The Relation Between Antibiotic Resistance and Biofilm Formation in *Klebsiella* Species Isolated from Human, Sheep and Goats at Matrouh Governorate, Egypt.

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ABSTRACT

Due to its antibiotic resistance and lack of effective therapy, *Klebsiella pneumoniae* has drawn attention from all around the world. Therefore, the current study was conducted at Matrouh Governorate to investigate the virulence and antibiotic resistance profiles of isolated *Klebsiella pneumoniae* from human (urinary system) and ovine (respiratory system) samples, as well as their relationship to biofilm formation. A total of 170 samples were obtained from private farms and the general hospital in Matrouh Governorate (90 ovine nasal swabs and 80 human urine samples). The samples were then subjected to bacterial isolation and identification using cultural methods, biochemical, PCR, antibiogram, phenotypic and genotypic detection of biofilm.. 57 (33.5%) *Klebsiella pneumoniae* isolates were found in 170 samples. The 16s-23SITS gene was detected in all 11 (100%) of the isolates that were examined. The 11 PCR-positive isolates were all deemed MDR because they demonstrated resistance to at least four different classes of antibiotics. Just one of the five human isolates exhibited resistance to five different antibiotic classes, whereas the other two isolates demonstrated resistance to seven different antibiotic classes and two isolates to six different antibiotic classes. Out of the six ovine isolates, four exhibited resistance to four different classes of antibiotics, whereas only two isolates demonstrated resistance to five separate classes. Among 11 *K. pneumoniae* isolates; seven (63.6%) were strong biofilm producers, including 4 (36.36%) from human isolates and 3 (27.27%) from ovine isolates, but 4 (36.36%) isolates were moderate biofilm producers, including one human isolate (9.09%) and 3 (27.27%) ovine isolates. All 11 isolates that exhibited phenotypic biofilm production were positive for *fim* A and *mrk* A genes. It was concluded that, there is a direct relation between antibiotic resistance and biofilm production, PCR is one of the most effective methods for identification of *Klebsiella pneumoniae isolates.*

1. INTRODUCTION

According to Zhang et al. (2021), *K. pneumoniae* is a pathogenic bacterium that is frequently linked to pneumonia in sheep and goats. In Egypt and Matrouh Governorate, small ruminants—especially sheep and goats—are economically significant due to their valuable meat, milk, wool, and hair products. According to Liu et al. (2023), pneumonia is a chronic disease that negatively impacts small ruminants' health, resulting in long-lasting effects and a general reduction in their quality of life. Superior sensitivity, specificity, and promptness have been shown by PCR. PCR provides accurate and sensitive results in 2–5 days

for testing urine and nasal specimens for *K. pneumoniae* infections (Xu et al., 2021).

Global attention is being drawn to *Klebsiella pneumoniae* because of the significant increase in opportunistic and severe infections that the organism causes, as well as the rise in antibiotic resistance that restricts effective treatment options and leads to a high mortality rate (27–37%), especially when multi-drug resistant (MDR) strains are involved (Cano et al., 2021 and Murray et al., 2022). Bacterial populations are protected by biofilms, which increase their ability to elude host defenses. Additionally, they limit the availability of certain antibiotics, which increases resistance in bacteria

(Singhai et al., 2012). Bacterial pathogens' formation of biofilms complicates the situation of bacterial resistance and lengthens the course of treatment (Wyres et al., 2020). The Tissue Culture Plate (TCP) is one of the detection techniques for biofilm formation (Sharma et al., 2022).

K. pneumoniae isolates use type 1 and type 3 pili to form biofilms, where the fimA and mrkA genes encode the main fimbrial components. These bacteria are significantly more resistant to antibacterial treatments than free-floating planktonic cells (Brusaferro et al., 2015). In this study, *Klebsiella pneumoniae* isolates from human (urinary system) and ovine (respiratory system) samples in the Matrouh Governorate will be examined for their virulence profile, antibiotic resistance, and relationship to biofilm formation.

2. MATERIAL AND METHODS

Ethical approved: The state ethics commission and Alexandria University's ethics committee in Egypt reviewed and authorized all specific animal and human operations (serial number 332 at date09/12 / 2024).

2.1. Samples:

A total of 170 samples, including 80 human urine samples from patients suffering from urinary tract infections (burning pain during urination , wanting to urinate in only few drops, pain or pressure in lower abdomen and turbid urine with bad odor), and 90 nasal swabs from sheep and goats(they are the most common animals in Matrouh) suffering from respiratory manifestations as difficult breathing , nasal discharges and pyrexia were collected from General Hospital and private

sheep and goat farms in Matrouh Governorate, respectively. The human urine samples were collected in sterile container while sheep and goat nasal swabs were immersed into tubes containing sterile nutrient broth.

2.2. Bacteriological cultivation:

As soon as feasible, the gathered samples were transported to the lab in an insulated ice box for bacteriological analysis. They were then streaked onto MacConkey's agar plates and cultured for 24 hours at 37˚C (Alcock et al., 2023).

2.3. Identification of *K. pneumoniae:*

The isolated bacterial strains were recognized by examining their morphological, cultural, and biochemical traits, along with their serological identification (all serological tests carried out in animal health research institute in Doki, Giza, Egypt.) as outlined by Boerlin et al. (2003)

2.4. Genotypic identification of isolated *K. pneumoniae* by PCR:

2.4.1. DNA extraction:

The boiling approach was used to extract DNA (Sambrook et al., 1989).

2.4.2. Using oligonucleotide primers, putative *K. pneumoniae* isolates can be identified and biofilm formation detected: **Tables 1 and 2** displayed the primer sequences and cycle conditions employed in this investigation.

2.4. 3. DNA Molecular weight marker

Pipetting up and down gently, the ladder was mixed. The necessary conductor was immediately added in 10 μl.

2. 4.4. Agarose gel electrophoresis (Sambrook et al., 1989).

Table (1): Oligonucleotide primers sequences used in this study:

Target genes	Genes	Sequence	Amplified product	Reference
K. pneumoniae species- specific gene	*16S-23S ITS*	ATTTGAAGAGGTTGCAAACGAT	130 bp	**Turton *et al*., 2010**
		TTCACTCTGAAGTTTTCTTGTGTTC		
Genes of biofilm formation	*FimA*	CGGACGGTACGCTGTATTTT	436 bp	**Alcántar-Curiel et al., 2013**
		GCTTCGGCGTTGTCTTTATC		
	MrkA	CGGTAAAGTTACCGACGTATCTTG TACTG	475 bp	
		GCTGTTAACCACACCGGTGGTAAC		

Table (2): Cycling conditions of the different primers used in this study:

Target	Gene	Initial denaturation	Denaturation	Annealing	Extension		No. of Cycles	Final extension	
K. pneumoniae species- specific gene	16S-23S ITS	94˚C/5 min	94˚C	55˚C 30 sec.	72˚C	30sec	35	72˚C	7 min.
Genes of biofilm formation	FimA			55˚C 40 sec.		45 sec.			10 min.
	mrkA			**55˚C 30 sec.**		**45sec**			10 min.

2.5. Antimicrobial susceptibility testing:

As indicated in **table (3),** the isolates were examined for susceptibility to eighteen antibiotic discs from seven distinct classes. The results were interpreted in accordance with the criteria issued by the Clinical and Laboratory Standards Institute (CLSI, 2019) based on the Kirby–Bauer (disc diffusion test) method utilizing a bacterial suspension with turbidity standards of 0.5 McFarland and Muller Hinton agar plates. MDR isolates were defined as those that exhibited resistance to a minimum of three distinct antibiotic classes (Magiorakos et al., 2012).

2.6 Phenotypic detection of biofilm formation by MDR *Klebsiella pneumoniae* isolates:

According to O'Toole & Kolter (1998), the tissue culture plate approach was used to identify biofilm development by *K. pneumoniae* isolates exhibiting MDR. Using a micro-ELISA auto-reader set to 620 nm, the optical density of the adherent-stained biofilm was determined. The strains were categorized as non, weak, moderate, and strong biofilm producers based on their optical densities.

Table (3): Antibiotic discs used in antibacterial sensitivity against *K. pneumoniae* isolates:

	Antibiotic	Class	Discs Code	Concenteration
1	Amoxicillin –clavulanic acid	Penicillins and B-lactam	Amc	**30 µg**
2	Ceftriaxone	Cephalosporin	Cro	**30 µg**
3	Cefepime		Fep	**30 µg**
4	Cefoxitin		Fox	**30 µg**
5	Ceftazidime		CAZ	**30 µg**
6	Cefotaxime		CTX	**30 µg**
7	Norfloxacin	Fluoroquniolone	NXN	**10 µg**
8	Ciprofloxacin		CIP	**5 µg**
9	Levofloxacin		LEV	**5 µg**
10	Ofloxacin		OFx	**5 µg**
11	Levofloxacin		LEV	**5 µg**
12	Gentamicin	Aminoglycosides	GEN	**10 µg**
13	Amikacin		AK	**30 µg**
14	Colistin	Polymyxins	Col	**10 µg**
15	Polymyxin- B		PXB	**300 µg**
16	Imipenem	Carbapenems	Imp	**10 µg**
17	Meropenem		MEM	**10 µg**
18	**Nitrofurantoin**	**Nitrofuron**	**FTN**	**300 µg**

3. RESULTS

Out of 170 samples (90 sheep and goat nasal swabs, 60 sheep samples, 30 goat samples, and 80 human urine samples), 57 (33.5%) *Klebsiella pneumoniae* isolates were found. These were divided into 40 isolates from human urine samples and 17 isolates (12 sheep and 5 goat) from sheep and goat nasal swabs as displayed in **table (4).** All 11 (5 from human urine samples and 6 from ovine nasal swabs) tested *Klebsiella pneumoniae* isolates (100%) showed that they tested positive for the 16s-23SITS gene (fig. 1). Since each of the 11 PCR-positive *K. pneumoniae* isolates showed resistance to at least four different antibiotic classes, they were all considered multidrug resistant. Just one of the five human isolates exhibited resistance to five different antibiotic classes, whereas the other two isolates demonstrated resistance to seven different antibiotic classes and two isolates to six different antibiotic classes. As seen in table (5), four isolates from six ovine isolates exhibited resistance to four different classes of antibiotics, whereas only two isolates demonstrated resistance to five separate classes. The percentages of resistance found in the estimates for multidrug-resistant *K. pneumoniae* isolates from five human urine samples were 72.2%, 66.6%, 88.8%, 72.2%, and 72.2% across 6, 6, 7, 5, and 7 classes respectively.as as shown in table (5). while the incidence of multidrug-resistant *K. pneumoniae* isolates within 6 ovine nasal swabs displayed resistance percentages of 50%, 44.4%, 44.4%, 44.4%, 44.4%, and 50%, and 5, 4, 4, 4, 4, 5 classes respectively.

Among 11 *K. pneumoniae* isolates; seven (63.6%) were strong biofilm producers, including 4 (36.36%) from human isolates and 3 (27.27%) from ovine isolates, but 4 (36.36%) isolates were moderate biofilm producers, including one human isolate (9.09%) and 3 (27.27%) ovine isolates. All 11 isolates that exhibited phenotypic biofilm production were positive for *fim* A and *mrk* A coding genes which responsible for biofilm formation as shown in fig (2, 3).

Table (4): The Prevalence of *Klebsiella pneumoniae* in human and ovine

Sample types	No. of isolates	Outcome		Total
		Positive	**Negative**	
Human urine samples	**Number**	40	40	**80**
	%	50 %	50 %	**100 %**
Nasal swabs from Sheep and goats	**Number**	17	73	**90**
	%	18.8 %	81.1 %	**100 %**
Total	**Number**	57	113	**170**
	%	**33.5**	**66.5**	**100**

%: according to total number of collected samples from each type.

Table (5). Results of antibiotic sensitivity pattern of suspected *K. pneumoniae* isolates

|	Source	Resistant antibiotics	Resistant Antibiotics classes	Resistance Percent %
1	Human urinary samples	AMC – CRO – FEP –FOX – CAZ – CIP- NXN – LEV – CTX – ATM- OFX – FTN – AK	6 Classes	72.2 %
2		AMC –FEP – FOX – GEN – CAZ – CRO – CTX – ATM – AK – IMP – MEM – FTN	6 Classes	66.6 %
3		AMC – CRO – CAZ – GEN – AK –FEP – ATM – FOX – CTX – FTN – OFX – CIP – IMP – MEM – NXN – LEV	7 Classes	88.8%
4		CRO – AMC – CAZ – FEP – FOX – ATM – FTN – CIP – CTX – LEV – OFX – NXN – MEM	5 Classes	72.2 %
5		AMC – CAZ – CTX – GEN – IMP – AK – CRO – MEM – FEP- ATM – FOX – FTN –NX	7 Classes	72.2 %
6	Nasal swabs from sheep and goats	AMC – CAZ – CTX – FEP – FTN – ATM – FOX – CRO – OFX	5 Classes	50 %
7		AMC – CAZ – CTX – FTN – ATM – FEP – FOX – CRO	4 Classes	44.4 %
8		AMC – CTX – CAZ – FOX – FEP–CRO – ATM – FTN – NXN – LEV	4 Classes	44.4 %
9		AMC- CAZ – CTX – CRO – FEP – ATM – FOX – FTN	4 Classes	44.4 %
10		AMC – CTX – CRO – CAZ – ATM – FOX – FEP – FTN	4 Classes	44.4 %
11		AMC- CTX – CRO – FEP – ATM – FOX –CAZ –AK – FTN	5 Classes	50 %
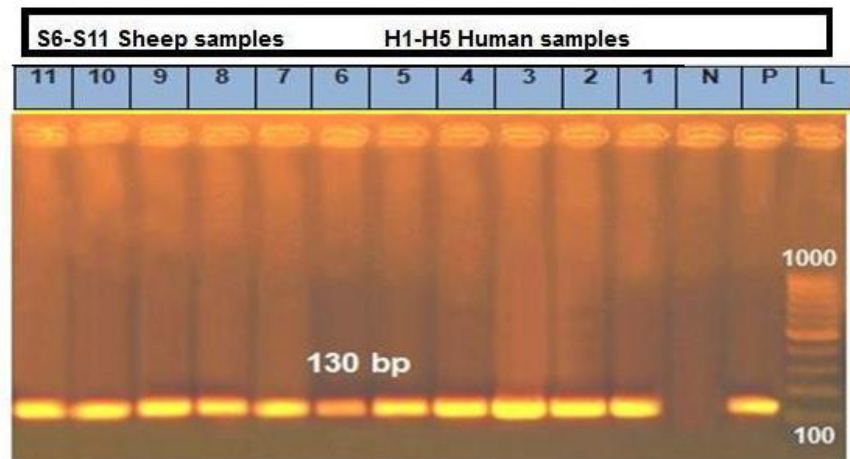
Fig (1): Amplification of the PCR result for the isolated *K. pneumoniae* using the 16S-23 SITS primer is demonstrated by ethidium bromide (0.1–0.5) µg/ml-stained agarose gel (1%) electrophoresis. Lane (L): 100 bp DNA molecular weight ladder, Positive isolates, 1–11,. The 16S-23 SITS coding gene's negative control is Lane (N), while its positive control is Lane (P) (specific band at 130 bp).
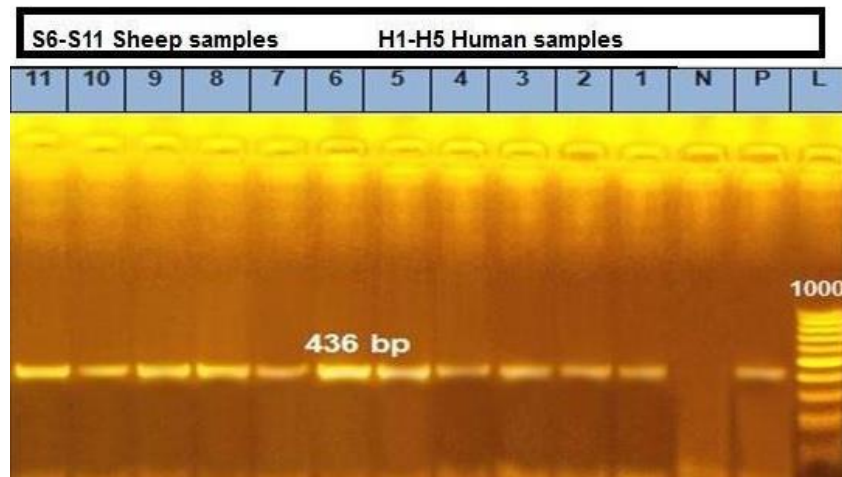

Fig (2): PCR product amplified with fimA is shown by ethidium bromide (0.1–0.5) µg/ml-stained agarose gel (1%) electrophoresis. A primer for *K. pneumoniae* fimA biofilm development genes unique to a certain species. Lanes (L): DNA molecular weight ladder (100 bp ladder); Lanes 1–11: positive isolates (specific band at 436 bp). A positive gene control is denoted by lane (P), and a negative gene control by lane (N).
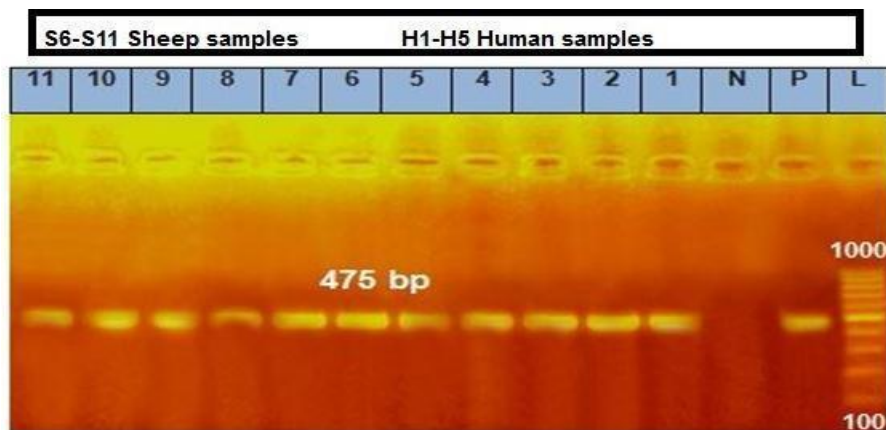

Fig (3): PCR product amplified with mrkA is shown by ethidium bromide (0.1–0.5) µg/ml-stained agarose gel (1%) electrophoresis. A primer for *K. pneumoniae* mrkA biofilm development genes unique to a certain species. Lanes (L): DNA molecular weight ladder (100 bp ladder); Lanes 1–11: positive isolates (specific band at 475 bp). A positive gene control is denoted by lane (P), and a negative gene control by lane (N).

4. DISCUSSION

The Gram-negative opportunistic pathogen *Klebsiella pneumoniae* is the cause of numerous nosocomial and community-acquired illnesses. In recent times, the escalating resistance of these microorganisms to antibiotics has emerged as a significant issue for the scientific community. Numerous scientific investigations and bacteriological inquiries have identified strains of *K. pneumoniae* from various animals and humans, (Ramadan, 2022; Aminul et al., 2021; and Gaballah et al., 2022).

Out of 170 samples (90 sheep and goat nasal swabs, 60 sheep samples, 30 goat samples, and 80 human urine samples), 57 (33.5%) *Klebsiella pneumoniae* isolates were found. These were divided into 40 isolates from human urine samples and 17 isolates (12 sheep and 5 goat) from sheep and goat nasal swabs as displayed in table (4). These findings like what found by El-Shehedi et al. (2017) who isolated *K. pneumoniae* from sheep and goat nasal swabs at a rate of 17%, which is lower than the findings of Claudia and Maria (2014), who observed that 70% of *K. pneumoniae* infections were detected in patients with urinary tract infections who were catheterized. On the other hand, these results were greater than those of Sukanta et al. (2018), who discovered *K. pneumoniae* in sheep at a rate of 3.84%, and Małgorzata et al. (2023), who reported a prevalence of up to 10% for *K. pneumoniae* nosocomial infections in humans.

Figure 1 illustrates that all 11 tested *Klebsiella pneumoniae* isolates (100%) tested positive for the 16s-23SITS gene (5 from human urine samples and 6 from ovine nasal swabs). These results are different from those reported by Arato et al. (2021), who discovered that 16S-23S ITS was present in about 20% of *K. pneumoniae* isolates. Table 5 shows the estimated resistance percentages for multidrug-resistant *K. pneumoniae* isolates from five human urine samples: 72.2%, 66.6%, 88.8%, 72.2%, and 72.2% across 6, 6, 7, 5, and 7 classes, respectively. These results align with those of Jing et al. (2023). The occurrence of multidrug-resistant *K. pneumoniae* isolates in six ovine nasal swabs, however, showed resistance percentages of 50%, 44.4%, 44.4%, 44.4%, and 50%, and 5, 4, 4, 4, 5, 5 classes, respectively, in contradiction to Moghadas et al. (2018). These findings align with those of Jing et al. (2023).

Seven (63.6%) of the 11 *K. pneumoniae* isolates produced strong biofilms, including four (36.36%) from human isolates and three (27.27%) from ovine isolates. Four (36.36%) isolates produced moderate biofilms, including one (9.09%) from a human isolate and three (27.27%) from an ovine isolate. According to our research, sputum isolates from sheep and goats had a substantially lower ability for biofilm formation than urine isolates from humans. Multidrug-resistant isolates generated biofilms at significantly higher rates than their non-multidrug-resistant counterparts, according to Sun et al. (2020) (Schurtz et al., 1994; Uddin et al., 2011; Ashwath et al., 2022).

These findings go counter to those of Ashwath et al. (2022), who found that biofilms formed in 97.1% of clinically isolated multidrug-resistant *K. pneumoniae* isolates from different samples and locales. This discrepancy may result from elements pertaining to regional differences, hospital prescription trends, and hygienic requirements. Additionally, Santiago et al. (2020) found that *K. pneumoniae* forms biofilms on inanimate surfaces including catheters and medical equipment as well as host tissues like the human respiratory system, urinary system, and gastrointestinal tract mucosal membranes. As seen in figs. (2,3), all 11 isolates that displayed phenotypic biofilm development tested positive for the genes that code for biofilm formation, fim A and mrk A. These results are consistent with those of PanelHan et al. (2023), who discovered that the entB, wabG, and ycfM genes were present in every isolate. Additionally, the mrkC, mrkD, fimH, and fimD genes were found in almost all strains (98.43%).

5. CONCLUSION

It was determined that PCR is one of the best techniques for identifying *Klebsiella pneumoniae* isolates and that there is a direct correlation between antibiotic resistance and biofilm formation.

Authors' Declarations

Authors' Contributions:
M.M.F: Contributed to conceptualization, formal analysis, investigation, supervision, resource provision, and drafting the original manuscript.

E.M.E: Responsible for data collection, formal analysis, project administration, resource provision, and manuscript review and editing.
S.A.K: Contributed to conceptualization, data curation, formal analysis, resource provision, supervision, and manuscript review and editing.

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