PCR detection and gene sequence of *Pseudomonas Aeruginosa* isolated from broiler chickens

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A B S T R A C T

A total of 372 samples was collected as follows, 224 samples from diseased broiler chickens, 84 samples from freshly dead and 64 samples from apparently healthy at different ages. The samples were examined bacteriologically. Seventeen isolates of *P. aeruginosa* (4.57%) were found and tested for their sensitivity against different antibiotics. The isolates were highly sensitive to Colstin sulphate (76.5%), Norfloxacin (52.9%), Amikacine (41.2%), Gentamicin (23.5%), Ciprofloxacin (17.6%) and Cefozone "cefoperazon" (11.8%). Polymerase chain reaction (PCR) for detection of *opr*L gene of *P. aeruginosa* was applied and showed positive amplification of 504 pb fragments, also gene sequence of *opr*L *gene of P. aeruginosa* was applied and had accession number KP056547 at GeneBank which was 99% identical to the corresponding GeneBank sequences.

Keywords: *P. aeruginosa*, *opr*L gene, PCR, Gene sequence, chickens.

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1. INTRODUCTION

Pseudomonas species play a very effective role in poultry industry in all ages. Morbidity and mortality varies from 2-100% therefore, the present study was conducted to isolate *P.aeruginosa* from internal organs of freshly dead, diseased and apparently healthy chickens at different ages in Kafr El-Sheikh and Albohaira governorates.

The most predominant pseudomonas species causing mortalities among birds specially chickens was *P.aeruginosa* which is gram negative, aerobic, motile, noncapsulated and nonsporulated bacilli (Abd Allah 1987). Septicaemic infection in poultry due to *P. aeruginosa* has been reported by Ray and Banerji (1969) and Narula and Kuppuswamy (1969). Birds of any age may be infected; young birds are most susceptible, also severely stressed or immunodeficient birds. Concurrent infections with viruses, other bacteria and mycoplasmas, enhance susceptibility to Pseudomonas infection (Stipkovits *et al.*, 1993). In chickens, *P. aeruginosa* produces dyspenia and cheesy deposits on the serous surfaces lining the air sacs and peritoneal cavity. In addition, congestion of internal organs, perihepatitis and pericarditis were reported (Riad, 1994). Many researchers have made attempts to develop molecular methods especially PCR for the detection of *P. aeruginosa* (Nikbin *et al.*, 2012).PCR is more rapid, simple and highly sensitive than cell culture assay for detection of microorganisms. It could be recommended as screening method in clinical laboratory (Schmidt *et al.*, 1995). Therefore, the present study was planned for bacteriological characterization of *P. aeruginosa* isolates and detection of sequence-specific target. The outer membrane protein (*opr*L) gene locus was used for the analysis by using PCR then sequence and submission them into Gene Bank.

2. MATERIAL AND METHODS

2.1. Samples collection

A total of 372 samples were collected from different ages of broiler chickens. Among them 224 samples were collected from diseased living birds showing profuse diarrhea and respiratory manifestations, 84 samples from freshly dead and 64 samples from apparently healthy birds. All samples were aseptically collected to prevent cross contamination and transferred immediately in ice box to the laboratory**.**

2.2.Bacteriological examination of P. aeruginosa

The samples were streaked onto selective media (pseudomonas cetrimide agar) and incubated aerobically for 24 hours at 37°C. The suspected colonies were inoculated for 24 hours at 37°C onto nutrient agar, sheep blood agar and MacConky's agar for purification and pigment production, haemolysis properties and lactose fermentation detection respectively. The plates containing characteristic colonies of *P. aeruginosa* were selected and the Gram staining was performed. Each colony showing typical colonial appearance were subjected to biochemical identification and examined for oxidase reaction, catalase reaction, urea hydrolysis, indole test, MR test, and Simmon's Citrate agar utilization. API 20 was used as a confirmatory biochemical test.

2.3. Antimicrobial susceptibility testing

By the Kirby- Bauer disk diffusion method (Finegold and Martin 1982), Muller Hinton broth, Muller Hinton agar and antibiotic discs are used (Colstin Sulphate, Norfloxacin, Amikacine, Gentamicin, Ciprofloxacin, Cefozone "Cefoperazon", lincomycin, Naldixic acid, Streptomycin, Florphenicol, Chloramphenicol and Doxycyclin). The results were interpreted according to NCCLS (2002).

2.4. Detection of P. aeruginosa by using PCR

DNA was extracted from the isolated *P. aeruginosa* using ABIO pure Genomic DNA extraction kit. The Oligonucleotide Primers that encoding for *opr*L gene for *P.aeruginosa* was used according to (Xu *et al*., 2004), sequence of forward primer was (ATG GAA ATG CTG AAA TTC GGC) and reverse primer was (CTT CTT CAG CTC GAC GCG ACG). DNA samples were amplified in a total of 25 µl as follows: 12.5 µl of Emerald Amp GT PCR Master Mix**,** 1 µl of each primer of 20 pmol concentrations, 4.5 µl of water, and 6 µl of template DNA. The reaction was performed in a Biometra thermal cycler. Aliquots of amplified PCR products were electrophoresed in 1.5% agarose gel (ABgene) in 1x TBE buffer at room temperature. For gel analysis, 15 µl of PCR products were loaded in each gel slot. A 100 bp DNA Ladder (QIAGEN Inc, Valencia, CA, USA) was used to determine the fragment sizes. The gel was photographed by a gel documentation system and the data was analyzed through computer software.

2.5.Gene Sequence of P. aeruginosa

PCR products were purified using QIA quick PCR Product extraction kit (QIAGEN Inc, Valencia, CA, USA). Big dye Terminator V3.1 cycle sequencing kit. (Perkin-Elmer/Applied Biosystems, Foster City, CA) was used for the sequence reaction and then it was purified using Centrisep (spin column). DNA sequences were obtained by Applied Biosystems 3130 genetic analyzer (HITACHI, Japan) and a comparative analysis of sequences was performed using CLUSTAL W. multiple sequence alignment program, version 1.83 of Meg Align module of Laser gene DNA Star software. Sequence submission was conducted by Bankit of GeneBank with *P. aeruginosa* srain YL84 complete genome, Bankit 1769629 Seq.

The sequence of forward template of *opr*L gene was:

AATGCCTGCGCTGGCTCTGGCATGGCTG
TGGCTGTGGGTTGCTCCTCCAAGGGCG
GCGATGCTTCCGGTGAAGGTGCCAATG
GCGGCGTCGACCCGAACGCAGGCTATG

GCGCCAACAGCGGTGCCGTTGACGGCA
GCCTGAGCGACGAAGCCGCTCTGCGTG
CGATCACCACCTTCTACTTCGAGTACGA
CAGCTCCGACCTGAAGCCGGAAGCCAT
GCGCGCTCTGGACGTACACGCGAAAGA
CCTGAAAGGCAGCGGTCAGCGCGTAGT
GCTGGAAGGCCACACCGACGAACGCGG
CACCCGCGAGTACAACATGGCTCTGGG
CGAGCGTCGTGCCAAGGCCGTTCAGCG
CTACCTGGTGCTGCAGGGTGTTTCGCCG
GCCCAGCTGGAACTGGTTTCCTATGGTA
AAGAGCGTCCGGTCGCTACCGGCCACG
ACGAGCAGTCCTGGGCTCAGAACCGTC
GCGTCGGCTTGGAAGAAG

Sequence of reverse template of *opr*L gene was:
AAAGGGATGCTCGTCGTGGCCGGTAGC
GACCGGACGCTCTTTACCATAGGAAAC
CAGTTCCAGCTGGGCCGGCGAAACACC
CTGCAGCACCAGGTAGCGCTGAACGGC
CTTGGCACGACGCTCGCCCAGAGCCAT
GTTGTACTCGCGGGTGCCGCGTTCGTCG
GTGTGGCCTTCCAGCACTACGCGCTGA
CCGCTGCCTTTCAGGTCTTTCGCGTGTA
CGTCCAGAGCGCGCATGGCTTCCGGCT
TCAGGTCGGAGCTGTCGTACTCGAAGT
AGAAGGTGGTGATCGCACGCAGAGCGG
CTTCGTCGCTCAGGCTGCCGTCAACGGC
ACCGCTGTTGGCGCCATAGCCTGCGTTC
GGGTCGACGCCGCCATTGGCACCTTCA
CCGGAAGCATCGCCGCCCTTGGAGGAG
CAACCCACAGCCACAGCCATGGCCAGA
GCCAGCGCAGCAAATTTGCCGAATTAG
GGATTTTTTCCATAGAAC

3. RESULTS

3.1. Identification of the isolated P. aeruginosa

P. aeruginosa on pseudomonas cetrimide agar medium appeared as small and smooth with different blue – green pigment colonies while it showed Beta hemolysis on blood agar medium. On MacConky's agar medium, the colonies were large, pale with greenish coloration. On nutrient broth, greenish yellow pigments were seen. All isolates showed similar pattern of reaction despite of the source of isolation. Oxidase, Citrate utilization, Urea hydrolysis and Catalase tests showed positive results while MR, Indole and H_2S production showed negative results.

3.2. Prevalence of P. aeruginosa in chickens

P. aeruginosa was isolated from diseased, freshly dead and apparently healthy chickens with a percentage of 14 (6.25%), 2 (2.38%) and 1(1.56%) respectively. Sixteen isolates of *P. aeruginosa* were isolated from a total of 330 samples obtained from broiler chickens (1-5 weeks) with a percentage of 4.85%. Meanwhile one isolate was reported in between 42 samples obtained from one day old chicks with a percentage of 2.38% (Table 1 & 2).

3.3. Antimicrobial sensitivity test for P. aeruginosa

The most effective antibiotics were Colstin sulphate (76.5%), Norfloxacin (52.9%), Amikacine (41.2%), Gentamicin (23.5%), Ciprofloxacin (17.6%) and Cefozone "Cefoperazon" (11.8%). On the other hand, all isolates were resistant to Lincomycin, Naldixic acid, Streptomycin, Florphenicol, Chloramphenicol and Doxycyclin (Table 3).

3.4. PCR results

Six isolates of *P.aeruginosa* were tested and gave a positive amplification of 504 bp fragment specific for the *opr*L gene of *P. aeruginosa* (species-specific gene) (Fig. 1).

3.5. Sequence of oprL gene of P.aeruginosa

GeneBank accession number for the studied nucleotide sequence (Bankit 1769629) is KP056547 and The results of the tested *P. aeruginosa* sequencing with *P. aeruginosa* YL84 complete genome showed that the forward and reverse sequence was 474/478 (99%), 457/458 (99%) identities and gaps 4/478 (0%), 1/458(0%) respectively.

Table (1): Prevalence of *P. aeruginosa* in chickens according to the site of isolation.

Type of sample	Diseased			recently dead			Apparent healthy			Total +ve%
	No.	No. of +ve result	% of +ve	No.	No. of +ve result	% of +ve	No.	No. of +ve Result	% of +ve	
Liver	29	1	3.45	14	-	-	9	-	-	1.9%
Air sac	29	1	3.45	14	-	-	9	-	-	1.9%
Lung	29	-	-	14	-	-	9	-	-	0%
Heart blood	29	1	3.45	14	1	7.14	9	-	-	3.8%
Gall bladder	29	-	-	14	-	-	9	-	-	0%
Intestine	29	1	3.45	14	1	7.14	9	-	-	3.8%
Cloacal Swabs	50	10	20	-	-	-	10	1	10	18.3%
Total	224	14	6.25	84	2	2.38	64	1	1.56	

Table (2): Prevalence of *P. aeruginosa* according to the age.

Source of samples	No. of samples				No. of +ve results	Percent %
	Diseased	recently dead	Apparent healthy	Total		
Broiler chickens (1-5 weeks)	200	78	52	330	16	4.85%
One day old chicks	24	6	12	42	1	2.38%
Total	224	84	64	372	17	4.57%

Table (3): antimicrobial sensitivity test for *P. aeruginosa*.

Antibacterial agents	Sensitive (S)		Resist (R)	
	No	%	No	%
Cefozone "cefoperazon"	2	11.8	6	35.3
Norfloxacin	9	52.9	5	29.4
Doxycyclin	-	-	17	100
Colstin sulphate	13	76.5	1	5.9
Chloramphenicol	-	-	17	100
Florphenicol	-	-	17	100
Gentamicin	4	23.5	5	29.4
Ciprofloxacin	3	17.6	5	29.4
Streptomycin	-	-	17	100
Naldixic acid	-	-	17	100
Amikacine	7	41.2	1	5.9
Lincomycin	-	-	17	100

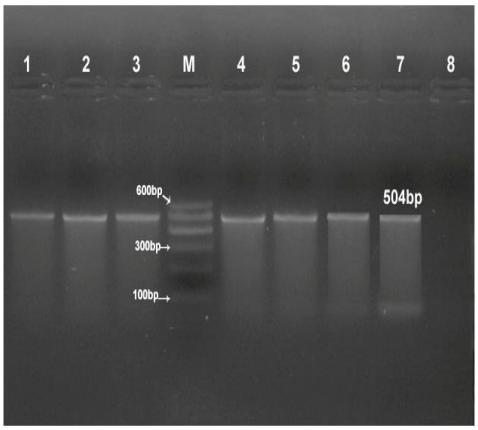

Figure 1: amplification of *opr*L gene of *P.aeruginosa*: Lane 1-6: positive tested *P. aeruginosa*, Lane: 7 positive control, lane: 8 negative control and lane M 100-600 pb DNA ladder.

4. DISCUSSION

In this study, 17 isolates of *P.aeruginosa* (4.57%) was obtained from 372 samples (apparently healthy, diseased and freshly dead chickens). These results were nearly similar to that recorded by Choudhury *et al*., (1993) who isolated *P. aeruginosa* in a percentage of 4.75%, Younes *et al*., (1990) who isolated *P. aeruginosa* from 20 dead chickens with percentage of 4.9%. Chakrabarty *et al.,* (1980) isolated *P. aeruginosa* with incidence of 8% from 100 chickens suffering from respiratory symptoms and Awaad *et al*., (1981) who isolated *P. aeruginosa* with an incidence of 2.9% from an outbreak in a broiler flock. These percentages differed greatly from the current results, which may be attributed to the cloacal swabs, which gave the highest recovery rate with an incidence of 18.3%; these results were similar to that recorded by El-Shafii (1992) and Panjoo *et al.*, (1984). The results of biochemical tests were in agreement with Awaad *et al.*, (1981) and El-Shafii (1992). *P.aeruginosa* isolates were highly sensitive to Colistin Sulphate (76.5%), Norfloxacin (52.9%) and Amikacin (41.2%) while Gentamicin, Ciprofloxacin and Cefozone "Cefoperazon" gave 23.5%, 17.6% and 11.8% respectively. These results were nearly agreed to Kim *et al*., (1982) and Abd El-Gawad *et al.,* (1998) who reported that *P. aeruginosa* isolates from chickens were highly sensitive to Garamycin, Neomycin, Danofloxacin, Colstin Sulphate, Amikacin and Tetracycline. In the current work all isolates were resistant to lincomycin, Naldixic acid, Streptomycin, Florphenicol, Chloramphenicol and Doxycyclin .These results came in accordance with Niilo (1959) and Bapat *et al*., (1985).

The target gene (*opr*L gene) was designed and evaluated for direct detection of *P. aeruginosa* , that was detected in all tested isolates, the size of amplicon for the gene of interest was 504 bp. PCR amplified fragment which agreed with a study performed and recorded the same results Xu *et al*., (2004), Abdullahi *et al.,* (2013) and Hassan (2013). So the PCR method can be used to provide a specific, rapid, simple, and highly sensitive detection of *P. aeruginosa* in clinical samples. The sequence obtained from *opr*L gene of *P. aeruginosa* with provided GeneBank accession number KP056547 was 99% identical to the corresponding GeneBank sequences EU286532 (Karanam *et al.*, 2008), CP002496 (Wu et al., 2011), CP007147.1(Chan *et al.*, 2014) and CP007224 (Deraspr *et al.*, 2014).

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تفاعل البلمرة المتسلسل وتحليل التتابع الجيني لسيدوموناس ايريجينوزا المعزولة من بدارى التسمين

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الملخص العربى

تلعب انواع السيدوموناس دوراً مؤثراً جداً فى صناعة الدواجن فى كل الاعمار. وتتراوح نسب الإمراض والنفوق من 2-100%. لذا أجريت هذه الدراسة لعزل السيدوموناس ايريجينوزا من الأعضاء الداخلية لدجاج حديث النفوق، ومريض واخر سليم ظاهرياً من أعمار مختلفة بمحافظتى كفر الشيخ والبحيرة. تم تجميع ٣٧٢ عينة كالتالى: ٢٢٤عينة من بدارى التسمين المريضة و84 من حديثة النفوق و٦٤ عينة من السليمه ظاهرياً من أعمار مختلفة. تم فحص العينات بكتريولوجيا وتم عزل سبعة عشرة معزولات من السيدوموناس ايريجينوزا واختبرت حساسيتها للمضادات الحيوية المختلفة حيث كانت المعزولات حساسة للكولستين سلفات (76.5%) نورفلوكساسين (52.9%) اميكاسين (41.2%) جنتاميسين (23.5%) سبروفلوكساسين- سيفوزون (11.8%) وجاءت كل المعزولات مقاومة للينكوميسين و ناليديكسك اسيد و ستربتوميسين و فلورفينيكول و كلورامفينيكول- دوكسي سيكللين. تم تطبيق تفاعل البلمره المتسلسل باستخدام البادئ للكشف عن جين L *opr* عند 504 bp وأيضا فحص التتابع الجيني للمادة الوراثية للسيدوموناس ايريجينوزا وتسجيلها ببنك الجينات واخذت الرقم KP056547، وكانت نسب التطابق مع التتابعات الجينية الأخرى تصل إلي 99%.

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