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Biofilm Detection in Multidrug-Resistant *Klebsiella pneumoniae* via Phenotypic and Genotypic Methods in Diverse Clinical Isolates

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Abstract

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Background: *Klebsiella pneumoniae* is a virulent pathogen that is resistant to several classes of antibiotics and has been responsible for severe hospital- and community-acquired infections. Physiologically found within the human gut, it can induce opportunistic infections, including pneumonia, urinary tract infection, bacteremia, and wound/burn infection. It is possible to isolate it from several clinical sources.

Methods: 190 samples were collected from different health centers of Diyala Governorate between September 2022 and January 2023. These health centers were Baquba Teaching Hospital, Al-Batoul Teaching Hospital, Consulting clinic, Burn and Wound wards, and Educational Laboratories.

Results: Biochemical tests and the VITEK® 2 compact system identified *K. pneumoniae* in 26.3% (n = 50) of isolates, with 34.0% (n = 17) being multidrug-resistant (MDR). MDR *K. pneumoniae* showed resistance to CTX (60%), MEM (48%), AMP (100%), AMC (68%), ATM (46%), CAZ (70%), IPM (40%), AK (38%), LEV (30%), CIP (38%), and SXT (60%). All 17 MDR isolates formed biofilms (100%) via the Microtiter Plate Method, with 76.48% (n = 13) being moderate and 23.52% (n = 4) strong producers. Molecular detection revealed 100% positivity for *luxS* and *mrkD* genes, and 41.17% (n = 7) for *traT*.

Conclusion: MDR *K. pneumoniae* with robust biofilm production, virulence genes, and high-level antimicrobial resistance creates significant infection control issues. Clonal hospital spread is indicated by the genetic similarity, and further epidemiologic studies are necessary to fully understand the strain behavior.

Keywords:
MDR, *Klebsiella pneumoniae*,
Virulence Factors Genes,
Biofilm

Editorial Note:
The editorial office provided
significant language and
presentation support to the
authors of this article to
improve its clarity.



Introduction

Klebsiella pneumoniae is a Gram-negative bacterium that is further categorized under the Enterobacteriaceae family. It is a capsulated, non-motile, facultative anaerobe, lactose-fermenting organism [1,2]. Among all the types of *Klebsiella*, *Klebsiella pneumoniae* is of the utmost significance. It is a normal resident of the human intestine and can cause many persistent infections such as urinary tract infections, Bacteremia, Purulent Liver abscess, wound infection, burns, and diarrhea. Therefore, Isolation and study of this bacterium are essential, as infants, older adults, and immunocompromised individuals are highly susceptible. However, the bacterium is also responsible for a growing number of community-acquired infections [1,3]. The mucous surfaces of the digestive system and pharynx are usually colonized in humans by these bacteria, and they can show high degrees of virulence and antibiotic resistance, where it is multi-resistant, meaning that it is resistant to more than one type or more than one group of antibiotics, and it is called Multidrug Resistance (MDR) [4,5]. The *Klebsiella pneumoniae* bacteria, which are highly virulent organisms, have the ability to form biofilms that enable them to cause chronic infections. This biofilm protects the bacteria from being phagocytosed and from the humoral and cellular immune responses, thus leading to persistent inflammation [6]. Biofilm formation has been documented in *K. pneumoniae* in 1988 [7]. *Klebsiella pneumoniae* is capable of biofilm formation: a phenomenon whereby cells adhere to and aggregate with other cells with the help of a matrix of polysaccharides, proteins, and DNA. These biofilms are of clinical importance on catheters and implanted devices and are known to colonize bodily tracts and cause severe infections in immunocompromised patients [8]. These bacteria often colonize hospital patients, complicating treatment and contributing to healthcare-associated infections, and biofilms are multiple or Monomicrobial formations that cause long-term infections that are problematic to treat and remove from the body. Most infections occur in hospitals due to contamination of medical equipment such as catheters and ventilators, which are coated with cellular components hosted on-site [9]. Genes involved in biofilm formation include *mrkD*, *mrkA*, *fimH*, and *fimA*, which enable adhesion [10]. One of the biofilm genes is the *luxS* gene, which belongs to QS quorum-sensing genes, and facilitates the production of biofilm. The transfer of genetic material between bacterial cells thus increases the resistance of bacteria to antibiotics [11]. The *traT* gene is another biofilm-producing gene in MDR *Klebsiella pneumoniae* that helps in conjugation and complement inhibition [12].

This study aims to analyze the characteristics of *K. pneumoniae* from varied clinical origins, in terms of appearance and genetic makeup, and explore the diversity of biofilm genes, such as *luxS*, *mrkD*, and *traT*, using molecular methods.

Methods

Sample Collection and Identification

190 samples were collected from different health institutions in Diyala Governorate, including Baquba Teaching Hospital, Al-Batoul Teaching Hospital, the consulting clinic, Burn and Wound wards, and Educational Laboratories for the period from September 2022 to January 2023. These samples were distributed between 80 Urine samples for people suffering from urinary tract infections and 45 Sputum samples for people with Respiratory infections, and 28 Wound samples were collected from the Wound wards, in addition to 22 Burn samples. Also, 15 Blood samples were collected from inpatients in Baquba General Hospital. All these samples were cultured on MacConkey Agar medium and Blood Agar medium, and then the samples were incubated at a temperature of 37°C for 24 hours. Bacteria were visually identified on MacConkey agar as lactose fermenters, then transferred to EMB medium for differentiation. HiCrome Agar, a selective medium, was used for easier isolation of *K. pneumoniae*, which appears light violet on this medium.

Several biochemical tests were performed on the collected samples, and the tests included the oxidase test, catalase test, urease test, indole test, and methyl red test. For final confirmation, Biochemical tests are embedded in the VITEK 2 compact system (BioMérieux / USA).

Antimicrobial Susceptibility Test

Antibiotics used included: Meropenem (MEM), Ampicillin (AMP), Ceftazidime (CAZ), Amoxicillin + Clavulanic acid (AMC), Aztreonam (ATM), Cefotaxime (CTX), Amikacin (AK), Ciprofloxacin (CIP), Levofloxacin (LEV), Imipenem (IPM), Azithromycin (AZM), and Trimethoprim-sulfamethoxazole (SXT). Antimicrobial susceptibility testing was performed using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar, in accordance with the Clinical and Laboratory Standards Institute (CLSI, 2020; M100) guidelines. The inhibition-zone diameters were measured and interpreted according to CLSI breakpoints.

Primers name	Sequence (5' - 3')	Annealing Temp °C	Product Size(bp)	References
<i>luxS-F</i>	GCCGTTGTTAGATAGTTTCACAG	53	475	Shadkam <i>et al.</i> , [20]
<i>luxS-R</i>	CAGTTCGTCGTTGCTGTTGATG			
<i>mrkD-F</i>	CCACCAACTATTCCCTCGAA	43	226	El Fertas-Aissani <i>et al.</i> , [24]
<i>mrkD-R</i>	ATGGAACCCACATCGACATT			
<i>traT-F</i>	GGTGTGGTGCATGAGCACAG	55	288	El Fertas- Aissani <i>et al.</i> , [24]
<i>traT-R</i>	CACGGTTCAGCCATCCCTGAG			

Table 1: Primers used for PCR amplification of biofilm genes in *Klebsiella pneumoniae*.

Phenotypic Detection of biofilm

Using the method described by Lamichhane *et al.*, [13], the microtiter plate technique was employed to assess biofilm formation:

1. The bacterial isolates were incubated on Nutrient agar at 37°C for a 24-hour period.
2. 2-4 colonies were transferred to the Nutrient broth medium supplemented with sucrose, and the turbidity was compared with the turbidity of the McFarland standard solution.
3. In a micropipette, 200 µl of bacterial stock for all isolates was transferred to a 96-well polystyrene plate, three replicates of each isolate were made, and nutrient broth was added to several wells and left unvaccinated, and negative control wells were considered. After that, the plate was covered and incubated for 24 hours at 37°C.
4. The cover was removed from the plate, and the contents of the wells were gently emptied from the liquid cultures, and the non-adhering cells were removed by washing the wells 2-4 times with distilled water.
5. For fixing the cells on the wells, Methanol 200 µl was used for a period of 10 minutes.
6. The plate was emptied of alcohol, and the cell membranes were then dyed by adding 200 µl of Crystal violet dye for 15 minutes.
7. Distilled water was used two to three times to strip excess dye, and then 200 µl of 95% Ethanol was introduced.

An ELISA reader measured relative absorption at 630 nm. Implant-associated wells were compared to controls, and the results were categorized according to the absorption strength: high, moderate, and non-biofilm forming. Absorbency classification is: OD ≤ OD_c, without biofilm; OD_c < OD ≤ 2OD_c, moderate biofilm; 2OD_c < OD, strong or high biofilm forming. "OD" is the mean optical density of the isolate; "OD_c" is the mean optical density of the control.

Molecular study of *Klebsiella pneumoniae*

DNA Extraction

DNA was extracted from bacterial cells growing on brain heart infusion broth, where these cells were grown on this medium 24 hours at a temperature of 37 °C, and the extraction process was carried out according to the ABIO pure Extraction protocol. DNA

extract's concentration and purity were assessed by measuring absorbance via a Quantus™ Fluorometer.

PCR detection of virulence-associated genes

Biofilm genes were detected via PCR, using gene-specific primers, from 17 of 50 multidrug-resistant isolates. Lyophilized primers, sourced from Macrogen Company, were reconstituted in nuclease-free water to yield a 100 pmol/µl stock. Solved 10 pmol/µl working solutions were also made by taking 10 µl of the -20°C stored cold stock and diluting it with 90 µl nuclease-free water. Table 1 presents the primer sequences for genes employed in the study.

Results

190 clinical samples were collected from 120 patients (63.15%) and non-inpatients (70 patients) (36.84%) from the health institutions of Diyala province, The samples included: Urine (80) samples, Sputum (45) samples, Wound swabs (28) samples, Burns swabs (22) samples and Blood samples (15) samples and the ages of patients ranged between (1-80) years, and these samples were plated on MacConkey agar and the medium of Blood Agar, where the results of the transplantation were that (121) samples by (63.70%) showed a clear bacterial growth on the culture medium and that (69) samples and by (36.31%) did not give any bacterial growth.

The results of bacterial culture on the medium of MacConkey according to the source of the sample, (Urine) out of a total of (80) samples appeared (57) positive for transplantation (71.3%), (Sputum) out of a total of (45) samples appeared (20) positive for transplantation by (44.4%), and (Wounds) out of a total of (28) samples appeared (19) positive for transplantation by (67.9%), (Burns) of a total of (22) samples appeared (15) positive for transplantation and by (68.2%), and (Blood) out of a total of (15) samples appeared (10) positive for transplantation by (66.6%).

The samples were distributed according to gender, 100 samples of females, where they were Urine 48 samples (60%), Sputum 25 samples (55.6%), Wounds 10 samples (35.7%), Burns 10 samples (45.5%), and Blood 7 samples (46.7%). As for males, 90 samples, among which there were Urine 32 samples (40%), Sputum 20 samples (44.4%), Wounds 18 samples (64.3%), Burns 12 samples (54.5%), and Blood 8 samples (53.3%).

Antibiotic Susceptibility Tests

Antimicrobial susceptibility testing was conducted for all 50 *K. pneumoniae* isolates obtained from various clinical sources. However, the resistance percentages presented below specifically correspond to the 17 multidrug-resistant (MDR) isolates identified among them, to maintain consistency with the data summarized in the Abstract. The sensitivity of *K. pneumoniae* isolates under study, which numbered 50, was studied, as they showed high resistance towards several antibiotics that are used in health institutions and are widely used to treat different types of infections. The inhibition zone diameters around the antibiotic discs were also measured, and the results were compared to the already established standard tables [14]. The results of the current study showed the sensitivity of *K. pneumoniae* isolates towards the group of β -lactam antibiotics, where the resistance to Ampicillin (AMP) was 100%, Amoxicillin + Clavulanic acid (AMC) 68%, Aztreonam (ATM) 46%, Imipenem (IPM) 40%, Ceftazidime (CAZ) 70%, Meropenem (MEM) 48%, and Cefotaxime (CTX) 60%. As for the percentage of isolates' resistance to Amikacin (AK), which belongs to the group of Aminoglycoside antibiotics, it was 38%, and the results of the resistance of *K. pneumoniae* to Quinolone antibiotics were 30% for Levofloxacin (LEV) and 38% for Ciprofloxacin (CIP). As for the Sulfonamide group, Trimethoprim-sulfamethoxazole (SXT) was 60%, and Azithromycin (AZM) was 40% [15]. The results of this study converge with the findings of El-Badawy *et al.*, [16], where the resistance rate to Levofloxacin was 38.59% and to Ciprofloxacin was 44.73%, respectively.

Biofilm detection by Micro-titer plate

The Microtiter Plate Method detected biofilm production in all 17 resistant *K. pneumoniae* isolates. Bacterial biofilm counts were determined via a microtiter plate assay and ELISA reader, yielding numerical absorbance at 630 nm. All isolates, as per the findings of current research work, were biofilm-producing by 100%. The absorbance (OD) values of the control wells were 0.03-0.04, and the biofilm-producing isolates were distributed into moderate producers, where 13 isolates out of 17 isolates were 76.48%, and 4 isolates were strong biofilm-producing isolates (23.52%). The classification of biofilms was based on the absorption values of the wells in which the isolates were distributed after the rate of the three readings for each isolation was extracted and compared with the absorbance (OD) values of the control wells, as shown in Table 2.

No. of isolates	Absorbency at 630 nm	Biofilm level compared to (ODc = 0.03-0.04) (2*ODc = 0.06-0.08)
13 (76.48%)	ODc < OD ≤ 2 x ODc	Moderate
4 (23.52%)	2 x ODc < OD	Strong

Table 2: Absorbency values and biofilm pattern by MTP method.

OD = Mean optical density reader average of Isolate

ODc = Mean optical density reader average of control

The results of the current study are consistent with some local studies, Al-Rubyai *et al.*, [17], where the study indicated that all isolates of *K. pneumoniae* bacteria are 100% biofilm producers, but in contrast with the current study, a study by Al-Timimi *et al.*, [18] found that among 50 isolates of *K. pneumoniae* bacteria, 46% were weak biofilm producers, 28% were moderate, 6% were strong, and 20% did not produce biofilms. Biofilm is an essential factor in the virulence of *K. pneumoniae*, which persists in infection due to its resistance to phagocytosis and immunity [6].

Molecular detection of biofilm genes of *K. pneumoniae*

All 17 isolates of *K. pneumoniae* (MDR) were subjected to molecular detection of some biofilm genes using the presets assigned to these genes and the polymerase chain reaction (PCR) technique; the genes detected are (*luxS*, *traT*, *mrkD*).

Detection of *luxS* gene

The results of the current study showed that all isolates were carriers of the *luxS* gene, the quorum-sensing gene (AI-2QS), as shown in Figure 1.

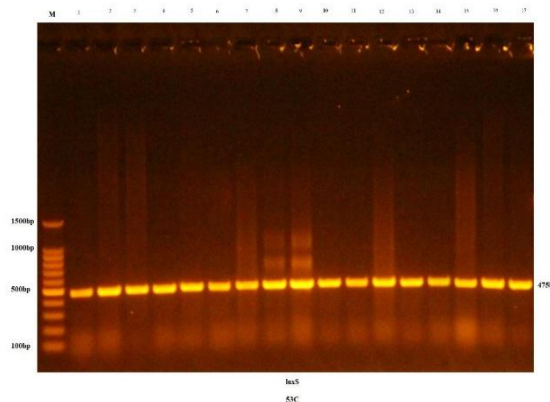


Figure 1: The amplification of *Klebsiella pneumoniae luxS* gene is shown using PCR, and the products are separated by 1.5% agarose gel electrophoresis and ethidium bromide (EthBr) staining. M: 100bp ladder marker.

The results of the current study are consistent with the results of a local study conducted by Mohamed *et al.*, [19]. All 17 isolates under study were 100% carriers of this gene, and these results are in agreement with the outcomes of Shadkam *et al.*, [20]. *K. pneumoniae* forms biofilms on different surfaces, where cell binding depends on the production of exopolysaccharides and adhesive proteins of type III. There are limited studies describing the relationship between Quorum sensing systems QS and biofilm formation in *K. pneumoniae* bacteria and this system (QS) type II was found in clinical isolation multi-drug resistance (MDR) on a

large scale as well as found that there is a relationship between the *luxS* gene and gene expression of LPS biosynthesis genes as well as the availability of nutrients and other environmental factors have an impact on the development of biofilms and the construction of polysaccharides and metabolic pathways and all these processes are coordinated within the network of Quorum sensing signals QS bacteria *K. pneumoniae* as well as gene characterization and other processes regulated by autoinducer-2 (AI-2) [21].

Detection of *mrkD* gene

mrkD, a biofilm gene, was detected (Figure 2). Its specific primer revealed the gene's presence in 100% of *K. pneumoniae* bacterial isolates.

The outcomes of this study are in consensus with those of Al-Saady *et al.*, [22], which showed the *mrkD* gene was present in 100% of the MDR *K. pneumoniae* isolates tested. The study of Compain *et al.*, [23] showed that all 65 isolates of *K. pneumoniae* bacteria are carriers of this gene by 100%. A study by El Fertas-Aissani *et al.*, [24] found *mrkD* in 96.3% of isolates, while current results align with Rastegar *et al.*, [25], which showed 138/146 *K. pneumoniae* isolates, or 94.5%, had the *mrkD* gene.

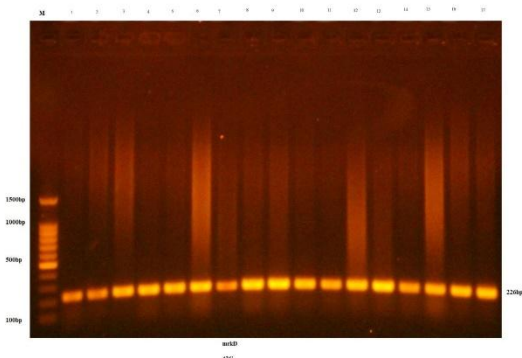


Figure 2: *mrkD* gene of primer amplification in *Klebsiella pneumoniae* fractionated on 1.5% electrophoresis of agarose gel stained with EthBr M: 100bp ladder marker.

Detection of *traT* gene

The outer membrane protein coding gene, *traT* gene, was the last biofilm gene detected in *K. pneumoniae* MDR isolates and is crucial for conjugation and complement inhibition [12]. The results of the PCR reaction of the gene *traT* using the primer assigned to it showed that out of 17 isolates of the bacteria *K. pneumoniae*, 7 isolates were carriers of this gene at a rate of 41.17%.

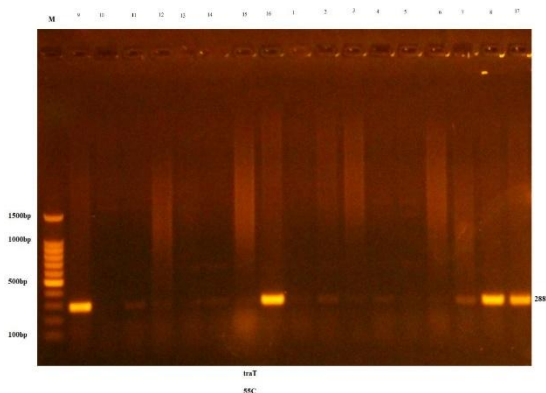


Figure 3: *traT* gene of primer amplification in *Klebsiella pneumoniae* fractionated on 1.5% electrophoresis of agarose gel stained with EthBr M: 100bp ladder marker.

The current results are consistent with the results of Mirzaie *et al.*, [26]. Where the presence of the *traT* gene in the isolates of the multi-drug-resistant *K. pneumoniae* bacteria MDR was 62%, it was found that 62 isolates out of 100 of the bacteria *K. pneumoniae* were carriers of this gene. The current results are inconsistent with those of El Fertas-Aissani *et al.*, [24], where the results showed that one isolate out of 54 isolates of *K. pneumoniae* was a carrier of the *traT* gene at 1.8% (Figure 3).

Discussion

This study demonstrates that MDR *Klebsiella pneumoniae* from Diyala hospitals combine extensive drug resistance with universal biofilm-forming capacity, amplifying their ability to persist and spread. Integrating genomic surveillance with strategies that disrupt biofilm formation could substantially reduce hospital transmission. Future research should evaluate anti-biofilm therapeutics and monitor genetic linkage between biofilm and resistance determinants.

The ability of bacteria to produce biofilm varies from one isolation to another due to several factors, such as the physical and chemical properties of *K. pneumoniae*, the physical interaction between components, the type of surface on which biofilms adhere, as well as temperature and pH [29].

The results of genetic detection of biofilm genes showed that all *K. pneumoniae* isolates under study carried the *luxS* gene and *mrkD* gene, while 7 isolates carried the *traT* gene.

In the current study, the results showed that the *K. pneumoniae* isolates exhibited resistance patterns commonly associated with β -lactamase-producing strains; however, specific phenotypic or genotypic tests for ESBL, MBL, and AmpC enzymes were not performed in this study, so these mechanisms should be verified in future research.

A high incidence of MDR *K. pneumoniae* is reported, which is resistant to many antimicrobials. All *K. pneumoniae* isolates were 100% biofilm producers and possessed virulence genes that made the treatment difficult, and the genes involved in biofilm formation can be transferred from one bacterium to another. The clonal dissemination of *K. pneumoniae* in all isolates was demonstrated by high genetic similarity in MDR strains. The *luxS* and *mrkD* genes were 100% molecularly detected through biofilm-producing genes. The *traT* gene was observed in 7 isolates, which means that measures should be taken. It is therefore important to conduct more epidemiological analysis of *K. pneumoniae* strains in Iraq.

Author Contributions

The authors isolated *Klebsiella pneumoniae* from various clinical sources from hospitalized and non-hospitalized patients and diagnosed them using traditional methods and diagnosis using VITEK2. The phenotypic detection of some virulence aspects was also carried out, the most significant of which is the production of biofilm. Genetic detection of biofilm genes (*luxS*, *mrkD*, *traT*) using their genetic primers.

Competing Interest

The authors declare that there are no competing interests in this manuscript.

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