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DETECTION OF SOME VIRULENCE AND ANTIBIOTIC RESISTANCE GENES OF *KLEBSIELLA PNEUMONIAE* ISOLATED FROM DISEASED BROILER CHICKENS

By

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ABSTRACT

Respiratory diseases affected broiler chickens is a problem threat their production and cause a great loses among them. This study was conducted to determine the prevalence and identification of *Klebsiella pneumoniae* isolates from varying age clinically diseased broiler chickens in Gharbia governorate. A total of 400 internal organs (heart, lung, liver and spleen) collected from 100 diseased chickens suffered from respiratory manifestation were subjected to detect rate of *Klebsiella pneumoniae* prevalence and detection of isolates susceptibility patterns to various antibiotics by disc diffusion method. In addition, these isolates were screened for the presence of selected virulence genes including, *rmp*A (regulator of the mucoid phenotype A), *mag*A (mucoviscosity associated gene), *wca*G and antibiotic resistance genes (bla_{TEM} and bla_{SHV}) by using PCR. Bacteriological examination revealed that isolation of *Klebsiella Pneumonia* with a total prevalence rate reached (13%) as 13 out of 100 examined cases where (27) isolates of *K. pneumoniae* were detected.

The highest percentage of isolation of *K. pneumoniae* was from lung (13%) followed by liver (7%) then spleen 5% and finally from heart (2%). All of the isolates were resistance to Ampicillin. The rate of resistance to other antibiotics was as follows, 25 (92.6%) to Lincomycin, 24 (88.9%) to Oxytetracycline, 22 (81.5%) to cefotaxime and Doxycycline. 9 (33.3%)to ciprofloxacin, 8 (29.6%) toAmoxicillin / clavulanic acid, 7 (25.9%) to Norfloxacin, 6 (22.2%) to gentamicin, and all of the isolates were sensitive to amikacin.

Klebsiella Pneumonia isolates were confirmed by PCR for detection of *gyr*A and *16S-23S* ITS genes, all isolates were positive for antibiotic resistance genes (bla_{TEM} and bla_{SHV}) and negative for *mag*A, *rmp*A and *wca*G genes.

INTRODUCTION

Klebsiella spp. is a normal intestinal flora of poultry, but could cause infections whenever the immune system of affected birds is compromised.

Klebsiella pneumoniae is opportunistic pathogens associated with severe nosocomial infections such as septicaemia, pneumonia, urinary tract infections and bloodstream infections. *K. pneumonia* has been taxonomically subdivided into three subspecies.

K. pneumonia subsp. pneumonia, *K. pneumonia* subsp. *Ozaenae and K. pneumonia subsp. Rhinoscleromatis* **(Brisse and Verhoef, 2001).**

Klebsiella pneumoniae is a Gram negative, encapsulated, non-motile, rod shape, lactose fermenting bacteria, belong to family *Enterobacteriaceae*. Members of this family are facultative anaerobic which cause infections whenever the immune system of affected bird is compromised **(Janda and Abbott, 2006).**

Klebsiella expresses two types of antigens on their cell surface. The first is smooth lipopolysaccharide (O-antigen); the other is capsular polysaccharide (K-antigen).

Both antigens contribute to the pathogenesis of this species. The major virulence factor of *Klebsiella* is the polysaccharide capsule, which is also responsible for the mucoid colony phenotype, **(Sikarwar and Batra, 2011).** *K. pneumoniae* can produce several virulence factors such as smooth LPS, pilli for adhesion to host cells, capsule that are antiphagocytic, siderophores that aid the bacterium in its competition with the host for iron uptake **(SAljanaby and Alhasani, 2016).**

DNA gyrase, which is composed of two A subunits and two B subunits, is encoded by the *gyr*A and *gyr*B genes. The genes encoding subunit A of DNA gyrase (*gyr*A), a protein that corresponds to the main target of fluroquinololnes in *Klebsiella* is used for detection of genus *Klebsiella* **(Brisse and Verhoef, 2001).**

This bacterium is a common saprophyte in many parts of the environment and occasionally causes embryonic mortality and excess losses in young chickens and turkeys **(Orajaka and Mohan, 1985*).*** *K. pneumoniae* has been frequently recovered from birds in which it functioned as a primary pathogen and was associated with respiratory tract disease, septicaemia, peritonitis, salpingitis, air sac disease, omphalitis, artheritis, panopthalmitis, intestinal disturbances and drop in egg production **(Sandra and Duarte, 1998); (Aly *et al.,* 2014) and (Saif *et al.,* 2003).** The genomic map of *K. pneumoniae* capsule contains gene

clusters as follows: *rmp*A (regulator of the mucoid phenotype A), *mag*A (mucoviscosity associated gene A), *cps* (capsular polysaccharide synthesis), *Wb* (O-specific polysaccharide is directed by the *wb* gene cluster) *kfu* gene (iron uptake system gene) **(Regue *et al.,* 2005 and Seidler *et al.,* 1975).** The *rmp*A gene regulates the synthesis of the *Klebsiella* polysaccharide capsule. The *mag*A gene is 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 *kfu* gene which codes for an iron uptake system, this gene is considered as a very important gene in iron up take from host cell **(Aher *et al.,* 2012**) which is a putative pathogenic gene, more prevalent in hypervirulent strains and purulent tissue infections **(Ma *et al.,* 2005).** The *wca*G virulence gene is located in the transferable regions of chromosome responsible for *K. pneumoniae* capsule biosynthesis and needed for the conversion of mannose to fucose, which may enhance the ability of bacteria to evade phagocytosis by macrophages **(Shu *et al.,* 2009).** Resistance and virulence are not independent properties, and their relationship may play an important role in the pathogenesis of *K. pneumoniae* infections **(Vila *et al.,* 2011**). Virulence and resistance are similar in that most of the determinants have been transmitted between bacteria by horizontal gene transfer and the transfer of DNA is probably the most important mechanism for dissemination and co-selection of virulence and resistance properties **(Da Silva and Mendonc, 2012).**

Klebsiella spp. is often multidrug resistant and an increasing proportion of strains produce extended-spectrum beta-lactamases (ESBLs). Extended-spectrum beta- 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 are multidrug-resistant **(Jacoby, 1997).**

Resistance to antimicrobial agents is often associated with the spread of transmissible plasmids, which may also carry virulence determinants, the acquisition of resistance and virulent traits may provide a survival benefit to the microorganism **(Da Silva and Mendonc, 2012).** The plasmid gene *rmp*A (regulator of mucoid phenotype A) confers a hypermucoviscous phenotype to *K. pneumonia* by enhancing capsular polysaccharide production. Also, as a result of the widespread use of these antimicrobial agents, bacteria have developed ways to

circumvent the effects of antibiotics through evolutionary adaptations **(Wright *et al.,* 2005).** Therefore, contamination of food with antibiotic-resistant bacteria poses a major threat to public health and the transfer of these antibiotic-resistant traits to pathogenic bacteria could potentially compromise the treatment of bacterial infections in the clinical setting **(Van *et al.,* 2007).** This study was aimed to investigate virulence and resistance factors of *K. pneumoniae* isolates from chickens suffered from respiratory manifestation Gharbia governorate.

MATERIAL AND METHODS

Samples:

A total of 400 samples (heart, lung, liver and spleen) were collected from 100 varying age broiler chickens suffered from respiratory manifestation from some farms in Gharbia governorate, Egypt, each sample was kept in sterile plastic bag in an ice box and send to the laboratory without delay.

Bacterial culture and identification:

All clinical specimens were inoculated directly in nutrient broth and incubated aerobically at 37°C for 24 hours and then a loopful from incubated broth was streaked onto MacConkey's bile salt agar and Xylose Lysine Desoxycholate (XLD) agar and incubated at 37°C for 24hours. The suspected mucoid lactose fermenting colonies were purified and identified biochemically by indole,methyl red,voges proskauer,citrate (IMViC),oxidase,H2S production, lysine decarboxylase, lactose fermentation,urea hydrolysis, catalase and coagulase production **(Harada *et al.,* 2013) and (Dashe *et al.,*2013).**

3-PCR Procedue:

a-DNA extraction:

DNA extraction from pure isolates (isolate from each organ) were performed using the QIAamp DNA Mini kit **(Qiagen, Germany, GmbH)** with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was thenwashed and centrifuged. Then nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

B-Oligonucleotide Primer:

Primers for Target genes under lest used were supplied from **(Metabion Germany)** were listed in (Table 1). For PCR, Primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol

concentrations, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler as shown in (Table 1).

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

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

Target gene	Primers sequences	Amplied segment (bp)	Primary denatu ration	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
Klebsiella gyrA	F. CGC GTA CTA TAC GCC ATG AAC GTA R. ACC GTT GAT CAC TTC GGT CAG G	441	94°C 5 min.	94°C 30 sec.	55°C 40 sec.	72°C 40 sec.	72° C 10 min.	Brisse and Verhoef, (2001)
magA	F. GGTGCTCTTTACATCAT TGC R. GCAATGGCCATTTGCGT TAG	1282	94°C5 min.	94°C 30 sec.	50°C 40 sec.	72°C 1.2 min.	72° C 12min.	Yeh *et al.*, (2007)
rmpA	F. ACTGGGCTACCTCTGCT TCA R. CTTGCATGAGCCATCTT TCA	535	94°C 5 min.	94°C 30 sec.	50°C 40 sec.	72°C 40 sec.	72° C 10 min.	
K. pneumoniae 16S-23S ITS	F. ATTTGAAGAGGTTGCA ACGAT R. TTCACTCTGAAGTTTC TTGTGTTC	130	94°C 5 min.	94°C 30 sec.	55°C 30 sec.	72°C 30 sec.	72° C 10 min.	Turton *et al.*, (2010)
WcaG	F. GGTTGGGTCAGCAC GTA R. ACTATTCCGCCAATT TGC	169	94°C 5 min.	94°C 30 sec.	58°C 30 sec.	72°C 30 sec.	72° C 10 min.	Derakhshan *et al.*, (2016)
BlaTEM	F. ATCAGCAATAAACCAGC R. CCCCGAAGAACGTTTTC	516	94°C 5 min.	94°C 30 sec	54°C 30 sec.	72°C 30 sec	72° C 10 min.	Colom *et al.*, (2003)
blaSHV	F. AGGATTGACTGCCT TTTTG R. ATTTGCTGATTTCGCTCG	392	94°C 5 min.	94°C 30 sec	54°C 30 sec.	72°C 30 sec	72° C 10 min.	

4-Antimicrobial susceptibity testing:

The bacterial resistance profile was determined using disc diffusion method in accordance with the clinical laboratory Standards Institute **(CLSI, 2007)** guidelines. **(Janet and John,**

2007) The antimicrobial agents tested were Ampicillin (Amp10 µg), Oxytetracycline (30 µg), Gentamicin (CN10 µg), Amoxicillin / Clavulinic acid (Amc 20 µg), Doxycycline (DO30 µg), Norfloxacin (NOR10 µg) Cefotaxime (CTX 30µg), Ciprofloxacin (CIP 5 µg), and Amikacin (Ak30µg) and Lincomycin (NY10µg).

RESULTS AND DISCUSSION

Table (2): Frequency of isolation rate of *Klebsiella Pneumonia* from different organs.

Samples	No. of examined samples	*Klebsiella spp*		*Klebsiella* Pneumonia	
		No	%	No	%
Liver	100	16	16%*	7	7%*
Lung	100	29	29%*	13	13%*
Spleen	100	13	13%*	5	5%*
Heart	100	7	7%*	2	2%*
Total	400	65	16.25%**	27	6.75%**

***The percentage was calculated according to the total number of each organ.**

****The percentage was calculated according to the total no. of organs.**

Bacterial cultures and identification:

The morphological examination showed that suspected colonies were observed as large, gray, mucoid, convex and circular colonies **(Dashe *et al.,* 2013).** The biochemical identification proved that, the isolated strains were indole-negative, methyl red-negative, voges proskauer-positive, citrate-positive, oxidase-negative, H2S production-negative, lysine decarboxylase-positive, lactose fermentation-positive, urea hydrolysis- positive, catalase-positive and coagulase-negative, so they were identified as *K. pneumoniae* **(Barbara *et al.,*1994).**

In the present study, 400 organ samples were collected from 100 clinically diseased broiler chickens (liver, lung, spleen, and heart), four samples per each bird were examined for the presence of *Klebsiella spp*.Bacteriological examination revealed that isolation of *K. pneumonia* with a total prevalence rate reached (13%) as 13 out of 100 examined cases, and *Klebsiella spp*. with a total prevalence rate (29%) as 29 out of 100 examined cases.

A low prevalence rate of *K. pneumonia* has been reported by **(Aher *et al.,* 2012)** at ratio of 5.6% and **(Yimer and Asseged, 2007)** revealed rate of *Klebsiella spp*. at ratio 1.3%. A total of 65 isolates of *Klebsiella spp*. were recovered from 400 organs (four samples per each bird), a total of 27 isolates of *K. pneumoniae* were recovered from 400 organs (four samples per each

bird). It was noticed that, the highest percentage of isolation of *K. pneumoniae* was from lung (13%) followed by liver (7%) then spleen (5%) and finally in heart (2%) (Table2).

These findings were lower than that reported by **(Aya *et al.,* 2017) and (Türkyilmaz, 2006)** who recovered *Klebsiella* species with a prevalence rate 22.78%. 47.1% respectively. While our results were higher than that reported by **(Hossain *et al.,*2013); (Khalda *et al.,* 2013); (Aly *et al.,* 2014) and (Younis *et al.,* 2016)** with a prevalence rate (6%, 8.69%, 10.2%, 10% and 15%) respectively. The isolation rate of *Klebsiella* from lung was higher than other organs. These results agree with **(Younis *et al.,* 2016)** who said that *Klebsiella* species recovered from lungs is higher percentage than from the other organs and disagrees with **(Aya *et al.,* 2017)** who said that *Klebsiella* species recovered from liver is higher percentage than the other organs. The wide distribution of *K. pneumoniae* in the lungs, spleen and liver of birds affected could probably indicate concurrent extra–intestinal infections. **(Türkyilmaz, 2006).** Also, **Dashe *et al.*, (2013**) isolated *K. pneumoniae* with a percentage of 8% from lungs and liver of 400 apparently healthy chickens. **Younis *et al.,*(2016)** reported that 30 (15%) *Klebsiella* species isolates were recovered from 200 tissue samples which differentiated into *K. pneumoniae* 11 % (22/200) and *K. oxytoca* 4 % (8/200), these results are higher than our results. **Aya *et al.,* (2017)** isolated *K. pneumoniae* with a percentage rate (7.78%),while higher isolation rate (64%, 73.33% and 18%) was reported by **(Ajayi and Egbebi, 2011), (Younis *et al.,*2016) and (Kumbish *et al.,*2006)** respectively. A lower incidence was recorded by **(Dashe *et al.,* 2008) and (Yehia and Riyadh, 2013)** with percentage rate (1.5% and 3.33%) respectively.

Table (3): Antimicrobial susceptibility pattern of *K. pneumoniae* isolated from diseased broiler chickens **(CLSI, 2007).**

Antimicrobial.	Resistance	Sensitive
Norfloxacin	**7 (25.9%)**	**20 (74.1%)**
Gentamicin	**6 (22.2%)**	**21(77.8%)**
Ciproflxacin	**9 (33.3%)**	**18 (66.7%)**
Amikacin	**- (0%)**	**27 (100%)**
Ampicillin	**27 (100%)**	**- (0%)**
Oxytetracycline	**24 (88.9%)**	**3 (11.1%)**
Cefotaxime	**22 (81.5%)**	**5 (18.5%)**
Lincomycin	**25 (92.6%)**	**2 (7.4%)**
Amoxicillin / clavulanic acid	**8 (29.6%)**	**21 (77.8%)**
Doxycycline	**22 (81.5%)**	**5 (18.5%)**

***%: calculated according to the No. of tested isolates (27).**

A total of 27 *Klebsiella* isolates were tested against 10 antimicrobial agents using the Kirby- Bauer disk diffusion assay. Analysis of the antimicrobial susceptibility profile showed that, all of the isolates were resistance to ampicillin. The rate of resistance to other antibiotics was as follows, 25 (92.6 %) to lincomycin, 24 (88.9%) to oxytetracycline, 22 (81.5%) to cefotaxime and doxycycline. 9 (33.3%) to ciprofloxacin, 8 (29.6%) to amoxicillin / clavulanic acid, 7 (25.9 %) to enrofloxacin, 6 (22.2%) to gentamicin, and all of the isolates were sensitive to amikacin as shown in (Table 4). The results showed that 18.5% (5/27) of the strains possessed multiple resistances (to three or more classes of antimicrobials). None of the isolates were sensitive to all antibiotics tested. Infections caused by multidrug- resistant bacteria are increasing in many countries. *K. pneumoniae* infection is often treated with β–lactam antibiotics; also, beta-lactam antibiotics are one of the most used resistant antibiotics that created a major crisis in medical clinic in the last two decades **(Fang *et al.,* 2004 and Amin *et al.,* 2009)**. In our study results of antibiotic sensitivity test showed that *K. pneumoniae* strains were resistant to different classes of antibiotics. Rate of resistance to ampicillin was high (100%), most of *Klebsiella* isolates are naturally resistant to ampicillin due to a constitutively expressed chromosomal class Ab-lactamase **(Livermore 1995),** 29.6% of the isolates were resistance to Amoxicillin/ clavulanic acid, that agrees with **(Beyene and Tsegaye, 2011) and (Behnam *et al.,* 2014)**. **Younis *et al.*, (2016)** reported that all of the isolates were resistant to amoxicillin and Amoxicillin/ clavulanic acid in contrast with our

finding. Amoxicillin resistance has been reported in veterinary clinical isolates **(Brisse and Van Duijkeren, 2005)**. About (60.5%) of *K. pneumoniae* isolates were resistant to amoxicillin/clavulanic acid **(Derakhshan *et al.,* 2016)**.77.8% of *K. pneumoniae* isolates were sensitive to gentamicin. These results agree with **(Behnam *et al.,* 2014)** who reported 65.89% of*K.pneumoniae*isolateswere sensitivetogentamicin.Alowprevalenceof *K. pneumoniae* isolates were sensitive (48.5%) to gentamicin **(Derakhshan *et al.,* 2016)** and Different rates of cephalosporin resistance had been reported.

Table (3) showed that, the resistance rate for cephalosporin (cefotaxime) was 81.5%, which was not far from those previously reported by **(Singh and Goyal 2003) and (Younis *et al.,* 2016)**, the resistance rates for cephalosporin were 86.67% for cefotaxime, 70 % for cefapime 66.67% for ceftriaxone. On the other hand, a lower rate of resistant to cephalosporins was recorded by **(Ullah *et al.,* 2009) and (Derakhshan *et al.,* 2016),** (60.0 %) to cefotaxime. Resistance of *Klebsiella pneumoniae* isolates to ciprofloxacin in our study was 33.3% which similar to those previously reported by **(Villegas *et al.,* 2004).**

Derakhshan *et al.,* (2016) and Younis *et al.,* (2016) reported a higher level of resistance of *Klebsiella pneumoniae* to ciprofloxacin, 50% and 66.67 % respectively, this agrees with the results reported by **Ullah *et al.,* (2009)**. Ciprofloxacin is a broad-spectrum fluoroquinolone antibacterial agent **(Periti *et al.,* 1998)** the resistance of *Klebsiella* to ciprofloxacin mainly due to a chromosomal mutation in the *gyrA* gene, which codes for the target of quinolone activity **(Bagel *et al.,* 1999**). Aminoglycosides are active against gram-negative bacilli which have a clinical important **(Ramirez and Tolmasky, 2010).** In this study, all isolates were sensitive to amikacin. *Klebsiella* isolates showing 10 % resistance to amikacin were reported by (**Younis *et al.,* 2016**). A low prevalence of amikacin resistance (7 %) to *K. pneumoniae* isolated from meat samples was also reported by **(Gundogan *et al.,* 2011).**

On the other hand, **(Ullah *et al.,* 2009) and (Derakhshan *et al.,* 2016)** reported that 63.04%, 68.5% of *Klebsiella* isolates were susceptible to amikacin respectively. A high prevalence of *K. pneumoniae* isolates were resistance to Oxytetracycline and Doxycycline (88.9%) and (81.5%) respectively. These data corroborate the findings reported by **(Kim *et al.,* 2005) and (Wu *et al.,* 2012)** who demonstrated high levels of resistance to beta-lactams and tetracycline, however antibiotic resistance rates in *K. pneumoniae* were also reported as 13 %, 25 %, 19.6% and 46.6% by **(Davies *et al.,* 2016; Bonnedahl *et al.,* 2014; Irajian *et al.,* 2009) and (Mohammadi-mehr and Feizabadi, 2011)** in Iran respectively. Other results of two studies

have shown the correlation between production of virulence factors and resistance phenotype **(Da Silva and Mendonc, 2012) and (Mansouri *et al.,* 2011)**. It has also been shown that ESBL producing isolates of *K. pneumoniae* are more invasive, yield more fimbrial adhesins, and are more resistant to bactericidal activity of serum **(Sahly *et al.,* 2004 and Sahly *et al.,* 2008).** The simultaneous presence of virulence genes and class 1 integrons enhances the possibility of spreading antibiotic resistance and virulence determinants through horizontal gene transfer. Further, the acquisition of resistance determinants by virulent strains may lead to long persistence of microorganisms in clinical settings **(Derakhshan *et al.,*2016)**.

Extended spectrum beta-lactamases (ESBLs) hydrolyse β-lactam ring, so it may inactivate cephalosporin and penicillin antibiotics **(Beyene and Tsegaye, 2011)**. Moreover, some genes are mobile among isolates and they spread in the environment. It is possible that a different mechanism of gene transfer such as horizontal gene transfer between serotypes may cause the spread of resistance genes **(Madhusudana and Surendran, 2010) and (Sharma and Navin 2006).** In the USA **(Kumar *et al.,* 2011),** showed that *K. pneumoniae* was multidrug- resistant (MDR) to fluoroquinolones, aminoglycosides and trimethoprim which are similar to the result of our study. Different reasons such as large component of the genetic and phenotypic diversity of clinical isolates,additional efflux pumps and multiplemechanisms of fluoroquinolone resistance cause antibiotic resistance in bacteria and this indicates an increase in resistance to antibiotics by this bacterium. *Klebsiella* strains recorded high antibiotic resistance with multiple antibiotic resistance (MAR). Rate of multiple antibiotic resistances was extremely high which may be due to the hazard routinely use antibiotics for treatment and control of bacterial diseases in poultry farms. When these antibiotics are administered to the birds at low levels for a long period, certain bacterial species become resistant **(Kilonzo-Nthenge *et al.,* 2008).** In view of all the findings, further investigation is needed to define the role of other host and pathogen factors which may assist in the progression of disease at the physiological and molecular levels.

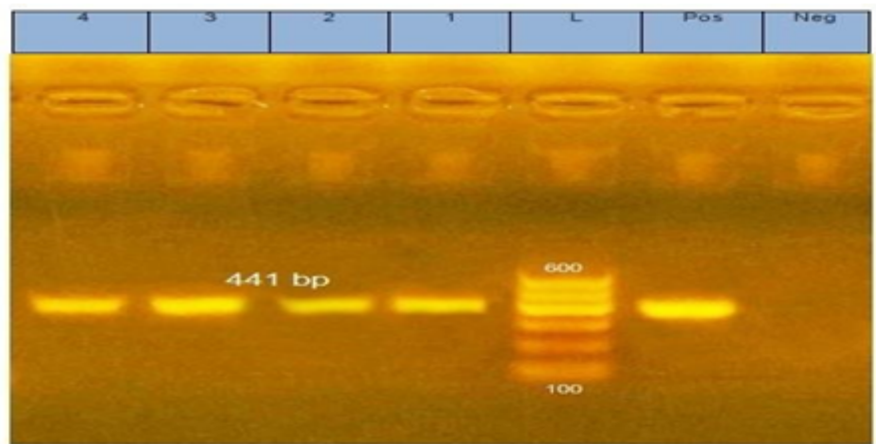

Fig. (1): Agar gel electrophoresis showing PCR amplification at 441 bp fragment for *gyr*A gene M) 100 bp DNA ladder; control positive *Klebsiella* species, control negative, lane (1, 2, 3, and 4) positive samples.

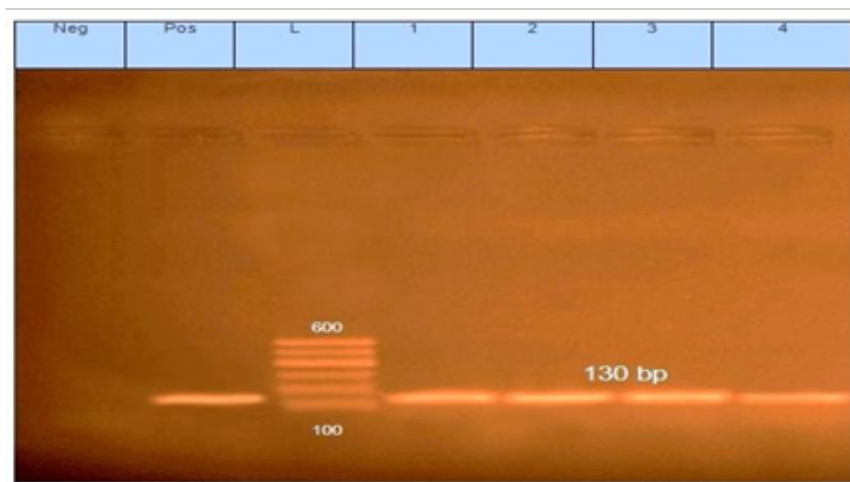

Fig. (2): Agar gel electrophoresis showing PCR amplification at 130 bp fragment for for 16S-23S ITS 100 bp DNA ladder; control positive, control negative, lane (1, 2, 3 and 4) positive for *K .pneumoniae*.

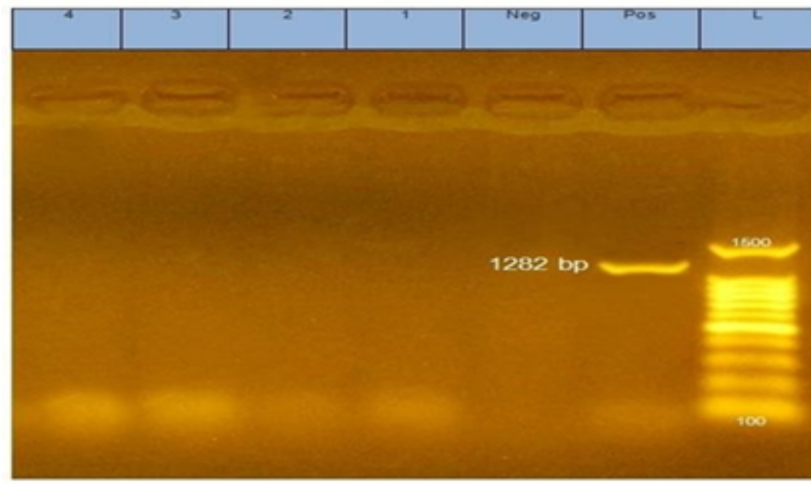

Fig. (3): Agarose gel electrophoresis showing amplification of 1282 bp fragment using *mag*A primer. M) 100 bp DNA Ladder, control positive, control negative; (Lane 1, 2, 3 and 4) negative samples.

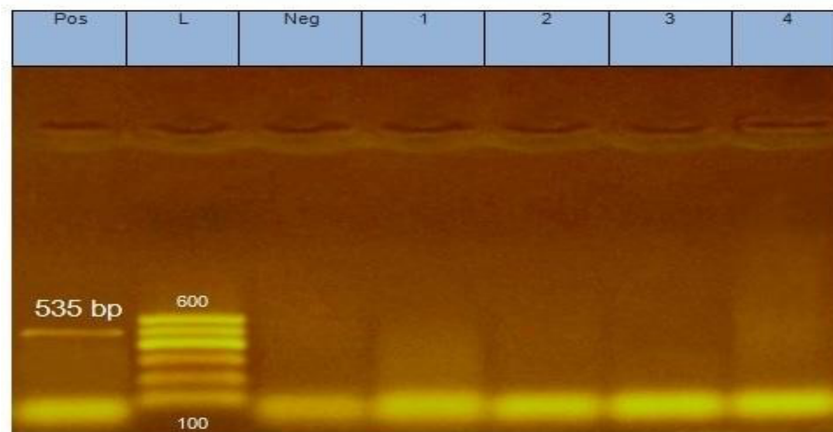

Fig. (4): Agarose gel electrophoresis showing Amplification of 535 bp fragment using *rmp*A primer. M) 100 bp DNA Ladder, control positive, control negative; (Lane 1, 2, 3 and 4) negative samples.

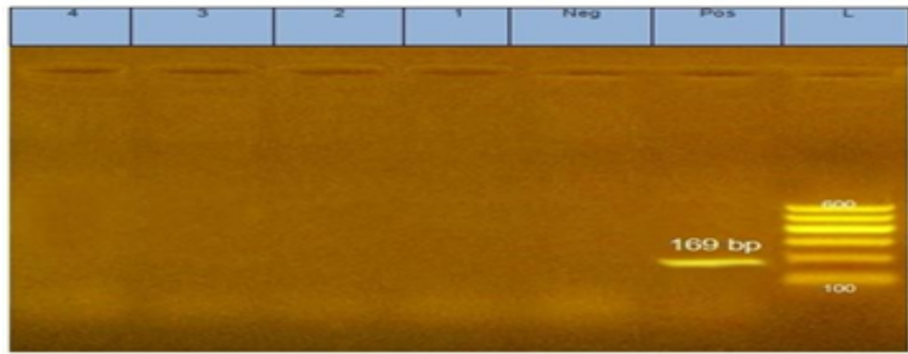

Fig. (5): Agarose gel electrophoresis showing amplification of 169 bp fragment using wcaG primer. M) 100 bp DNA Ladder, control positive, control .negative; (Lane 1, 2, 3 and 4) negative samples.

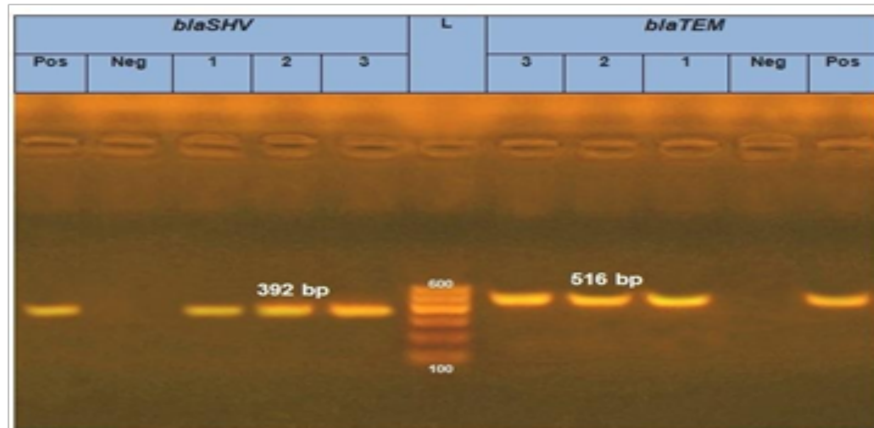

Fig. (6): Agarose gel electrophoresis showing amplifica of 516 bp and 392bp fragment using blaTEM and blaSHV primers. M) respectively, 100 bp DNA Ladder, control positive, control negative; (Lane 1, 2, and 3) positive samples for both genes.

*Gyr*A primers were designed based on the alignment of the *gyr*A sequences with those of other bacteria available in public sequence database. These primers were amplifying a portion of the genes broader than those generally amplified to determine solely mutations in the quinolone's resistance determining region that were used for all *Klebsiella species***(Brisse and Verhoef, 2001).** Some of isolates were confirmed by polymerase chain reaction (PCR) using genus specific primer sequences *(gyr*A*)* and confirm the identification of *K. pneumoniae* subsp. *pneumoniae*, using primers for the 16S-23S internal transcribed spacer region (Table 1), which yielded product sizes of 441 bp and 130 bp respectively Fig. (1,2). Current biochemical methods of identification are time consuming and often are inconclusive because related species often present similar biochemical patterns lopes **(Lopes *et al.,* 2007)**.

The entire isolates were screened by PCR to identify the *mag* A *and rmp* A genes by using specific primer sequences which amplify a region of 1282 bp and 535 bp, respectively. The *mag*A and *rmp*A, genes were not detected. Fig. (3, 4). The absence of *rmp*A and *mag*A genes (related to extracapsular polysaccharides and mucoviscosity) were an unexpected finding. These results similar to results of **Davies *et al.,* (2016). (El -Fertas-Aissani *et al.,* 2013)** assessed strains from different human clinical samples and observed a low prevalence of *rmp*A and no samples were positive for *mag*A. These genes play an important role in the pathogenesis of *K. pneumoniae* related to the invasiveness and resistance and they are predominant in human patients presenting invasive diseases in certain regions of the world. Considering the negative results for *mag*A and *rmp*A, additional studies are needed to clarify the precise role of *mag*A and *rmp*A, as well as its interaction with components of bacterial surface envelopes such as exopolysaccharide, capsular polysaccharides and LPSs **(Fang *et al.,* 2004).** Since *mag*A was unique to serotype K1 strains and all of the serotype K1 and K2 strains were *rmp*A positive, **(Yeh *et al.,* 2007)** classified all strains into four groups: serotype K1 (*mag*A positive and *rmp*A positive), serotype K2 (*mag*A negative and *rmpA* positive), *rmpA*-positive non-K1/K2 (*mag*A negative), and *rmp*A-negative non-K1/K2 (*mag*A negative). Detection of these genes may indicate the virulence potential of *Klebsiella* isolates. Capsular serotypes K1 and K2 that carry *mag* A and *rmp*A genes make the bacteria more invasive and more resistant to phagocytosis. **Yu *et al.,* (2006)** in Taiwan showed that prevalence of *rmp*A, and *mag*A were 48% and 17% respectively.

Prevalence studies showed an association of *rmp*A with *K.pneumoniae* virulence **(Fang *et al.,* 2004; Ku *et al.,* 2008; Cheng *et al.,* 2010 and Yu *et al.,*2007)** reported that there is association between phenotypic evidence of mucoidity and presence of *rmp*A gene. **Derakhshan *et al.,* (2016)** reported that out of 200 isolates of *K. pneumoniae*, *rmp*A was detected in 14 isolates (7.0%). Several putative virulence factors have been described in *K. pneumoniae*, including the plasmid-borne *rmp*A (regulator of mucoid phenotype A), which regulates extracapsular polysaccharide synthesis and mediates the expression of the hypermucoviscous phenotype. The *rmp*A virulence gene has been associated with a distinctive syndrome of liver abscess **(Vila *et al.,* 2011).** The *mag*A gene is the part of capsular polysaccharide gene of *K. pneumoniae* serotype k1 **Fang *et al.,* (2005) and Struve *et al.*, (2005)** described *mag* A as a novel virulence factor responsible for the increased virulence of certain *K. pneumoniae* strains.

Using PCR, **Zamani *et al.,* (2013),** detected *mag*A gene in 4 (3.8%) isolates from 101 strains of *K. pneumoniae* and they proved that *mag*A gene can specially belong to *K. pneumoniae*, while **(Struve *et al.,* 2005)** in Denmark showed that *mag*A is restricted to the gene cluster of *K. pneumoniae* capsule serotype K1. In Taiwan **Fang *et al.*, (2004)** reported the prevalence of *mag*A gene in invasive and non-invasive *K. pneumoniae* isolates as 98% and 29%, respectively. **Behnam *et al.,* (2014),** proved that, of 173 isolates of *K. pneumoniae*, 4 (2.31%) were positive and 169 (97.68 %) were negative for *mag*A gene. The lack of concordance among this gene frequency with other studies might be partly due to this reason that *mag*A gene rarely can be seen in other infections caused by *K. pneumoniae* except liver abscesses.

In addition, not detection of *mag*A among our isolates might be due to its low index of iron-uptake system (*kfu*) which is a proprietary system to absorb acquire iron on the chromosome of this bacterium, because it can be seen mostly on strains of positive *mag*A which induced hepatic abscesses **(Fang *et al.,*2005**).

When cultured in iron-deficient medium, all analysed strains exhibited at least one gene related to iron uptake. The most common gene was *kfu*. In addition, *kfu* has an important role in sepsis and respiratory infections **(El-Fertas-Aissani *et al.,* 2013)**.

By PCR *wca*G gene was identified by using specific primer sequences which amplify a region of 169 bp. All strains were negative for this gene Fig. (5) which is disagree with **(Derakhshan *et al.,* 2016),** of the 200 isolates, *wcaG* was detected in 47 isolates (23.5%) and *rmp*A in 14 isolates (7.0%). The *wcaG* gene is located on the transferable regions of the chromosome **(Shu *et al.,* 2009).** In relation to beta-lactamases, bla_{TEM} and bla_{SHV} were detected by PCR using specific primer sequences which yielded product size of 516 bp , 392bp respectively, all strains were positive to the bla_{TEM} and bla_{SHV} genes Fig. (6). this resistance to ampicillin was high correlated to production of acquired beta-lactamases, more than 340 b-lactamases have been described the genes that encode ESBLs are usually found in plasmids, and those encoding ESBLs of types *CTX-M (gene* $bla_{CTX\text{-}M}$*), TEM* (bla_{TEM}*), PER* (bla_{PER}*), VEB* (bla_{VER}*)* and *SHV* (bla_{SHV}*)* are the main groups **(Jemima and Verghese 2008). (Brinas *et al.,* 2002)** examined the occurrence of bla_{TEM} -,bla_{SHV} and bla_{OXA}-type beta-lactamases, suggesting either *TEM* hyperproduction **(Shannon *et al.,* 1990 and Wu *et al.,* 1995)** or the possible presence of inhibitor-resistant *TEM* enzymes. *TEM*-1 is a broad-spectrum beta-lactamase with activity against penicillins, first described in *Klebsiella* genus **(Heritage *et al.,* 1999).** Genes harboring ESBLs are associated with several specific genetic

structures. A variety of mobile genetic elements, such as transposons, insertion sequences, and integrons, play important roles in the dissemination of ESBL genes. TEM-type ESBL genes are acquired by the mutation of plasmid-mediated, parent TEM-1 and -2 genes, and the main producer of TEM-type ESBLs is in *Enterobacteriaceae* these genes occur within the earliest bacterial transposons identified **(Chong *et al.,* 2011).**

The molecular detection of these genes provides an avenue for early diagnosis of the infection from susceptible hosts. In view of all the findings, further investigation is needed to define the role of other host and pathogen factors which may assist in the progression of disease at the physiological and molecular levels.

CONCLUSION

-*K. pneumoniae* is a major cause of respiratory infection in broiler chickens cause severe losses among them.

-In spite of the absence of some virulence genes of *K. pneumoniae* under study, the pathogenic potential of the isolated strains and their association with clinical manifestations in respiratory tract infections of broiler chicken were confirmed. There is evidence that, the routine use of antibiotics in animal husbandry leads to antibiotic resistance in bacteria which complicate the management and treatment of the infection. Detection of virulence genes is required and it can be useful in tracking, treating and knowledge of *K. pneumoniae* infection prevalence rate.

-These antibiotic-resistant bacteria can infect or reach the human population not only by direct contact, but also by food products of animal origin. Therefore, the reduction and eventual elimination of antibiotics for purposes other than veterinary therapy or treatment of infections in animals is essential. This can be achieved by improving methods of animal husbandry, the eradication of diseases in animals, the optimal use of existing vaccines. Importantly, a good programmed for prevention of antibiotic resistance also includes an active system of surveillance for resistance, an active and effective infection control programme to minimize secondary spread of resistance, and the sensible use of antimicrobials in animal production systems. Despite increasing resistance over the years, there is still high sensitivity to certain antibiotics. More studies are necessary in different geographical regions to investigate the sensitivity of organisms to antibiotics. Strict hygienic measures and disinfection programs in broiler chicken farms should be applied. We should avoid miss use of antibiotics that would increase resistance of bacteria to antibiotics.

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