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# MOLECULAR CHARACTERIZATION OF SOME VIRULENCE GENES IN *KLEBSIELLA PNEUMONIAE* ISOLATED FROM BROILERS

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## ABSTRACT

In the present study, a total of 160 samples (lung, air sacs, liver and kidneys, 40 samples each) were collected from clinically diseased broiler chickens. Clinical signs were weakness, gasping, ruffled feather, nasal mucoid discharge, poor growth, facial oedema and conjunctivitis, while postmortem findings were signs of septicemia, pneumonia, air sacculitis, nephritis, sinusitis and liver and lung abscesses. Isolation and biochemical identification of *K. pneumoniae* were done. Results of bacteriological examination revealed that *K. pneumonia*e isolates were recovered from 160 samples with overall prevalence (14.4%). The isolation rates from lung, air sacs, liver and kidneys were 30 %, 12.5%, 10% and 5%, respectively. The isolates of *K. pneumonia*e were found to be virulent by using PCR assay incorporating *mag*A, *fim*H and *tra*T genes primers and were found to be resistant to some antibiotics by using PCR assay incorporating *tetA*(A), *bla*TEM and *mph*A genes primers. Antibiogram for 20 recovered *K. pneumoniae* isolates against 10 commercially used antibiotics in broiler chicken farms revealed that *K. pneumoniae* isolates were completey resistant to oxytetracycline (100%) and ampicillin (100%) followed by erythromycin (90%), streptomycin (80%), cefotaxim (70%) and gentamycin (65%) and moderate resistance to neomycin (45%) and chloramphenicol (30%). On the other hand *K. pneumoniae* isolates showed the lowest resistance to ciprofloxacin (20%) and norfloxacin (10%).

***Key words:*** *K. pneumoniae,* PCR, Virulence genes, antibiotic resistance genes.

## INTRODUCTION

*Klebsiella species* are gram-negative, encapsulated, non-motile, rod shape, lactose fermenting bacteria, belong to *family Enterobacteriaceae*. Members of this family are facultative anaerobic. This genus consists of 77 capsular antigens (K antigens), leading to different sero-groups (Janda and Abbott, 2006). The organism expresses both O-antigen (smooth lipopolysaccharide) and K-antigen (capsular polysaccharide) and both antigens contribute to its pathogenicity. A major virulence factor of *K. pneumoniae* is the capsule, which protects Klebsiella from lethal serum factors and phagocytosis (Fung *et al.,* 2002 and Mizuta *et al.,* 1983).

The genomic map of *K. pneumoniae* capsule contains gene clusters as follows: *rmp*A, *rmp*A1 and *rmp*A2 (regulator of the mucoid phenotype A, Al and A2, respectively), *mag*A (mucoviscosity associated gene A), *cps* (capsular polysaccharide synthesis), *Wb* (O-specific polysaccharide is directed by the *Wb* gene cluster) (Regue *et al.,* 2005 and Seidler *et al.,* 1975).

The *rmp*A and *rmp*Al genes regulate the synthesis of the Klebsiella polysaccharide capsule and they are conserved in most isolates of *K. pneumoniae.* The *mag*A gene is a part of the *K. pneumoniae* serotype K1 capsular polysaccharide gene cluster and contributes to the bacterial virulence (Fang *et al.,* 2004). The *mag*A plays an important role in serious infection of Klebsiella such as septicemia, bacteremia, pneumonia and liver and lung abscesses (Chan *et al.,* 2005 and Chung *et al.,* 2007). The chromosomal *mag*A gene causes increased levels of resistance to phagocytosis and has hyperviscous phenotype, which is characterized by forming a mucoviscous string during passing loop through a colony *(*Struve *et al.,* 2005).

Saif *et al.* (2003) related the clinical signs of weakness, gasping, oxidative pneumonia, mucoid discharge and poor egg quality and decrease egg production, pleuritis and air saculitis to *Klebsiella spp.* infection.

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In humans, *Klebsiella spp.* causing infections are often multidrug resistant and an increasing proportion of strains produce extended-spectrum beta-lactamases (ESBLs). Extended-spectrum β-lactamases confer resistance to penicillins and cephalosporins. ESBLs are most commonly detected in *K. pneumoniae*, they are plasmid-mediated enzymes, and these plasmids also carry resistance genes to other antibiotics. Thus, Gram negative bacilli containing these plasmids were multidrug-resistant. In contrast, the prevalence of antimicrobial resistance in animal and poultry Klebsiella isolates was poorly documented (Jacoby, 1997).

The present study aimed to determine some virulence and antimicrobial resistance genes associated with *K. pneumoniae* infection and to Study antimicrobial resistance profile to prevent the spread of resistant *K. pneumoniae* among the diseased chickens via planning a proper control program.

## MATERIALS AND METHODS

**Samples:**
A total of 40 clinically diseased broiler chickens were obtained from different private chicken farms in Dakahlia province and also from cases which were arrived to Mansoura Provincial Laboratory. Four samples consisting of lungs, air sacs, liver and kidneys were collected from each diseased bird. The samples were dispatched to the Laboratory without delay to be examined bacteriologically for isolation and identification of causative agent.

**Clinical and Postmortem examination:**
All chickens were examined clinically, then sacrified and immersed in a disinfectant before being autopsied. Gross pathological changes were recorded, summarized and presented with results for both freshly dead and clinically diseased broiler chickens.

**Media:**
**a -**Liquid media: Tryptose broth, peptone water and nutrient broth.
**b-** Solid media: Sheep blood agar, MacConkey's agar and Xylose lysine desoxycholate (XLD) agar (Oxoid).

**Isolation and identification:**
Bacterial isolation was carried out by inoculating aseptically collected samples from lungs, air sacs, liver and kidneys directly on sheep blood agar and MacConkey's agar and incubated at 37°C for 24-48 hrs (Quinn *et al.*, 1994). After incubation, colonies culture characters and morphological characters were studied. Biochemical tests including, catalase, oxidase, indole production, methyl red, Voges-Proskauer, citrate utilization, lysine decarboxylase, urea hydrolysis, lactose fermentation and $H_2S$ production were used for *Klebsiella spp.* Identification (Trivedi *et al.*, 2015).

**Molecular characterization of *Klebsiella pneumoniae* by PCR:**
Five *K. pneumoniae* isolates were subjected to PCR test in PCR unit in Animal Health Research Institute, AHRI according to Olivera *et al.* (2003).

**DNA extraction:**
Chromosomal DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200µl of the sample suspension was incubated with 10µl of proteinase K 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. Nucleic acid was eluted with 100µl of elution buffer provided in the kit.

**Oligonucleotide Primer:**
Primers used were supplied from Metabion (Germany) are listed in table (1)
For PCR, Primers were utilized in a 25-µl reaction containing 12.5µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5µl of water, and 6µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler (Table 1).

**Analysis of the PCR Products:**
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, 20µl of the uniplex PCR products were loaded in each gel slot. Gelpilot 100bp and 100bp plus DNA ladders (Qiagen, Germany, GmbH) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

**Table (1):** Primers sequences, target genes, amplicon sizes and cycling conditions:

| Target gene | Primers sequences ( 5'- 3' ) | Amplified segment (bp) | Primary denaturation | Amplification (35 cycles) | | | Final extension | Reference |
|---|---|---|---|---|---|---|---|---|
| | | | | Secondary denaturation | Annealing | Extension | | |
| ***K. pneumonia* 16S-23S ITS** | **F:**ATTTGAA GAGGTTGC AAACGAT | 130 | 94˚C 5 min. | 94˚C 30 sec. | 55˚C 30 sec. | 72˚C 30 sec. | 72˚C 7 min. | ***Turton et al., 2010*** |
| | **R:**TTCACTC TGAAGTTT TCTTGTGT TC | | | | | | | |
| ***magA*** | **F:**GGTGCTC TTTACATC ATTGC | 1282 | 94˚C 5 min. | 94˚C 30 sec. | 50˚C 40 sec. | 72˚C 1.2 min. | 72˚C 12 min. | ***Yeh et al., 2007*** |
| | **R:**GCAATG GCCATTTG CGTTAG | | | | | | | |
| ***fimH*** | **F:**TGCAGA ACGGATAA GCCGTGG | 508 | 94˚C 5 min. | 94˚C 30 sec. | 50˚C 40 sec. | 72˚C 45 sec. | 72˚C 10 min. | ***Ghanba -rpour and Salehi, 2010*** |
| | **R:**GCAGTC ACCTGCCC TCCGGTA | | | | | | | |
| ***TraT*** | **F:**GATGGC TGAACCGT GGTTATG | 307 | 94˚C 5 min. | 94˚C 30 sec. | 55˚C 30 sec. | 72˚C 30 sec. | 72˚C 7 min. | ***Kaipain-en et al., 2002*** |
| | **R:**CACACG GGTCTGGT ATTTATGC | | | | | | | |
| ***mphA*** | **F:**GTGAGG AGGAGC TTCGCGAG | 403 | 94˚C 5 min. | 94˚C 30 sec. | 58˚C 40 sec. | 72˚C 40 sec. | 72˚C 10 min. | ***Nguyen et al., 2009*** |
| | **R:**TGCCGC AGGACTC GGAGGTC | | | | | | | |
| ***TetA(A)*** | **F:**GGTTCAC TCGAAC GACGTCA | 576 | 94˚C 5 min. | 94˚C 30 sec. | 50˚C 40 sec. | 72˚C 45 sec. | 72˚C 10 min. | ***Randall et al., 2004*** |
| | **R:**CTGTCC GACAAGT TGCATGA | | | | | | | |
| ***blaTEM*** | **F:**ATCAGC AATAAA CCAGC | 516 | 94˚C 5 min. | 94˚C 30 sec. | 54˚C 40 sec. | 72˚C 45 sec. | 72˚C 10 min. | ***Colom et al., 2003*** |
| | **R:**CCCCGA AGAACG TTTTC | | | | | | | |

***In vitro* Antibiotic Susceptibility Test:**

Twenty *K. peumoniae* isolates were subjected to antibiotic sensitivity test against 10 commonly used antibiotics in chicken farms. The antimicrobial susceptibility profile against oxytetracycline, ampicillin, erythromycin, gentamycin, streptomycin, neomycin, cefotaxim, chloramphenicol, ciprofloxacin and norfloxacin were tested by disk diffusion methods according to Clinical and Laboratory Standards Institute (CLSI, 2012).

## RESULTS

Results are illustrated in tables (2-4) and figures (1-7)

**Table (2):** Prevalence of *K. peumoniae* in examined broiler chickens:

| Organs | No. of examined samples | No. of positive samples | Percentage of positive samples |
|---|---|---|---|
| Lung | 40 | 12 | 30 % |
| Air sacs | 40 | 5 | 12.5 % |
| Liver | 40 | 4 | 10 % |
| Kidney | 40 | 2 | 5 % |
| Total | 160 | 23 | 14.4% |

Twenty *K. peumoniae* isolates were subjected to antibiotic sensitivity test against 10 commonly used antibiotics in chicken farms. Results are shown in table (3).

**Table (3):** Antibiotic sensitivity and resistance pattern for (20) *K. pneumoniae* isolates.

| | Antibiotic | Sensitive | | Intermediate | | Resistant | |
|---|---|---|---|---|---|---|---|
| | | No. | (%) | No. | (%) | No. | (%) |
| 1 | Oxytetracycline | 0 | 0 | 0 | 0 | 20 | 100 |
| 2 | Ampicillin | 0 | 0 | 0 | 0 | 20 | 100 |
| 3 | Erythromycin | 1 | 5 | 1 | 5 | 18 | 90 |
| 4 | Streptomycin | 1 | 5 | 3 | 15 | 16 | 80 |
| 5 | Cefotaxim | 2 | 10 | 4 | 20 | 14 | 70 |
| 6 | Gentamycin | 2 | 10 | 5 | 25 | 13 | 65 |
| 7 | Neomycin | 5 | 25 | 6 | 30 | 9 | 45 |
| 8 | Chloramphenicol | 8 | 40 | 6 | 30 | 6 | 30 |
| 9 | Ciprofloxacin | 11 | 55 | 5 | 25 | 4 | 20 |
| 10 | Norofloxacin | 13 | 65 | 5 | 25 | 2 | 10 |

**Table (4):** Results of PCR assay for detection of *K. peumoniae* virulence and antimicrobial resistance genes.

| **Isolate** | **Results** | | | | | | |
|---|---|---|---|---|---|---|---|
| | ***K. pneumoniae 16S-23S ITS*** | **Virulence genes** | | | **Antimicrobial resistance genes** | | |
| | | ***mag*A** | ***fim*H** | ***tra*T** | ***tet*A** | ***bla*TEM** | ***mph*A** |
| 1 | + | + | + | + | + | + | + |
| 2 | + | + | + | + | + | + | + |
| 3 | + | - | + | + | + | + | + |
| 4 | + | + | + | + | + | + | + |
| 5 | + | + | + | + | + | + | + |

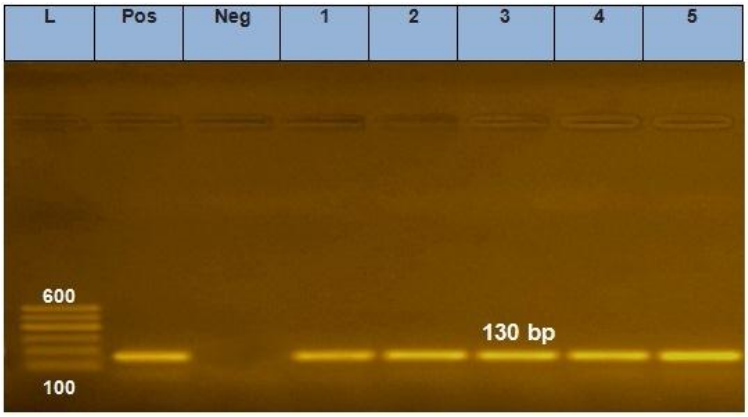


**Figure (1):** Agarose gel electrophoresis showing amplification of 130 bp fragment using *16S-23S ITS* primer of *K. pneumoniae*.
L: 100 - 600 bp ladder
Lane (1-5): Positive samples

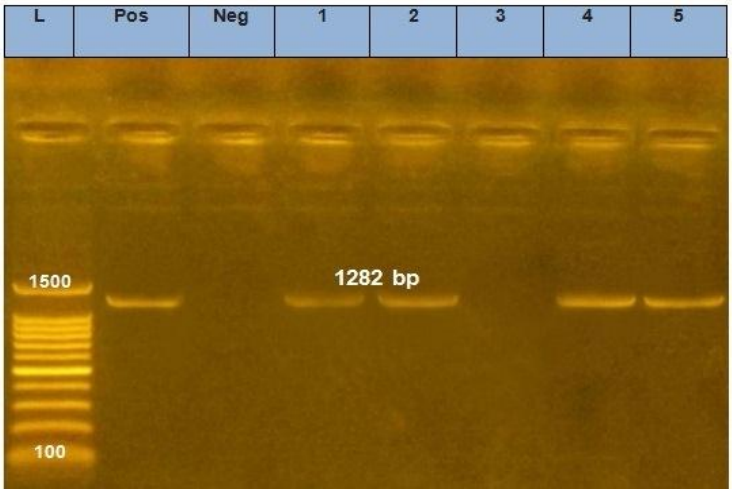


**Figure (2):** Agarose gel electrophoresis showing amplification of 1282 bp fragment using *mag*A primer.
L: 100 - 1500 bpladder
Lane (1, 2, 4 and 5): Positive samples
Lane (3): Negative sample

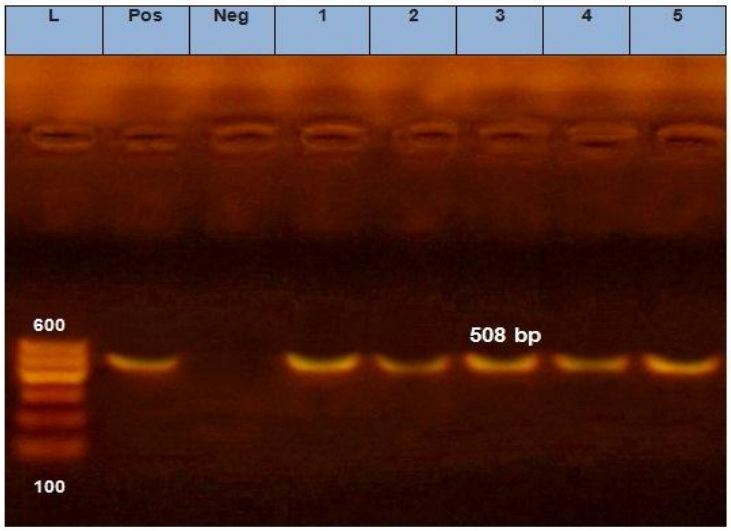


**Figure (3):** Agarose gel electrophoresis showing amplification of 508 bp fragment using *fim*H primer.
L: 100 - 600 bp ladder
Lane (1-5): Positive samples

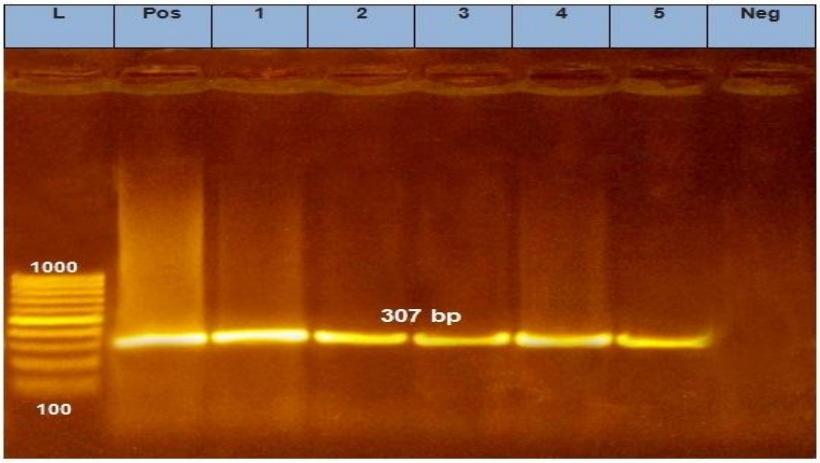


**Figure (4):** Agarose gel electrophoresis showing amplification of 307 bp fragment using *Tra*T primer.
L: 100 - 1000 bp ladder
Lane (1- 5): Positive sample

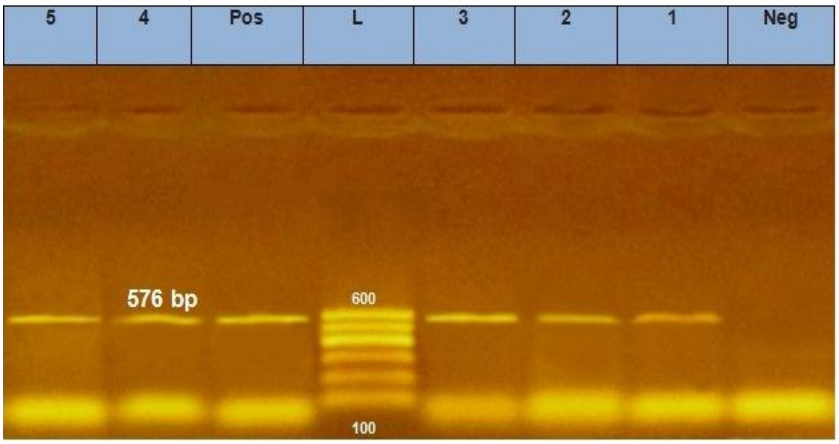


**Figure (5):** Agarose gel electrophoresis showing amplification of 576bp fragment using *tetA* (A) primer.

L: 100 - 600 bp ladder
Lane (1-5): positive samples

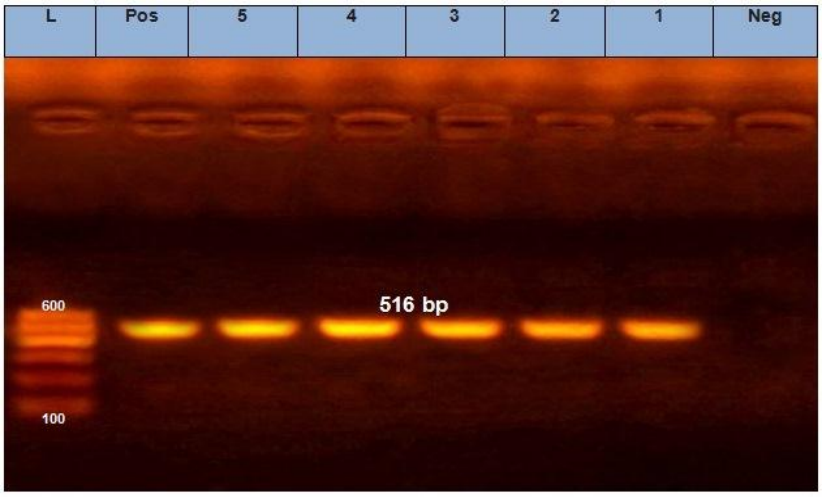


**Figure (6):** Agarose gel electrophoresis showing amplification of 516 bp fragment using *bla*TEM primer.
L: 100 - 600 bp ladder
Lane (1-5): positive samples

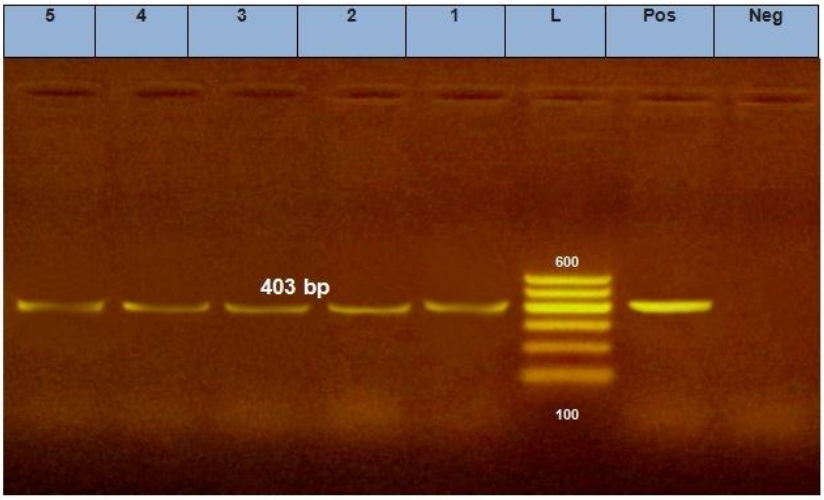


**Figure (7):** Agarose gel electrophoresis showing amplification of 403 bp fragment using *mph*Aprimer.
L: 100 - 600bp ladder
Lane (1-5): positive samples

## DISCUSSION

Bacterial pathogens play an important role in causing respiratory disease in domestic poultry species (Glisson, 1998). In many cases, the bacterial pathogens colonize the respiratory system as a secondary bacterial invasion only after a primary viral or environmental insult. *Klebsiella pneumoniae* has been frequently recovered from birds in which it functioned as a primary pathogen and was associated with respiratory tract disease (Sandra and Duarte, 1998). *Klebsiella pneumoniae* infection of young poultry increased the severity of respiratory disease (Saif *et al*., 2003).

In the present study, the clinical findings of *K. peumoniae* infected broilers were weakness, gasping, ruffled feather, nasal mucoid discharge, poor growth, facial oedema and conjunctivitis. These findings were similar to that observed by Popy *et al.* (2011).

Regarding the postmortem lesions of *K. peumoniae* infected broilers, there were signs of septicemia, pneumonia, air sacculitis, nephritis, sinusitis and liver and lung abscesses. These findings agreed with that observed by Chung *et al.* (2007).

In general, the investigation of 160 samples collected from clinically diseased broiler chickens revealed that the prevalence rate of *K. peumoniae* was (14.4%) as shown in (Table 2). Nearly similar results were recorded by Aly *et al.* (2014) and Khalda *et al.* (2000) who recorded that the prevalence rate of *K. peumoniae* in broiler chickens was (10%) and (10.2%), respectively.On the other hand, Turkyilmaz (2005) recorded a higher prevalence rate (47.1%). Meanwhile, Dashe *et al.* (2013) and Abdulrazzaq *et al.* (2014) reported that the prevalence of *K. peumoniae* in broiler chickens was (8%) and (7%), respectively.

In the present study, the isolation rate of *K. peumoniae* from lungs (30%) was higher than that of the other internal organs (air sacs, liver and kidneys, 12.5, 10 and 5 %, respectively) as shown in (Table 2). It was in the same direction with Younis *et al.* (2016).

PCR detection based on 16S-23S rDNA internal transcribed spacer (ITS) of *K. pneumoniae* was carried out in the present study. Five isolates of *K. pneumoniae* were positive to the PCR detection (Figure 1). This agree with that reported by Yin Liu *et al.* (2008).

PCR assay was conducted for detection of some virulence genes of *K. pneumoniae*. PCR assay could identify *mag*A, *fim*H and *tra*T genes by using specific primer sequences which yielded product sizes of 1282bp, 508bp and 307bp, respectively. Out of the tested isolates, four isolates were positive and one isolate was negative for *mag*A gene (figure 2), five isolates were positive for *fim*H gene (Figure 3) and five isolates were positive for *tra*T gene (Figure 4). Detection of these genes may indicate the virulence potential of *K. pneumoniae* isolates. Struve *et al.* (2005) described *mag*A as a novel virulence factor responsible for the increased virulence of certain *K. pneumoniae* strains. They provided evidence that the *mag*A gene, so far believed to be a specific virulence factor in highly virulent Klebsiella strains. El Fertas *et al.* (2013) concluded that *fim*H gene is the most common virulence gene of *K. pneumoniae* and *tra*T gene was detected at a lower prevalence rate in *K. pneumoniae* isolates.

*K. pneumoniae* is an important multidrug-resistant (MDR) pathogen affecting both humans and animals. PCR assay was conducted for detection of some antimicrobial resistance genes of *K.*

*pneumoniae*. PCR assay could identify *tetA*(A), *bla*TEM and *mph*A genes by using specific primer sequences which yielded product sizes of 576bp, 516bp and 403bp, respectively. Out of the tested isolates, five isolates were positive for *tetA*(A) gene (figure 5), five isolates were positive for *bla*TEM gene (Figure 6) and five isolates were positive for *mph*A gene (Figure 7). Detection of these genes may indicate the high multiple antibiotic resistances of *K. pneumoniae* isolates. Weixia Wang *et al.* (2014) found that the class A tet determinants *tet*(A) and *te*t(A)-1 could confer high-level tetracycline resistance. Ojdana *et al.* (2014) found that the prevalence of *bla*TEM genes was responsible for the production of broad-spectrum β-lactamases among *K. pneumoniae*. Soge *et al.* (2006) found that all the large CTX-M plasmids of *K. pneumoniae* carried several drug resistance genes including *bla*TEM-1gene (ampicillin resistance) and *tet*(A) gene (tetracycline resistance) while 65 % of plasmids carried *mph*(A) gene (macrolide resistance).

Twenty *K. peumoniae* isolates were subjected to antibiotic sensitivity test against ten commonly used antibiotics in chicken farms. All isolates were resistant to ampicillin and oxytetracyclines. This was agreed with Gundogan and Avci (2013) who reported that *klebsiella species* showed 100 % resistant to ampicillin and Rasool *et al.* (2003) who found that *K. peumoniae* was resistant to tetracyclines. Brisse *et al.* (2006) discussed that *klebsiella species* were resistant to ampicillin as a result of chromosomal class-A β-Lactamase production. Also, *K. peumoniae* isolates showed 90% resistance rate against erythromycin. It was in agreement with that reported by Kilonzo *et al.* (2007). These results were supported by PCR assay which detected *tet*A (A), *blaTEM* and *mph*A antimicrobial resistance genes against tetracyclines, ampicillin and erythromycin, respectively. These results run parallel with that reported by Guo *et al.* (2016) and Hou *et al.* (2015). On the other hand, *K. pneumoniae* isolates showed moderate resistance rate (65% and 45%) against gentamycin and neomycin, respectively. This might run parallel with Chang *et al.* (2000) who recorded that *K. pneumoniae* were moderately susceptible to aminoglycosides. Also, *K. pneumoniae* isolates showed lower resistance rate (20% and 10%) against ciprofloxacin and norfloxacin, respectively. It was agreed with Gundogan and Avci (2013) who reported 23.8% resistance rate against ciprofloxacin. While, Olufemi *et al.* (2012) reported (54.5% and 63.6%) resistance rates against ciprofloxacin and norfloxacin, respectively.

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## التوصيف الجزيئي لبعض جينات الضراوه للكلبسيلا نيموني المعزوله من بداري التسمين

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أجريت هذه الدراسه علي عدد ١٦٠ عينه (٤٠ من كلا من الرئتين، الأكياس الهوائيه، الكبد، الكلي) تم تجميعهم من عدد ٤٠ من بداري التسمين المصابه ظاهريا من عدة مزارع مختلفه بمحافظة الدقهليه. بأجراء الفحص الظاهري لهم تبين وجود ضعف عام ، صعوبه في التنفس، تورم بالوجه، التهاب بالعين وارتشاح بالأنف مع وجود سوائل بها وأوضحت الصفه التشريحيه وجود احتقان بالأعضاء الداخليه ، التهابات رئويه ، التهاب بالأكياس الهوائيه ،التهاب الكلي مع وجود خراريج في الرئه والكبد. وقد أظهرت نتائج الفحص البكتيريولوجي أن نسبة الأصابه العامه بميكروب الكلبسيلا نيموني كانت (١٤،٤ %). وسجلت نسبة العزل من الرئه ، الأكياس الهوائيه ، الكبد والكلي 30 %، 12.5%، 10% و 5% علي التوالي . وقد تم تصنيف ميكروب الكلبسيلا نيموني بالطرق البيوكيميائيه. وباجراء اختبار تفاعل انزيم البلمره المتسلسل لبعض المعزولات تم تحديد والكشف عن بعض جينات الضراوه ( *mag*A, *FimH*, *Tra*T) وكذلك بعض الجينات المقاومه للمضادات الحيويه ( *Tet*A (A) , *Bla*TEM , *mph*A ).وباجراء اختبار الحساسيه لعدد ٢٠ معزوله لقياس نسبة مقاومتها لعدد (١٠) مضادات حيويه من المستخدمه في مزارع بداري التسمين تبين أن نسبة المقاومه كانت ١٠٠% لكلا من الأمبيسلين والأوكسي تتراسيكلين ، ٩٠% للارثروميسين ، ٨٠% للاستربتومايسين ، ٧٠% للسيفوتاكسيم ،٦٥% للجنتاميسين ،٤٥% للنيومايسين ،٣٠% للكلورامفينيكول بينما كانت أقل نسبة مقاومه هي ٢٠% و ١٠% للسيبروفلوكساسين والنورفلوكساسين علي التوالي .