Prevalence of *Klebsiella Species* in Broiler Chickens with Special Reference to Antimicrobial Resistance and Virulence of *K. Pneumoniae*

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Abstract

In the present research work, 800 tissue samples were collected from lungs, air sacs, liver and spleen of 200 diseased broiler chickens (35-50 days old). These samples were used to isolate and identify *Klebsiella species* by traditional methods. The results showed that *Klebsiellae* were recovered from 28 (3.5%) out of 800 examined samples and the isolates were differentiated into *K. pneumoniae,* 75 % (21/28) and *K. oxytoca,* 25 *%* (7/28). The rate of recovery of *Klebsiellae* was 6.5%, 3%, 2.5% and 2% from lungs, air sacs, liver and spleen, respectively. PCR assay was conducted to confirm the isolation results by detection of the common genes of *Genus Klebsiella.* PCR assay was used to detect whether *K. pneumoniae* isolates have virulence and antimicrobial resistance genes or not. For that, five isolates that were strongly positive for biochemical tests, were selected. All 5 (100%) isolates were found to have *fimH* and *traT* virulence genes and 4 (80%) isolates were found to have *magA* virulence gene. While, all 5 (100%) isolates were found to have *tetA(A)* (Tetracycline resistance gene), *blaTEM* (ampicillin resistance gene) and *mphA* (erythromycin resistance gene) antibiotic resistance genes. Antimicrobial sensitivity testing for some *K. pneumoniae* isolates revealed that 20 out of 20 (100%) tested *K. pneumoniae* isolates, were resistant to ampicillin and oxytetracycline, 18 out of 20 (90%) tested isolates, were resistant to erythromycin, 16 out of 20 (80%) tested isolates, were resistant to streptomycin, 14 out of 20 (70%) tested isolates, were resistant to cefotaxim, 13 out of 20 (65%) tested isolates, were resistant to gentamycin, 9 out of 20 (45%) tested isolates, were resistant to neomycin and 6 out of 20 (30%) tested isolates, were resistant to chloramphenicol. While, the isolates were less resistant to ciprofloxacin and norfloxacin by a percentage of 20% and 10%, respectively.

Key words: Polymerase chain reaction, *Klebsiella spp.*, antimicrobial resistance.

Introduction

Genus Klebsiella belongs to *Family Enterobacteriaceae* (One of the largest Gram –Ve bacteria families). Among *Genus Klebsiella*, *K. pneumoniae* is the clinically most important species which cause severe illness in both animals and humans, so, they are of public health concern. *K. pneumoniae* is now one of the disturbing pathogens that has been rising in poultry production because of its high economic losses and its public health importance. The most important cause that make this pathogen of great interest is the increasing antimicrobial resistance of this bacteria which makes its control difficult. The most important factors contributing to the pathogenicity of *K. pneumoniae* are the smooth lipopolysaccharide (LPS) antigen (O antigen) and the capsular polysaccharide antigen (K antigen). *Klebsiella spp.* are often multidrug resistant with high resistance to penicillins and cephalosporins due to production of ESBLs.

This work is planned to study the prevalence of *Klebsiella spp.*in broiler chickens. To achieve that, the following steps were fulfilled:

1- Isolation of *Klebsiella spp.* from clinically diseased broiler chickens.

2- Identification of *Klebsiella pneumoniae* isolates using traditional methods as biochemical reactions and API 20 E system.

3- Invitro antimicrobial sensitivity testing for *K. pneumoniae* isolates.

4- Detection of common genes, some virulence associated genes and some antimicrobial resistance genes in *K. pneumoniae* isolates by PCR assay.

Material and Methods

Samples:

A total of 800 tissue samples (representing lungs, air sacs, liver and spleen) of 200 diseased broiler chickens (35-50 days old), obtained from poultry clinics and farms located in Mansoura, Dakahlia Governorate, Egypt. Diseased chickens were sacrificed and packaged in separate sterile strong plastic bags and delivered without delay to the bacteriology laboratory where tissue samples, were aseptically collected for bacteriological examination.

Isolation and identification:

Isolation of *Klebsiella species* was carried out according to ***Quinn et al., (1994)***.

For *Klebsiella* identification, the API-20 E was used according to the manufacturer's instruction ***(BioMerieux, France).***

Antimicrobial sensitivity testing for *K. pneumoniae* isolates by disc diffusion according to *ISO 6579 (2002)* method:

Antibiotic sensitivity testing of 20 *K. pneumoniae* isolates was carried

out according to ***Finegold and Martin* (1982).**

Testing of *K. pneumoniae* isolates by PCR:

Five strong positive *K. pneumoniae* isolates, were selected and tested by PCR according to ***Olivera et al., (2003)***.

Oligonucleotide Primers:

Primers used were obtained from ***Metabion (Germany)***.

Analysis of the PCR Products:

It was carried out by gel electrophoresis according to ***Sambrook et al., (1989).***

Results

Table (1): *Prevalence of Klebsiella spp. in diseased chicken organs:*

Examined samples	No. of samples	Number and percentage of *Klebsiella* isolates				
		K. pneumoniae		*K.oxytoca.*		Total
		No.	%	No.	%	
Lungs	200	10	5%	3	1.5%	13 (6.5%)
Air sacs	200	4	2%	2	1%	6 (3%)
Liver	200	4	2%	1	0.5%	5 (2.5%)
Spleen	200	3	1.5%	1	0.5%	4 (2%)
Total	800	21	2.62	7	0.87	28 (3.5%)

Table (2): *Results of antibiotic sensitivity testing for (20) K. pneumoniae isolates.*

	Antimicrobial	Antimicrobial disc	Antimicrobial class	Sensitive		Intermediate		Resistant	
				No.	(%)	No.	(%)	No.	(%)
1	Oxytetracycline	OT	Tetracyclines	0	0	0	0	20	100
2	Ampicillin	AMP	β- Lactams	0	0	0	0	20	100
3	Erythromycin	E	Macrolides	1	5	1	5	18	90
4	Streptomycin	S	Aminoglycosides	1	5	3	15	16	80
5	Cefotaxim	CTX	Cephalosporins	2	10	4	20	14	70
6	Gentamicin	GM	Aminoglycosides	2	10	5	25	13	65
7	Neomycin	N	Aminoglycosides	5	25	6	30	9	45
8	Chloramphenicol	C	Phenicols	8	40	6	30	6	30
9	Ciprofloxacin	CIP	2nd generation Quinolones	11	55	5	25	4	20
10	Norofloxacin	NOR	2nd generation Quinolones	13	65	5	25	2	10

Table (3): *Results of PCR test for detection of K. pneumoniae common genes, virulence genes and antibiotic resistance genes.*

Isolate No.	Results of PCR assay						
	K. pneumoniae common genes	Virulence genes			Antibiotic resistance genes		
		magA	*fimH*	*traT*	*tetA(A)*	*blaTEM*	*mphA*
1	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+
3	+	-	+	+	+	+	+
4	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+

Results are shown in **Photoes (1-7).**

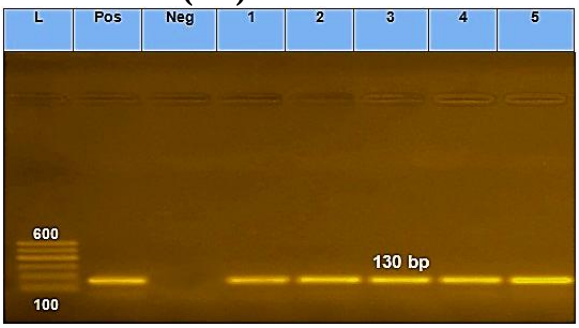

Photo (1): Electrophoretic pattern of *K. pneumoniae* common gene (130 bp fragment).
L: DNA size marker (100 – 600 bp).
Lane (pos.): *K. pneumoniae* (Positive control).
Lane (neg.): Saline (Negative control).
Lanes (from 1 to 5): Positive *K. pneumoniae*.

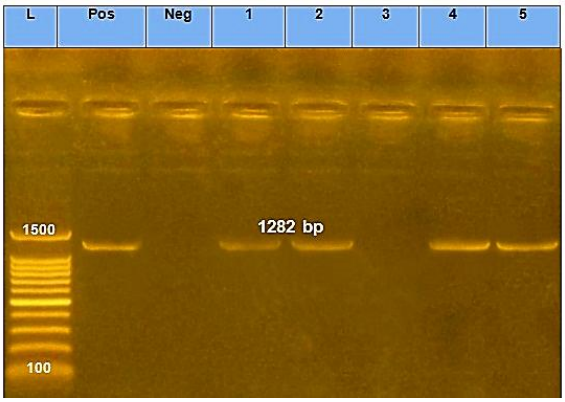

Photo (2): Electrophoretic pattern of *magA* gene (1282 bp fragment).
L: DNA size marker (100-1500 bp)
Lane (pos.): *K. pneumoniae* (Positive control).
Lane (neg.): Saline (Negative control).
Lanes (1, 2, 4, 5): Positive samples (*K. pneumoniae*).
Lane 3: Negative sample.

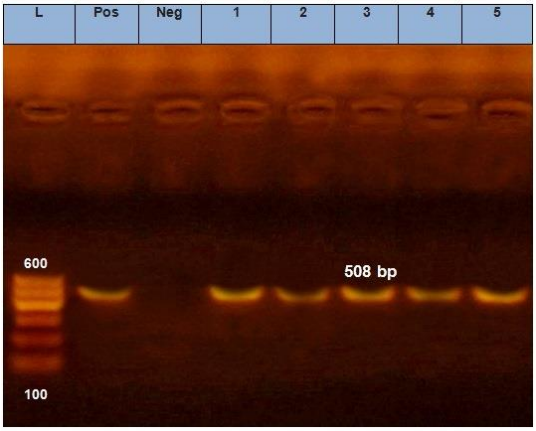

Photo (3): Electrophoretic pattern of *fimH* gene (508bp fragment).
L: DNA size marker (100 – 600 bp).
Lane (pos.): *K. pneumoniae* (Positive control).
Lane (neg.): Saline (Negative control).
Lanes (from 1 to 5): Positive *K. pneumoniae*.

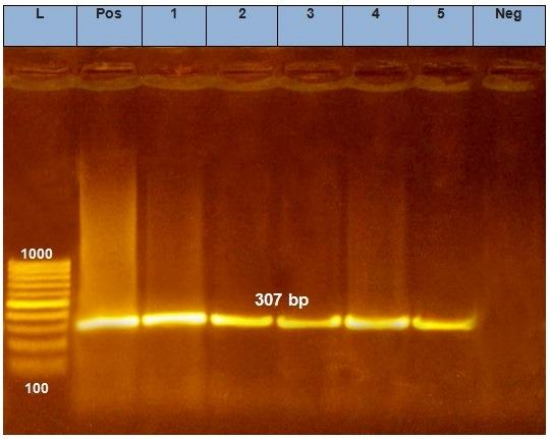

Photo (4): Electrophoretic pattern of *traT* gene (307bp fragment).
L: DNA size marker (100 – 1000 bp).
Lane (pos.): *K. pneumoniae* (Positive control).
Lane (neg.): Saline (Negative control).
Lanes (from 1 to 5): Positive *K. pneumoniae*.

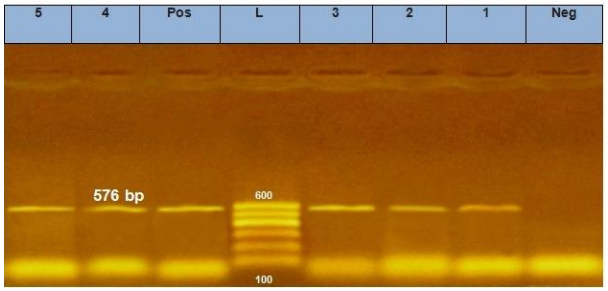

Photo (5): Electrophoretic pattern of *tetA (A)* gene (576bp fragment).
L: DNA size marker (100 – 600 bp).
Lane (pos.): *K. pneumoniae* (Positive control).
Lane (neg.): Saline (Negative control).
Lanes (from 1 to 5): Positive *K. pneumoniae*.

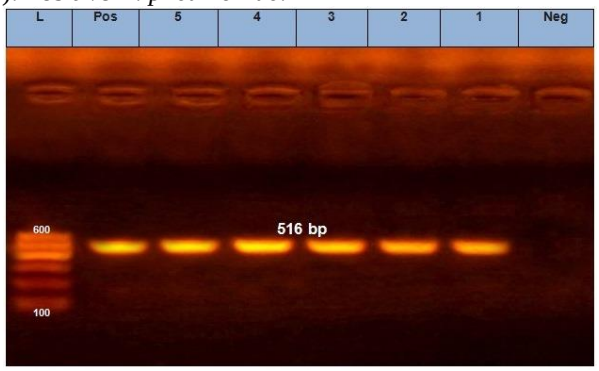

Photo (6): Electrophoretic pattern of *blaTEM* gene (516 bp fragment).
L: DNA size marker (100 – 600 bp).
Lane (pos.): *K. pneumoniae* (Positive control).
Lane (neg.): Saline (Negative control).
Lanes (from 1 to 5): Positive *K. pneumoniae*.

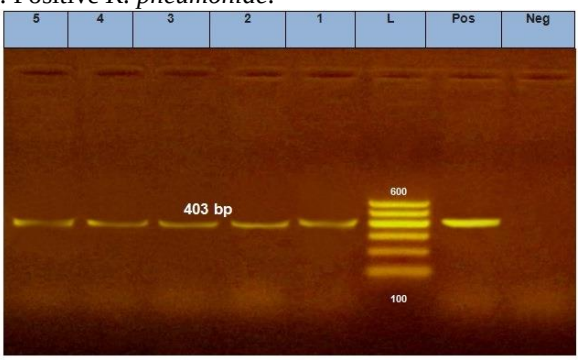

Photo (7): Electrophoretic pattern of ***mphA*** gene (**403 bp fragment).**
L: DNA size marker (100 – 600 bp).
Lane (pos.): *K. pneumoniae* (Positive control).
Lane (neg.): Saline (Negative control).
Lanes (from 1 to 5): Positive *K. pneumoniae*.

Discussion

In the present study, 28 (3.5%) *Klebsiella* isolates were recovered from 800 examined tissue samples and the isolates were differentiated into *K. pneumoniae,* 75 % (21/28) and *K. oxytoca,* 25 % (7/28) as shown in **(Table 1)**. To confirm the isolation results, A PCR assay was conducted and it could idenrtify *K. pneumoniae* common genes in all examined isolates. *Klebsiella spp.* were isolated with a percentage of 6.5%, 3%, 2.5% and 2% from lungs, air sacs, liver and spleen, respectively, **(Table 1)**. These results revealed that lungs were found to be the highest organ for isolation of *Klebsiella spp*. These results are very close to that recorded by ***Abd El hafez (2011)*** and ***Popy et al. (2011)***. But, results were dissimilar to that reported by ***Turkyilmaz (2005)*** who could isolate *Klebsiella spp.* from broiler chickens by a high percentage (about 47%). Meanwhile, ***Khalda et al. (2000), Dashe et al. (2013)*** and ***Aly et al. (2014)*** could isolate *K. peumoniae* from broiler chickens by a percentage of 10.2%, 8% and 10%, respectively.

In this research work, 14 out 28 (50%) *Klebsiella* isolates were found to produce hyperviscous colonies. This result is very close to that reported by ***Amraie et al. (2014)*** who recorded hyperviscous colonies in 42.19% of samples.

In this research work, 11 out of 28 (39.28%) *Klebsiella* isolates were found to be hemolytic. These results agreed with that reported by ***Gundogan and Yakar (2007).***

Results of Antimicrobial sensitivity testing of 20 *K. pneumoniae* isolates revealed that 20 0ut of 20 (100%) tested isolates, were resistant to ampicillin and oxytetracyclines. This was very close to that reported by ***Abdelrahman (2019)***, ***Rasool et al., (2003)*** and ***Gundogan and Avci (2013)***. Also, it was found that 18 out of 20 (90%) tested isolates were resistant to erythromycin. This result was nearly similar to that concluded by ***Kilonzo et al., (2007)***. To confirm these results, a PCR assay was conducted using primers for some antibiotic resistance genes, the reaction could detect *tetA(A), blaTeM* and *mphA* genes. PCR results for antibiotic resistance gene were very close to that obtained by ***Guo et al., (2016)*** and ***Hou et al., (2015)***. Results of Antimicrobial sensitivity testing of *K. pneumoniae* isolates against three antibiotics that are belonging to the aminoglycosides group (streptomycin, gentamycin and neomycin) showed that 80%, 65% and 45% of the examined isolates, were resistant to streptomycin, gentamycin and neomycin, respectively. This is very close to the result concluded by ***Chang et al., (2000)***. Also, 4 (20%) and 2 (10%) out of 20 *K. pneumoniae* isolates were found to be resistant to ciprofloxacin and norofloxacin, respectively. It was nearly similar to

that reported by ***Gundogan and Avci, (2013)*** who reported about 24% resistance of *K. pneumoniae* isolates to ciprofloxacin. But, ***Olufemi et al., (2012)*** reported very dissimilar results as they reported about (54.5% and 63.6%) resistance to ciprofloxacin and norofloxacin, respectively. Results of antimicrobial resistance test are supplied in **Table (2).**

Results of PCR assay for detection of *K. pneumoniae* common gene showed that all the five examined were found to have common gene as shown in **(Table 3 and Photo 1)**. These results run close to that were obtained by ***Yin et al., (2008)***.

Also, PCR test could detect *mag*A, *fim*H and *tra*T virulence genes where the results showed that 4 isolates out of the 5 (80%) examined isolates, were found to have *magA* gene **(Table 3 and Photo 2)** and all the tested five isolates (100%) were found to have *fimH* gene **(Table 3 and Photo 3)** and *traT* gene **(Photo 4).** These results confirmed that *K. pneumoniae* isolates were potentially virulent. PCR test results were very close to the results reported by ***Struve et al., (2005)*** and ***El Fertas-Aissani et al., (2013)***.

To confirm the results of antimicrobial sensitivity testing, a PCR test was carried out to identify some genes that were responsible for the high antimicrobial resistance of *K. pneumoniae* isolates. PCR could detect *tetA(A)*, *blaTEM* and *mphA* genes. It was found that the 5 (100%) examined isolates, were found to have *tetA(A)* gene, *blaTEM* gene and *mphA* gene **(Table 3 and Photoes 5, 6 and 7)**. These genes are responsible for resistance against tetracyclins, ampicillin and erythromycin, respectively. Similar results were concluded by ***Weixia et al., (2014)***, ***Dominika Ojdana et al., (2014)*** and ***Olusegun et al., (2006)***.

Conclusion

1. *K. pneumoniae* is an important bacterial pathogen that causes high economic losses in poultry production, beside, its zoonotic importance.

2. *K. pneumoniae* isolates may vary in their pathogenicity according to their virulence.

3. *Klebsiella spp.* are highly drug resistant bacteria. This high drug resistance may be attributed to the misuse of antibiotis in poultry farms in Egypt, So, this research work recommended a more rational use of antibiotics in poultry farms and a more censorship to control this phenomenon.

4. PCR assay is still holding a high position for accurate and rapid diagnosis of *Klebsiella spp.*

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الملخص العربي

انتشار ميكروب الكلبسيلا في دجاج التسمين مع اشاره خاصه الي مقاومة مضادات الميكروبات وضراوة الكلبسيلا نيموني

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في هذه الدراسه تم تجميع عدد 800 عينه من الرئتين , الأكياس الهوائيه, الكبد , الطحال من عدد 200 من بداري التسمين المصابه ظاهريا والتي كانت أعمارها تتراوح ما بين 35 الي 50 يوما والتي كانت تعاني من أعراض تنفسيه واسهال وفقدان للوزن مع التهابات في الرئه, الأكياس الهوائيه والكبد مع تضخم في الكبد. وقد أظهرت نتائج العزل والفحص البيوكيميائي أن ميكروب الكلبسيلا تم عزله من 28 عينه من اجمالي 800 عينه تم فحصها بنسبة (3.5 %). صنفت هذه المعزولات الي 21 (75%) معزوله من ميكروب الكلبسيلا نيموني و7 (25%) معزولات من ميكروب الكلبسيلا أوكسيتوكا وسجلت نسبة العزل 6.5% , 3% ,2.5%,2% من الرئه و الأكياس الهوائيه والكبد والطحال علي التوالي. وقد تم تأكيد هذه النتائج باجراء اختبار تفاعل انزيم البلمره المتسلسل. وقد أجري اختبار تفاعل انزيم البلمره المتسلسل علي 5 من معزولات ميكروب الكلبسيلا نيموني لاكتشاف بعض جينات الضراوه وجينات مقاومة المضادات الميكروبيه. وقد أظهرت النتائج أن 5 معزولات احتوت علي جينات الضراوه *FimH,Tra*T . بينما احتوت 4 معزولات علي جين الضراوه *mag*A و احتوت المعزولات الخمسه علي بعض الجينات المقاومه للمضادات الميكروبيه وهي (*Tet*A(A) ,*Bla*TEM , *mph*A)
وأجري اختبار الحساسيه لعدد 20 معزوله لقياس نسبة مقاومتها لعدد (10) مضادات ميكروبيه شائعة الاستخدام وقد أظهرت النتائج أن نسبة المقاومه كانت 100% لكلا من الأمبيسلين والأوكسي تتراسيكلين , 90% للارثروميسين , 80% للاستربتومايسين , 70% للسيفوتاكسيم ,65% للجنتاميسين ,45% للنيومايسين ,30% للكلورامفينيكول بينما كانت أقل نسبة مقاومه هي 20% و 10% للسيبروفلوكساسين والنوروفلوكساسين علي التوالي.