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VIRULENCE OF ISOLATED *PSEUDOMONAS AERUGINOSA* INFECTING DUCKLING AND ANTIBIOTIC RESISTANCE WITH AN EXPERIMENTAL TREATMENT TRIAL

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ABSTRACT

The present study was conducted in Kafrelsheikh governorate to isolate *P.aeruginosa* from 120 ducks, sampling from cloacal swab, internal organs and tracheal swab of freshly dead, healthy and diseased ducklings at different ages which showed depression, emaciation, ocular and or nasal discharges, diarrhea and sometimes enlargement of hock joint and had lesions of airsacculitis, congested liver, catarrhal enteritis and sometimes presence of gelatinous material in the hock joints with rate of isolation of 16.66% (20 samples were positive). Serological identification of suspected isolates of *P. aeruginosa* showed that 16 isolates of polyvalent I group I and 4 isolates of polyvalent II group J. Susceptibility of the isolated *P. aeruginosa* for 11 antibiotics demonstrated that the highest resistance was noted against Erythromycin, Oxytetracycline, Ampicillin and Amoxicillin (95%). The highest sensitivity of *P. aeruginosa* was observed against Florphenicol (80%) and it used for treatment in experimental design and give good results. Multiplex PCR used to identify virulence represented by toxA gene, lasI gene and oprL gene of *P. aeruginos*a show that all isolates where positive for the 3 examined virulence genes and these indicate that all isolates were highly virulent strains and antibiotic resistance genes represented by qnrS gene, blaCTX gene and mexR gene of *P. aeruginos*a showed that all isolates where positive for the 3 examined AB resistance exept isolate No 4 were negative for *blaCTX* and these indicate that all isolates were highly AB resistance exept isolate No 4 was not have *blaCTX* resistance gene. Experimental infection was done to study the Pathogenicity of *P. aeruginosa* which isolated from diseased ducklings on newly hatched ducklings with dose of infection of 0.2ml of $1x10^7$ cfu/ml s/c and use the potent antibiotic florphenicol for treatment of experimentally infected ducks. Our study proved that virulence genes owned by the *P. aeruginosa* confirming its pathogenicity for ducks, especially in the presence of oprL gene which plays a great role in antimicrobial resistance, so biosafety was recommended for hatcheries and farms, the hygiene, cleaning and disinfection will reduce *P. aeruginosa* spreading in the farms.

Keyword: *Pseudomonas aeruginosa*, duckling, Virulence genes, antibiotic resistance genes, sensitivity test, Experimental infection.

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INTRODUCTION

Pseudomonas aeruginosa an opportunistic pathogen, decrease host defenses causeing infection of yolk sac and omphalitis resulting in deaths of young ducks and hatched ducks. Mortality begins from hatching and continue for 10-14 days or more, and the infection with *P. aeruginosa* is responsible for mortality and clinical signs including respiratory signs and septicaemia (Bapat *et al.* 1985). P. aeruginosa can infect all tissues in poultry (Khattab *et al.,* 2015). P. aeruginosa causes problems in ducks as septicemia, respiratory manifestation, lameness, conjunctivitis and diarrhea.

(Saif *et al.,* 2008) Stated that the clinical signs include lameness; incoordination; swelling of head, and hock joint or footpads; diarrhea, and septicemia. (Barnes, 2003) mention that The affected ducks showed greenish watery diarrhea and paralytic signs with lameness in some birds and necropsy revealed echymotic and petechial haemorrhages on heart and liver, respectively (Qureshi, S.D. *et al.,* 2010 and Hamza, M. Eid *et al.,* 2019) isolated *P. aeruginosa* from trachea, heart, lung, liver and spleen of the affected ducks and it is gram-negative, aerobic gammaproteo-bacteria that can cause disease in animals and humans. *P. aeruginosa* is one of bacteria responsible for drug-resistant nosocomial infections. (Mena and Gerba, 2009).

P. aeruginosa is important in the etiology of many infectious diseases seen in humans (Silby *et al.,* 2011). *P. aeruginosa* is one of the environmental associated diseases and serious diseases in poultry farms which has multifarious virulence genes and plays a major role in poultry outbreaks. P. aeruginosa produces cheesy deposits on air sacs, dyspnea and congestion of internal organs, pericarditis and peri-hepatitis (Kheir El din and Awaad 1986). The development of antibiotic resistance due to low permeability of outer membrane thus potentially compromises the effective therapeutic use of antimicrobial agents (Davies and Davies, 2010). Pseudomonas species demonstrated a high resistance to monotherapy of penicillins, tetracyclines, cephalosporins, fluoroquinolones, and macrolides. Only combination of drugs as Ticarcillin + Clavulanic acid, Cefoperazone + Sulbactum, Cefotaxime + Sulbactum, Piperacillin + Tazobactum, Ceftriaxome + Sulbactum according to (Javiya *et al.,* 2008). Emergence and spread of resistance in bacteria may be due to mutational events introduced during bacteria replication and vertical transmission of genetic variants through generations in a particular bacteria strain. (Walker *et al.,* 2002) Reported that P. aeruginosa is non-spore forming rod, motile and gram-negative. This organism is resistant to many antimicrobials and can invade hatched ducks. Virulent strains of this bacterium can cause diarrhea, dehydration, dyspnea, septicemia, and death. *P. aeruginosa* produce hemolysis on blood agar due to activity of phospholipase C which is potent toxins can damage cells including red blood cells in vivo, resulting in hemorrhage, edema, and tissue necrosis.

P. aeruginosa has proteolytic enzymes degrade lipoproteins in the yolk, this permits its proliferation, enhance other bacteria to multiply (Garcia and Isenberg, 2007).

PCR is simple highly sensitive more rapid, than cell culture assay in detection of bacteria. So it could be recommended as screening method in laboratory (Schmidt *et al.,* 1995) .

MATERIALS AND METHODS

I. Samples Collection according to (**Middleton *et al.*, 2005**)**:** A total of 120 samples were collected from diseased ducklings showing profuse diarrhea, respiratory manifestations, yellowish nasal secretion, ruffled feather and conjunctivitis

and samples included liver, heart, yolk sac, cloacal swab and tracheal swabs were collected from many different farms in Kafrelsheikh governorate for isolation and identification of *P. aeruginosa*. All samples were handled and collected aseptically to prevent cross contamination using sterile sampling materials and transmitted immediately in ice box to be examined in the laboratory.

II. Isolation and Biochemical identification of *p. aeruginosa*:

Samples inoculated in nutrient broth and incubated at 37 °c for 24 hours, then sub cultured onto selective medium (MacConkey agar and Pseudomonas agar base) incubated at 37 °C for 24 hours and observe the non-lactose fermenting colonies and sub cultured onto tryptic soya agar plate to observe the pigmentation. Suspected colonies were picked up and subjected to further identifications based on colonial and cellular morphology, pigment production detection of fruity smell, oxidase test and Gram staining. Cultivation on laboratory media and all biochemical tests were performed according to (Quinn *et al.*, 2002) and (Shukla and Mishra, 2015). Oxidase test, catalase test, arginine hydrolysis test, gelatin liquefaction, Indol, methyl red and urease test identified by API 20 typing system to confirm isolates (Cheesbrough, 2000).

III. Serological identification:

Serotyping of the isolated *P. aeruginosa* was applied by using slide agglutination technique (specific 4 polyvalent and 16 monovalent antisera) according to the recommendation of the manufacturer's protocol (Bio-Rad®, France) according to Glupczynski *et al*. (2010). The division of *P. aeruginosa* to groups based on O antisera of *P.aeruginosa*, depend on the International Antigen Typing Scheme (IATS) according to Legakis *et al.* (1982).

IV. Antibiotic Sensitivity Test: The antimicrobial sensitivity test was performed as reported by Finegold and Martin (1982) by disc diffusion method. Different antimicrobials were used such as En: Enrofloxacin (5µg), Nor: norfloxacin (10 µg), Tl: tylosin (15 µg), Fl: florphenicol (30µg), Do: doxycycline (30µg), Amp: ampicillin (10 µg), Aml: amoxicillin (10 µg), CTX: cefotaxime (30 µg), E: Erythromycin (15µg), CIP: Ciprofloxacin (5µg) and T: Oxytetracycline (30µg). The interpretation of the measured zone was done according to CLSI (2018).

V. Multiplex PCR:

1 - Extraction of DNA: samples was performed by QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations, as in the kit. Oligonucleotide Primer. Primers were supplied from Metabion as in table (1).

2 - PCR amplification: For multiplex PCR.

3 - Analysis of the PCR Products: The products of PCR were separated by electrophoresis and the data analyzed through computer software, Primers sequences, target genes, amplicon sizes and cycling conditions for conventional PCR.

Table 1: Target genes, amplicon sizes, primers sequences, and cycling conditions for conventional PCR.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
ToxA	**GACAACG CCCTCAG CATCACC AGC**	396	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Matar *et al.*, 2002**
	CGCTGGC CCATTCG CTCCAGC GCT							
LasI	**ATGATCG TACAAATT GGTCGGC**	606						**Bratu *et al.*, 2006**
	GTCATGA AACCGCC AGTCG							
oprL	**ATG GAA ATG CTG AAA TTC GGC**	504						**Xu *et al.*, 2004**
	CTT CTT CAG CTC GAC GCG ACG							
qnrS	**ACGACAT TCGTCAA CTGCAA**	417	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Robicsek *et al.*, 2006**
	TAAATTG GCACCCT GTAGGC							
blaCTX	**ATG TGC AGY ACC AGT AAR GTK ATG GC**	593						**Archambault *et al.*, 2006**
	TGG GTR AAR TAR GTS ACC AGA AYC AGC GG							
mexR	**GCGCCAT GGCCCAT ATTCAG**	637						**Sánchez *et al.*, 2002**
	GGCATTC GCCAGTA AGCGG							

VI. Pathogenicity of *P. aeruginosa*:
To study the Pathogenicity of *P. aeruginosa* isolates from diseased ducklings on newly hatched ducklings with dose of infection of 0.2ml of $1x10^7$ cfu/ml s/c.

VII. Experimental trials:
It was carried out at Animal Health Research Institute, provisional lab in Kafr el-Sheikh governorate. seventy ducklings one day old were placed into isolation units, feed and water were provided ad libitum, 10 ducks were sacrificed and examined bacteriologically to be free from *P. aeruginosa*, and the other ducklings were divided to 3 groups; first group as a control group, second group was infected (injected with 0.2 ml of 1 x 10^7 colony-forming units/bird) without treatment and the third group was infected with *P. aeruginosa* as in second group and treated with the most effective antibiotic according to sensitivity test , Florphenicol , for 5 days, symptoms, postmortem lesions and mortalities were recorded for 4 weeks. Mortality was recorded daily, and the ducks that died were necropsied and yolk sacs and liver were cultured. After 14 days, the remaining ducks were euthanatized and necropsied. Bacterial isolates from yolk sacs and liver were identified by using the API 20 system to confirm that it was as the challenged isolated bacterial. All bacterial isolates were identified morphologically, culturally, biochemically and serologically for *P. aeruginosa.* The challenge bacterial isolates produced somewhat different and often distinctive postmortem lesion.

RESULTS

In this study, from a total of 120 samples were collected from diseased and freshly dead ducklings, 20 samples were positive for *P. aeruginosa* with percentage of 16.66%

Symptoms of examined duckling:
The diseased or freshly dead ducklings showed depression, emaciation, ocular and or nasal discharges, diarrhea and sometimes enlargement of hock joint with lameness

Postmortem lesions of examined duckling:
The diseased or freshly dead ducklings showed airsacculitis, congested liver, catarrhal enteritis and sometimes presence of gelatinous material in the hock joints

Table 2: result of serotyping of isolated *P.aeruginos*a

Type of samples	No. of positive samples	% of positive samples	no .of isolates Serotypes
liver	4	3.33	polyvalent **I** group I 3 polyvalent **II** group J 1
heart	4	3.33	polyvalent **I** group I 3 polyvalent **II** group J 1
yolk sac	2	1.66	polyvalent **I** group I 1 polyvalent **II** group J 1
Cloacal swab	7	5.83	6 polyvalent **I** group I polyvalent **II** group J 1
Tracheal swab	3	2.50	polyvalent **I** group I 3
total	20	16.66	16 polyvalent **I** group I 4 polyvalent **II** group J

Total No. of samples were 120 for each type.

Table 3: Susceptibility of isolated *P.aeruginosa* (20 isolates) to antimicrobial agents.

Antimicrobial agent	(isolates)			
	Sensitive		Resistant	
	No.	%	No.	%
Enrofloxacin	10	50%	10	50%
norfloxacin	9	45%	11	55%
Ciprofloxacin	11	55%	9	45%
tylosin	2	10%	18	90%
Erythromycin	1	5%	19	95%
Oxytetracycline	1	5%	19	95%
doxycycline	3	15%	17	85%
ampicillin	1	5%	19	95%
amoxicillin	1	5%	19	95%
cefotaxim	3	15%	17	85%
Florphenicol	16	80%	4	20%

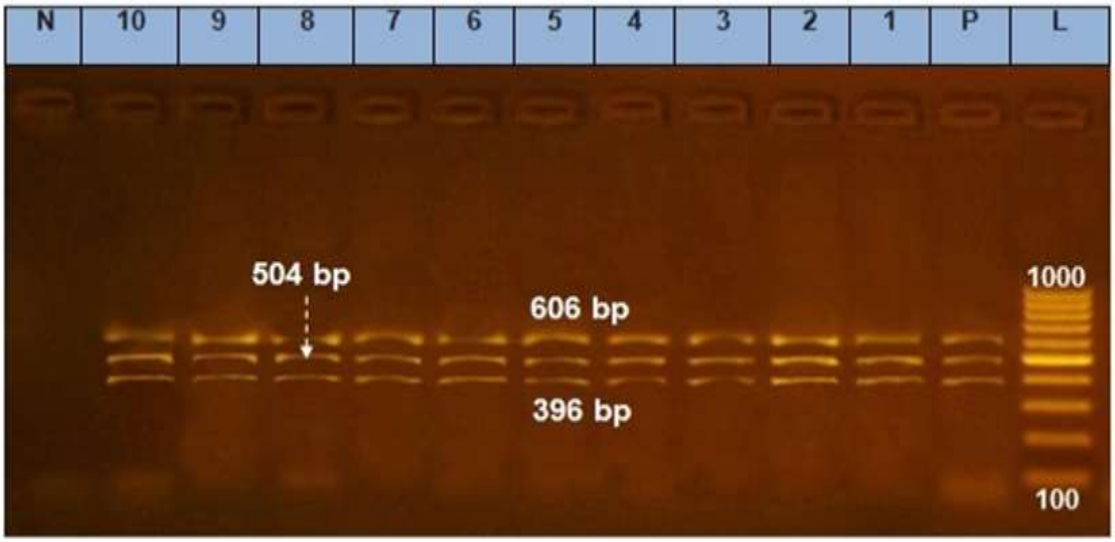

Photo (1): Agarose gel electrophoresis of multiplex PCR for virulence genes toxA (396 bp), lasI (606 bp) and oprL (504bp) *P. aeruginos*a

Photo details
Lane L: 100 bp ladder as molecular size DNA marker.
Lane P: Control positive genes.
Lane N: Control negative.
Lanes 1 to 10: Positive for 3 genes.

Table 4: Result of multiplex PCR for virulence genes toxA (396 bp), lasI (606 bp) and oprL (504bp) *P. aeruginos*a

Pseudomonas sample	*toxA*	*lasI*	*oprL*
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+
5	+	+	+
6	+	+	+
7	+	+	+
8	+	+	+
9	+	+	+
10	+	+	+

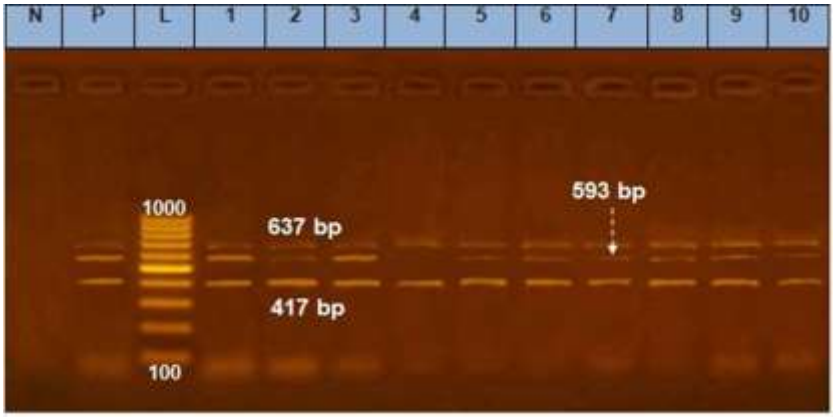

Photo 2: Agarose gel electrophoresis of multiplex PCR for AB resistance genes represented by qnrS (417 bp) blaCTX (593 bp) and mexR (637 bp) of *P. aeruginos*a**.**

Photo details

Lane L: 100 bp ladder as molecular size DNA marker.

Lane P: Control positive genes.

Lane N: Control negative.

Lane 1 to 10: Positive for 3 genes except no 4 negative for *blaCTX***.**

Table 5: Result of multiplex PCR for AB resistance genes represented by qnrS (417 bp) blaCTX (593 bp) and mexR (637 bp) of *P. aeruginos*a.

Pseudomonas sample	*qnrS*	*blaCTX*	*mexR*
1	+	+	+
2	+	+	+
3	+	+	+
4	+	-	+
5	+	+	+
6	+	+	+
7	+	+	+
8	+	+	+
9	+	+	+
10	+	+	+

Table 6: Symptoms of *Pseudomones aeruginosa* challenged ducklings.

Group	treatment	general signs of illness, (decreased appetite, loss of weight)	respiratory distress	diarrhea	Enlarged hock joint &lameness
1	Non challenged, non-treated	1st week: ---- 2nd week : --- 3rd week : ----	1st week: ---- 2nd week : --- 3rd week : ----	1st week: ---- 2nd week : --- 3rd week : ----	1st week: ---- 2nd week : --- 3rd week : ----
2	Challenged, non-treated	1st week: ++ 2nd week : +++ 3rd week : ++	1st week: ++ 2nd week : +++ 3rd week : ++	1st week: + 2nd week : ++ 3rd week : ++	1st week: ++ 2nd week : +++ 3rd week : +++
3	Challenged, treated with florphenicol	1st week: ++ 2nd week : + 3rd week : --	1st week: ++ 2nd week : + 3rd week : +	1st week: + 2nd week : + 3rd week : +	1st week: ++ 2nd week : + 3rd week : +

Table 7: Post mortem lesions in weekly sacrificed ducklings.

Group	treatment	airsacculitis	congested liver & spleen	pericarditis, perihepatitis	mortality
1	Non challenged, non-treated	1st week: ---- 2nd week : --- 3rd week : ----	1st week: ---- 2nd week : --- 3rd week : ----	1st week: ---- 2nd week : --- 3rd week : ----	No
2	Challenged, non-treated	1st week: ++ 2nd week : +++ 3rd week : ++++	1st week: ++ 2nd week : +++ 3rd week : ++++	1st week: + 2nd week : ++ 3rd week : ++++	2
3	Challenged, treated with florphenicol	1st week: + 2nd week : ++ 3rd week : ++	1st week: + 2nd week : + 3rd week : +	1st week: + 2nd week : + 3rd week :+	No

Table 8: *P. aeruginosa* re-isolation rate from challenged ducklings.

Group	Treatments	Number of examined ducklings	Weeks after challenged		
			1 st wk	2 nd wk	3 rd wk
1	Non challenged, non-treated	20	0/20 (0%)	0/20 (0%)	0/20 (0%)
2	Challenged, non-treated	20	18/20 (90%)	16/18 (88.89%)	12/18 (66.67%)
3	Challenged, treated with florphenicol	20	10/20 (50%)	6/20 (30%)	4/20 (20%)

DISCUSSION

Pseudomonas infection was considered an extensive economic problem in poultry farms, especially *P. aeruginosa* Shahat *et al.* (2019) which causing a high mortality in birds (Elsayed *et al*., 2016). The complications caused by *P. aeruginosa* in birds have appeared in the form of respiratory signs, septicemia, keratitis and sinusitis (Hai-ping, 2009).

In our study, the prevalence of *Pseudomones aeruginosa* was 16.66% which was higher than that recorded by Asawy and El-Latif (2010), Hamza *et al.* (2019) and Odoi, Hayford (2016) that was (15.8%), 10% and (9.7%) respectively. The affected ducklings showed emaciation, ocular and or nasal discharges, diarrhea and sometimes enlargement of hock joint with lameness, these symptoms nearly similar to that recorded by (Qureshi *et al.*, 2010).

Virulent isolates of this bacteria cause dehydration, diarrhea, septicemia, dyspnea, and death as reported by (Harbottle *et al*., 2007) Regarding to lesions showed on freshly dead ducklings were airsacculitis, congested liver, catarrhal enteritis and sometimes presence of gelatinous material in the hock joints P. aeruginosa causes sinusitis, respiratory infection, keratoconjuctivitis, keratitis and septicemia and responsible for septicemia, pyogenic infections, endocarditis and lameness De Vos *et al.* (2009). Infections may also occur through contaminated vaccines, skin wounds and antibiotic solutions or syringe used for injection and it may be systemic affecting tissues, multiple organs or localized in air sacs or infraorbital sinus producing swelling of the wattles, head, sinuses and joints in affected birds. *P. aeruginosa* were isolated from birds and poultry farms all over the worled Sams (2001). *P.aeruginosa* is gram-negative, motile, non-spore forming rods (Elsayed *et al*., 2016). It is characterized by producing of watery soluble green pigment with a specific fruity odor. (Barnes, 2003) reported that *P. aeruginosa* produces cheesy deposits and dyspnea. Congestion of internal organs, peri-hepatitis and pericarditis were reported by (Kheir El din and Awaad 1986). The identification of these strains should be considered during microbiological examination. In this study, identification showed typical colorless colonies on MacConkey agar media and green-blue color colonies for Pseudomonas spp. on Pseudomonas agar media similar to Haleem *et al.* (2011), also the morphological features with gram stain showed a gram-negative rods of pseudomonas spp. these findings were supported by Tripathi *et al.* (2011).

The of bacteriological examination for samples showed a green-blue color colonies with an odor like sweet grape were clear on Pseudomonas agar and didn't ferment lactose sugar in MacConkey agar. Different biochemical tests were used to identify *p. aeruginosa* which showed a clear positive result for catalase test, oxidase test, Citrate reaction, arginine hydrolysis (gives brown color) and gelatin liquefaction but is negative to indole production, methyl red reaction and Voges Proskauer test. *P. aeruginosa* produces pyocyanin and pyoverdin pigments, grows well at 42˚C and 4˚C and gives red butt and slant without H2S production on triple sugar iron agar. But the biochemical scheme cannot separate other species due to the high resemblance among the results of isolates so further identification was done by serological test and PCR to reach to accurate species.

Result of serological identification of suspected isolates of *P. aeruginosa* were explained in table (2) showed that serologically identified isolates into 16 isolates of polyvalent I group I and 4 isolates of polyvalent II group J

The highest rate of isolation was from cloacal swabs, so fecal matter considered as the most dangerous source for spreading of *P. aeruginosa* inside ducks farms, then the second rate of isolation was from heart and liver and these indicate the ability of *P. aeruginosa* to cause septicemia, respiratory infections, septicemia and mortalities and these agree whith (Eman *et al*., 2017), and the last rate of isolation was from yolk sac and this indicate that *P. aeruginosa* can be transmitted vertically from diseased dam.

P. aeruginosa was listed as the head of most three frequent Gram-negative pathogens and is linked to the worst visual diseases. Its outbreak varies from 2 to 100% (Saad *et al*., 2017). Serotypes O10 H, O6 G, O11 E, and O2 G E of *P. aeruginosa* were detected by Shahat *et al.* (2019).

In table (3): The interpretation of antimicrobial resistance of *P. aeruginosa* isolates according to CLSI (2018).

Susceptibility of *P. aeruginosa* for different antimicrobials demonstrated that an obvious resistance was noted against

Erythromycin, Oxytetracycline, Ampicillin and Amoxicillin (95%) and was followed by Tylosin (90%) and Doxycycline and Cefotaxim (85%) and the highest sensitivity was observed against Florphenicol (80%), Ciprofloxacin (55%), Enrofloxacin (50%) and Norfloxacin (45%), so the most influential antibiotics was Florphenicol (80%) and it used for treatment in experimental design. Shahat *et al.* (2019) recorded that antimicrobial resistance is important problems confronting the world and it is elevating in developing countries. Therefore, it's important to detect *P. aeruginosa* quickly and identify its susceptibility pattern; this may avoid useless antibiotic treatment which presents antibiotic-resistant pathogens (Hamisi *et al.*, 2012). The antimicrobial susceptibility reported that the identification of *P. aeruginosa* with traditional methods takes a long time to perform. Shahat *et al.* (2019) detect that Antimicrobial agents Resistance *P.aeruginosa* isolates where Intermediate Sensitive to Sulphamethazole, Gentamycin, Erythromycin, Tetracycline, Ciprofloxacin, Amoxicillin, Ampicillin, Streptomycin, Nalidixic acid, Norfloxacin. Atlas and Synder (2006). Reported that antibiotic sensitivity tests for all isolates were resist to ceftiofur, sulfisoxazole, lincomycin, bacitracin, oxytetracycline, penicillin, naladixic acid, erythromycin, and tetracycline, varied to other antibiotics, sensitive to gentamicin. The isolates were identified by biochemical tests as urease production test, motility test, catalase test, oxidase test, citrate utilization test, triple sugar iron agar test, indole test, nitrate reduction test, gelatinase liquefaction, oxidative-fermentative test, haemolysin production, alkaline protease production, lecithinase production and Sugar Fermentation. In Ghana a study carried out show that *P. aeruginosa* all isolated from poultry were susceptible to antibiotic levofloxacin from 20 to100% and 75% were intermediate susceptibility to aztreonam. and resistance to carbapenems, cephalosporins, penicillins, monobactam, quinolones and aminoglycoside and β-Lactamase encoding genes as (blaIMP, blaVIM) not detected in any isolates and the class 1 integron carry resistant genes detected in 89.4% of the multi-drug resistant strains Odoi (2016) Identified blaVIM gene in *P. aeruginosa* from poultry resembled corresponding regions in its clinical isolates of *P. aeruginosa* and these isolates were resistant to all β-lactam tested, as, imipenem, meropenem, aztreonam, and ceftazidime Bassetti *et al.* (2013) and Zhang *et al.* (2017). In Nigeria *P. aeruginosa* was resist to tobramycin, β-lactams, nitrofurantoin, tetracycline and sulfamethoxazole-trimethoprim, while ofloxacin, ertapenem and imipenem were effective against the bacterial pathogens Aniokette *et al.* (2016). In Pakistan, a study investigated the causative bacteria for necropsy in chicken, about 28% prevalence were P. aeruginosaand it was 100% resistant towards meropenem, colistin, erythromycin, ciprofloxacin and ceftriaxone, while 60% was sensitivit against ampicillin, cefoperazone, ceftazidime, sulbactam and rifampicin. Isolates were multidrug resistance to other antibiotics Sharma *et al.* (2017). Ahmed (2016) and Tartor and Elnaenaeey (2016) who mentioned that *P. aeruginosa* was highly resistance to Tetracycline, Erythromycin and Ampicillin, Sulphamethazone, Erythromycin, Ampicillin, Tetracycline, Amoxicillin and Erythromycin followed by Nalidixic acid (57.1%), Streptomycin (42.9%). Abd El-Gawad *et al.* (1998) reported that P. aeruginosa isolates of chickens were sensitive to Tetracycline. Abdel-Tawab *et al.* (2016) found that P.aeruginosa isolates were resistant to Nalidixic acid (80%). Mohammad (2013) recorded a high sensitivity with Ciprofloxacin and Norfloxacin but low sensitivity of P. aeruginosa to Ciprofloxacin and Norfloxacin was recorded by Abd El-Tawabet *et al.* (2014). Abd El-Gawad *et al.* (1998) and Kurkure *et al.* (2001) reported a high sensitivity to antibiotic Gentamycin (88.6% and100% respectively). These variations among the results may be

attributable to the difference in many conditions surrounding hatcheries or may be a result of hyper-mutation which occurred frequently in *P.aeruginosa* strains and leading to the development of various antimicrobial resistance as reported by Zheng *et al.* (2019). Antibiotic-resistant bacteria (ARB) can easily spread alongside the food chain and cause most of public health hazards (Da Costa *et al.*, 2013, FAO, 2015 and WHO, 2015).

PCR method has been used to provide a specific, rapid, simple, and vastly identifiction of *P. aeruginosa* which has got an enormous numbers of some extracellular virulence factors and cellular components which implicated in pathogenesis Qin *et al.* (2003) and (Habeeb *et al*., 2012). Molecular examination of *P. aeruginosa* serologically identified ten isolates in this study for detection of virulence genes toxA gene, lasI gene and oprL gene of *P. aeruginos*a using multiplex PCR.

In photo No (1) and table No (4) Result of multiplex PCR for virulence genes represented by toxA gene at 396 bp, lasI gene at 606 bp and oprL gene at 504bp of *P. aeruginos*a show that all isolates where positive for the 3 examined virulence genes and these indicate that all isoletes were highly virulent strains. *P. aeruginosa* has virulence repertoire such as lipopolysaccharide, elastase, alkaline proteases, pyocyanin, pyoverdin, hemolysins, phospholipase C and rhamnolipids. Some factors are coordinated by a global regulatory system activated by autoinducers involved (lasI) gene (Habeeb *et al.,* 2012). Also some genes as exoS, exoT, exoU, and exoY genes which regulate the action of *P. aeruginosa* secretion system which injects toxic effectors proteins into the cytosol of host cells and accompanied by inferior clinical outcomes and elevated mortality rates (Hauser, 2009). *P. aeruginosa* uses the virulence factor exotoxin A to inactivate eukaryotic elongation factor 2 in the cell, such as the diphtheria toxin does, hence eukaryotes can't synthesize protein and necrotize (Eman *et al.,* 2017). The powerful toxins of bacteria released during bacteremia continu after *P. aeruginosa* killed by antimicrobial (Kirienko *et al.,* 2015). The main troublesome characters of *P. aeruginosa* is a minimal susceptibility to many types of antibiotics, making it a very hard bacteria to be eliminated and this due to *P. aeruginosa* genome contains the largest known resistance island genes (Khattab *et al.,* 2015). The important reason for antimicrobial resistance was impermeability which belongs to the outer membrane lipoprotein (oprL gene) that implicated in efflux transport systems and its effects on cell permeability (De Vos *et al.,* 1997). Antibiotics are profusely administered for therapeutic and prophylaxis purposes in veterinary field (Dandachi *et al.,* 2018).

Concerning the results of virulence factors we found that the detection of oprL gene in all isolates (100%) confirmed the existence of *P aeruginosa* DNA which considerd a specific marker for molecular detection of *P.aeruginosa* and encodes a protein in the inner and outer membranes, which is essential for the invasion of epithelial cells (De Vos *et al*., 1997 and Shahat *et al*., 2019), the same result obtained by Hassan (2013) and in efflux transport systems affecting cell permeability so there is a strong relation between detection of oprL and phenotypic antibiotic resistance that reported by Qin *et al*. (2003) and Lavenir *et al.*, (2007). In this study, the incidence rate of toxA gene was 71.42%, as shown in similar results of toxA reported by Qin *et al*. (2003) and Lavenir *et al*. (2007). Khan and Cerniglia (1994) showed that 96% of tested *P.aeruginosa* isolates contained a toxA gene. Furthermore, the exoS and lasB genes were detected in five isolates of P.aeruginosa (71.42% for each of them) and this percentage was nearly similar to Tartor and El-naenaeey (2016) who found that the colossal majority of *P. aeruginosa* isolates showed exoS gene (78.6%). The higher percentage was recorded by Nikbin *et al.*

(2012) who detected lasB in all strains of *P.aeruginosa* (100%). The mentioned virulence genes in this work such as, toxA, exoS and lasB were coordinated by a critical global regulatory systems consisted of transcriptional activator protein (LasR) and Pseudomonas autoinducer, (PAI), the central gene responsible for activation of this system was putative autoinducer synthase (lasI) (Habeeb *et al.*, 2012). The lasI gene which is a quorum sensing Regulation gene was detected. Venturi (2006) reported that the lasI is not detected in any Pseudomonas spp. otherwise *P. aeruginosa* strain. Percentage of lasI gene was less than that was detected by Alshalah *et al.* (2017) who succeeded in the amplification of lasI gene in all clinical isolates of *P.aeruginosa*. In addition to, Nikbin *et al.* (2012) whom explained that the possession of *P. aeruginosa* for several virulence genes make it a reason for various levels of virulence and pathogenicity.

In photo No (2) and table No (5) Result of multiplex PCR for AB resistance genes represented by qnrS gene at 417 bp, blaCTX gene at 593 bp and mexR gene at 637 bp of *P. aeruginos*a show that all isolates where positive for the 3 examined AB resistance except isolate No 4 were negative for blaCTX and these indicate that all isolates were highly AB resistance exept isolate No 4 was not have blaCTX resistance gene and this results differ with Shahat *et al.* (2019) which detect virulence and disinfectant resistance genes in *P.aeruginosa* isolates such as (oprL, toxA, exoS, lasB and lasI) which result in amplicons 504bp, 396bp, 118bp, 1220bp and 606bp respectively, and cleared that oprL gene was disclosed in all P. aeruginosa isolates with percentage of 100%, while other genes were detected with the same percentage 71.4%, which it was Serotypes of P. aeruginosa Group P. aeruginosa O10 H *P. aeruginosa* O6 G P. aeruginosa O11 E P. aeruginosa O2 G and found also resistant genes in *P.aeruginosa* isolates (no.7 isolates) OprL gene 7 (100%) toxA gene 5 (71.42%) lasI gene 5 (71.42%) lasB gene 5 (71.42%) exoS gene 5 (71.42%) qacA/B gene 1 (14.28%) qacC/D gene 1 (14.28%) qacED1 gene 7 (100%).

Regarding the results of our study in tables (6, 7), The infected ducklings showed general signs of illness (decreased appetite and loss of body weight), respiratory distress, diarrhea and sometimes enlargement of hock joint with lameness, the severity of symptoms appeared in the 2nd challenged groups in the first week and began to decline after treatment with florphenicol in the 3rd group from the 2nd week without mortalities, however these symptoms continued in the non- treated group till the3rd week with mortality 10% which had lesions of air-sacculitis, congested liver, congested spleen, pericarditis and peri-hepatitis.

In table (8) re-isolation rate of *P. aeruginosa* from challenged ducklings was negative in group1 and gradually decreased in group 2 due to normal immunity and also decreased in group 3 but it more decreased than group 2 due to use of effective antibiotic in treatment in the third group, so use of effective antibiotic after sensitivity test was important for rapid cure of diseased ducks and effectively decrease rate of shedding of *P. aeruginosa* to surrounding environment and control disease. The prevalence of *P. aeruginosa* was 16.66% which was higher than that recorded by Asawy and El-Latif (2010), Hamza *et al.* 2019 and Odoi (2016) that was (15.8%), 10% and (9.7%) respectively. The affected ducklings showed emaciation, ocular and or nasal discharges, diarrhea and sometimes enlargement of hock joint with lameness, these symptoms nearly similar to that recorded by (Qureshi *et al.*, 2010). The lesions showed on freshly dead ducklings were air-sacculitis, congested liver, catarrhal enteritis and sometimes presence of gelatinous material in the hock joints.

CONCLUSION

Our study proved that virulence genes owned by the *P. aeruginosa* confirming its pathogenicity for ducks, especially in the presence of oprL gene which plays a great role in antimicrobial resistance, so biosafety was recommended for hatcheries and farms, hygiene, cleaning and disinfection will reduce *P. aeruginosa* spreading in the farms.

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الضراوة لبكتريا السودوموناس ايرجينوزا المعزولة من البط الصغير ومقاومته للمضادات الحيوية مع محاوله تجريبيه للعلاج

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أجريت الدراسة الحالية في محافظة كفر الشيخ لعزل ميكروب السيدوموناس ايروجونازا من ١٢٠ بطه من الأعضاء الداخلية لصغار البط النافق والمريض في أعمار مختلفة والتي أظهرت الاكتئاب والهزال وإفرازات العين والأنف والإسهال وأحيانا تضخم مفصل العرقوب. وكانت لديهم آفات التهاب الأوعية الدموية ، واحتقان الكبد ، والتهاب الأمعاء وأحيانًا وجود مادة هلامية في مفاصل العرقوب وكانت هناك ٢٠ عينه ايجابيه للعزل بمعدل عزل ١٦,٦٦% .

تم عمل التصنيف السيرولوجى للمعزولات حيث وجد ان ١٦ معزوله منهم تنتمى الى السيروتيب بولى فيلنت **I** مجموعه **I** أما الاربعه معزولات الباقيه فكانت تنتمى الى السيروتيب بولى فيلنت **II مجموعه** J . تم عمل اختبار الحساسيه لمعزولات السيدومونس ايروجينوزا لعدد ١١ مضاد حيوى وكان الميكروب أعلى مقاومه للمضادات الحيويه اريثرومايسين و الاوكسى تتراسيكلين والامبسللين والاموكسسيللين بنسبه ٩٥% فى حين كانت أعلى حساسيه للمضاد الحيوى الفلورفنيكول بنسبه ٨٠% حيث استخدم فى العلاج فى العدوى التجريبيه وأعطى نتائج جيده .

ولمزيد من التعريف بالميكروب تم عمل تفاعل البلمره المتسلسل المتعدد لتحديد ٣ جينات للضراوة (toxA gene, lasI gene , oprL gene) وكانت الجينات موجوده بكل المعزولات وهذا يدل على شده ضراوه المعزولات وأيضا تم فحص ٣ جينات لمقاومة المضادات الحيوية (qnrS gene, blaCTX gene , mexR gene) وكانت الجينات موجوده فى كل المعزولات باستثناء معزوله واحده كانت لا تحتوى على جين (blaCTX) وهذا يعنى ان المعزولات شديده المقاومه للمضادات الحيويه.

أجريت عدوى تجريبية لدراسة الإمراضية للسيدوموناس ايروجونازا المعزولة من فراخ البط المريضة على فراخ البط حديث الفقس بجرعة إصابة ٠,٢ مل من ١ x ١٠ ^{٧} CFU بالحقن تحت الجلد واستخدام المضاد الحيوي الفعال (الفلورفينيكول) لعلاج البط المصاب تجريبياً. ومن دراستنا يتضح ان جينات الضراوه الموجوده بميكروب السيدوموناس ايروجينوزا تؤكد قوه هذا الميكروب على احداث المرض بالبط الصغير وخاصه فى وجود جين oprL الذى يلعب دورا مهما فى مقاومه المضادات الحيويه ولذا ننصح بتطبيق نظام الامان الحيوى فى المفرخات والمزارع والاهتمام بالصحه العامه والنظافه والتطهير للتقليل من انتشار ميكروب السيدوموناس ايروجينوزا .