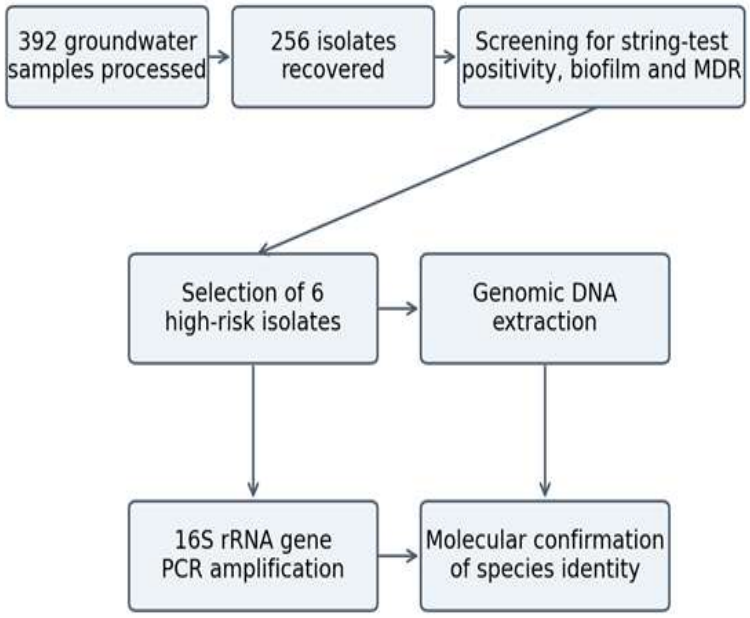

Figure 1. Workflow for selection and 16S rRNA-based molecular characterization of representative high-risk groundwater isolates.

3.2 Phenotypic screening context supporting isolate prioritization

The broader dataset was used only as a screening framework to justify molecular follow-up. Within that framework, *Klebsiella spp*. showed the strongest hypermucoid signal, whereas *Klebsiella spp.* and *Pseudomonas spp*. showed greater biofilm tendency than the other taxa. Enterococcus faecalis did not emerge as a priority group for molecular follow-up in this study. These patterns informed prioritization of isolates for downstream molecular work-up, but detailed species-wide prevalence and full phenotype distribution were intentionally not expanded here because they belong to the broader surveillance paper rather than the present 16S rRNA-focused manuscript.

Bacterial group	Screening context relevant to molecular prioritization	Selected for molecular work-up

Escherichia coli	Dominant in broader dataset; minority showed hypermucoid phenotype; representative MDR isolates identified	GW70, GW141
Klebsiella spp.	Strongest mucoviscosity signal in broader dataset; notable biofilm-forming tendency; representative MDR isolates identified	GW109, GW155, GW188
Pseudomonas spp.	No hypermucoid signal but important biofilm-forming tendency; one representative MDR isolate identified	GW132
Enterococcus faecalis	Detected in broader screening but not prioritized for molecular follow-up in the present study	None

Table 2. Phenotypic screening context used to prioritize isolates for molecular characterization.

3.3 Multidrug-resistant profile of the selected isolate panel

All six isolates selected for molecular analysis demonstrated multidrug-resistant phenotypes against the antibiotic screening panel used in the study and were therefore prioritized for 16S rRNA-based molecular characterization. The selected panel comprised GW70 and GW141 (*Escherichia coli*), GW109 and GW155 (*Klebsiella pneumoniae*), GW188 (*Klebsiella oxytoca*), and GW132 (*Pseudomonas aeruginosa*).

3.4 Genomic DNA yield and quality of selected isolates

Genomic DNA extraction yielded adequate DNA from all selected isolates. DNA concentrations ranged from 85.3 to 110.2 ng/µL, while A260/A280 ratios ranged from 1.79 to 1.88, indicating acceptable high purity DNA suitable for downstream PCR amplification. The highest DNA concentration was recorded for GW155 (*K. pneumoniae*), whereas GW132 (*P. aeruginosa*) yielded the lowest concentration but remained within an acceptable range for molecular analysis.

Isolate ID	**Species**	**DNA concentration (ng/µL)**	**A260/A280 ratio**	**Interpretation**
GW70	*E. coli*	92.4	1.84	High purity DNA
GW141	*E. coli*	88.7	1.81	High purity DNA
GW109	*K. pneumoniae*	105.6	1.86	High purity DNA
GW155	*K. pneumoniae*	110.2	1.88	High purity DNA
GW188	*K. oxytoca*	97.9	1.83	High purity DNA
GW132	*P. aeruginosa*	85.3	1.79	Acceptable purity DNA

Table 3. Spectrophotometric assessment of genomic DNA from selected high-risk isolates.

3.5 16S rRNA PCR product quality and molecular confirmation

PCR amplification of the 16S rRNA gene was successful for all selected isolates. PCR product concentrations ranged from 37.9 to 50.1 ng/µL, and A260/A280 ratios ranged from 1.82 to 1.90, indicating high-quality or acceptable amplicons for downstream molecular confirmation. Final molecular confirmation supported the selected isolated identities as *E. coli, K. pneumoniae, K. oxytoca,* and *P. aeruginosa*.

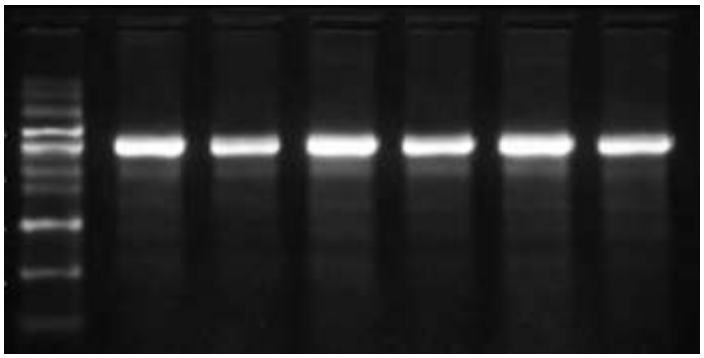

Figure 2 16S rRNA PCR gel image of the six selected isolates

Isolate ID	Phenotypic ID	PCR product (ng/µL)	A260/A280 ratio	Quality interpretation	Final molecular confirmation
GW70	*E. coli*	42.6	1.87	High-quality PCR product	*E. coli*
GW141	*E. coli*	39.8	1.85	High-quality PCR product	*E. coli*
GW109	*K. pneumoniae*	48.3	1.89	High-quality PCR product	*K. pneumoniae*
GW155	*K. pneumoniae*	50.1	1.90	High-quality PCR product	*K. pneumoniae*
GW188	*K. oxytoca*	44.7	1.86	High-quality PCR product	*K. oxytoca*
GW132	*P. aeruginosa*	37.9	1.82	Acceptable quality PCR product	*P. aeruginosa*

Table 4. Quality assessment of 16S rRNA PCR products and final molecular confirmation of selected isolates.

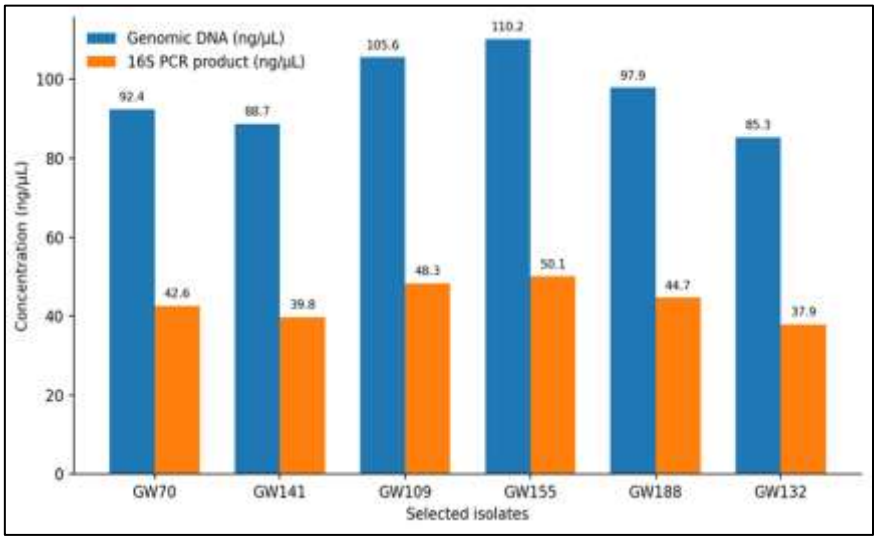

Figure 3. Genomic DNA concentration and 16S PCR product concentration among the selected isolates.

4. DISCUSSION

The present study is deliberately centered on the molecular characterization of a selected high-risk isolate panel. Within the screening framework, *Klebsiella spp.* emerged as the most important phenotypic signal for prioritization because of their strong mucoviscosity-associated phenotype and substantial biofilm tendency. *Pseudomonas spp.*, although less dominant in the broader isolate collection, also remained relevant because of their biofilm-associated persistence profile

(Mukhopadhyay et al., 2025). The selected isolate panel therefore captured a rational spread of representative high-risk organisms from the screened groundwater population rather than a random subset of culture positives.

The finding that all six selected isolates were resistant to ceftazidime, cefotaxime-clavulanic acid, meropenem, gentamicin, and ciprofloxacin is epidemiologically important. Even though this article does not attempt to provide a full surveillance-style resistance analysis of the broader isolate pool, the presence of a representative groundwater-associated isolate panel with this degree of resistance is enough to support concern regarding environmental persistence and potential exposure through domestic water use (Flores-Vargas et al., 2021).

Adequate DNA yield and purity were obtained from all selected isolates, 16S amplification was successful across the panel, and molecular confirmation supported the final identities of *Escherichia coli, Klebsiella pneumoniae, Klebsiella oxytoca,* and *Pseudomonas aeruginosa*. This strengthens the study by showing that the selected groundwater-associated high-risk isolates were not merely presumptive culture entities but taxonomically confirmed bacterial species (Pandey et al., 2014).

A 16S rRNA-based workflow confirms identity; it does not establish resistance genes, virulence determinants, plasmid content, or clonal relatedness. For that reason, the scientifically defensible conclusion is that groundwater in the study setting harbors phenotypically high-risk multidrug-resistant bacteria that were molecularly confirmed at the species level. Stronger mechanistic claims would require targeted resistance-gene and virulence-gene analysis or whole-genome sequencing (Sruthy et al., 2025).

These findings support a shift from narrow indicator-based groundwater surveillance toward a more risk-oriented framework that integrates phenotypic screening with molecular confirmation. Such an approach is more useful for identifying representative bacteria that may persist in groundwater systems and pose greater public-health significance than routine indicator counts alone.

5. CONCLUSION

The present 16S rRNA-based molecular study confirms that groundwater in Kozhikode contains a selected subset of phenotypically high-risk multidrug-resistant bacteria worthy of molecular follow-up. Six representative isolates prioritized through screening were successfully characterized by genomic DNA assessment, 16S rRNA gene amplification, and final molecular confirmation as *E. coli, K. pneumoniae, K. oxytoca,* and *P. aeruginosa*. These findings support the need for groundwater surveillance strategies that move beyond routine indicator bacteriology and incorporate targeted phenotypic screening plus molecular confirmation for high-risk environmental isolates.

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