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POTENTIAL RISK OF PSEUDOMONAS INFECTION IN BROILER CHICKENS WITH DETECTION OF THE ANTIBIOTIC RESISTANT GENES

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ABSTRACT:*Pseudomonas aeruginosa* is a bacterium causing high morbidity and mortality rates in chicken and other birds' particularly young age resulting in great economic losses. In this study a total of 100 samples of tracheal swabs, nasal swabs, liver, lung and heart (20 of each) were collected from birds at 7, 14, 21 and 35 days of ages from freshly dead and diseased broiler chickens broilers chicken Showing yellowish diarrhea, ruffled feather, respiratory manifestations, and conjunctivitis . Bacteriological examination for the prevalence of *Pseudomonas aeroginousa* isolation of (20) well identified strains of with 20 % higher isolation from birds 7, 14 days than 21 and 35 Days old. The results of antimicrobial sensitivity of the isolates by vitek2 system showed that the isolates were resistant to Tetracycline, Ampicillin, Amoxicillin/Clavulanic Acid, Chloramphenicol, Nitrofurantoin, and Trimethoprim while, they were sensitive to Amikacin, Cefpirome, Impenem, Piperacillin, Gentamicin and Tobramycin. The presence of the antibiotic resistance genes such as *blaTEM* (4/4) and *mexR* (4/4) for *Pseudomonas aeruginosa* were detected by Polymerase Chain Reaction in some *P. aeruginosa* isolates.

Key word: Pseudomonas aeruginosa- broiler chicken - vitek2 system- PCR- antibiotic sensitivity genes.

INTRODUCTION

P aeruginosa is an opportunistic organism produces septicemia and respiratory infections. It causesperihepatitis, pericarditis, congestion of internal organs, cheesy materials on the serous surfaces of, peritoneum. And airsacs in chickens Riad, (1994) and Abd El- Tawab *et al.,* (2014). Infection with *(P. aeruginosa*) Associated with environment is mainly correlated with water, soil, and humid environments Finlayson and Brown, (2011) and Dinev *et al.,* (2013). This bacterium is counted as one of the major bacteria causing severe problems in poultry farms. *P aeruginosa* is Gram -negative, motile, non-spore forming rod present in short chain under microscope, grows on ordinary media, specialized with producing soluble green pigment and characterize with fruity odor Adams and Moss, (2008). *P aureginosa* that causing infection and mortality rate of 50-100% especially in baby chicks. Saad *et al.,* (2017). The Vitek2 system can detect dozens of specimens automatically at the same time. Therefore, Vitek2 system gave reliable, rapid and highly reproducible results Ling, *et al.,* (2001). The virulence of *P aureginosa* is due to cellular and extra cellular factors as elastase, lipopolysaccharide, pyocyanin, alkaline proteases, phospholipase C, haemolysins, pyoverdin, rhamnolipids, biofilm, flagella, and Pilli. *Also,* secrets toxins such as: exoenzyme S, exoenzyme T, exoenzyme Y, and exoenzyme U into the infected host. As these exotoxins excreted from pseudomonas which causes continuous infection even after the organism has been killed by antibiotics Kebede, (2010) and, Kirienko *et al.,* (2015). There are many causes which increase the coincidence infection of Pseudomonas As stress conditions, infection with other diseases, viruses, mycoplasma and bacteria. All of these causes increase the chance of Pseudomonas infection which can occur at any age especially young age Stipkovits *et al*., (1993).

So, the aim of this work was identification of P. *aeruginosa* from broilers chicken samples. And identify its antibiotic susceptibility of the isolates using Vitek2 compact system. Besides, and resistance genes of the *P. aeruginosa* isolates.

MATERIAL AND METHODS

Sample collection:

A total of 100 samples of tracheal swabs, nasal swabs, liver, lung and heart (20 of each) were aseptically collected from freshly dead and diseased living broiler chickens at 7,14,21 and 35 days of age showing yellowish diarrhea, ruffled feather, respiratory manifestations, and conjunctivitis. Then, these samples were transferred in an ice box to laboratory for isolation and identification of *Pseudomonas aeruginosa*, According to Middleton et *al.,* (2005).

Isolation and identification:

Collected samples were inoculated in nutrient broth tubes and incubated aerobically at 37ºC for 24 hours, A loopfull was taken from this inoculated broth and stricked on MacConkey agar and *Pseudomonas* agar medium plates and incubated aerobically for 24-48 hours at 37°C; pigment production and lactose fermentation were detected respectively. The plates containing characteristic colonies of *Pseudomonas aeruginosa* (large, irregular, translucent and produced greenish diffusible pigment and characterized by its fruity smell) were selected and the Gram staining test was performed then subjected to biochemical identification according to Quinn *et al.,* (2002). The method of isolates were also identified biochemically by vitek2 compact system according to Chatzigeorgiou *et al.,* (2011).

Sensitivity test of *Pseudomonas aeruginosa* using Vitek2 compact system:

The antibiotic sensitivity test for identification of *P. aeruginosa* by Vitek 2 compact system and antimicrobial susceptibility test (AST-GN card) were done according to the manufactures' instructions Chatzigeorgiou *et al.,* (2011). The microorganism suspension

was transferred in to the identification AST-GN cards. This card identifies 47 different biochemically tests, and, the test tubes containing the microorganism suspensions were placed to special rack (cassette), then, this filled cassette placed into a vacuum chamber station where vacuum was done and reintroduced air. After the vacuum was applied and air was reintroduced into the station, the suspension of organism was transferred into micro-channels that fill all the test wells. *Pseudomonas aeruginosa* antibiotic sensitivity test was done against Amikacin, Tetracycline, Oxacillin, Ceftriaxone, Cefotaxime, Cefoperazone, Ceftazidime, Ciprofloxacin, Gentamicin, Impenem, and Piperacillin.

Detection of *blaTEM* and *mexR genes* in some *Pseudomonas aeroginousa* isolates using PCR:

DNA extraction was done from the 4 isolates using QIAamp DNA Mini kit (Qiagen, Germany, GmbH) and modifications according the manufacturers' recommendations. Briefly, 200 µl of the sample suspension were added to 200 µl of the lysis buffer and 10 µl of the proteinase K and incubated at 56˚C for 10 min, then, 200 µl of 100% ethyl alcohol were added to the lysate. After that the sample was washing and centrifuging, and 100 µl of the elution buffer which provided by the kit were used to elute the nucleic acid.

Amplification of PCR

Primers used for PCR amplification for *blaTEM* genes. These primers were utilized for the PCR reaction and for the analysis using forward and reverse PCR primers for *blaTEM* antibiotic resistant gene which was done according to Colom *et al.,* (2003). The *mexR* antibiotic resistant gene was done according to Sánchez *et al.,* (2002). As concluded in Table (1). The primers were utilized in a 25 µl reaction containing 12.5 µl of PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of nuclease-free water, and 6 µl of DNA template. The reaction was performed in an Applied Biosystem Thermal Cycler. Cycling conditions of the different primers during the PCR amplification as the manufacturer's recommendations as described in Table (2).

PCR product analysis

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 products was loaded in each gel slot. A gelpilot 100bp and 100bp plus DNA Ladders (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra).

RESULTS AND DISCUSSION

Pseudomonas infections of birds are of great importance because epidemics may spread rapidly through poultry flocks causing mortality in all ages Shukla and Mishra (2015). This bacterium is counted as one of the major bacteria causing severe problems in poultry farms.

The bacteriological examination results for the 100 samples showed 20 positive isolates as 20 % for *Pseudomonas aeruginosa,* as shown in Table (3) which appeared in a green-blue color colonies with odor like a sweet grape. These results are in line with that obtained by Haleem *et al.,* (2011), Abdel-Tawab *et al.,* (2016), and Bakheet *et al.,* (2017).

Result in Table (4), shown P. aeruginosa was highly isolated from liver followed from tracheal swabs, lung nasal swabs and finally from heart the results reported by Abd El-Tawab *et al.,* (2014), who mentioned that high prevalence with liver and negative with heart samples.

Table (5), reveals that *Pseudomonas aeruginosa* was in higher rate (10 positive) isolates from chicks of 7 day baby chicks, (6 positive) isolates from chicks of 14 day, (3 positive) isolates from 21 day chicken age which agreed with Abd El- Tawab *et al.,* (2014), who reported that *Pseudomonas* spp. higher in baby chicks in the age ranged from 1 day to 10 days than older ages. Also, Kebede,

(2010) found that the main reason of high mortalities in baby chicks was due to the infection by *P. aeruginosa* during hatching time from invading shells of egg or from the environment.

Table (6). The results Vitek2 system identification which proved by many laboratories worldwide who adopted the Vitek2 automated system for the identification of gram negative strains in ordinary clinical microbiology is rapid. Vitek2 system has many advantages. There is one negative control well and 47 biochemical that give final results in approximately 10 hours or less bioMérieux. (2006) and Chatzigeorgiou *et al.*, (2011). First, it can avoid environmental contamination or cross-contamination as it is a closed system. Second, Vitek2 system can detect specimen card if it is misplaced on its cartridge. So, during its operation it owns a dependable recheck system. Third, it is easy for to prepare and load bacterial specimens by laboratory staff and the Vitek2 system can detect dozens of specimens automatically at the same time. So, Vitek2 system gives reliable, rapid and highly reproducible results Ling *et al.,* (2001).

Table (7). The results of antibiotic sensitivity test proved that P. aeruginosa strains were resistant to Tetracycline, Ampicillin, Amoxicillin/Clavulanic Acid, Chloramphenicol, Nitrofurantoin, Trimethoprim, Cefpodoxime and Ceftiofur. While they were sensitive to Amikacin, Cefpirome, Impenem ,Piperacillin Gentamicin, Marbofloxacin and Tobramycin, which the reported with Kurkure *et al.,* (2001), and Abdel-Tawab *et al.* (2016) proved high sensitivity with Ciprofloxacin and Gentamycin.

Detection of the antibiotic resistant gene of the tested *Pseudomonas aeruginosa* isolates by PCR showed that selected ones were positive for *blaTEM* gene giving amplicon at (516 bp)as shown in Fig (1). These results are in line with results obtained by Mombini *et al*.,(2019), and Giriyapur *et al*.,(2011) who found that almost of the *Pseudomonas aeruginosa* isolates were positives for *blaTEM* gene because the miss use of antimicrobial therapy as broad spectrum betalactams lead to mutations of genes for beta-lactams specially *blaTEM* .

Also, all the tested *Pseudomonas aeruginosa* isolates showed to be positive for antibiotic resistant *mexR* gene as shown in Fig (2). These results are in agreement with the results obtained by Walid *et al.,* (2020). The most critical point is that bacteria could acquire virulence factors from the surrounding environment resulting in more alteration and causing cellular damages. Moss *et al.,* (2019).

CONCLUSION AND RECOMMENDATIONS

P. aureginosa this bacteria causing severe problems in poultry farms. So, for minimize the economic losses in the poultry production firm hygienic measures should be applied. A strict antibiotic policy and establishment of infection control programs will help to lower the incidence of resistance in *P. aureginosa*.

Table (1): Primers sequences, target genes and amplicons sizes.

Target gene	**Primers sequences** Forward (5`-3`) Reverse (3`-5`)	**Amplified segment (bp)**	**Reference**
blaTEM	ATCAGCAATAAACCAGC	516	Colom *et al*., 2003
	CCCCGAAGAACGTTTTC		
mexR	GCGCCATGGCCCATATTCAG	637	Sánchez *et al*., 2002
	GGCATTCGCCAGTAAGCGG		

Table (2): Cycling conditions for each primer during PCR.

Target Gene	**Primary Denaturation**	**Secondary Denaturation**	**Annealing**	**Extension**	**No. of cycles**	**Final extension**
blaTEM	94˚C 5 min	94˚C 30 sec	54˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.
mexR	94˚C 5 min	94˚C 30 sec	55˚C 30 sec.	72˚C 30 sec	35	72˚C 7 min

Table (3): Prevalence of P. aeruginosa in the examined samples**.**

No. of examined samples	**No. of +ve samples**	**%. of +ve samples**	**No. of -ve samples**	**%. of -ve samples**
100	20	20%	80	80%

+ ve (Positive samples), -ve (Negative samples)

Table (4): Distribution of *Pseudomonas aeruginosa* isolates in examined samples.

Source of sample	**No. of +Ve samples**	**% of +Ve samples**	**No. of -Ve samples**	**% of -Ve samples**
tracheal swabs	5	25	15	18.75
Nasal swabs	3	15	17	21.25
Heart	2	10	18	22.5
Lung	4	20	16	20
Liver	6	30	14	17.5
Total	20	100	80	100

Percentage calculated according to the number of isolates.

Table (5): Distribution of *Pseudomonas aeruginosa* isolates in the examined samples according to broiler ages**.**

broiler age (day)	No. of + Ve samples	% of +Ve samples	No. of -Ve samples	% of -Ve samples
7	10	50	10	12.5
14	6	30	14	17.5
21	3	15	27	33.75
35	1	5	29	36.25
Total	20	100	80	100

Percentage calculated according to the number of isolates.

Table (6): biochemical of Pseudomonas aeruginosa using Vitek2 system.

2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	-	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTP	-	13	dGLU	+	14	GGT	+	15	OFF	-
17	BGLU	-	18	dMAL	-	19	dMAN	-	20	dMNE	-	21	BXYL	-	22	BAlap	+
23	ProA	+	26	LIP	+	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTER	-	36	CIT	+	37	MNT	+	39	5KG	-
40	ILATK	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	IHISa	-						

APPA: Ala-Phe-pro arylamidase , ADO: Adonitol, PyrA: L-pyrrolydonyl-Arylamidase, IARL: L-Arabitol, dCEL: D-Cellobiose, BGAL: Beta-Glactosidase, H2S:H2S production, BNAG: Beta-N-Acetyl-Glucamindase, AGLTP: GlutamyleArylamidasepNA, dGLU: D-glucose, GGT: Gamma-Glutamyle-Transferase, OFF: Fermentation/ Glucose, BGLU: Beta-Glucosidase, dMAL: D-Maltose, dMAN: D-Mannitol, dMNE: D-Mannose , BXYL:Beta-Xylosidase, BAlap: Beta- Alanine Arylamidase Pna, ProA: L-Proline Arylamidase, LIP: Lipase, PLE: Palationose, TyrA: Tyrosine arylamidase, URE: Urease, dSOR: D-sorbitol, SAC: Saccharose/Sucrose, dTAG: Tagatose, dTER: D-Terhalose, CIT: Citrate (sodium), MNT: Malonate, 5KG: 5-Keto-D-Gluconate, ILATK: L-Lactate alkanization, AGLU: Alpha-Glcosidase, SUCT:Succinate alkanization, NAGA: Beta-N-Acetyl, AGAL: Alpha-Glactosidase, PHOS: Phophatase, GlyA: Glycine Aryamidase, ODC: Ornithine Decarboxylase, LDC: Lysine decarboxylase, IHISa: L-Histidine assimilation.

+ ve (Positive), -ve (Negative).

Table (7): antibiogram pattern of Pseudomonas aeruginosa isolates against different antibiotics.

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Ampicillin	>=32	R	Gentamicin	<=1	S
Amoxicillin/Clavulanic Acid	>=32	R	Tobramycin	<=1	S
Pipracillin	8	S	Enrofoxacin	2	I
Cifatexin	>=64	R	Marbofloxacin	<=0.5	S
Cefpodoxime	>=8	R	Tetracycline	>=16	R
Ceftiofur	>=8	R	Nitrofurantoin	>=512	R
Cefpirome	4	S	Chloramphenicol	>=64	R
Imipenem	2	S	Polmyxin B	2	
Amikacin	<=2	S	Trimethoprim/Sulfamethoxazole	160	R

R: Resistance, I: Intermediate, S: Sensitivity. (MIC). Minimum inhibitory concentration.

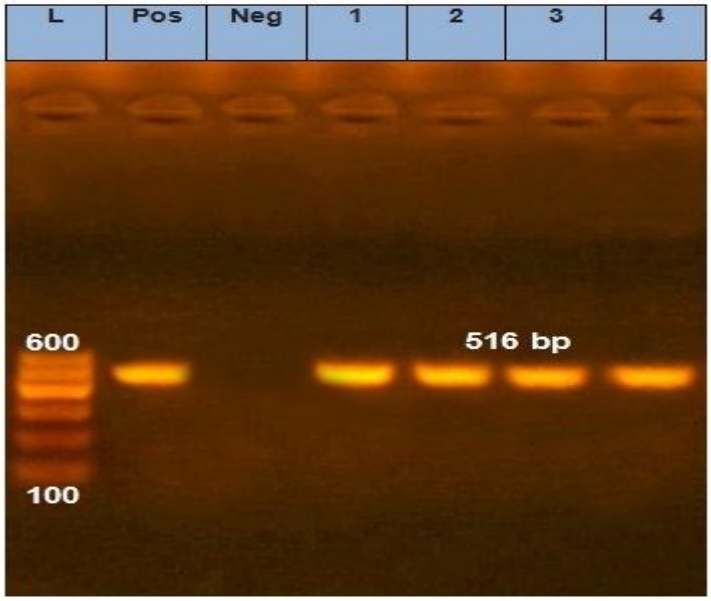

Figure (1) : Agarose gel electrophoresis of multiplex PCR amplification of *Pseudomonas aeruginosa* extracted DNA for *blaTEM* gene .L: represents the molecular size marker (DNA ladder-516bp). 1, 2, 3 and 4: are positive for *blaTEM* gene of *Pseudomonas aeruginosa* isolates.

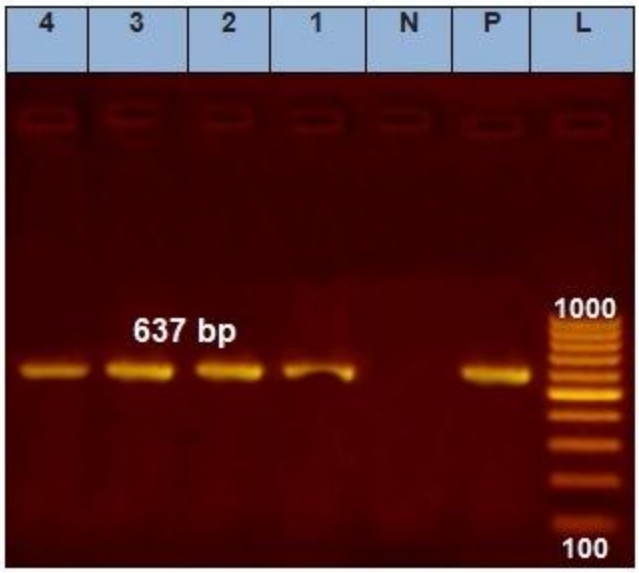

Figure (2) : Agarose gel electrophoresis of multiplex PCR amplification of *Pseudomonas aeruginosa* extracted DNA for *mexR* gene .L: represents the molecular size marker (DNA ladder-637bp). 1, 2, 3 and 4: are positive for *mexR* gene of *Pseudomonas aeruginosa* isolates.

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الملخص العربي

المخاطر المحتملة لعدوي السيدومونس في دجاج التسمين مع الكشف على جينات المقاومة للمضادات الحيوية

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يسبب ميكروب السيدومونس ارجينوزا نسبه عاليه من الامراض و النفوق في الطيور وخاصه الاعمار الصغيره. تم في هذا العمل تجميع عدد 100 عينه من دجاج التسمين مريضه ونافقة في اعمار مختلفه (7 و 14 و 21 و 35 يوم) و كانت العينات من طيور نافقه حديثا وتظهر علية اعراض اسهال اصفر واعراض تنفسيه والتهاب الملتحمه. أظهرت نتائج الفحص البكتريولوجي للعينات عن عزل ميكروب السيدومونس ارجينوزا بعدد 20 عينه ايجابيه بنسبه مئويه 20% وكانت نسبه العزل من الدجاج الصغير اعلي من الكبير. كما اظهرت إختبارات الحساسيه باستخدام جهاز الفيتك علي مقاومه جميع المعزولات للتتراسيكلين، الامبسلين ،الاموكسيسيلين، الكلورامفينيكول و الترايميثوبريم. كما كانت حساسه بنسبه متوازنه للاميكين و السيفوبريم و الجنتاميسين. تم باستخدام تفاعل انزيم البلمره المتسلسل الكشف عن جينات المقاومه للمضادات الحيويه *blaTEM* 4\4 و4/4 mexR.