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BIOFILM AND ANTIMICROBIAL RESISTANCE OF *KLEBSIELLA PNEUMONIAE* ISOLATED FROM SHEEP

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ABSTRACT

Sheep plays a significant economic role in the breeding and production of raw materials, such as meat, milk, wool, and hair in Qena City, Egypt. K. pneumoniae is an opportunistic bacterium able to cause diseases in humans and animals. K. pneumoniae is mostly resistant to antibiotics due to the formation of biofilm that causes huge losses in sheep breeding farms in Qana City. This study aimed to find the best antibiotic to treat bacterial infections caused by K. penumoniae and the relationship between antimicrobial resistance and biofilm formation. 150 nasal swabs were collected from various locations in Qena City and classified into three groups (Fifty nasal swabs from sheep suffering from respiratory infestations, 50 samples from apparently clinical health sheep and 50 from sheep dead or slaughtered accidentally). The samples were plated onto MacConkey agar and then confirmed by PCR assay. Antibiotic sensitivity test of identified K. pneumoniae isolates were done. Diagnosis of biofilm formation by tissue culture plates among isolates showed multi-drug resistances and detection of virulence genes (Fim A and Mrk A) responsible for biofilm formation by PCR. It was found that out of 150 samples 104 isolates were identified biochemically as K. pneumoniae . Using PCR technique for 11 K. pneumoniae isolates out of 104 biochemically identified K. pneumoniae isolates were K. pneumoniae 16s-23SITS coding gene (species–specific gene). It was also found that Fim A and Mrk A were the virulence genes that were responsible for biofilm formation in this study.

Keywords: *K. pneumoniae*, susceptible, biofilm formation, PCR, Antimicrobial resistance.

INTRODUCTION

The bacteria are almost isolated from various infections in sheep. Particularly, K. pneumoniae is an important clinical pathogen that is highly associated with immunosuppression and secondary infection that consider the main cause of mortality and morbidity in sheep breeding Abdel-Halium *et al*. (2019) recorded that *K. pneumoniae* and *K. oxytoca* are highly related to small ruminants' pneumonic cases. Vuotto *et al*. (2017) farms. *K. pneumoniae* represent a more significant healthcare-associated pathogen. In animals housed under stress factors and unhygienic conditions, *K. pneumoniae* causes upper respiratory tract infection. Xu *et al*. (2018) recorded that *K. pneumoniae* is considered acted as the most important

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recorded that sheep and goats play a significant economic role because they are bred to obtain resources like meat, milk, wool, and hair production, especially in villages and desert areas. Scandorieiro *et al*. (2023) revealed that pneumonia is a persistent problem affecting the health of small ruminants, in particular causing long-term health effects and an overall decline in health. Including host physiology and immunology, various factors such as bacteria, viruses, parasites, environmental factors, and poor management Nirwati *et al*. (2019) Bisso Ndezo *et al*. (2021) revealed that the most important symptoms of pneumonia in sheep are rapid and shallow breathing, high body temperature, loss of appetite, severe shortness of breath, and purulent and mucous nasal discharge. Zhao *et al*. (2022) reported that *K. pneumoniae* is of great interest worldwide due to the dramatic rise in severe infections, as well as antibiotic resistance, along with biofilm formation, which presents major challenges in obtaining effective treatment therapies. Ghaith *et al*. (2020) recorded that biofilm is protected from phagocytosis, antibodies are neutralized, and cilia of epithelial cells are eliminated. In addition, biofilm bacteria are more resistant to antibacterial treatments than free-living planktonic cells. Singh *et al*. (2019) reported that *K. pneumoniae* produces biofilms through type 1 and type 3 filaments, in which genes known as *Fim A* and *Mrk A* code for the major fimbrial subunits, respectively. Wang *et al*. (2014) studied that an increasing number of studies have shown that *K. pneumoniae* causes a variety of animal diseases, including pneumonia, bacteremia, and septicemia. Franco *et al*. (2019) studied that sheep diseases represent a major limiting factor for such a huge industry where pneumonia and neonatal diarrhea represent the leading health problems. Zhao *et al*. (2020) recorded that due to the extensive use and abuse of antimicrobial agents for promoting growth and treating diseases in small ruminants, *K. pneumoniae* has become strongly resistant to most antibiotic agents. Yu *et al*. (2023) reported that the respiratory tract in sheep is frequently exposed to pathogenic bacteria, and most sheep remain healthy due to pulmonary defenses that effectively clear these organisms. If there is any defect in the mucociliary mechanism of the lung and its lung defensive function becomes weak or damage occurs in the lung tissue, it allows *K. pneumoniae* to enter the lower respiratory tract. This is where infections can begin. Saha *et al*. (2023) have reported that the most effective antibiotics for treating pneumonia in sheep and goats caused by *K pneumoniae* are ciprofloxacin, ceftriaxone, and oxytetracycline. Antibiotic resistance among Gram-positive and Gram-negative pathogens is a serious matter of concern and alternative strategic and therapeutic solutions must be available. Accordingly, this study aims to first isolate and classify *K. pneumoniae* from sheep with symptoms of respiratory distress, apparently healthy sheep, and dead or maliciously slaughtered sheep. Second, identifying the antibiotic resistance patterns and their abilities to form biofilms of *K. pneumoniae* isolates, and third, verifying the genes responsible for the antimicrobial and biofilm effects of *K. pneumoniae* isolates. Other putative virulence factors have been described, including plasmid-borne factors (*Fim A* and *Mrk A*)

MATERIALS AND METHODS

1. Isolation and identification of pathogenic bacteria:

One hundred and fifty nasal swabs were collected from sheep displaying respiratory infections, from apparently healthy sheep and sheep dead or slaughtered accidentally collected randomly from different locations in Qena City including (diseased, healthy and dead) 50 for each group. The samples were transferred into sterile plastic bags and transported in an ice box to the Department of Microbiology. They were cultured within 2 hours of collection on MacConkey agar plates (Oxoid, UK). The agar plates were incubated for 18–24 hours at 37°C then after

24 hours, if no growth had developed, the incubation period was extended for an additional 24 hours before a decision was made as a negative growth. To create pure cultures, isolates from growth-positive plates were sub-cultured at 37°C for 24 hours on nutrient agar plates (Oxoid, UK). Based on colony shape, and Gram staining, microorganisms were identified. Gram-negative bacteria were confirmed based on lactose fermentation on MacConkey Agar, catalase test, urease test, and IMViC reaction test (Alekish, 2015).

Table 1: Antimicrobial discs used in this study.

	Antibiotic	Discs Code	Concentration	Class
1	Amoxicillin – clavulanic acid	AMC	30 µg	Penicillins and B-lactam
2	Ceftriaxone	CRO	30 µg	Cephalosporin
3	Cefepime	FEP	30 µg	Cephalosporin
4	Cefoxitin	FOX	30 µg	Cephalosporin
5	Ceftazidime	CAZ	10 µg	Cephalosporin
6	Ciprofloxacin	CIP	35 µg	Fluoroquinolone
7	Norfloxacin	NXN	10 µg	Fluoroquniolone
8	Cefotaxime	CTX	30 µg	Cephalosporin
9	Aztreonam	ATM	30 µg	Monobactam
10	Ofloxacin	OFX	5 µg	Fluoroquniolone
11	Levofloxacin	LEV	5 µg g	Fluoroquniolone
12	Nitrofurantoin	FTN	300 µg	Nitrofuron
13	Amikacin	AK	30 µg	Aminoglycosides
14	Colistin	COL	10 µg	Polymyxins
15	Polymyxin- B	PXB	300 µg	Polymyxins
16	Imipenem	IMP	10 µg	Carbapenems
17	Meropenem	MEM	10 µg	Carbapenems
18	Gentamicin	GEN	10 µg	Aminoglycosides

2. Antibacterial sensitivity testing:

The isolated bacteria were subjected to sensitivity testing against eighteen antibacterial agents on Mueller-Hinton (MH) agar plates using the disk diffusion method (Reller *et al*., 2009,) and according to their sensitivity patterns to the antibiotic groups, these isolates were categorized into sensitive ,intermediate and resistant. Multidrug resistance (MDR) isolates are those isolates that are resistant or intermediate susceptible to more than three antimicrobial agents (Gurunathan *et al*., 2018).

The antibacterial agents and their quantities on the disks are listed in Table 1. The inhibition zones around the antibacterial agent disks were measured after 24 and 48 hours of incubation at 37°C, and the bacteria were classified as susceptible, intermediate, or resistant based on the criteria outlined in the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI., 2023).

The isolates showing resistance to at least 3 different antimicrobial classes were categorized as Multidrug Resistance (MDR).

The multiple antibiotic resistances (MAR) index for each isolate was calculated as following: Number of antimicrobials to which the isolate showed resistance / Number of antimicrobials to which the isolate had been tested. Whereas, the MAR index for each antimicrobial was calculated as follows: Total number of resistance detected / (total number of antimicrobials tested × Total number of isolates).

3. Polymerase Chain Reaction (PCR). Molecular characterization of isolated *K. pneumoniae*.

Random eleven isolates (4 isolates from (group 1) and 1 isolates from (group 2) and 6 isolates from (group 3) of biochemically identified *K. pneumoniae* isolates were subjected to detection of *K. pneumoniae* 16s-23SITS coding gene and biofilm producing genes *Fim A and Mrk A* by using PCR technique.

DNA extraction from *K.Pneumonia* isolates was performed using the QIAamp DNA Mini Kit (Qiagen, USA) according to QIAamp DNA mini kit instructions.

Oligonucleotide primers used for the detection of *K. pneumoniae* 16s-23SITS coding gene and detection of biofilm formation genes by suspected *K. pneumoniae* isolates have specific sequences and amplify a specific product as shown in **Table (2).**

PCR Products analysis

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of each uniplex PCR product and 30 µl of each multiplex PCR product were loaded in each gel slot. To gauge the sizes of the fragments, a generic 100 bp DNA ladder (Fermentas, Thermo) was employed. A gel documentation system (Alpha Innotech, Biometra) took pictures of the gel, and computer software was used to analyze the information (Sambrook, 1989).

Table 2: Oligonucleotide primers sequences Source: Metabion (Germany).

Target agent	Genes	Sequence	Amplified product bp	Ref.
Target agent *K.Pneumonia*	*16S-23S ITS*	ATTTGAAGAGGTTGCAAACGAT TTCACTCTGAAGTTTTCTTGTGTTCGCT-GTA-AAC-GAA-CTC-GCC-AC	130	Turton *et al*., 2010
	Fim A	CGGACGGTACGCTGTATTTT GCTTCGGCGTTGTCTTTATC	436	Alcántar-Curiel *et al*., 2018
	Mrk A	CGGTAAAGTTACCGACGTATCTTG TACTGGCTGTTAACCACACCGGTGGTAAC	475	

Table 3: PCR reaction

Component	Volume/reaction
Emerald Amp GT PCR mastermix (2x premix)	12.5 µl
PCR grade water	4.5 µl
Forward primer (20 pmol)	1 µl
Reverse primer (20 pmol)	1 µl
Template DNA	6 µl
Total	25 µl

Table 4: Cycling conditions of the primers during PCR

Target	Gene	Prim. denaturation	Sec. denaturation	Annealing	Extension		No. of cycles	Final extension	
K. Pneumoniae	16S-23S ITS	94˚C/5 min	94˚C	55˚C 30 sec.	72˚C	30 sec	35	72˚ C	7 min.
	Fim A			55˚C 40 sec.		45 sec.			10 min.
	Mrk A			55˚C 30 sec.		45 sec			10 min.

4. Detection of biofilm formation by *K. pneumoniae* isolates showed MDR using Tissue culture plate Method:

Detection of biofilm formation using the Tissue Culture Plate Method (TCP) (O'Toole and Kolter 1998).

1-Using a microplate of 96 wells (flat bottom plate) (ELISA plate). Aliquots of 200 µL of bacterial culture in TSB (107 CFU·mL−1) were added to each well, and TSB alone was used as the negative control. All sets were incubated at 37˚C for 24h.

2-Media were removed from the microplate by inversion; wells were washed four times with 0.2 mL of phosphate buffer saline (PBS pH 7.2) to remove free-floating planktonic cells.

3- Cells adhered to the microplate were stained with 200 µL of violet crystal solution (0.1%) for 30 min.

4- Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying at 40˚C for 15min.

5-Biofilm was quantified by adding 200 µL of 95% of ethanol to each well.

6-Optical density (OD) of stained adherent bacteria were determined with ELISA reader (model: sunrise R4, serial No: 610000079) at a wave length of 620 nm (OD 620 nm) after the adjustment to zero of the negative control.

7-Experiment was performed in triplicate and repeated three times, the data was then averaged and standard deviation was calculated. To compensate for background absorbance, OD readings from sterile medium, fixative and dye were averaged and subtracted from all test values. The mean OD value obtained from the media control well was deducted from all the test OD values. (Biofilm OD = OD1 –ODc). These OD values were considered as an index of bacteria adhering to the surface and forming biofilms.

8-The data obtained were used to classify the strains as non, weak, moderate and strong biofilm producers as shown in table (5)

Table 5: Equations used to classify the strains as non, weak, moderate and strong biofilm producers.

	Results	Equations
1	Non biofilm producer (0)	OD ≤ ODc
2	Weak biofilm producer (+ or 1)	ODc <OD ≤ 2×ODc
3	Moderate biofilm producer (++ or 2)	2×ODc <OD≤ 4×ODc
4	Strong biofilm producer (+++or3)	3×ODc <OD≤ 4×ODc

RESULTS

were 4 isolates sensitive to Amikacin(AK) (36.4%) but 11 isolates were highly resistant to Ceftazidime (CAZ), Amoxicillin –clavulanic acid (AMC), Ceftriaxone (CRO), Cefoxitin (FOX), Aztreonam (ATM), Cefotaxime (CTX), Cefepime (FEP) and Nitrofurantoin (FTN) (100% all of them) but 4 isolates were resistant to Meropenem (MEM) (36.4%) and 3 isolates were low resistant to Ciprofloxacin (CIP) and Imipenem (IMP), Levofloxacin (LEV) and Gentamycin (GEN) (27.3% all of them).

Results of antibiotic sensitivity among *K. pneumoniae* isolates from sheep samples.
Out of eleven PCR-positive *K. pneumoniae* isolates (Four isolates from diseased sheep with respiratory infections (SA1-SA4) and one isolate From apparently healthy Sheep (SH5) and 7 isolates from Sheep dead or slaughtered accidentally (SD6-SD11) were more susceptible to Polymyxin- B (PXB) and Colistin (COL) (100%), 8 isolates were sensitive to (IMP) (72.7%) then 7 isolates were sensitive to Ofloxacin (OFX) , Levofloxacin (LEV) and Gentamycin (GEN) (63.6 % all of them) whereas, they

Table 6: Prevalence of *K. Pneumoniae* in sheep.

Types of sheep Nasal swabs	Group	No. of samples	No. of isolates	% of group	% of the total No.
respiratory infected sheep	G 1	50	45	90	30
Apparently healthy Sheep	G 2	50	12	24	8
dead Sheep or accidentally slaughtered.	G 3	50	47	94	31.33
Total	3	150	104		69.33

Table 7: Antibiotic susceptibility results (percentage) of the *K. pneumoniae* isolates from Sheep (3 groups).

	Sheep suffering from respiratory infestations (No. 45)			Apparently healthy Sheep (No. 12)			Sheep dead or slaughtered accidentally (No. 47)		
	R	S	I	R	S	I	R	S	I
AMC	45(100%)	0(0%)	0(0%)	0(0%)	12(100%)	0(0%)	47(100%)	0(0%)	0(0%)
CRO	45(100%)	0(0%)	0(0%)	0(0%)	12(100%)	0(0%)	47(100%)	0(0%)	0(0%)
FEP	45(100%)	0(0%)	0(0%)	12(100%)	0(0%)	0(0%)	47(100%)	0(0%)	0(0%)
FOX	45(100%)	0(0%)	0(0%)	12(100%)	0(0%)	0(0%)	47(100%)	0(0%)	0(0%)
CAZ	45(100%)	0(0%)	0(0%)	0(0%)	12(100%)	0(0%)	47(100%)	0(0%)	0(0%)
CIP	3(6.7%)	40(89%)	2(4.4%)	0(0%)	12(100%)	0(0%)	5(10.6%)	42(89.4%)	0(0%)
NXN	4(8.9%)	33(73%)	8(17.8)	6(50%)	6(50%)	0(0%)	5(10.6%)	39(8٣%)	3(6.38%)
CTX	45(100%)	0(0%)	0(0%)	2(16.7%)	8(66.7%)	2(16.7%)	47(100%)	0(0%)	0(0%)
ATM	45(100%)	0(0%)	0(0%)	5(41.7%)	3(25%)	4(33.3%)	47(100%)	0(0%)	0(0%)
OFX	40(8.9%)	3(6.7%)	2(4.4%)	2(16.7%)	3(25%)	7(58.3%)	5(10.6%)	32(68%)	10(17%)
LEV	30(67%)	13(29%)	2(4.4%)	2(16.7%)	2(16.7%)	8(66.7%)	2(16.7%)	40(85%)	3(6.38%)
FTN	45(100%)	0(0%)	0(0%)	0(0%)	12(100%)	0(0%)	47(100%)	0(0%)	0(0%)
AK	5(11.1%)	28(62%)	12(27%)	0(0%)	6(50%)	6(50%)	7(14.9%)	37(7٩%)	7(1٥%)
GEN	3(6.7%)	42(93%)	0(0%)	3(25%)	2(16.7%)	7(58.3%)	6(12.8%)	33(70.2%)	8(17%)
COL	00(0%)	45(100%)	0(0%)	2(16.7%)	8(66.7%)	2(16.7%)	0(0%)	47 (100%)	0(0%)
PXB	00(0%)	45(100%)	0(0%)	0(0%)	6(50%)	6(50%)	0(0%)	47 (100%)	0(0%)
IMP	3(6.7%)	35(78%)	7(15.5%)	3(25%)	4 (33.3%)	5 (41.7%)	8(17.02%	35(74.5%)	5(10.6%)
MEM	4(8.9%)	41(0%)	4(8.9%)	3(25%)	6(52%)	3(25%)	8(17.02%	29(61.7%)	10(21.3%)

Table 8: Percentages of antibiotic sensitivity among *Klebsiella pneumoniae* isolated from Sheep samples.

Antibiotics				**The number of isolates of *K. pnemoniae***										
				Diseased				**Healthy**	**Dead**					
				SA 1	**SA 2**	**SA 3**	**SA 4**	**SH 5**	**SD 6**	**SD 7**	**SD 8**	**SD 9**	**SD 10**	**SD 11**
	R	**S**	**I**											
AMC	11 (100%)	0 (0%)	0 (0%)	R	R	R	R	R	R	R	R	R	R	R
CRO	11 (100%)	0 (0%)	0 (0%)	R	R	R	R	R	R	R	R	R	R	R
FEP	11 (100%)	0 (0%)	0 (0%)	R	R	R	R	R	R	R	R	R	R	R
FOX	11 (100%)	0 (0%)	0 (0%)	R	R	R	R	R	R	R	R	R	R	R
CAZ	11 (100%)	0 (0%)	0 (0%)	R	R	R	R	R	R	R	R	R	R	R
CIP	3 (27.3%)	6 (54.5 %)	2 (18.2%)	R	I	R	R	S	I	S	S	S	S	S
NXN	4 (36.4%)	5 (45.4 %)	2 (18.2%)	R	I	R	R	R	I	S	S	S	S	S
CTX	11 (100%)	0 (0%)	0 (0%)	R	R	R	R	R	R	R	R	R	R	R
ATM	11 (100%)	0 (0%)	0 (0%)	R	R	R	R	R	R	R	R	R	R	R
OFX	4 (36.4%)	7 (63.6 %)	0 (0%)	R	S	R	R	S	R	S	S	S	S	S
LEV	3 (27.3%)	7 (63.6 %)	1 (9.1%)	R	S	R	R	S	I	S	S	S	S	S
FTN	11 (100%)	0 (0%)	0 (0%)	R	R	R	R	R	R	R	R	R	R	R
AK	5 (45.5%)	4 (36.4%)	2(18.2%	R	R	R	S	R	S	S	I	S	I	R
GEN	3 (27.3%)	7 (63.6%)	1 (9.1%)	I	R	R	S	R	S	S	S	S	S	S
COL	0 (0%)	11 (100%)	0 (0%)	S	S	S	S	S	S	S	S	S	S	S
PXB	0 (0%)	11 (100%)	0 (0%)	S	S	S	S	S	S	S	S	S	S	S
IMP	3 (27.3%)	8 (72.7%)	0 (0%)	S	R	R	S	R	S	S	S	S	S	S
MEM	4(36.4%)	7 (63.6 %)	0 (0%)	S	R	R	R	R	S	S	S	S	S	S

S=Sensitive **R**= Resistant **I** = Intermediate

Results of molecular characterization of the isolated *K. pneumoniae*:

Results of amplification of *K. pneumoniae* 16s-23 SITS coding gene (species-specific gene): Random eleven isolates (4 isolates from (group 1) and 1 isolate from (group 2) and 6 isolates from (group 3) of biochemically identified *K. pneumoniae* isolates were subjected to detection of *K. pneumoniae* 16s-23SITS coding gene by using technique of PCR. The specificity of the primers was confirmed by positive amplification of fragment with the extracted DNA of the bacterial isolates. All 11(100%) tested isolates were positive for the K. pneumoniae16s-23SITS coding gene. The PCR assay yielded amplified products at 130 bp specific for *K. pneumoniae* 16s-23SITS coding genes shown in figure (1).

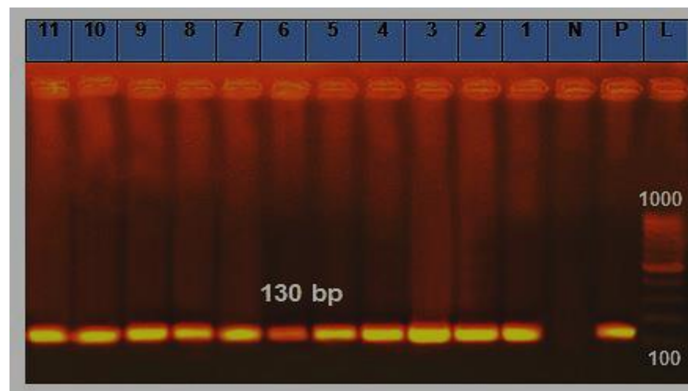

Fig. (1): Amplified PCR product using 16S-23 SITS primer for the isolated ***K. pneumonia* 16S-23 S ITS genes. Lane (L):** DNA molecular weight ladder (100bp ladder), **Lanes (1-11):** positive isolates (specific band at 130 bp). **Lane (P):** positive control for 16S-23 SITS coding gene. **Lane (N):** negative control for 16S-23 SITS coding gene.

Results of PCR detection of *FimA* gene from *K. pneumoniae* isolates for detection of biofilm formation.

The eleven *K. pneumoniae* isolates were subjected to the detection of the *Fim A* coding gene responsible for biofilm formation using the PCR technique. The specificity of the primers was confirmed by the successful amplification of fragments from the extracted DNA of the bacterial isolates at the 436 bp specific band. All 11 tested isolates were positive for the *Fim A* coding gene (100%), and the PCR assay yielded amplified products at 436 bp specific for *the Fim A* coding gene (Figure 2).

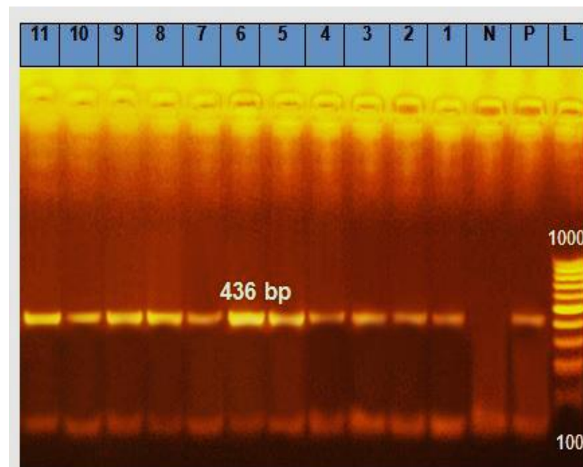

Fig. (2): Amplified PCR product using *Fim A* primer for specific species-specific biofilm formation of ***K. pneumoniae Fim A* gene**. **Lane (L):** DNA molecular weight ladder (100 bp ladder), **Lanes (1-11):** positive isolates (specific band at 436 bp). **Lane (P):** positive control for the gene. **Lane (N):** negative control for *Fim A* gene

Results of PCR detection of *Mrk A* gene from *K. pneumoniae* isolates for detection of biofilm formation.

The eleven *K. pneumoniae* isolates were subjected to the detection of *Mrk A* coding gene responsible For biofilm formation using the PCR technique. The specificity of the primers was confirmed by positive amplification of fragments with the extracted DNA of the bacterial isolates. All 11 tested isolates were positive for *Mrk A* coding gene (100%), The PCR assay yielded amplified products at 476 bp specific for *Mrk A* gene As shown in Figure (3):

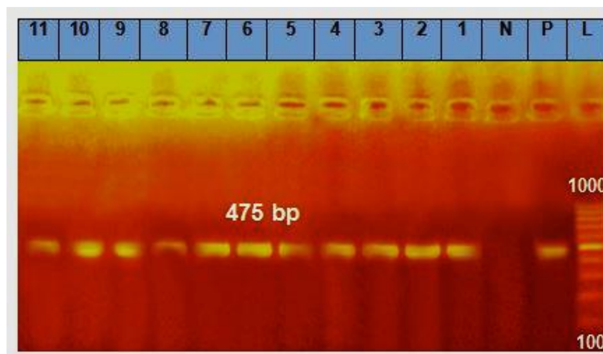

Fig. (3): Amplified PCR product using *Mrk A* primer for specific species-specific biofilm formation of ***K. pneumoniae Mrk A* gene**. **Lane (L):** DNA molecular weight ladder (100-1000 bp ladder), **Lane (1-11):** positive isolates (specific band at 475 bp.). **Lane (P):** positive control for gene, **Lane (N):** negative control for gene.

Results of biofilm formation by *K. pneumoniae* isolates using the Tissue Culture Plate Method (TCP):

Out of 11 *K. pneumoniae* isolates, Six (54.5%) isolates were strong biofilm producers, among them 3 (27.3 %) from sheep suffering from respiratory infections and 3 (27.3 %) from sheep dead or slaughtered accidentally and 3 (27.3 %) isolates were moderate biofilm producer among them 1 from sheep suffering from respiratory infections (9.09%) and 2 (27.27%) from dead sheep or slaughtered accidentally and there are two weak biofilm producers From apparently healthy Sheep and one from dead sheep or slaughtered accidentally, as shown in Table (9).

Table 9: The biofilm formation by *K. pneumoniae* isolates.

From diseased sheep with respiratory infections (Group 1)		From apparently healthy Sheep (Group 2)		from dead sheep or slaughtered accidentally (Group 3)	
No	Degree	No	Degree	No	Degree
1	Moderate	5	Weak	6	weak
2	Strong			7	Strong
3	Strong			8	Moderate
4	Strong			9	Strong
				10	Strong
				11	Moderate

Relationship between antimicrobial resistance and biofilm formation in *K. penumoniae* isolated from different sources in sheep breeding farms. 150 nasal swabs were collected from various locations in Qena City, as shown in Table (10).

Table 10: Multidrugs resistance pattern of biofilm production by *K. pneumoniae* isolates:

Number of isolates forming the biofilm	Biofilm degree	Multidrug resistance Combination
6	Strong	AMC, CRO, FEP, FOX, CAZ, CTX, ATM, FTN
3	Moderate	AMC, CRO, FEP, FOX, CAZ, CTX, ATM, FTN
2	Weak	AMC, CRO, FEP, FOX, CAZ, CTX, ATM, FTN

DISCUSSION

Sheep play an important role in the lives and economies of rural populations in Egypt, (Gaballah *et al*., 2022, Ramadan 2022 and Aminul *et al*., 2021). *K. pneumoniae* represented the main bacterial agents causing pneumonia (Amrane and Lagier, 2020 and Wang *et al*., 2020). Intrinsic resistance to antimicrobial agents dramatically increases when *K. pneumoniae* strains grow as a biofilm (Almalki and Varghese *et al*., 2020).

In this study, 150 animals, consisting of 3 groups of sheep. The results revealed an infection rate of 69.33% (104 out of 150) with *K. pneumoniae* in the studied animals (3 groups). A previously conducted study in Egypt reported a 36% infection rate of *K. pneumoniae* in pneumonic sheep (Metawi *et al*., 2019). However, lower infection rates of 27.15% were reported in sheep with respiratory infections in Egypt reported by (Ali and Abu-Zaid, 2019) nearly the same in our studies which was recorded 30% (45 out of 150) as shown in Table (6). These variations in infection rates could be

attributed to differences in sample size, study population, and epidemiological and ecological characteristics (Fouad *et al*., 2022). In our study, the infection rate of *K. pneumoniae* was slightly higher in sheep. However, these differences did not reach statistical significance, consistent with findings reported by (Kattimani *et al*., 2020). The higher susceptibility of sheep can be attributed to the immune system, increased vulnerability to transportation stress, sudden environmental changes, and viral infections, (Yadav, 2020). (Pavan *et al*., 2021).

The antimicrobial susceptibility of the 11 *K. pneumoniae* isolates was resistant to at least three antimicrobials. Previous studies on the antimicrobial susceptibility of *K. pneumoniae* isolated from pneumonic sheep have reported different prevalence and patterns (Patel *et al*., 2017) which can be attributed to regional variations in antimicrobial use, the availability of over-the-counter antibiotics without prescriptions, and the level of veterinary services provided (Qasim, 2019 and El Damaty *et al*., 2023) may explain the high resistance rate observed in this study.

Out of eleven PCR-positive *K. pneumoniae* isolates were highly susceptible to Polymyxin- B (PXB) and Colistin (COL) (100%) and 8 isolates were susceptible to Imipenem (IMP) (72.7%) then 7 isolates were sensitive to Ofloxacin (OFX), Levofloxacin (LEV) and Gentamycin (GEN) (63.7%)) whereas 4 isolates were intermediate susceptible to Amikacin(AK) (36.4%), but 11 isolates were highly resistant to Ceftazidime (CAZ), Amoxicillin –clavulanic acid (AMC), Ceftriaxone (CRO), Cefoxitin (FOX), Aztreonam (ATM), Cefotaxime (CTX), Cefepime (FEP) and Nitrofurantoin (FTN) (100% all of them) but were intermediate resistant to Meropenem (MEM) (36.4%) and 3 isolates show low resistant to Ciprofloxacin (CIP) and Imipenem (IMP), Levofloxacin (LEV) and Gentamycin (GEN) (27.3% all of them). This resistance is a result of its frequent use in the veterinary field in Egypt.

This study showed that 100% of the 11 *K. pneumoniae* isolates with a MAR index had high biofilm-forming ability, with 36.4% showing strong biofilm-forming ability. It has similar results in other studies (Banerjee *et al*., 2020 and Zaghloul *et al*., 2021).

This link between biofilm formation and antimicrobial resistance is due to the emergence of mutations within biofilm-forming genes (Mah *et al.,* 2003 and Zhang *et al*., 2013).

All 11(100%) tested isolates were positive for *K. pneumoniae* 16s-23SITS coding gene (species-specific gene). *Fim A* coding gene and *Mrk A* coding gene are responsible for biofilm formation using the PCR technique.

CONCLUSIONS

The present study, conducted in Qena Governorate, Egypt, revealed a high *prevalence of Klebsiella pneumoniae* infections in pneumonic sheep. Notably, these isolates exhibited multidrug resistance (MDR). The Klebsiella microbe is highly resistant to most antibiotics due to its formation of biofilms that increase its resistance to antibiotics. The purpose of the research is to obtain the antibiotic of choice through an antibiotic sensitivity test to avoid using weak antibiotics in the treatment. K. pneumoniae isolates were highly susceptible to Polymyxin- B (PXB) and Colistin (COL) (100%)

One of the most important methods to effectively control K. pneumoniae infection and limit its impact is implementing a sustainable control strategy and enhancing knowledge and health awareness among sheep breeders.

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الغشاء الحيوى والمقاومة للمضادات البكترية لميكروب الكلبسيلا الرئوية من الأغنام

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تلعب الأغنام دورًا اقتصاديًا مهمًا في محافظة قنا بجمهورية مصر العربية حيث يتم تربيتها في المقام الأول للحصول على موارد قيمة مثل اللحوم والحليب والصوف وإنتاج الشعر. تعد الكلبسيلا الرئوية. أحد مسببات الأمراض الانتهازية القادرة على التسبب في مجموعة واسعة من الأمراض لدى البشر والحيوانات. تعد زيادة وانتشارمقاومة الكلبسيلا الرئوية للمضادات البكترية الرئوية من أهم المشاكل الصحية في جميع أنحاء العالم. كما يؤدي إنتاج الأغشية الحيوية بواسطة الكلبسيلا الرئوية إلى تفاقم وتعقيد المقاومة البكتيرية وإطالة وقت العلاج. الهدف من البحث هو محاولة العثور على أفضل مضاد حيوي يستخدم لعلاج الالتهابات البكتيرية الناتجة عن الاصابة بعدوى الكلبسيلا الرئوية المقاومة للمضادات الحيوية بسبب تكوين الأغشية الحيوية التي تسبب خسائر كبيرة في مزارع تربية الأغنام في مدينة قنا. وقد قامت هذه الدراسة بتحليل العلاقة المحتملة بين مقاومة مضادات الميكروبات وتكوين الأغشية الحيوية في الكلبسيلا الرئوية المعزولة من مصادر مختلفة في مزارع تربية الأغنام. تم جمع ١٥٠ مسحة أنفية من مواقع مختلفة في مدينة قنا وتم تصنيفها إلى ثلاث مجموعات. تم إثراء العينات أولاً باستخدام مرق المغذيات المخصب، يليه الفرد على أجار ماكونكي. تم جمع خمسين مسحة أنفية من الأغنام التي تعاني من إصابات الجهاز التنفسي، و٥٠ عينة من أغنام تبدو سليمة ظاهريا و٥٠ عينة من حيوانات ميتة أو مذبوحة اضطراريا. سجلت نتائج الفحص البكتريولوجي وجود عدوى الكلبسيلا الرئوية في ٤٥ مسحة (٣٠,٩٠%) حسب المجموعة والعدد الإجمالي على التوالي من الأغنام التي تعاني من إصابات الجهاز التنفسي، و١٢ مسحة (٨,٢٤%) من الأغنام السليمة ظاهريا و٤٧ (٣٣,٣١,٩٤%) من الأغنام النافقة أو تم ذبحها اضراريا. من أجل التحديد النهائي للمستعمرات المشتبه فيها، تم استخدام تقنية PCR لتقدير حدوث ومستويات الكلبسيلا الرئوية الكشف عن الحساسية المضادة للبكتيريا لعزلات الكلبسيلا الرئوية المحددة (المقاومة للمضادات الحيوية). أظهر الكشف عن الأنماط الظاهرية لتكوين الأغشية الحيوية بواسطة طرق زراعة الأنسجة بين العزلات مقاومة متعددة الأدوية والكشف عن جينات *Fim A* و*Mrk A* المسؤولان عن تكوين الأغشية الحيوية بواسطة .PCR وقد وجد أنه من أصل ١٥٠ عينة (٥٠ مسحة أنف من أغنام تعاني من إصابات الجهاز التنفسي (المجموعة ١)، و٥٠ مسحة أنف من أغنام تبدو سليمة ظاهريا (المجموعة ٢) و٥٠ مسحة أنف من حيوانات ميتة أو تم ذبحها اضراريا (المجموعة ٣)، تم عزل ١٠٤ عزلة . تم عزل ١١ عزلة من بكتيريا *K. pneumoniae* كيميائيا باستخدام تقنيةPCR ، وتم عزل (٥) عزلات من أغنام مصابة بالتهابات تنفسية و(١) مسحات أنفية من أغنام سليمة ظاهريا و(٥) عزلات من الأنف كانت مسحات الأغنام الميتة أو المذبوحة اضطراريا (S6-S11)من الكلبسيلا الرئوية جين 16s-23SITSوقد وجد أيضاً أن *Fim A* و *Mrk A* هما جينات الضراوة المسؤولة عن تكوين الأغشية الحيوية في هذه الدراسة. من ناحية أخرى من بين إحدى عشرة عزلة إيجابية لتفاعل البوليميراز المتسلسل كانت عزلات الكلبسيلا الرئوية شديدة الحساسية للبوليميكسين- ب (PXB) والكوليستين (COL) (100٪) وكانت ٨ عزلات حساسة للـ 72.7) Imipenem (IMP)٪) وكانت ٧ عزلات حساسة للأوفلوكساسين (OFX) و الليفوفلوكساسين (LEV) و الجنتامايسين (63.7%) (GEN))، في حين كانت ٤ عزلات حساسة بشكل متوسط لمضاد الأميكاسين (36.4%) (AK)، لكن ١١ عزلة كانت شديدة المقاومة للسيفتازيديم (CAZ)، أموكسيسيلين – حمض الكلافولانيك (AMC)، سيفترياكسون (CRO)، سيفوكسيتين (FOX)، أزتريونام (ATM)، سيفوتاكسيم (CTX)، سيفيبيم (FEP) ونيتروفورانتوين (FTN) (جميعها بنسبة ١٠٠٪) ولكنها كانت مقاومة متوسطة للميروبينيم (MEM) (36.4 %) و٣ عزلات أظهرت مقاومة منخفضة للسيبروفلوكساسين (CIP) والإيميبينيم (IMP)، الليفوفلوكساسين (LEV) والجنتاميسين (27.3%) (GEN جميعها). وتعزى هذه المقاومة نتيجة لكثرة استخدامه في المجال البيطري في مصر.