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Isolation and identification of Klebsiella pneumoniae from raw milk samples from urban Peshawar: An observational study from Pakistan

Muhammad Jawad Ullah
Iqra National University
Usman Ali Shams
isuasbe@gmail.com

University of Health Sciences Lahore
Noor Ullah
Khyber Medical University
Faheema Yousafzai
Iqra National University
Muhammad Umar Nafees
University of Veterinary and Animal Sciences
Muhammad Ziad
Solid Waste Management Water and Sanitation Services
Muhammad Asif Zeb
Khyber Medical University
Fawad Inayat
Abdul Wali Khan University Mardan

Article

Keywords: Klebsiella pneumoniae, antibiotic resistance, biofilm formation, qnrS gene, qnrB gene, milk samples, urban Peshawar

Posted Date: April 27th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-9130001/v1

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Additional Declarations: No competing interests reported.

Abstract

Background

Klebsiella pneumoniae is a Gram-negative opportunistic pathogen known to cause both nosocomial and community-acquired infections. This study focused on the isolation, phenotypic characterization, antibiotic resistance patterns, and biofilm formation capabilities of *K. pneumoniae* isolated from milk samples collected in urban Peshawar, Pakistan. In addition, the molecular characterization of quinolone resistance genes (*qnrS* and *qnrB*) was performed to better understand the antibiotic resistance profile of these isolates.

Methods

Seventy-eight milk samples were collected from various dairy farms in urban Peshawar. These samples were cultured on MacConkey agar, and lactose-fermenting colonies were isolated and identified using Gram staining and biochemical tests. Antibiotic susceptibility was assessed using the disc diffusion method following CLSI guidelines. Biofilm formation was evaluated on brain-heart infusion (BHI) agar supplemented with Congo Red dye. Molecular characterization of antibiotic resistance genes was conducted through PCR, followed by gel electrophoresis for visualization of *qnrS* and *qnrB* genes.

Results

Out of the 101 bacterial isolates obtained, 77% were identified as *Klebsiella pneumoniae*. The isolates exhibited resistance to cephalosporin (32.67%) and meropenem (24.75%) but remained sensitive to ampicillin (7.92%) and tazobactam (8.91%). PCR analysis revealed that 58% of the isolates carried the *qnrS* gene, while 46% were positive for the *qnrB* gene. The formation of biofilms was confirmed by the presence of black crystalline colonies on BHI agar.

Conclusions

This study highlights the prevalence of antibiotic-resistant *K. pneumoniae* in milk samples from urban Peshawar, emphasizing the public health risks associated with the consumption of contaminated dairy products. The detection of *qnrS* and *qnrB* genes and the ability of the isolates to form biofilms underscore the potential for these strains to persist in the environment and resist treatment with antibiotics. These findings call for stricter hygiene protocols in dairy production and further molecular surveillance to monitor emerging antibiotic resistance in bacterial populations.

Introduction

Klebsiella pneumoniae is a gram-negative rods that belongs to the Enterobacteriaceae family [1]. This widely distributed bacterium has both clinical and ecological importance [2]. Although it is a commensal bacterium inside the human gastrointestinal system, it has gained recognition as a prominent opportunistic pathogen linked to nosocomial and community-acquired infections. In addition to its clinical significance, *K. pneumoniae* has been detected in several ecological habitats, such as water, soil, and food, underscoring its capacity for adaptation and potential as a reservoir. The initial description of *K. pneumoniae* was published in 1882 by Carl Friedlander. He isolated the bacterium from the lungs of individuals who died as a result of complications related to pneumonia, and he determined that the organism was an encapsulated bacillus [1].

As a fundamental nutritional resource, milk plays a crucial role in connecting the environment to human consumption. Globally, the presence of *K. pneumoniae* in milk has been shown in multiple research studies [3]. The presence of contamination in milk not only gives rise to apprehensions about the safety of consuming milk but also emphasizes the possible function of milk as a medium for transmitting *K. pneumoniae* to the community [4].

In the urban context of Peshawar, Pakistan, where the production and distribution of dairy products play a crucial role in everyday life, it becomes essential to gain knowledge about the occurrence, observable traits, and resistance to antimicrobial agents of *K. pneumoniae* in milk samples [5]. The acquisition of this information may provide valuable insights for public health initiatives that seek to mitigate the prevalence of milkborne illnesses and enhance our comprehension of the epidemiological patterns of this bacterium within urban settings [6].

The *qnrS* and *qnrB* genes are responsible for bacteria becoming resistant to quinolone antibiotics. These genes are found in different types of bacteria, such as *E. coli, K. pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. In some bacteria, such as Enterobacteriaceae, the *qnr* gene provides moderate resistance to fluoroquinolone antibiotics. These genes are often found on identical plasmids as extended-spectrum beta-lactamase (ESBL) genes associated with antibiotic resistance. Quinolones and beta-lactams are two types of antibiotics used to treat various infections caused by certain types of bacteria [7].

This research aimed to phenotypically determine and describe *K. pneumoniae* strains isolated from raw milk samples collected in urban Peshawar. This study aimed to gain insight into the variety and possible pathogenicity of *K. pneumoniae* strains found in this crucial food source by thoroughly examining their phenotypic characteristics, including colony shape, metabolic profiles, and antibiotic susceptibility patterns.

Materials and methods

Study Setting and Ethical Approval

The research was conducted at the Department of Allied Health Sciences at Iqra National University Peshawar and the study was ethically approved by the Ethical Research Committee of Iqra National University, Peshawar, Pakistan (Ref No: INU/AHS/20697-23).

Sample Collection and Processing

Seventy-eight unpasteurized milk samples were collected from dairy farms in urban Peshawar using sterile Falcon tubes. These samples were transported in liquid form to the microbiology laboratory. The samples were cultured on MacConkey agar to encourage the growth of Gram-negative bacteria, particularly Klebsiella species. Each sample was homogenized in peptone buffer solution and serially diluted to 10^{-7}. Dilutions at 10^{-5}, 10^{-6}, and 10^{-7} were spread onto MacConkey agar plates, with 100 µL of the sample used for each dilution. The plates were incubated at 37°C overnight. Lactose-fermenting colonies were isolated and further subcultured on nutrient agar to obtain pure cultures.

Isolation and Purification of Klebsiella Species

After incubation at 37°C for 24 hours, isolated lactose-fermenting colonies were examined. Purification was carried out by growing the isolates in nutrient broth, followed by Gram staining and microscopic examination to confirm the presence of Klebsiella species. Selected colonies were re-cultured in nutrient broth for further testing and confirmation.

Biochemical Identification

The identification of Klebsiella species was performed using biochemical tests, including Simmon's citrate, oxidase, indole, and the Triple Sugar Iron (TSI) tests. Gram staining was also carried out to verify bacterial morphology.

Antibiotic Susceptibility Testing

The antibiotic susceptibility of the isolates was assessed using the disc diffusion method on Mueller-Hinton agar (MHA). Sterile cotton swabs were dipped in bacterial suspensions and spread evenly on MHA plates. After drying, antibiotic discs (such as cephalosporin and ciprofloxacin) were placed on the plates, which were incubated at 37°C overnight. Zones of inhibition around the antibiotic discs were measured to determine bacterial resistance patterns.

Biofilm Formation Assay

Biofilm formation, a virulence factor of Klebsiella species, was evaluated using brain-heart infusion (BHI) agar modified with 0.8 g/L Congo red dye. The dye was added after autoclaving and cooling the medium.

The inoculated plates were incubated, and biofilm formation was indicated by the appearance of black crystalline colonies onto the modified BHI agar.

Molecular Analysis of Klebsiella Genes

Molecular analysis focused on identifying Klebsiella pneumoniae and detecting *qnrS* and *qnrB* genes. DNA was extracted using the method described by Lu *et al*., where bacterial cells grown overnight in nutrient broth were centrifuged to collect pellets. The cells were lysed using lysis buffer that contains 1% Triton-X 100, 100mM Tris (pH 8.0) and 1mM of EDTA to release DNA. To obtain a purified DNA, proteinase K treatment for protein degradation was carried out. Cells were centrifuged at 13000 rpm for 5 minutes and resuspended in 100 µl of lysis buffer and boiled for 30 minutes. The solution was again centrifuged at maximum rpm i.e. 13000 rpm for 5 minutes. Supernatant was transferred into a clean tube and stored at -20 ºC for further use [8].

PCR amplification was performed to detect *qnrS* and *qnrB* genes. Each reaction mixture contained

primers, PCR master mix, DNA template, and nuclease-free water. The optimized PCR conditions for the *qnrB* gene included an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation (95°C for 1 minute), annealing (54°C for 1 minute), and extension (72°C for 1 minute), with a final extension at 72°C for 7 minutes. For the *qnrS* gene, annealing was performed at 56°C, with the other conditions remaining the same.

Table 1
Shows the primers used for amplification of genes

Target Gene	Primer sequences (5’-3’)	Gene Product Size (bp)
qnrS_417_bp_fw	ACGACATTCGTCAACTGCAA	417
qnrS_417_bp_rev	TAAATTGGCACCCTGTAGGC	
qnrB_469_bp_fw	GATCGTGAAAGCCAGAAAGG	469
qnrB_469_bp_rev	ACGATGCCTGGTAGTTGTCC	

Gel Electrophoresis and Visualization

The PCR products were analyzed using 1.5% agarose gel electrophoresis in 1× TAE buffer. The gel was prepared by dissolving 1.2 g of agarose in 80 mL TAE buffer, followed by cooling and the addition of ethidium bromide. The samples were mixed with 6X loading dye and loaded into wells in the gel. The gel was run at 100 V for 20–25 minutes, and the DNA bands were visualized using a gel documentation system.

Results

Growth on MacConkey agar media and phenotypic identification

A total of 101 isolates were obtained from 78 milk samples and initially cultured on MacConkey agar. Isolates producing mucoid, pinkish colonies, indicative of lactose fermentation, were subsequently subcultured on fresh MacConkey agar to achieve pure isolates for further analysis. These purified colonies underwent a series of biochemical assays. Among the isolates, 77% were confirmed as *Klebsiella pneumoniae*. Gram staining of the subcultured isolates demonstrated that 89% were Gram-negative bacilli. Biochemical testing further supported the identification: both the oxidase and indole tests yielded negative results, consistent with *K. pneumoniae*. In contrast, the Simmon's citrate test was positive, confirming citrate utilization, while the Triple Sugar Iron (TSI) test produced a negative result, aligning with the metabolic profile of *K. pneumoniae*.

Antibiotic susceptibility results

An antibiotic susceptibility assay was performed to validate the identification of *Klebsiella pneumoniae*, following the Clinical and Laboratory Standards Institute (CLSI) guidelines. All 101 isolates underwent antimicrobial susceptibility testing. The highest resistance was observed against cephalosporin (n = 33; 32.67%), followed by meropenem (n = 25; 24.75%). In contrast, the lowest resistance rates were noted for ampicillin (n = 8; 7.92%) and tazobactam (n = 9; 8.91%). The isolates exhibited the highest sensitivity to tazobactam and ampicillin, with 85 isolates (84.15%) demonstrating susceptibility to these antibiotics.

Table 2
The antibiogram pattern for the antibiotics used according to CLSI guidelines.

Antibiotics (ug)	Sensitive	Resistant	Intermediate
Cefepime	79.2%	14.85%	5.94%
Ampicillin	84.5%	7.92%	7.92%
Meropenem	68.3%	24.75%	6.93%
Azithromycin	84.1%	8.91%	6.93%
Ceftazidime	80.1%	19.80%	4.95%
Gentamycin	80.1%	14.85%	-
Imipenem	69.30%	20.79%	9.90%
Ciprofloxacin	87.12%	12.87%	-
Cephalosporin	57.4%	32.67%	9.90%
Tazobactam	91.08%	8.91%	-

Molecular characterization results

Following phenotypic analysis, all isolates underwent molecular characterization to detect the presence of the *qnrS* and *qnrB* genes. Genomic DNA extraction was performed manually following the method described by Lu et al. The extracted DNA was observed on 1% agarose gel, and the results were visualized through gel electrophoresis. All *Klebsiella pneumoniae* isolates were screened for the *qnrB* gene, encoding quinolone resistance protein B (469 bp). Among the isolates, 46% tested positive for the *qnrB* gene, as illustrated in Fig. 1. Additionally, all 101 *K. pneumoniae* isolates were analyzed for the *qnrS* gene (417 bp), which encodes a quinolone-resistant NADH oxidoreductase. Approximately 58% of the isolates were positive for the *qnrS* gene, as depicted in Fig. 2.

Biofilm Formation

Biofilms are intricate microbial communities encased within a self-produced extracellular matrix, which provides structural support and enhances microbial survival and resistance. The objective of this study was to confirm biofilm formation on brain heart infusion (BHI) agar supplemented with Congo Red dye. The development of distinct black crystalline colonies on the agar surface confirmed the ability of the bacterial isolates to form organized biofilm structures, as illustrated in Fig. 3.

Discussion

This study provides a comprehensive analysis of the prevalence of *K. pneumoniae* and its antibiotic resistance based on 78 milk samples collected from various areas of Peshawar. A study by Hendra et al. (2022) revealed significant variation in bacterial flora among different regions compared to research conducted in other countries. Another study conducted in Malaysia showed a high prevalence of *K. pneumoniae* in raw milk samples, suggesting that geographical and environmental factors may significantly influence bacterial contamination [9].

In a review of MBLs in worldwide healthcare systems, Behzadi et al. highlighted their clinical importance in Enterobacteriaceae. Our investigation of clinical isolates with MBLs also revealed their prevalence and influence on antibiotic resistance in healthcare. Behzadi et al. emphasized the necessity for ongoing surveillance and powerful bioinformatics techniques to track and comprehend the spread of MBL.

According to Behzadi et al., the number of β-lactamase enzymes has increased significantly, with over 1800 such enzymes being discovered. This finding is consistent with our finding that clinical isolates have a high MBL incidence [10]. Haryani et al. (2007) reported regional variability in MBLs in Malaysia, suggesting that environmental and geographical factors are essential. Our results show regional MBL prevalence variations, supporting this finding. [11]. Our study and the review of Behzadi et al. emphasize molecular testing for MBLs. As noted by Cho SY et al. (2015) and validated by our findings, PCR and other molecular approaches are more trustworthy and accurate than biochemical testing. Resistance genes such as *qnrS* and *qnrB*, which were common in our study and global investigations, demonstrated this [11].

According to our study, *K. pneumoniae* strains produce biofilms, increasing their resistance. Nirwati et al. (2019) reported that *K. pneumoniae* biofilm development increases antibiotic resistance. According to Guerra et al. (2022), biofilm development helps clinical germs survive and resist biofilms. Compared to Haryani et al. (2007), who reported a reduced prevalence of MBLs despite positive Simmon citrate tests, regional testing outcomes vary, and standardized procedures are needed. Hussein and Al-Meani (2022) and our data show that MBL-producing strains are common in Iran and China, underlining the worldwide scope of this issue.

As part of our investigation, we conducted a thorough analysis of isolated colonies and employed many tests to determine the features of the *Klebsiella* strains. Interestingly, our results differed from those of the study conducted in Malaysia by Haryani et al. (2007), where most *K. pneumoniae* results were negative despite the good outcomes of the Simmon citrate test. This difference underscores the importance of considering regional characteristics and distinct testing procedures in various research studies [12].

The analysis unequivocally showed that the kind of sample significantly impacted the frequency of detecting *K. pneumoniae*. Research conducted in northwestern Iran and China also yielded comparable results [13]. The evaluation of antibiotic resistance genes in northwestern Iran is remarkable because it revealed a substantial presence of *K. pneumoniae* strains carrying the CTX, SHV, and TEM genes, highlighting the worldwide apprehension regarding antibiotic resistance.

The analysis revealed a substantial level of antibiotic resistance in *K. pneumoniae*. The observations revealed in our research on resistance to ampicillin and other antibiotics align with the global tendencies that have been observed in studies conducted in China, Iran, and Morocco [14, 15]The emergence of multidrug-resistant bacteria, which are resistant to commonly used antibiotics, raises concerns about the efficiency of current treatment regimens.

The depth of our work increased with the identification of the *qnrS* and *qnrB* genes, together with the molecular verification of *K. pneumoniae*. According to Cho SY et al. (2015), polymerase chain reaction (PCR) was found to be a superior and dependable method for genetic confirmation compared to traditional biochemical assays [16]. Consistent with a concerning antibiotic resistance pattern, our study revealed a notable prevalence of *qnrS* and *qnrB* resistance genes. Research conducted in Togo and Morocco revealed differences in the distribution of these genes among *Klebsiella* species worldwide, as demonstrated by Kim et al. (2010) [17].

In addition, in the present study, *K. pneumoniae* formed colonies of crystals, showing that they can form biofilms that are attributed to extra resistance in these strains [18, 19]. A study conducted by Nirwati et al. (2019) also revealed that most *K. pneumoniae* isolates were resistant to a wide range of antibiotics. Most of these strains were also shown to be biofilm producers of various capacities. Global efforts should be intensified to prevent the spread of multidrug-resistant bacteria and eliminate the hospital-born microbes that are causing a dramatic increase in mortality [19].

Conclusion

This study highlights the significant prevalence of *Klebsiella pneumoniae* in milk samples collected from urban Peshawar, underscoring the potential health risks associated with contaminated dairy products. The high resistance rates observed, particularly against cephalosporins and meropenem, raise concerns about the increasing antibiotic resistance of these isolates, which could complicate clinical treatment options. The detection of *qnrS* and *qnrB* genes further emphasizes the genetic basis for quinolone resistance within these strains, suggesting the need for continuous molecular surveillance. Additionally, the ability of these isolates to form biofilms not only enhances their survival in adverse environments but also contributes to their resistance to antibiotics, posing an even greater challenge to public health.

Our findings underscore the importance of implementing stringent hygiene protocols in dairy production and distribution to mitigate the transmission of resistant pathogens through food sources. Public health initiatives must focus on routine monitoring of antimicrobial resistance in bacterial populations associated with food products. Future research should also explore alternative therapeutic strategies and effective preventive measures to address the growing issue of multidrug-resistant *K. pneumoniae*.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethical Research Committee of Iqra National University, Peshawar, Pakistan (Ref No: INU/AHS/20697-23). Informed consent was not applicable, as the study involved raw milk samples and did not directly involve human or animal subjects.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

This research received no external funding.

Acknowledgements

The authors acknowledge the Department of Allied Health Sciences, Iqra National University, Peshawar, and the Centre of Biotechnology and Microbiology, University of Peshawar, for providing laboratory

facilities and technical support.

Author Contributions

- **Muhammad Jawad Ullah**: Conceptualization, methodology design, laboratory work, data curation, and drafting of the manuscript.
- **Noor Ullah**: Sample collection, laboratory analysis, and contribution to methods section.
- **Faheema Yousafzai**: Performed biochemical testing, biofilm assays, and assisted in data interpretation.
- **Muhammad Umar Nafees**: Contributed to microbiological analysis and assisted in PCR experiments.
- **Muhammad Ziad**: Data entry, statistical analysis, and preparation of tables/figures.
- **Muhammad Asif Zeb**: Assisted in molecular characterization and gel electrophoresis visualization.
- **Usman Ali Shams**: Contributed to literature review, background writing, and editing of the manuscript.
- **Fawad Inayat** (Corresponding Author): Supervision, project administration, manuscript critical review, editing, and final approval of the version to be submitted.

All authors read and approved the final manuscript.

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Figures

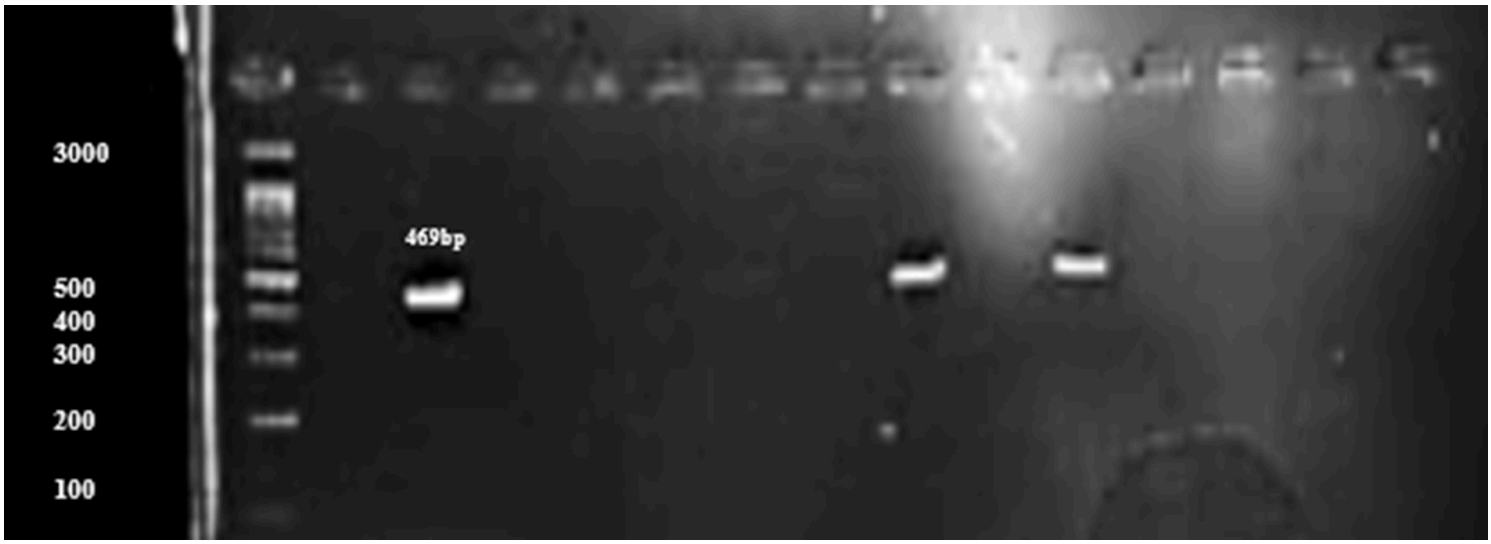


Figure 1

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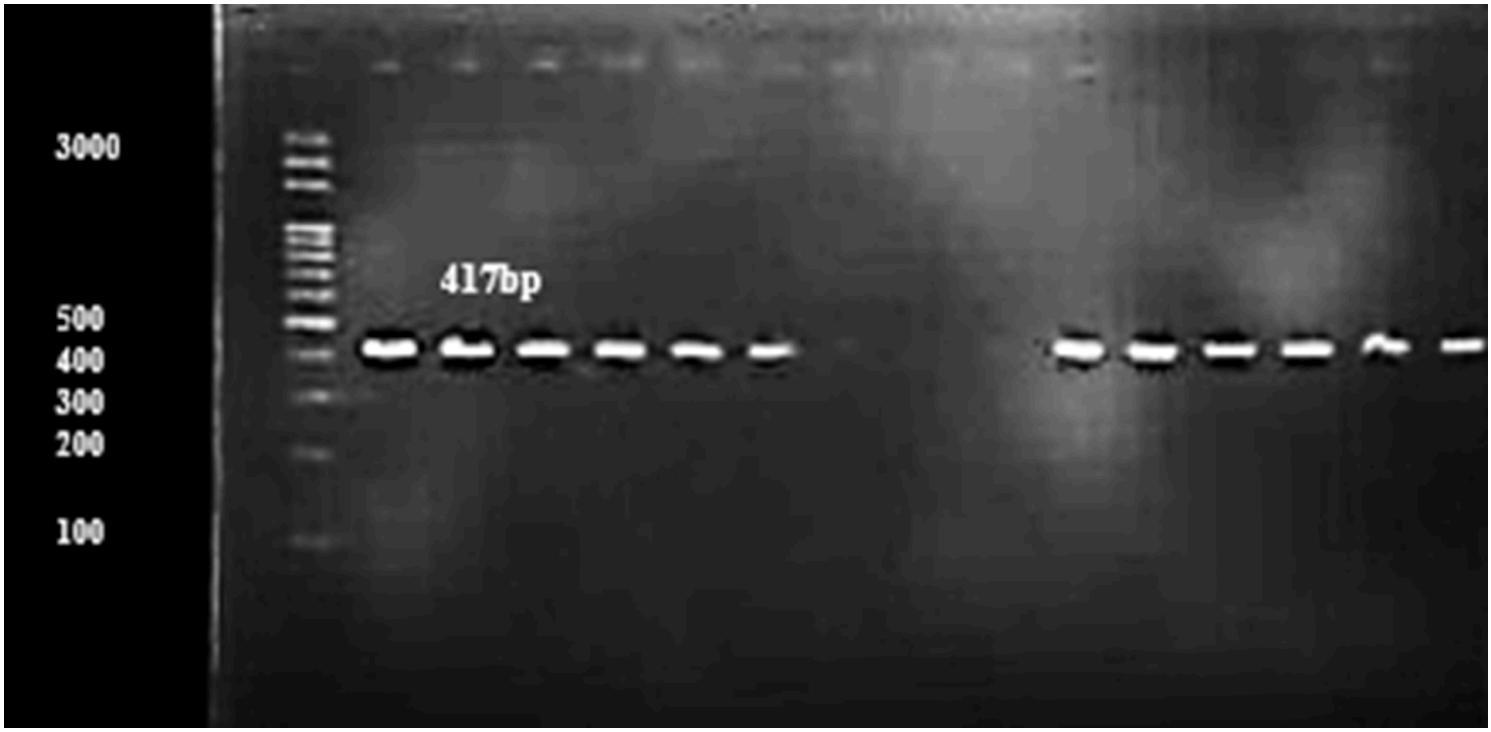


Figure 2

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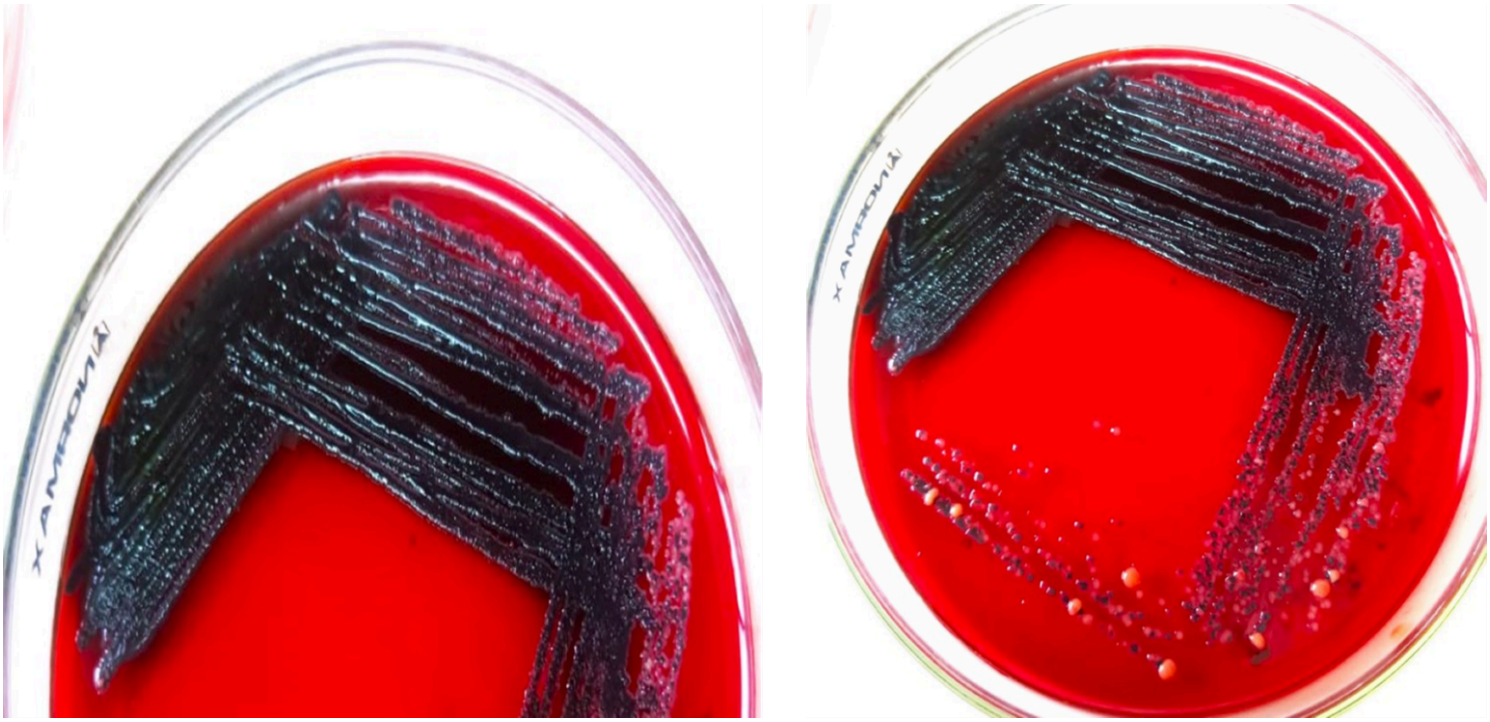


Figure 3

Formation of biofilms by *K. pneumoniae*.