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Identification and characterization of *klebsiella pneumonia* isolated from farm animals and their biofilm production estimation

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ABSTRACT

Klebsiella pneumoniae considered one of the most commensal bacteria which cause Economical problems in farm animals as causing mastitis and respiratory disorders in ruminants and neonatal septicemia in calves. Antibiotic are commonly used for disease control in animals and human. Multidrug resistant (MDR) *K. pneumoniae* strain show resistance to more than one antibiotic. In the present study *K. pneumoniae* were isolated and identified from different clinical samples (nasal swabs, milk sample, anal and vaginal swabs). A total of 293 samples from 258 animals (190 sample from clinically diseased cattle, 60 from clinically diseased buffaloes and 43 from clinically diseased calves). Results showed that the isolation rates were 20 /190 samples taken from clinically diseased cattle(10.5%), 5 /60 samples from clinically diseased buffaloes (8.33%) and 5 /43 samples from calfs (11.6%). *Klebsiella* was isolated between samples type as 24 /233 nasal swabs, 4/45 mastitic milk sample and 2 /10 anal swabs sample with percentage of 10.3%, 8.8, and 20%, respectivly. The anti-microbial susceptability test were done for 30 isolates of *klebsiella* using disc diffusion method. The results showed that the highest resistance was to ampicillin (100%), followed by chloramohinicol (76.7%) and the highest sensitivity was to gentamycin (100%), followed by ciprofluxacin and erythromycin (76.7%), nalidexic (73.33%), and streptomycin (53.4%). The biofilm formation ability of *k. pneumoniae* protects bacteria from immune system of the host. The present study of biofim formation module indicated that all strains were biofilm formed strains and among them, 11 (36.66%), 12 (40%), and 7 (23,33%) were strong, moderate, and weak biofilm producers, respectively. In conclusion, the detection of MDR klebsiella pneumoni from animals the isolates showed different ability for biofilm formation

1. INTRODUCTION

Klebsiella (K.) is one of the most pathogenic bacteria that are Gram negative bacilli capsulated, non motile ,non-sporulated arranged singly, in pairs or in short chains and sometimes arranged in clusters (Podschun and Ullmann, 1998)

Members of the genus Klebsiella, belongs to family Enterobacteriaceae, live freshwater environments including surface water, sewage and soil, as well as the mucosal surfaces of mammals K. pneumoniae is the most medically important species in the genus and is responsible, together with K. oxytoca (KO), for nosocomial infections in humans (Harada et al., 2016).

K. pneumoniae is belonging to the ESKAPE group, which includes five other nosocomial microorganism that exhibit multidrug resistance and virulence Enterococcus faecium, Staphylococcus aureus, Acinetobacter baumannii, Pseudomonas aeruginosa, and *Enterobacter spp. K. pneumoniae* is a major cause of nearly 10% of nosocomial infections in Western countries and is known for high phenotypic and genetic diversity, in particular regarding antimicrobial-resistance genes (ARGs) and plasmid burden (Chiaverini et al., 2022). K. pneumoniae is of interest because it commonly causes clinical mastitis in dairy herds. Which is a highly prevalent disease in dairy cows and creates an incontestable economic burden in the dairy industry, in addition to being a dangerous animal welfare issue because of the pain associated with the infection (Cheng et al., 2020).

The incidence of microbial infections has been increasing in the past few decades. This has led to the uncontrolled use of antimicrobial drugs for prevention and treatment in several countries. This, in turn, cause the emergence of specific drug and multidrug resistance among various strains of microorganisms including K. pneumonia (Wasfi et al., 2016). In recent years it has been observed that a drastic increase of pathogenic bacterial resistant to multiple antibiotics including *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and other Gram-negative pathogenic species (Finley et al., 2013). In Gram-negative bacteria, one of the most important mechanisms of antibiotic resistance is the production of β-lactamases (Hawkey and Jones, 2009). These enzymes are usually acquired by horizontal gene transfer and confer resistance to β-lactams, the most commonly used class of antibiotics for the treatment of human and animal infections (Henriques et al., 2006).

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Biofilms can be defined as a dynamic biological system of microbial cells that are strongly associated with a surface and embedded in an organic polymeric matrix of microbial origin. It is recorded that more than 65% of microbial infections are caused by microorganisms when they grow in biofilms (Cook and Dunny, 2014). Microorganisms universally attach to surfaces and produce extracellular polysaccharides, resulting in the formation of a biofilm. Biofilms pose a serious problem for public health because of the increased resistance of biofilm-associated organisms to antimicrobial agents (Revdiwala et al., 2012).

The aim of the present study is isolation and identification of *klebsiella K. pneumonia* from clinical samples as well as Determination of antimicrobial sensitivity of isolated *klebsiella* against different antibiotics using disc diffusion method and investigation of *klebsiella* isolates ability to form biofilm.

2. MATERIAL AND METHODS

2.1. Ethical approval

The Ethics Review Committee of Benha University's Faculty of Veterinary Medicine accepted this work with ethical approval number (BUFVTM 16-06-2023).

2.2. Sample collection.

During the period from March to December 2020 a total of 293 samples (nasal swaps, milk samples, anal swabs, and vaginal swaps) were collected from 258 clinically diseased animal showing respiratory signs diarrhea and mastitis from different localities in Egypt (Behera, Gharbia and Qalyubia) (Table 1). Samples were collected separately in peptone water under hygienic measure as possible and transferred directly in ice box to the laboratory for bacteriological examination.

Table 1 Type and number of samples collected from different animals.

Animal	No. of animals	Samples			
		Nasal swabs	Milk	Vaginal Swabs	Anal swabs
Cattle	160	140	45	5	-
Buffaloes	60	60	-	-	-
Calf	38	33	-	-	10
Total	258	293 sample			

2.3. Isolation of klebsiella species.

All collected swaps incubated at 37ºc for 24 hours. all samples were streaked on MacConkey agar plates, blood agar and TSI media at 37ºc for 24-48 hours and examined for presence of lactose fermented *klebsiella spp*. (Manual, 1998), on blood agar for detection of hemolytic activity of isolates, and on TSI media.

2.4. Purification of klebsiella isolates

The suspected colonies were picked up and subcultured on sterile nutrient agar incubated at 37oc for 24 hours. The colonies were examined for cultural characters and microscopically for morphological characters by Gram stain. one single colony representing typical colonial appearance and morphological character was picked up and propagated in semi-solid nutrient agar tubes for further identification (Manual, 1998).

2.5. Biochemical identification.

The suspected mucoid colonies were subjected to the following biochemical tests, Urease production, Citrate utilization, Indole production test and H2S production on TSI agar (Manual, 1998).

2.6. Antimicrobial sensitivity test.

The isolated organisms were screened for antibiotic sensitivity tests according to The Clinical & Laboratory Standards Institute (CLSI). The following antibiotics discs were used; ciprofloxacin (CIP5), Ampicillin (AMP10), Erythromycin (E15), Chloramphenicol (C30), Streptomycin (S10), Enrofloxacin (EX5), Nalidix (NA30), and Gentamycin (GEN30) (Oxoid, UK.). Nutrient broth cultured with klebsiella isolates was matched visually with standard McFarland tube, Sterile cotton swab was dipped into the broth squeezed and streaked into muller hinton agar plate. Antibacterial discs were applied aseptically to the surface of the plate at an appropriate distance with the help of sterile forceps and incubated at 37˚C for 24 hours, aerobically. Antibiotic sensitivity patterns of isolated klebsiella were performed against 8 commonly used antibiotics belonging to different groups. Using a metric ruler, measured the diameter of the zone of inhibition (if present) for each antibiotic used. By comparing the measurement obtained from the individual antibiotics to the table of standards to determine if the bacterial species tested is resistant or sensitive to the antibiotic (Wheat, 2001).

2.7. Biofilm formation.

Biofilm formation was investigated using crystal violet quantitative microtiter plate method. Each *K. pneumoniae* isolate was cultured overnight at 37°C in trypticase soy broth (TSB; Himedia, India). Following that, 2µL of cell suspension were injected into sterile 96-well polystyrene microtiter plates containing 198µL of TSB. Each test comprised negative control wells containing 100 mL of uninoculated TSB. The cells were incubated for 24 h. at 37 °C. The wells were gently washed three times with 100µL phosphate-buffered saline (PBS). The wells were dried upside down. The biofilm mass was stained with 50µL of 0.1% crystal violet (Oxoid, UK). The wells were gently washed with 200µL of distilled water three times and dried in inverted positions. Finally, the wells were dissolved in 200µL of 30% Acetic acid to solubilize the stain. A microplate reader (Tecan sunrise, Jencons, UK) was used to measure biofilm mass optical density (OD) at 595 nm. The OD cut-off (ODc) was defined as three standard deviations above the mean OD of the negative control. All the isolates were classified based on the adherence capabilities into the following categories: non-biofilm producers (OD ≤ ODc), weak biofilm producers (ODc< OD ≤ 2xODc), moderate biofilm producers (2ODc < OD ≤ 4xODc), and strong biofilm producers (4xODc < OD) (Stepanovic´ et al., 2007).

3. RESULTS

3.1. Isolation of klebsiella from different animal and samples types.

Klebsiella isolates appeared as large, regular, round, smooth, raised, moist, and cream yellow colonies grew on TSA. While on MacConkey agar appears as large, pink, regular, round, smooth, and raised colonies. Non hemolytic colonies on blood agar media were observed.

The Gram staining result showed that the isolate were gram-negative bacilli arranged Single or in short chain.

Bateriological examination (Table 2) revealeded that isolation of *klebsiella* species 20/190 samples taken from clinically diseased cattle (10.5%), 5/60 samples from clinically diseased buffaloes (8.33%) and 5/43 samples from calves (11.6%). The prevalance of *klebsiella* isolation

between samples type was 24/233nasal swabs (10.3%), 4/45 mastitic milk samples (8.8%) and 2/10 anal swabs.

Table (2) prevalance of *klebsiella* isolated from different animal spp, and different samples types.

Animal	No.of +ve/no of sample	Type of sample	No.of +ve/type of sample
Cattle	20/190(10.5%)	Nasal swaps	24/233(10.3%)
Buffaloes	5/60(8.33%)	Milk	4/45(8.8)
Calves	5/43(11.6%)	Anal swabs	2/10(20%)

3.2. Biochimical identification of isolated klebsiella.

Biochimical characters of klebsiella isolats showed positive results for: Catalase, Urease and Citrate utilization test, Also showed negative results for Indole and Oxidas test.

3.3. Antimicrobial sensitivity testing of klebsiella isolates:

Invitro antimicrobial sensitivity test of 30 *klebsiella* isolates against different antibiotics using disc diffusion method were showed different degree of resistance to the used antimicrobials (Table 3). All tested strains showed complete resistance to ampicillin (100%)followed by chloramphinicol (76.6). All of them were completely sensitive to gentamycin by (100%) followed by ciprofluxacin (76.6%), nalidexix acid (73.33%) and erythromycin (70%). the strains resist at least three groups of antimicrobials are considered as ; multi drug resistance (MDR) strains. highest multi drug resistance rate were found among klebsiella isoltes from cattle(63.1%) followed by calfs (21%) and buffaloes (15.7%).

Table 3 Intrpretation of antimicrobial susceptability testing of *klebsiella* isolates.

Antimicrobial agent	Sensitive		Intermediate		Resistant	
	No	%	No	%	No	%
Ampicillin	0	0	0	0	30	100
Chloramphenicol	5	16.7	2	6.6	23	76.6
Streptomycin	16	53.4	1	3.3	13	43.3
Ciprofloxacin	23	76.6	0	0	7	23.4
Gentamycin	30	100	0	0	0	0
Nalidixic	22	73.33	2	6.66	6	20
Erythromycin	21	70	0	0	9	30

3.4. Biofilm formation modelof klebsiella.

In this study biofilm production by 30 *K. pneumoniae* isolates were evaluated using the crystal violet staining method, and the results showed a range of absorbance values from 0.122 to 0.545. All analyzed isolates were biofilm producers; among them, 11 (36.66%), 12 (40%), and 7 (23,33%) were strong, moderate, and weak biofilm producers, respectively (Table 4).

Table 4 results of biofilm formation of *klebsiell*a isolated from different hosts.

Examined animals	Strong	Moderat	Weak	Non adherant
	No.(%)	No.(%)	No.(%)	No.(%)
Cattle (20)	11 (55)	7 (35)	2(10)	-
Buffaloes (5)	-	3(60)	2(40)	-
Calves (5)	-	2(40)	3(60)	-
Total (30)	11(36.6)	12(40)	7(23.4)	-

4- DISCUSSION

*K.pneumoniae*is recorded one of the most important opprtunistic pathogen in animals cuasing mainly respiratory affection and mastitis. In current study, The samples were examined for presence of klebsiella isolates using bacteriological methods, the obtained isolation rate are nearly similar to that recoded by Chiaverini et al. (2022) who examined samples from wild animals. In addition, He et al. (2022) detected the same cultural characters of *klebsiella* as and recorded that the isolated bacteria were cultured on TSA, blood agar, and McConkey agar for 24 h and grew well. Large, regular, round, smooth, raised, moist, and cream yellow colonies grew on TSA. There was no hemolysis on the blood agar and the colonies were smooth, round, moist, ivory, and raised colonies, while large, pink, regular, round, smooth, and raised colonies grew on McConkey agar. Higher isolation rate of klebsiella species from different sources most of them were from pig, cattle, pork and milk and the rest of isolates were isolated from vegetables, pets, livestock and farm animals (Klaper et al., 2021)

The results of anti microbial susceptability test is coordinated with that reported by Harada et al. (2016) who demonstrated that the resistance rates to ampicillin, cephalothin, enrofloxacin, ciprofloxacin, trimethoprim/sulfamethoxazole, cefotaxime, gentamicin, tetracycline, chloramphenicol, amoxicillin-clavulanic acid, and cefmetazole were 98.1, 37.9, 37.9, 35.9, 35.0, 34.0, 31.1, 30.1, 28.2, 14.6, and 6.8%, respectively.

In addition, Gomaa (2021) confirmed that all *K. pneumonia* isolates were resistant to ampicillin and amoxacillin-clavulinic acid (100%) followed by cefepime (72.72%), tetracycline, trimethoprime and trimethoprime/sulphamethaxole (54.54% each), while they were sensitive to imipenem (82%) followed by aztreonam (55%) then amikacin and azithromycin (45%). All 17 isolates (100%) were resistant at least to one antimicrobial. Regarding β-lactams, all K. pneumoniae isolates were resistant to ampicillin, cloxacillin, and cefazolin. All the 16 K. pneumoniae isolates were susceptible to aminoglycosides (amikacin, gentamicin, netilmicin, and kanamycin), tigecycline, and quinolones (nalidixic acid) (Chiaverini et al., 2022). Cheng et al. (2018) recorded 33 K. pneumoniae isolates resistance rates to ampicillin, amoxicillin, Ciprofloxacin, Cefotaxime, Ceftriaxone, Gentamicin, Ceftazidime and Amikacin were 93.9%, 81.8%, 60.6%, 57.6%, 33.3%, 27.3%, 18.2% and 9.1%, respectively.

In the current study multi drug resistance(MDR) strains were detected. The highest multi drug resistance rate were found among klebsiella isoltes from cattle followed by calfs and buffaloes. This results agreed with that reported by Montso et al. (2019) who found that large proportions (85–100%) of the isolates from all the sampling sites except for those from samples from Mafikeng (54.5%) and Boshoek (66.7%) were resistant to Ampicillin. In addition, significant proportions (66.7–100%) of the isolates from Stella and Boshoek were resistant to Cefotaxime, Piperacillin, Ceftazidime, and Aztreonam. Also , large proportions (90%) of the isolates from Potchefstroom were resistant to amoxicillin, cephalothin, and Piperacillin. Brisse and Duijkeren (2005) found that from 78 isolates, resistance was common against ampicillin (99%) and cephalexin (43%) but not against ceftazidime, ceftiofur, tetracycline, enrofloxacin, gentamicin and trimethoprim-sulfamethoxazole. Thirteen isolates resistant to three or more antimicrobials or combinations of there were found, but acquired antimicrobial resistance remains lower among animal isolates than among human nosocomial isolates.

In the present study, the biofim formation module indicated that all strains were biofilm formed strains. This results is coordinated with that reported by Massé et al. (2020). While, Gomaa (2021) stated that 81.81% of *K. pneumonia* isolates could produce biofilms, those categorized as strong (33.33%), moderate (22.22%) or weak (44.44%) biofilm producers, whereas 18.18% of the isolates were non-biofilm producers.

5. CONCLUSION

In conclusion, the detection of MDR *K. pneumoniae* from animals the isolates showed different ability for biofilm formation.

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