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# Occurrence of *Klebsiella pneumoniae* in the soil samples of selected dairy farms in Thrissur, Kerala#

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**Abstract**

*Klebsiella pneumoniae is a Gram-negative bacterium commonly found in the environment, especially soil. This study aimed to explore the prevalence and molecular detection of K. pneumoniae in soil samples from 50 dairy farms in Thrissur, Kerala. A total of 50 soil samples were collected, processed and analysed using conventional culture techniques, followed by molecular confirmation through polymerase chain reaction (PCR) targeting the rpoB gene. Out of the 50 samples, 38 (76 %) tested positive for K. pneumoniae using cultural methods and 36 (94.73 %) were confirmed by PCR. Additionally, eight per cent of the isolates exhibited the hypermucoviscosity phenotype, a key indicator of heightened virulence. This study found an occurrence of K. pneumoniae in soil, with a detection rate of 72 per cent. The results suggest that soil in dairy farms could act as an environmental reservoir for K. pneumoniae, potentially impacting environmental, animal and human systems. Its increasing resistance to antimicrobials also poses a serious public health threat due to limited treatment options. This research emphasises the need for long-term surveillance and further investigation into the environmental factors promoting the persistence and virulence of K. pneumoniae in soil ecosystems.*

**Keywords:** *Klebsiella pneumoniae, soil reservoirs, hypervirulent strains*

*Klebsiella pneumoniae* is a Gram-negative bacterium that is commonly found in the environment, especially in soil ecosystems. Its occurrence in soil is of significant interest due to its role as a potential reservoir for virulent strains and its implications for both environmental and public health. This bacterium is part of the normal flora of the gastrointestinal tract of humans and animals; however, its survival and proliferation in soil highlight its adaptability to various ecological niches (Bagley, 1985).

The ability of bacterium to persist in soil can be attributed to several pivotal factors. Primarily, *K. pneumoniae* demonstrates a robust capacity to form biofilms, thereby conferring protection against desiccation and nutrient scarcity. Its polysaccharide capsule plays a critical role in augmenting its virulence and environmental resilience, enabling it to endure adverse conditions. Furthermore, soil enriched with organic matter, such as animal waste and decomposing plant

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material, provides a nutrient-dense substrate that fosters the proliferation of this opportunistic pathogen (Guerra *et al*., 2022).

*Klebsiella pneumoniae* in soil may also have implications for the transmission of infections, particularly in agricultural settings where animals, humans and crops may be in close contact. Limited research has been conducted on the occurrence of *K. pneumoniae* in the soil of Thrissur, especially in dairy farms. By studying its occurrence in the soil, researchers can gain insights into the environmental reservoirs of *K. pneumoniae* and assess the risks associated with its occurrence, especially concerning the hypervirulent strains.

## Materials and methods

### *Sample collection*

A total of 50 dairy farms were surveyed to obtain soil samples from each farm. Soil samples weighing approximately 100 g were collected from the dairy farm. Prior to collection, any surface debris at the sampling location was meticulously cleared, and the soil samples were collected from a depth ranging from one to six inches below the surface, utilising a sterile container. The site for the collection of soil samples was determined based on the areas with the highest frequency of interactions between animals and humans with the soil. Great care was taken to ensure that the samples were free of any extraneous materials such as stones and roots (Parul *et al*., 2014).

The aseptically collected samples were brought to the laboratory under refrigerated conditions and processed for analysis. All the samples were processed in the Quality Control Laboratory, Department of Veterinary Public Health, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, within 24 h of collection.

### *Isolation and identification of K. pneumoniae by culture methods*

The samples were initially pre-enriched, followed by streaking onto selective agar. All the samples collected were subjected to isolation and identification of *K. pneumoniae* (Kumar and Kumari, 2013) by conventional culture techniques with certain modifications. Initially, five grams of soil sample was transferred to 45 mL Tryptone Soy Broth (TSB) and incubated at 37°C for 24 h. The samples pre-enriched in TSB were primarily streaked onto MacConkey agar (MCA) and incubated at 37° C for 24 h. Characteristic large, pink mucoid colonies were selected for secondary selective streaking on Simmon's Citrate-Inositol agar (SCIA), which were further incubated at 37° C for 48 h. The colonies, which appeared to be large yellowish mucoid, were then selected for biochemical tests for further confirmation. The various biochemical tests used in the study included Gram's staining, motility test, oxidase test, catalase test, IMViC test and various sugar fermentation tests.

### *Hypermucoviscosity of the positive isolates*

The hypermucoviscosity phenotype is linked to higher virulence in *K. pneumoniae*. The string test was conducted to identify this phenotype. This entailed streaking *K. pneumoniae* isolates on MCA and then incubating them for 24 h at 37°C. A positive outcome was indicated by the development of a viscous string that was at least one centimetre long (Mohammed and Flayyih, 2018).

### *Molecular confirmation of K. p neumoniae isolates by PCR*

Genomic DNA extraction was performed using the snap-chill or boiling technique. Four to five colonies were picked from the pure culture plate and suspended in 100 µL of nuclease-free water in 1.5 mL Eppendorf tubes. The contents in the Eppendorf tubes were mixed well and boiled at 100°C for 15 min, followed by immediate chilling at -20°C for 15 min. The DNA-containing solution was then centrifuged at 12,000 rpm for 10 min. The supernatant was collected in a new Eppendorf tube and stored at -20°C for further use as template DNA (Singh *et al*., 2022).

The oligonucleotide primers were custom-synthesised and obtained in lyophilised form from Sigma-Aldrich Chemical in Bangalore. The specific forward and reverse primers utilised in this study are detailed in Table 1.

The polymerase chain reaction was executed using a 25 µL reaction mixture in an automated thermal cycler (BioRad T100 Thermal Cycler). In each polymerase chain reaction (PCR) tube, for 25 µL of reaction mixture, 12.5 µL prepared master mix, 3 µL of template DNA, 1 µL each of forward and reverse primer and 7,5 µL of nuclease-free water (NFW) were added. The PCR process began with an initial denaturation at 95°C for 5 min, performed as a single step. This was followed by 32 amplification cycles, each consisting of denaturation at 95°C for 45 sec, annealing at 58°C for 1 minute, and extension at 72°C for 1 minute. The reaction concluded with a final extension step

**Table 1.** Primers used for the identification of *K. pneumoniae rpo*B gene

| Gene | Primer | Primer sequences | Size (bp) | Reference |
|---|---|---|---|---|
| *rpo*B | F | 5'CAACGGTGTGGTTACTGACG3' | 108 | Bobbadi *et al.* (2019) |
| | R | 5'TCTACGAAGTGGCCGTTTTC3' | | |

at 72°C for 10 minutes.

The PCR products were detected by electrophoresis in 1.5 per cent gel in TBE electrophoresis buffer (1X). The gel was visualised, and the images were documented on a gel documentation system (Syngene, USA).

## Results and discussion

*Klebsiella pneumoniae* is widely distributed in nature, particularly inhabiting environments such as soil, water and plants, as well as various mucosal surfaces of humans and animals (Podschun and Ullmann, 1998)

A total of 38 isolates (76 %) of the 50 samples collected were positive for *K. pneumoniae* by culture techniques. The approach used in the present investigation was akin to the methodologies employed in the studies conducted by Iwu *et al.* (2020), Bobbadi *et al.* (2019), and Swetha *et al.* (2021), with minor adjustments. In this study, a secondary selective agar, SCIA, was utilised (Kregten *et al.*, 1984). The various biochemical tests used in the study are given in Table 2.

**Table. 2.** Biochemical tests for *K. pneumoniae*

| Biochemical tests | *K. pneumoniae* |
|---|---|
| Gram's staining | Negative |
| Motility | Negative |
| Oxidase | Negative |
| Catalase | Positive |
| Indole | Negative |
| Methyl red | Negative |
| Voges Proskauer | Positive |
| Citrate | Positive |
| Urease | Positive |
| Triple Sugar Iron (TSI) Agar | A/A |
| SUGAR FERMENTATION TESTS | |
| Inositol | + |
| Lactose | + |
| Sorbitol | + |
| Arabinose | + |
| Erythritol | - |

A study in Oklahoma by Kim *et al.* (2005) detected the occurrence of *K. pneumoniae* in 25 per cent of samples isolated from the farm environment, which is lower than the current study. A lower isolation rate of *K. pneumoniae* was obtained from the soil samples collected from soil (44.72 %) near river water, pond water and paddy fields in the study conducted by Swetha in 2021.

A total of four isolates (eight %) displayed positive results by string test for hypermucoviscosity. A

**Fig. 1.** *K. pneumoniae* on MacConkey Agar

**Fig. 2.** *K. pneumoniae* on Simmon's Citrate Inositol Agar

study conducted by Mai *et al*. (2023) reported a higher percentage, with 12.03 per cent of isolates displaying hypermucoviscosity. In the study by Podschun *et al*. (2001), the *K. pneumoniae* isolates from the environmental samples and the clinical samples resembled the expression of virulence factors. They studied the distribution of virulence factors that included fimbriae, siderophores and serum resistance properties and found that there was no significant difference between the occurrence of these virulence factors in *K. pneumoniae* from environmental samples and clinical samples. The finding highlighted the importance of monitoring environmental strains of *K. pneumoniae*, especially in agricultural settings, as they might contribute to the emergence of hypervirulent strains capable of causing outbreaks or infections in livestock farms.

The hypermucoviscosity strains of *K. pneumoniae* exhibit heightened virulence, contributing to their increased pathogenicity. The string test serves as a distinctive characteristic of hypermucoviscous *K. pneumoniae* strains, as it illustrates the bacteria's capacity to generate an excessive quantity of capsular polysaccharide, resulting in colonies with a "sticky" or mucoid appearance. This test is a cost-effective and straightforward method that does not require specialised equipment. Its rapid results enable surveillance for monitoring the prevalence of such strains.

Polymerase chain reaction was performed to confirm the isolates, and out of 38 culturally identified isolates from the soil, 36 (94.73 %) were confirmed as *K. pneumoniae*.

The overall occurrence of *K. pneumoniae* after molecular confirmation was observed in the soil of dairy farms at 72 per cent (36 isolates of 50 samples).

In a research study conducted in 2021 in the Thrissur district of Kerala, Swetha observed findings consistent with the current study, indicating that the prevalence of *K. pneumoniae* identified through PCR analysis was 89.02 per cent. Additionally, Bobbadi *et al.* (2021) reported a 90 per cent positive rate for the *rpo*B gene in *K. pneumoniae* isolates obtained from environmental samples in Andhra Pradesh using PCR testing.

The study conducted in Africa by Iwu *et al.* (2020) found that only 20 per cent of the soil samples collected from two district municipalities in South Africa contained *K. pneumoniae*, which is contrary to the current study. Swetha *et al.* (2022) had identified different rates of *K. pneumoniae* prevalence in various soil samples. The prevalence was 53.33 per cent from soil near pond water, 36.67 per cent from soil near river water, and 48.89 per cent in soil from paddy fields from Thrissur. The molecular confirmation of these rates was performed using molecular techniques that targeted the *rpo*B gene. The studies indicated the varying rates of occurrence of *K. pneumoniae* in different locations of soil in Thrissur. The study revealed that the environment acts as a reservoir for *K. pneumoniae*.

Thirty-eight isolates were initially identified as *K. pneumoniae* using phenotypic methods, but PCR analysis targeting the *rpo*B gene revealed that only 36 samples (94.73 %) were confirmed as *K. pneumoniae*. This discrepancy underscores the limitations of relying solely on phenotypic identification methods, which can lead to the misidentification of closely related species or strains; for example, *K. pneumoniae* is phylogenetically closely related to *K. variicola* and *K. quasipneumoniae* that are associated with agricultural niches, and they have similar biochemical expressions. This consequently may result in false positives in biochemical identification (Long *et al*., 2017).

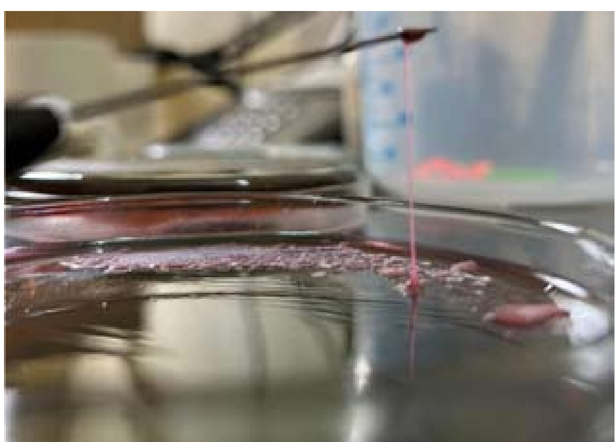

**Fig. 3.** Positive string test indicating hypermucoviscosity of *K. pneumoniae*

The detection of *K. pneumoniae* in 72 per cent of soil samples indicated that soil, particularly in dairy farms, may serve as a significant environmental reservoir for this bacterium. This observation suggests that *K. pneumoniae* is not confined to mucosal surfaces of human or animal sources but could persist in ecological environments such as soil, potentially contributing to its dissemination and survival. The widespread presence of *K. pneumoniae* also indicated that it has a broad environmental niche, enabling it to thrive in various habitats. (Bagley, 1985).

The detection of *K. pneumoniae* in soil samples from dairy farms may also be due to contamination from animal faeces, as the organism is known to colonise the gastrointestinal tracts of animals. This finding points out that agricultural practices, particularly those involving livestock, may play a role in the dissemination of *K. pneumoniae* in the environment. Soil could serve as a long-term reservoir for *K. pneumoniae*, contributing to its circulation between environmental, clinical, and animal settings.

## Conclusion

The findings of this study revealed a significant presence of *K. pneumoniae* in the soil of dairy farms, as evidenced by a 72 per cent detection rate using molecular methods. Notably, the prevalence of hypermucoviscous strains among the isolates emphasises the potential pathogenicity of these environmental bacteria, indicating their ability to thrive in ecological niches outside of clinical environments. Given the substantial public health implications of *K. pneumoniae* as a multidrug-resistant pathogen, it is imperative that future research focuses on elucidating the ecological dynamics contributing to its survival in soil. Investigating the environmental factors that influence its virulence, such as soil composition, moisture content, and microbial interactions, will yield valuable insights into its environmental ecology. Furthermore, ongoing surveillance of *K. pneumoniae* in agricultural settings has the potential to generate crucial data regarding its transmission dynamics and resistance mechanisms.

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## Conflict of interest

The authors declare that they have no conflict of interest.

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