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Molecular studies on some antibiotic-resistant genes of *Klebsiella* species isolated from chicken

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ABSTRACT

klebsiella species are one of the key issues that have been steadily rising in intensive poultry production, causing great economic losses. The main cause of this uncomfortable condition is the increasing resistance of bacteria to antibiotics and has food safety risks because it can act as a source of contamination for chicken meat and eggs. In the present study, we aim to isolation and identification of *klebsiella spp*. and Molecular screening of antibiotics resistance genes that present in isolated *Klebsiella spp*. Twenty-Eight *klebsiella* species obtained from 50 healthy chickens differentiated into 21/28 (75%) *Klebsiella pneumoniae* and 7/28 (25%) *Klebsiella oxytoca*. Antimicrobial sensitivity testing against 12 commonly used antibiotics in chicken farms revealed that *klebsiella* species were fully resistant to oxytetracycline (100%) and penicillin (100%) and trimethoprim (100%) and moderate resistance to cefadroxil, doxycycline, amoxicillin/clavulanic acid, chloramphenicol, cefotaxime and lowest resistance to meropenem and *klebsiella* species extremely susceptible to amikacin (100%), tobramycin (100%) and norfloxacin (100%). A total of 12 *klebsiella* species differentiated into 9 *klebsiella pneumoniae* and 3 *klebsiella oxytoca* screened to find *ESBL* coding gene in the *klebsiella* species. The isolates were found to have *bla SHV* (100%), *bla TEM* (91.7%), and *bla CTX-M* (83.4%).

1. INTRODUCTION

Klebsiella pathogens are Gram-negative bacteria, encapsulated; rod-shaped opportunistic facultative anaerobic, bacteria can produce potentially fatal diseases in humans and animals, and can be transmitted from one person to another. In the genus *Klebsiella*, the most common pathogenic member is *Klebsiella pneumoniae*. *Klebsiella* species belong to the family *Enterobacteriaceae* which is characterized by widths ranging from 1.0 to 1.0 mm and lengths ranging from 0.6 to 6.0 mm. *Klebsiella* species are frequently found in mucoid colonies. The genus has 77 capsular antigens (K antigens), which result in various serogroups (Paczosa and Mecsas, 2016; Jensen et al., 2020).

Klebsiella species cause a wide range of illnesses in both people and animals. They are most well-known as microorganisms that cause infectious diseases such as urinary tract infections and pneumonia as well as rising the percent of death between patients (Cabral et al., 2012).

Klebsiella species are present in the gastrointestinal tracts of animals and the environment, particularly those reared for human consumption. *Klebsiella oxytoca* is an enterotoxigenic bacterium that can make hemorrhages in the intestine (Gundogan et al. 2011). *Klebsiella* species are observed in human and animal stools, containers of water, and personal water (slama et al., 2010). *Klebsiella pneumoniae* is a prevalent infectious illness that affects chicks and causes significant economic losses (Aly et al., 2014). The respiratory illness is characterized by dyspnea, pump handled respiration, gasping, mucous discharge, facial edema, sinus enlargement, tracheitis, exudative pneumonia, pleuritis, air saculitis, pericarditis, reduced egg production, and low egg quality (Tantawy et al., 2018).

In the presence of other bacteria, *Klebsiella* infections can develop as a secondary infection (Paczosa and Mecsas, 2016).

Cephalosporins of the third generation (3GCs) (e.g., cefotaxime, ceftriaxone) were founded in 1977 and first used in humans in the early 1980s. They were a major step forward in the treatment of illnesses caused by multi-resistant Gram-negative bacteria like *Klebsiella pneumoniae.* High-level resistance to all of these drugs developed shortly after the introduction of Cephalosporins in the protocol of treatment and this led to establishment of the term extended-spectrum B-lactamase production is the predominant mechanism for resistance to B-lactam antibiotics in Gram-negative bacteria. Extended-spectrum B-lactamases (*ESBL*) were discovered in *Klebsiella* species and then in other Gram-negative bacteria in the 1980s (Cheng et al., 2008; Kiratisin et al., 2008). *ESBL* genes are constantly being mutated, resulting in the creation of novel enzymes with extended substrate proles. There are currently over 300 distinct *ESBL* genes, which have been classified into nine different structural and evolutionary groups based on amino acid sequence. The most common kinds were TEM and sulphydryl variable SHV. However, in certain nations, the CTX-M variety is more frequent

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(Paterson et al., 2003). Temoniera (TEM), sulfhydryl variable (SHV), and cefotaximase are the three types of proteins (Cabral et al., 2012).

The identification of TEM and SHV genes by molecular methods in ESBL generating bacteria, as well as their antibiotic resistance pattern, might provide important information regarding their epidemiology (Jain and Mondal, 2008).

PCR techniques identify pathogens in a sensitive and specific manner, and they may distinguish virulent bacteria from virulent individuals of the same species. Because they are highly established and, when employed as culture confirmation tests, are reliable, quick, and sensitive, PCR-based methods are increasingly being used in microbiology research (Olsen, 2000).

The goal of this study was to find out more about Klebsiella species obtained from poultry farms in Menoufia Governorate by monitoring the development of antibiotic resistance and evaluation the diversity of *ESBL* genes in *Klebsiella oxytoca* and *Klebsiella pneumoniae* isolated from chicken.

2. MATERIAL AND METHODS

2.1. Collection of samples

Lung, kidney, liver, intestine were collected from 50 chicken under sterile conditions and sent to the laboratory in an ice box as quickly as possible.

2.2. Isolation and identification of Klebsiella species

Samples were cultivated into nutrient broth and incubated aerobically at 37℃ for 18-24 hours. A loopful of inoculated nutrient broth was streaked onto MacConkey agar medium. the inoculated medium was incubated aerobically at 37℃ for 24-48 hours and then examined for bacterial growth. Suspected colonies were sub cultured onto XLD and EMB. After incubation, colonies culture characters and morphological characters were studied. Biochemical tests including, catalase, oxidase, indole production, methyl red, Voges- Proskauer, citrate utilization, lactose fermentation and H2S production were used for *Klebsiella spp*. Identification (Trivedi et al., 2015).

2.3. Antibiotic sensitivity Test (Chess brough, 2000)

All Isolated *Klebsiella spp.* were tested for their sensitivity to antibiotics with the disc diffusion test on Mueller-Hinton Agar. All disks used in the disc diffusion test were obtained from Oxoid, England. The culture turbidity was adjusted to 0.5 McFarland standards. The sterile cotton swab was dipped into the suspension and spread evenly over the entire Mueller Hinton Agar surface. The antibiotics discs were placed onto the surface of the inoculated plates and incubated at 37°C for 16-18 hrs. The diameter of inhibitory zones on several antibiotic discs was measured and compared to an antibiotic sensitivity testing sheet to obtain the result (resistant or sensitive) for isolated *klebsiella* species. This testing sheet is based on CLSI (2017) (Clinical Laboratory Standards Institute).

2.4. Molecular characterization of some antibiotic-resistance gene of klebsiella species by polymerase chain reaction (PCR)

Twelve *klebsiella* species differentiated into nine *klebsiella pneumoniae* and three *klebsiella oxytoca* were subjected to PCR test in PCR unit in Animal Health Research Institute, Dokki, Giza, Egypt.

2.5. DNA extraction

Chromosomal DNA extraction from samples was performed using the QIAamp DNA Mini kit instructions. Briefly, 200µl of the sample suspension was incubated with 20 µl QIAGEN protease and 200µl of lysis buffer at 56°C for 10 min. After incubation, 200µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations.

2.6. PCR primers, Master Mix and cycling conditions

The PCR primers sequences and their amplified products were indicated in table 1. These primers were synthesized by Metabion Company, (Germany). The preparation of master mix was based on Emerald Amp GT PCR master mix Code No. RR310Akit (Takara), as stated in table 2

For all genes the PCR cycling conditions were Frist denaturation at 94˚C/5 min, second denaturation 94˚C/30 sec, annealing at 54˚C/40 sec and extension at 72˚C/45 sec for 35 cycles with final extension at 72˚C/10 min.

Table (1) the primer sequences and their amplified products for antibiotic-resistance gene of *klebsiella* species genes

Gene	Sequence (5'-3')	PCR product	Reference
blaTEM	F- ATCAGCAATAA-ACCAGC R-CCCCGAAGAAC-GTTTTC	516 bp	Colom *et al.*, 2003
blaSHV	F- AGGATTGACTG-CCTTTTTG R-ATTTGCTGATT-TCGCTCG	392 bp	
blaCTX-M	F- ATG TGC AGYA-CC AGT AAR GTK ATG GC R TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	593 bp	Archambault *et a.l*, 2006

F: Forward, R: Reverse

Table (2) the master mix preparation

Component	Volume/reaction
PCR master mix Emerald Amp GT (2x premix)	12.5 µl
Water of PCR grade	5.5 µl
Primer for the Forward (20 pmol)	1 µl
Primer for the Reverse (20 pmol)	1 µl
DNA template	5 µl
The Total	25 µl

2.7. Analysis of the PCR Products

Electrophoresis in agarose gel (Sambrook et al., 1989) Warm agarose was put directly into the gel casting equipment with the appropriate comb in apposition and allowed to polymerize at room temperature. After removing the comb, the electrophoresis tank was filled with TBE buffer. Each uniplex PCR product requires twenty µl, negative control and positive control were loaded to the gel. The power supply ranged from 1 to 5 volts per centimeter of tank length. After around 30 minutes, the run was terminated and the gel was moved to the UV cabinet. A gel documentation system photographed the gel, and the data was evaluated using computer software.

3. RESULTS

3.1. Isolation and identification of klebsiella species

Bacteriological examination revealed that *Klebsiella* species isolates were recovered from 200 samples with overall prevalence 28 (14%). Isolates of *klebsiella* species were differentiated into 21/28 (75%) *Klebsiella pneumoniae* and 7/28 (25%) *Klebsiella oxytoca*. *Klebsiella* isolates produced pink colored mucoid colony on MacConkey agar that give positive reaction for catalase test, vogues Proskauer test,

citrate test and urease test. Meanwhile the isolates were negative for indole, oxidase and methyl red tests.

3.2. Antibiotic sensitivity test

Results of twenty-one *klebsiella peumoniae* and seven *klebsiella oxytoca* tested by antibiotic sensitivity test against 12 commonly used antibiotics in chicken farms were demonstrated by table 3 and 4.

Table (3): Patterns of antibiotic sensitivity and resistance for (21) *klebsiella pneumoniae*

Antibiotic (oxoid)	Sensitive		Intermediate		Resistant	
	No.	(%)	No.	(%)	No.	(%)
T (30 µg)	0	0	0	0	21	100
TR (5 µg)	0	0	0	0	21	100
P(10 units)	0	0	0	0	21	100
CFD (30 µg)	1	5	4	19	16	76
DO (30 µg)	9	43	7	33	5	24
CTX (30 µg)	13	62	7	33	1	5
AMC (30 µg)	14	67	3	14	4	19
C (30 µg)	16	76	2	10	3	14
MEM (10 µg	19	90	2	10	0	0
Tob (10)	21	100	0	0	0	0
Ak (30 µg)	21	100	0	0	0	0
Nor (10 µg	21	100	0	0	0	0

T**:** Oxytetracycline**,** TR: Trimethoprim**,** P: Penicillin G, CFD: Cefadroxil**,** DO: Doxycycline, CTX: Cefotaxime, AMC: Amoxyclav, C: Chloramphenicol, MEM: Meropenem, Tob: Tobramycin, Ak: Amikacin, Nor: Norfloxacin

Table (4): Patterns of antibiotic sensitivity and resistance for (7) *klebsiella oxytoca*

Antibiotic (oxoid)	Sensitive		Intermediate		Resistant	
	No.	(%)	No.	(%)	No.	(%)
T (30 µg)	0	0	0	0	7	100
TR (5 µg)	0	0	0	0	7	100
P(10 units)	0	0	0	0	7	100
CFD (30 µg)	0	0	4	57	3	43
DO (30 µg)	3	43	1	14	3	43
CTX (30 µg)	5	72	1	14	1	14
AMC (30 µg)	4	58	2	28	1	14
C (30 µg)	6	86	0	0	1	14
MEM (10 µg	5	72	2	28	0	0
Tob (10)	7	100	0	0	0	0
Ak (30 µg)	7	100	0	0	0	0
Nor (10 µg	7	100	0	0	0	0

T**:** Oxytetracycline**,** TR: Trimethoprim**,** P: Penicillin G, CFD: Cefadroxil**,** DO: Doxycycline, CTX: Cefotaxime, AMC: Amoxyclav, C: Chloramphenicol, MEM: Meropenem, Tob: Tobramycin, Ak: Amikacin, Nor: Norfloxacin

3.3. PCR for ESBL Genes in Klebsiella species

A total of 12 *klebsiella* species isolates were differentiated into 9 *klebsiella pneumoniae* and 3 *klebsiella oxytoca* were screened to look for the *ESBL* coding gene inside the *klebsiella* species. Resistance gene analysis revealed the isolates were found to have 100% *bla* SHV, 83.4% *bla* CTX-M and 91.7% *bla* TEM as revealed in table 5 and detected by Figure 1, 2 and 3 respectively

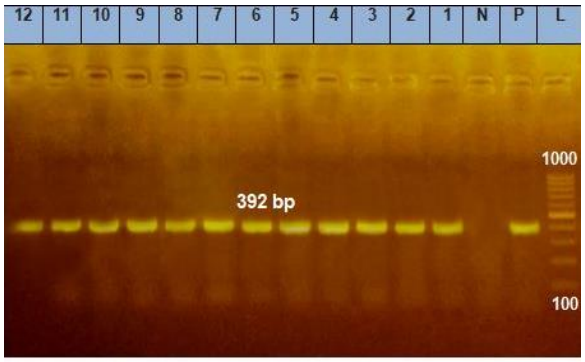

Fig (1): Amplification of 392 bp fragment employing *bla* SHV primeron agarose gel electrophoresis. L: 100 - 1000 bp ladder Lane (1-12): positive sample *klebsiella oxytoca* (1-3) *klebsiella pneumoniae* (4-12), P: Positive control N: negative control

Table (5): Patterns of *PCR for ESBL Genes in Klebsiella species*

Type of *klebsiella* species	No. of examined samples	*bla* TEM		*bla* SHV		*bla* CTX-M	
		No.	*(%)*	*No.*	*(%)*	*No.*	*(%)*
K. Pneumoniae	9	8	88.9	9	100	8	88.9
K. Oxytoca	3	3	100	3	100	2	67
Total	12	11	91.7	12	100	10	83.4

% calculated according to total number of positive samples = 12

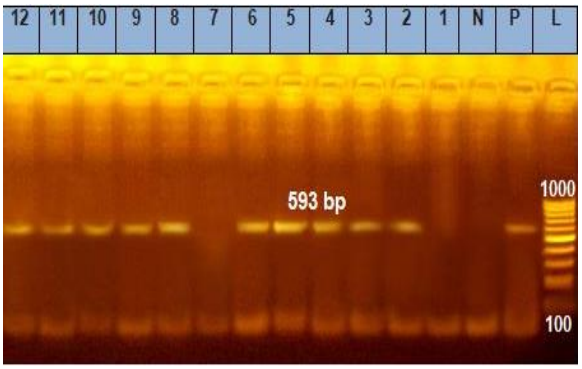

Fig (2): Amplification of 593 bp fragment employing *bla* CTX-M primeron agarose gel electrophoresis. L: 100 - 1000 bp ladder Lane (1-12): positive sample *klebsiella oxytoca* (1-3) klebsiella *pneumoniae* (4-12), P: Positive control N: negative control

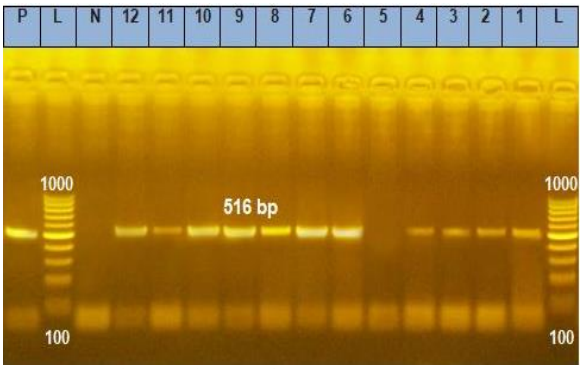

Fig (3): Amplification of 593 bp fragment employing *bla* TEM primeron agarose gel electrophoresis. L: 100 - 1000 bp ladder Lane (1-12): positive sample *klebsiella oxytoca* (1-3) *klebsiella pneumoniae* (4-12), P: Positive control N: negative control

4. DISCUSSION

In this research the Results demonstrated by examination of 200 samples according to clinical observation and isolation revealed that the percentage of *klebsiella* species was 14%. *Klebsiella* isolates differentiated into *Klebsiella pneumoniae* 21/28 (75%) and *Klebsiella oxytoca* 7/28 (25%) The obtained results agree with that of Younis et al., 2016; Elgaos et al., 2019 and Abdelrhman 2019 that were 15%, 14.4% and 16.88% respectively.

On the other hand, Turkyilmaz (2005) recorded a higher prevalence rate (47.1%). Meanwhile, Aly et al. (2014), Khalda et al. (2000) and Dashe et al. (2013) reported that the prevalence of *Klebsiella peumoniae* in broiler chickens was 10%, 10.2% and 8% respectively.

Antimicrobial sensitivity testing revealed that all *klebsiella* species isolates are MDR bacteria. MDR in *Klebsiella* species leads to extended treatment times and more difficult cures. The pattern of antibiotic resistance was supposed to serve as a guide for selecting the best medicines for therapy. Antibiotic usage without proper monitoring may result in antibiotic resistance. Until now, antibiotics are inexpensive and may be used without a veterinarian's supervision (Hayati 2019) as many as, chicken farmers in Egypt antibiotics are still being used without the approval of a veterinarian. According to FAO (2008), regardless of dose, an estimated

75% of antimicrobial drugs given to intensively raised broiler chickens may be excreted into the environment, resulting in the emergence of antibiotic-resistant bacterial strains in humans. Furthermore, research suggests that antimicrobial residues in manure may be to blame for the pollution of soil, surface water, and groundwater resources near farms operating in intensive broiler rearing activities.

Twenty-eight *klebsiella* species were tested by antibiotic sensitivity test against twelve commonly used antibiotics in chicken farms. All isolates were resistant to Penicillin G 100%, Trimethoprim 100%, Oxytetracycline 100% this was agreed with Elgaos *et al.* (2019), Abdelrahman (2019) who reported that *klebsiella* species showed 100 % resistance to Penicillin G, Oxytetracycline. Because of its broad-spectrum action, this antibiotic is frequently utilized in a variety of applications. Since its introduction, the usage of Penicillin G, Trimethoprim, Oxytetracycline is quite high in farms. They are used for individual and flock therapy, as well as antimicrobial growth promoters administered through feed or drinking water.

Tetracycline is a kind of antibiotic that is frequently used in Egypt and globally dueto its efficacy as a broad-spectrum antibiotic that is quickly absorbed, inexpensive, and has few adverse effects. In human bacterial infections, nations that have prohibited or never used fluoroquinolones in poultry have substantially lower levels of resistance than countries that continue to use the antibiotics in poultry (WHO 2011).

Cephalosporin resistance has been documented at various rates. Cephalosporin resistance rates were measured in this investigation 76% for cefadroxil, 5% for cefotaxime They were not dissimilar to those previously mentioned by Salem *et al.* (2019), Ullah et al. (2009), On the contrary, the rate of cephalosporin resistance was measured by Younis *et al.* (2016), Singh and Goyal (2003).

Isolates of *Klebsiella* are resistant to Doxycycline and Chloramphenicol in our research was 24%, 14% respectively and this was agreed with Younis et al. (2016), Abdelrahman (2019).

On the contrary, a decreased prevalence of resistance to Chloramphenicol reported by Elgaos et al. (2019). *Klebsiella* isolates' resistance to Amoxy clavulanic was 19% in our study, On the other hand Complete resistant to Amoxy clavulanic was measured by Younis et al. (2016).

Klebsiella isolates' resistance to Amikacin, Tobramycin, Norfloxacin In our research was 0%. All isolates were sensitive to Amikacin, Tobramycin, Norfloxacin, this result was agreed with Younis *et al.* (2016) for Amikacin but differ with him for Norfloxacin, while it was agreed with Elgaos *et al.* (2019) for Norfloxacin.

The presence of *ESBL* encoding genes can be used to identify *ESBL* bacteria. PCR assay was conducted in order to find certain antimicrobial resistance genes of *Klebsiella* species. PCR assay could recognize *bla* CTX-M, *bla* TEM, *bla* SHV, and genes utilizing certain primer sequences that resulted in product sizes of 516 bp, 392bp and 593bp, respectively (Overdevest 2011)

Among the examined isolates the findings of this study revealed that the *bla* TEM gene was discovered at (91.7%) and then the *bla* CTX-M (83.4%). The obtained results which were not dissimilar to those previously reported by Hayati (2019), who found *bla* TEM gene 100% and so on *bla* CTX-M (90.9%). Al-Agamy et al. (2009), found that *bla* TEM in (84.1) %. Newire et al. (2013) bla TEM and bla SHV genes were discovered in 98 percent of *Klebsiella pneumoniae* isolates, *bla* CTX-M was carried by 11%, The dominant genotype discovered was the *bla* CTX-M gene, according to some research, The *ESBL* type was frequently viewed as a single or combined entity (Ibrahim and Hameed 2015).

The frequency of the *bla* SHV gene obtained in the current research 100% was not far from those previously reported by than Al-Agamy et al., (2009), where *bla* SHV genes were discovered in 97.3% of the examined samples.

Much lower values for these genes were 13% (Salem et al., 2019) for SHV, 60% for TEM and 33% for CTX-M β-lactamase genes (Messai et al. 2008), whereas Bali et al. (2010) found that the most frequent genotype was TEM (73.43%), which was followed by SHV (21.87%), and CTX-M (17.18%). Hayati et al. (2019) found that *bla* SHV genes were detected in only one isolate from 11 *Klebsiella* species isolates.

The *ESBL* gene moves fast from animal to human due to genetic elements that are mobile including Bacterial transposons, insertion sequences, and integrons. Genetic factors may potentially spread resistance to other bacteria in the gastrointestinal tract of the animal. The pathogens are subsequently spread from the farm to the surrounding environment via trash, which is contaminated soil and water due to poor hygiene and sanitation. Around the farm and market, *ESBL* bacteria have been discovered in crops, soil, and water (Wu 2016)

All *Klebsiella pneumoniae* isolates with an *ESBL* gene were also MDR bacteria, based on antibiotic sensitivity testing results. The presence of Multidrug resistance bacteria puts human and animal health at risk. As a result of these problems, therapeutic choices may be limited. MDR-ESBL microorganisms also prompted the usage of antibiotics like colistin, which are no longer utilized owing to toxicity (Fard 2004)

Building surveillance systems and performing feed and livestock surveillance are examples of actions that might be performed. Poultry farms must also strengthen their biosecurity procedures. In intensive production systems, litter and manure waste must be carefully handled to avoid pollution of the air, land, and water, as well as severe health implications (Thyagarajan 2014).

5. CONCLUSION

The current study concluded that, All *Klebsiella* species were classified as MDR bacteria and harbored *ESBL* genes. The presence of antibiotic resistance genes in bacteria has the potential to spread its resistance properties. Antibiotic sensitivity Test showed high multiple antibiotic resistances which require strict regulations of the use of antibiotics in veterinary therapy to minimize the emergence of resistant bacteria in animals which may increase the public health problem.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest for current data

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