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The role of hatcheries, hatching eggs and one day old chicks in dissemination of beta lactam antibiotic resistance *Pseudomonas aeruginosa* (ESBL)

Ghada Allam Abd EL –Dayem [*]; Ahmed H. Ramadan [**] and Hanaa S. Ali [***]

[*] Poultry Diseases Department, Animal Health Research Institute (AHRI) (Mansoura Branch), Agriculture Research Center (ARC), Dokki, Giza, Egypt.

[**] Bacteriology Department, Animal Health Research Institute (AHRI) (Mansoura Branch), Agriculture Research Center (ARC), Dokki, Giza, Egypt.

[***] Pathology Department, Animal Health Research Institute (AHRI) (Mansoura Branch), Agriculture Research Center (ARC), Dokki, Giza, Egypt.

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ABSTRACT

Out of 450 samples collected from infertile egg ,dead in shell embryo ,baby chicks with omphalitis and hatcheries environment *P. aeruginosa* was identified in 12. 22% (55/450) of the total examined samples. Two strains (4%) were isolated from infertile egg,18 strains (12%) from dead in shell embryo ,33 strain(15%) from baby chicks and 2 strains (6.7%%) from hatcheries environment. Extended spectrum beta lactam (ESBL) production was confirmed in 8 isolates(14.55%) by double disk diffusion test . *bla*SHV and *bla*TEM genes were detected in (*8/8)and* (7/8) of ESBL-*P. aeruginosa* isolates respectively, whereas none of these isolates harbored, *bla*OXA and *bla*CTX-M genes. Molecular detection of (*opr*L) and (toxA) genes were amplified in in all eight studied strains. The results of antibiotic sensitivity for ESBL producing *P. aeruginosa* isolates indicated that the isolates were susceptible to norfloxacin, ciprofloxacin and colistin sulphate .On the other hand, all isolates were completely resistant to ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazol and most of the isolates were resistant to streptomycin and tetracycline. The pathogenicity test of the isolated field strain of ESBL-*P. aeruginosa* proved to be highly pathogenic induced mortality rate of 43.33% . The predominant lesions of dead and sacrificed chicks were septicemia, congestion of internal organs (liver, heart& lung) and inflamed intestine. Histopathological findings supported the previous gross lesions. In conclusion, it can be said that there is an upsurge of beta lactamase producing *Pseudomonas aeruginosa* infer that one day old chicks and hatcheries can serves as a reservoir for growth and dissemination of clinically significant antibiotic resistance among bacterial species.

*Corresponding author: Ghada Abd EL –Dayem, Mansoura Lab., Animal Health Research Institute, Dokki
E-mail: Anasesra@gmail.com
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INTRODUCTION

Hatcheries are vulnerable to being reservoirs of infectious agents because of their central **position within the poultry production chain (Wales and Davies 2020).** The problem usually starts with contaminated eggs which are incubated under optimal condition for microbiological growth. The rate of embryonic death and vitality of newly hatched chicks are influenced by the extent of bacterial contamination in poultry hatcheries (**Gehan 2009, Amer et al. 2017).** Various bacterial pathogens *include Escherichia coli, Salmonella* species*, Pseudomonas* species *Proteus* species*, and Enterobacter* species*,* have been isolated from dead in shell embryos, baby chicks with omphalitis and hatcheries (**Elsayed et al. 2016, Amer et al. 2017, Bakheet et al. 2017).**

P.aeruginosa is a profiteer pathogen leads to respiratory infections, septicemia and high mortality of embryos and newly hatched chicks **(Dinev et al. 2013 Farghal et al. 2017**). Infection of yolk sac by pseudomonas is enhanced by its ability to degrade of other pathogen **(Elsayed et al. 2016).** Death of the chicks exposed to *P. aeruginosa often due to* Combinations of several bacteria multiplying in the yolk sac (**Sarma 1985)**

P .aeruginosa declares plenty of virulence factors, which share in its toxicity and pathogenicity **(Fadhil et al. 2016)** .These include enterotoxins, exocytotoxins, and toxins produced by protein secretion systems, as a result of expression of certain virulence operons. Consequently, many of these virulence have been implemented in infection, septicemia, and fatal condition **(McCarty et al. 2012; Tayabali et al. 2015)**

The introduction of extended-spectrum cephalosporins (ESCs) improved the treatment options in both veterinary and human medicine **(Pfeifer et al. 2010)** .Extended spectrum cephalosporins are one of the most important antibiotics in treatment of infections produced by *P. aeruginosa* isolates. However, resistance to these antimicrobials , mainly conferred by extended-spectrum β-lactamases (ESBL) and Amp C. β-lactamases, has recently gained importance and is considered as an emerging public health concern (Reich et al. 2013) .One-day -old chicks are shown to be a major risk factor for the introduction of ESBL/AmpC-producing bacteria in the broiler production chain (**Dierikx et al. 2013**)

One of the main trouble characters of *P. aeruginosa* is a minor susceptibility to a lot of types of antimicrobials, making it a very hard pathogen to eliminate **(Balasubramanian et al. 2013, Khattab et al. 2015)** There are several mechanisms of resistance to antimicrobial agents ranging from efflux pump to mobile genetic elements and hydrolyzing enzymes have been described in clinical isolates of *P. aeruginosa* **(Mesaros et al. 2007, Odumosu et al. 2013) .**Unfortunately, it can expand their antibiotic resistance by mutations or by acquiring resistance through horizontal gene transfer causing therapeutic failures in humans (**Cholley et al. 2010**). Various class A ESBLs, such as TEM-, and SHV-, -type ESBLs, and class D ESBLs such as OXA-type ESBLs have been identified in *P. aeruginosa* **(Nordmann and Guibert 1998, Livermore 2002).** Therefore, this study was designed to investigate the role of hatcheries, hatching eggs and baby chicks in dissemination of ESBL producing pseudomonas. Moreover, characterization of their susceptibility to antimicrobial agents and evaluate their pathogenicity for baby chicks.

MATERIAL AND METHODS

Samples collection:

A total of 450 samples were collected from different hatcheries and farms in Dakahlia governorate. Out of them, 50 samples from the yolk of infertile egg,150 samples from internal organs (heart, liver, and yolk) of dead in shell embryo, 220samples from (heart, liver, and yolk) of diseased and freshly dead chicks (1-7 day) t hat most of them shown omphalitis and 30 samples from hatcheries environment (walls and equipments).

Isolation and Biochemical identification of pseudomonas spp.:

Samples were collected in sterile plastic containers, kept in ice box and transported as soon as possible to the laboratory. Isolation of Pseudomonas isolates was done according to **Shukla and Mishra (2015) .**The samples were aerobically inoculated into nutrient broth for

24h at 37°C. A loop-full of inoculated broth were streaked onto MacConkey agar and Pseudomonas agar base media and incubated aerobically for 24h at 37°C. The non-lactose fermented colonies were randomly selected and sub-cultured onto nutrient agar plates to observe the pigmentation**.** Identification of suspected colonies was carried out through Gram staining**,** biochemical reactions and sugar fermentation.

Preliminary determination of ESBL producing *Pseudomonas*:

Pseudomonas isolates were subjected to antibiotic sensitivity testing by disc diffusion method on Mueller Hinton agar (Bayer et al. 1966)**.** Inhibition zone diameters were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines. CLSI recommends an initial 'screening test' to detect resistance against one or more indicator substrates followed by 'confirmatory test' using one or more of the indicator substrates in combination with a betalactamase inhibitor, looking for synergy effects. All isolates were screened for resistance against four antimicrobial agents: cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 30 µg), ceftriaxone (CTR, 30 µg) and aztreonam (AT, 30 µg). Resistance to at least one of the indicator antibiotics was considered as 'positive' screening test (Drieux et al. 2008; CLSI 2014)**.**

Phenotypic confirmation of ESBL resistance:

Isolates that were found positive in 'screening test' were subjected to 'confirmatory test' by Modified Double Disc Synergy Test (MDDST) by antibiotic discs of amoxicillin /clavulanic acid (30 µg) was placed at the center of the MH agar plate, and antibiotic discs containing cefotaxime (30 µg) and ceftazidime (30 µg) were placed at a distance of 15 mm (centre to centre) from the central disk, amoxycillin/clavulanic acid (30 µg). The plate was incubated at 37°C for 18-24 h. Any distortion or increase in the zone towards the disc of amoxicillin-clavulanate was considered as positive for the ESBL production (Paterson and Bonomo 2005) .

Antimicrobial susceptibility test:

Antibiotic susceptibility tests were performed for ESBL producing *P. aeruginosa* isolates by using the standard disc diffusion method (Kirby–Bauer) on Mueller–Hinton agar plates. Different antimicrobials were used such as Tetracycline (30 µg), chloramphenicol (30 µg), Ampicillin (10 µg), trimethoprim/ sulphamethoxazole (1.25-23.75µg) , Streptomycin (10 µg), colistin sulphate (10 µg) , Ciprofloxacin (5 µg), and Norfloxacin (10 µg). The interpretation of the measured zone was done according to CLSI (2014).

DNA extraction: DNA extraction from samples was 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 $56^{O}C$ for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Oligonucleotide Primer. Primers used were supplied from **Metabion (Germany)** are listed in table (1).

PCR amplification. For PCR, primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmolconcentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler.

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 PCR products were loaded in each gel slot. Generuler 100 bp ladder (Fermentas, Thermo Scientific, Germany) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table (1) Primers sequences, target genes, amplicon sizes and cycling conditions for conventional PCR

Target gene	Primers sequences	Amplified segment (bp)	Amplification (35 cycles)				Final extension	Reference
			Primary denaturation	Secondary denaturation	Annealing	Extension		
toxA	GACAACGCCCTCAGCATCAC-CAGC CGCTGGCCCATTCGCTCCAGCG CT	396 bp	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	(Matar et al. 2002)
oprL	ATG GAA ATG CTG AAA TTC GGC CTT CTT CAG CTC GAC GCG ACG	504 bp	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	(Xu et al. 2004)
blaTEM	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516 bp	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	(Colom et al. 2003)
blaSHV	AGGATTGACTGCCTTTTTG ATTTGCTGATTTCGCTCG	392 bp	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	
blaCTX	ATG TGC AGY ACC AGT AAR GTK ATG GC TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	593 bp	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	(Archambault et al. 2006)
BlaOXA-1	ATATCTCTACTGTTGCATCTCC	619 bp	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	(Colom et al. 2003)

Pathogenicity of isolated *Pseudomonas aeruginosa*:

Chicks: A total of 95, day-old Cobb chicks were obtained from local hatcheries and 5 birds were subjected for bacteriological examination to confirm absence of *P. aeruginosa* and other pathologic bacteria. Chicks were reared on thoroughly cleaned and disinfected semi closed houses and vaccinated using standard protocol for vaccination. Feed and water was given *ad libitum*.

Experimental infection*: Pseudomonas aeruginosa* field strain (beta lactam resistance) was isolated from baby chicks flock suffering from septicemia and high mortality. At 4 day old chick each chick was challenged intramuscularly with 0.2 ml of 24 hr broth culture contained 10^3 viable cell of *P. aeruginosa* /ml (**Joh et al. 2005).**

Medication: Norfloxacin were added as10 mg/kg BW every 12 h for five consecutive day's .The treatment started 48 hour post infection (after appearance of clinical sings).

Experimental design .Ninety, one day old broiler chicks were randomly divided into three equal groups of 30 birds. Each group subdivided into 2 replicates of 15 birds for each.

Group A (GA): Non infected non-treated group considered as control negative

Group B (GB): Infected with *Pseudomonas aeruginosa* (10^3) and non-treated group considered as positive control.

Group C (GC): The birds were infected with *Pseudomonas aeruginosa* (10^3) and treated with Norfloxacin (10mg/kg body weight every 12 h) in the drinking water after appearance of signs for five successive days.

Evaluation parameters*:*

Morbidity and mortality rates: The birds in the challenged group were observed daily after the challenge for any symptoms and deaths all the period of experiment (3 weeks). Dead birds were necropsied immediately after detection of their death and macroscopical lesion were registered. Also, on weekly basis, at 7, 14, 21days post experimental infection 4birds of surviving

chicks from each group were sacrificed and necropsied.

Bacteriological and histopathological investigation: For bacterial re-isolation swabs from heart, liver, lung and intestine from 4 sacrificed birds in each group at the 1st, 2ndand3rd weeks after the challenge. In addition, at 1st and 2nd week after challenge tissue samples from these organs were fixed in 10% neutral buffered formalin, fixed tissues were dehydrated in methanol, cleared in xylene, trimmed, embedded in paraffin sections at 4um and stained with hematoxyline and eosin **(Bancroft and Gamble 2008).**

RESULTS

Prevalence of P. aeruginosa:

Out of 450 examined samples, *P. aeruginosa* was identified in 12. 22% (55/450) of the total examined samples based on morphological and biochemical characteristics. Two strains (4%) were isolated from infertile egg, 18 strains (12%) from dead in shell embryo, 33 strain (15%) from baby chicks and 2 strains (6.7 %) from hatcheries environment (table 2).

Determination of ESBL producing *Pseudomonas*

Twenty five out of 55 *Pseudomonas* isolates screened were found to be resistant to one or more of the third generation cephalosporins (cefotaxime, ceftriaxone and ceftazidime), and monobactam (aztreonam), with an overall incidence of 45.45 % (25/55) beta-lactam resistance and were designated as 'suspect ESBL producers'. Incidence of resistance to ceftazidime , cefotaxime, ceftriaxone, and aztreonam was found to be 45.45 % (25/55), 41.82 (23/55), 29.1% (16/55),and 34.55% (19/55), respectively(table3).

Phenotypic confirmation of ESBL by combination disc method:

ESBLs was confirmed in 8 isolates by modified double disc diffusion test, which includes 1 isolates from infertile egg ,3 from dead in shell embryo and 4 from baby chicks . The overall incidence of ESBL resistance phenotype in *Pseudomonas* species was found to be 32% (8/25) (table 4).

Antimicrobial susceptibility test:

The results of antimicrobial susceptibility testing for the 8 ESBL *P. aeruginosa* showed that all 8 strains were resistance to ampicillin, chloramphenicol and trimethoprim/ sulphamethoxazole, 7 strains resistant to tetracycline and 5 strains resistance for streptomycin. On the other hand, the highest sensitivity was observed against Norfloxacin (7), Ciprofloxacin (6) and colstin sulphate (5). So, they were considered the most influential antibiotics (table 5).

Detection of beta-lactamase (*bla*) genes and virulence genes:

The obtained result revealed that (*opr*L) and (toxA) genes were detected in (8/8) of all *P. aeruginosa (*fig1&2). In addition, *bla*SHV and *bla*TEM genes were detected in (8/8) *and* (7/8) of studied isolates respectively (fig 3&4), whereas none of these isolates harbored, *bla*OXA and *bla*CTX-M genes (fig 5&6).

Results of experimental infection:

Clinical signs:

No clinical signs were observed in the uninfected-untreated group (GA) while in infected groups (B&C) clinical signs appeared within 48 hour post challenge. The clinical symptoms observed were depression, inappetance, ruffled feather, emaciation, and greenish diarrhea .Within 24 hours following initiation of norfloxacin treatment, infected birds treated with norfloxacin (GC) improved clinically after the 3-days treatment period and clinical signs disappeared within 5 to 7 days following treatment.

Post-mortem findings:

Dead and sacrificed birds of infected-untreated group (GB) revealed a picture of septicemia, congestion of internal organs (liver, lung and heart), and inflamed intestine (fig7-A). While infected and treated groups with norfloxacin (GC) showed less severe lesions (fig8-A) .After two weeks following challenge lesions in infected group were less severe (fig7-B) and in infected treated virtually disappear (fig 8-B) **.**

Mortalities: In *Pseudomonas aeruginosa* challenged groups, mortalities started at the first day post-challenge and were (43.33 %)

while in group (C) treated with norfloxacin reduced into (16.66%) (Table7).

Re-isolation of *Pseudomonas aeruginosa* from organs: All chicks in uninfected-untreated group (GA) were negative for re-isolation of the challenge bacteria. *Pseudomonas aeruginosa* could be recovered from tissues in some birds which infected and treated with norfloxacin in the drinking water (GC) with re-isolation rate of (29.41%). While infected-untreated group (GB) had a higher re-isolation rate *of Pseudomonas aeruginosa* were (64%) from different organs. (Table8).

Histopathological finding:

The results of histopathological examination of liver, lung, heart and intestine from dead and sacrificed chicks in infected groups (GB) and treated groups (GC) are shown in fig (9-12).

Lung in infected untreated group (GB) at first week showed severe congested pulmonary blood vessel with edema in its wall (fig.9 -A). Hyperplasia of the lining epithelium of secondary bronchiole with thickening of the mucosal and submucosal layers due to edema and few inflammatory cells infiltration with foci of fibrinous material (fig.9 -B). At 2nd week lung showed blood vessel containing fibrinous material with interstitial edema, mononuclear cells, heterophilis infiltration and congested blood capillaries (fig 9-C). While in infected treated group (GC) at first week showed mild congestion and hemorrhage (fig 9-D). At 2nd week showed marked improvement in histological structure of pulmonary tissue (fig 9-E).

Liver in infected untreated group (GB) at first week showed multiple areas of coagulate necrosis and various degenerative changes in hepatic cells (fig.10 -A). Also, focal replacement by intense erythrocytes and edema with or without bacterial masses (fig.10 -B).At 2nd week, liver showed vacuolar degeneration of hepatocytes and dilated sinusoids (fig.10 -C). While in infected treated group (GC) at first week showed mild degenerative changes or apparently normal hepatic cells without bacterial emboli. Mild vacuolation of few hepatic cells (fig.10 -D). At 2nd week, liver showed marked improvement in histological structure of the liver (fig.10 -E).

Heart in infected untreated group (GB) at first week showed intense inter and intra muscular edema and inflammatory cells aggregations among degenerated myocardial muscles (fig.11 -A). Fibrinopurulent pericarditis with marked edema, numerous heterophils, deposition of pale eosinophilic fibrin threads and severe vacuolation in cardiac muscle fibers (fig.11-B). At 2nd week, heart showed focal proliferation of connective fibrous tissues with diffuse interstitial infiltration with mononuclear cells, some heterophiles and edema (fig.11 -C). While in infected treated group (GC) at first week showed mild swelling of some muscle fibers with little interstitial edema among degenerated muscle fibers. Some blood vessels and capillaries were hyperemic with perivascular edema (fig.11 -D) and mild myocardial degeneration (fig.11 -E). At 2nd week showed apparently normal appearance of the cardiac muscle (fig.11 -F).

Intestine in infected untreated group (GB) at first week showed dilation of some crypts, denuded of epithelial cells and lamina propria mildly expanded with edema, mononuclear cells and few heterophils infiltration were seen (fig.12- A). Focal destruction of superficial villus tips with intense desquamation of villous enterocytes beside epithelial sheets and a few inflammatory cells in intestinal lumen (fig.12 -B). Sub mucosa and mucosa showed a few inflammatory cells infiltration with mononuclear cells, heterophils, and edema with destructed crypt epithelium .At 2nd week, atrophy, fusion or sloughing of villi (fig. 12-C). On the other hand, in infected treated group (GC) at first week showed mild degeneration of epithelium and crypts and few granulocytes, heterophils in submucosa (fig.12 -D). Also, mild inflammatory cell infiltration, edema in mucosa and sub mucosa and mild inflammatory exudate in the intestinal lumen (fig. 12-E). At 2nd week showed apparently normal appearance of the intestine (fig.12 -F).

Table (2) The incidence of *Pseudomonas* isolated from examined samples:

Source of sample	Type of sample	No. of examined samples	No. of positive sample	Frequency%
Infertile egg	Yolk	50	2	4
Dead in shell embryo	Yolk, Liver and heart blood	150	18	12
Young chicks (1-7 day)	Yolk, Liver and heart blood	220	33	15
Hatchery	Walls and equipment	30	2	6.7
Total		450	55	12.22

Table (3) Preliminary screening for ESBL pseudomonas

Antibiotic used	Total No. of isolates	Sensitive No.(%)	Resistance No.(%)
Ceftazidime	55	30(54.55)	25(45.45)
cefotaxime	55	32 (58.49)	23 (41.82)
Ceftriaxone	55	39(70.91)	16 (29.1)
Aztreonam	55	36 (65.45)	19 (34.55)

Table (4) Phenotypic confirming for ESBL producing pseudomonas

No.of screened isolate	**ESBL-ve**	**ESBL +ve**
25	**17(68%)**	**8(32%)**

Table (5) Results of antimicrobial susceptibility test for 8 ESBL producing pseudomonas

Antibiotic	No. resistance	No. sensitive
chloramphenicol	8	0
norfloxacin	1	7
trimethoprim/ sulphamethoxazole	8	0
Colistin sulphate	3	5
tetracycline	7	1
streptomycin	5	3
ampicillin	8	0
ciprofloxacin	2	6

Table (6) virulence and beta lactam resistance genes in 8 ESBL producing pseudomonas

	Virulence genes		Antibiotic resistance genes			
	oprL	toxA	*bla***SHV**	*bla***TEM**	*bla***CTX-M**	*bla***OXA**
1	+	+	+	+	-	-
2	+	+	+	+	-	-
3	+	+	+	+	-	-
4	+	+	+	+	-	-
5	+	+	+	+	-	-
6	+	+	+	+	-	-
7	+	+	+	+	-	-
8	+	+	+	-	-	-

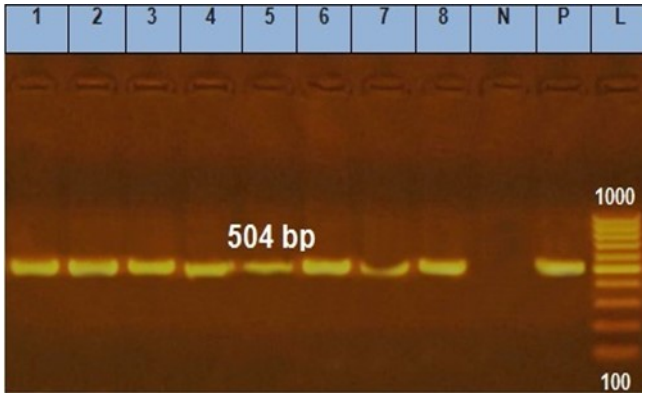

Fig. (1): Amplification profile of (*oprL* gene) of *P.aeruginosa* isolates at 504bp.L:100 bp represents ladder, lanes 1-8 represent positive isolates, P= positive control, N= negative control

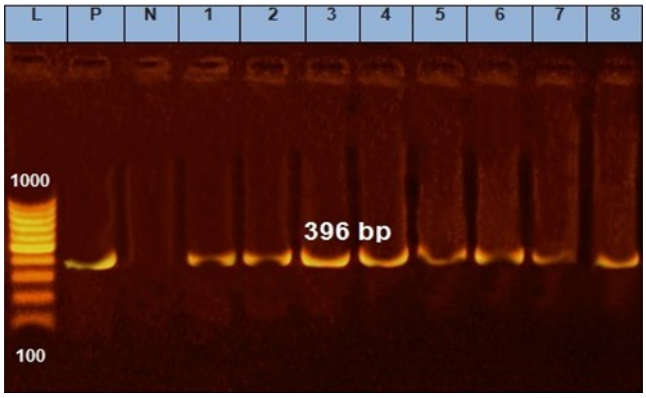

Fig. (2): Amplification profile of (*toxA* gene) gene of *P.aeruginosa* isolates at 396bp.L:100 bp represents ladder, lanes 1-8 represent positive isolates, P= positive control, N= negative control

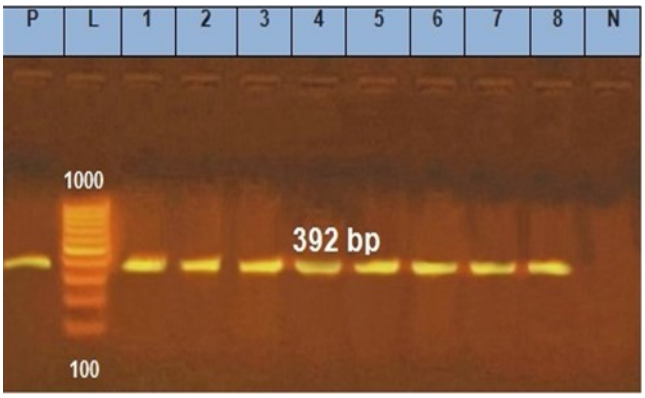

Fig. (3): Amplification profile of (*SHV* gene) of *P.aeruginosa* isolates at 392bp.L:100 bp represents ladder, lanes 1-8 represent positive isolates, P= positive control, N= negative control

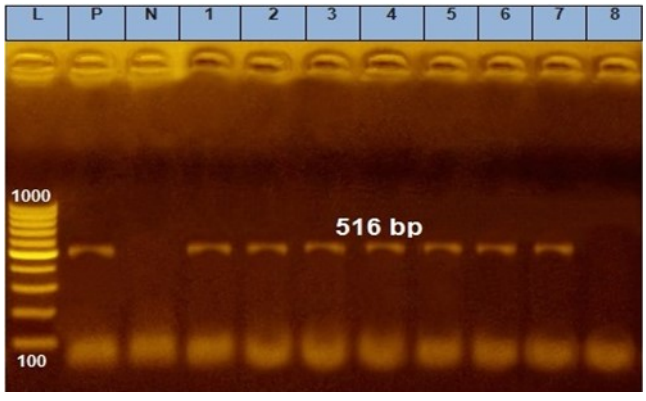

Fig. (4): Amplification profile of (*TEM* gene) of *P.aeruginosa* isolates at 516bp.L:100 bp represents ladder, lanes 1-7 represent positive isolates, P= positive control, N= negative control

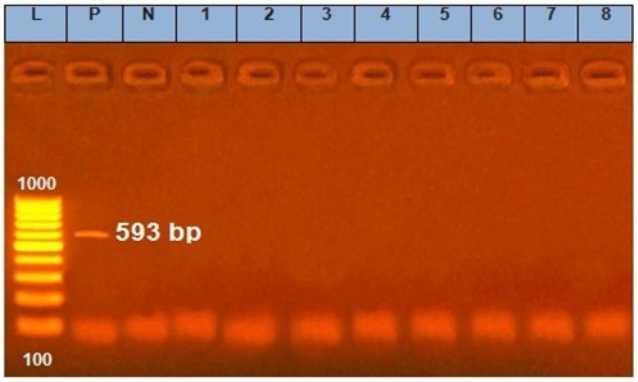

Fig. (5): Amplification profile of (*CTX* gene) of *P.aeruginosa* isolates at 516bp.L:100 bp represents ladder, lanes 1-8 represent negative isolates, P= positive control, N= negative control

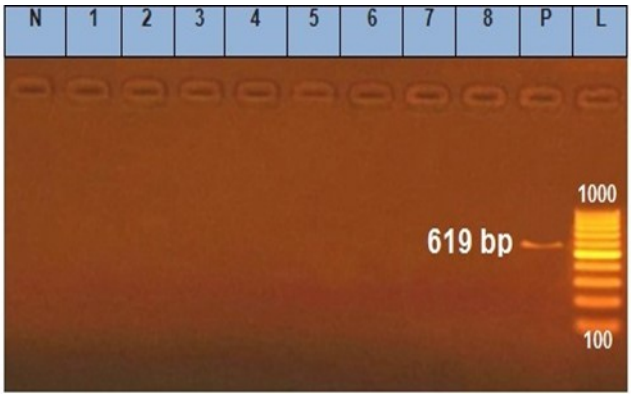

Fig. (6): Amplification profile of (*OXA* gene) of *P.aeruginosa* isolates at 619bp.L:100 bp represents ladder, lanes 1-8 represent negative isolates, P= positive control, N= negative control

Table (7) The Mortality rate in experimentally infected chicks

Experimental group	No .of examined chicks	Mortality rate number Weeks PC*			Mortality rate
		1	2	3	
GA	30	0	0	0	0%
GB	30	6	4	3	43.33%
BC	30	4	1	0	16.66%

*Post challenge

Table (8) Reisolation rate of pseudomonas experimentally from infected chicks

Experimental group	**Weeks PC *(positive / total examined birds**)**			**Total positive chicks**	
	1	**2**	**3**	**No**	**%**
GA	**0/4**	**0/4**	**0/4**	**0**	**0**
GB	**8/10**	**5/8**	**3/7**	**16**	**64**
GC	**3/8**	**2/5**	**0/4**	**6**	**29.41**

*post challenge
**Dead and scarified birds

Fig (7) post mortem lesions of chicks experimentally infected with pseudomona showing congested heart , liver , lung and inflamed intestine

Fig(8) post mortem lesions of chicks experimentally infected with pseudomona and treated showing mild congested heart , liver , lung and inflamed intestine

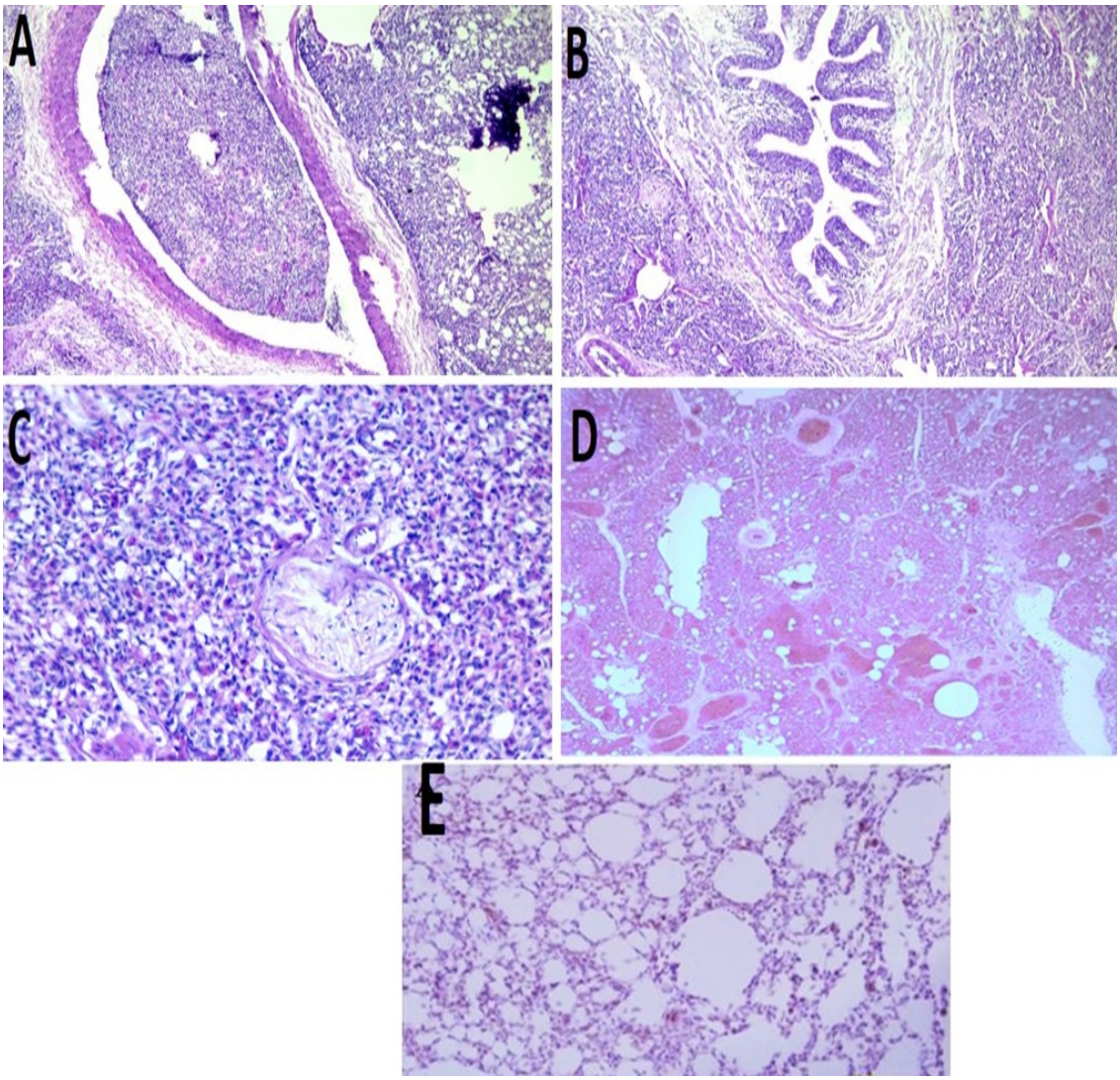

Fig (9) Photomicrograph of sections from lung stained with H&E.
(A) Lung of infected chick with pseudomonas at first week showing sever congested pulmonary blood vessel with edema in its wall x100.
(B)Lung of infected chick with pseudomonas at first week showing showing hyperplasia of the lining epithelium of secondary bronchiole with thickening of the mucosal and submucosal layers due to edema and few inflammatory cells infiltration with foci of fibrinous material x100.
(C) Lung of infected chick at 2nd week showing blood vessel containing fibrinous material with interstitial edema, mononuclear cells, heterophilis infiltration and congested blood capillaries x400.
D) Lung of treated chick at first week showing mild congestion and hemorrhage X200.
(E) Lung of treated chick at 2nd week showing marked improvement in histological structure of pulmonary tissue x 100.

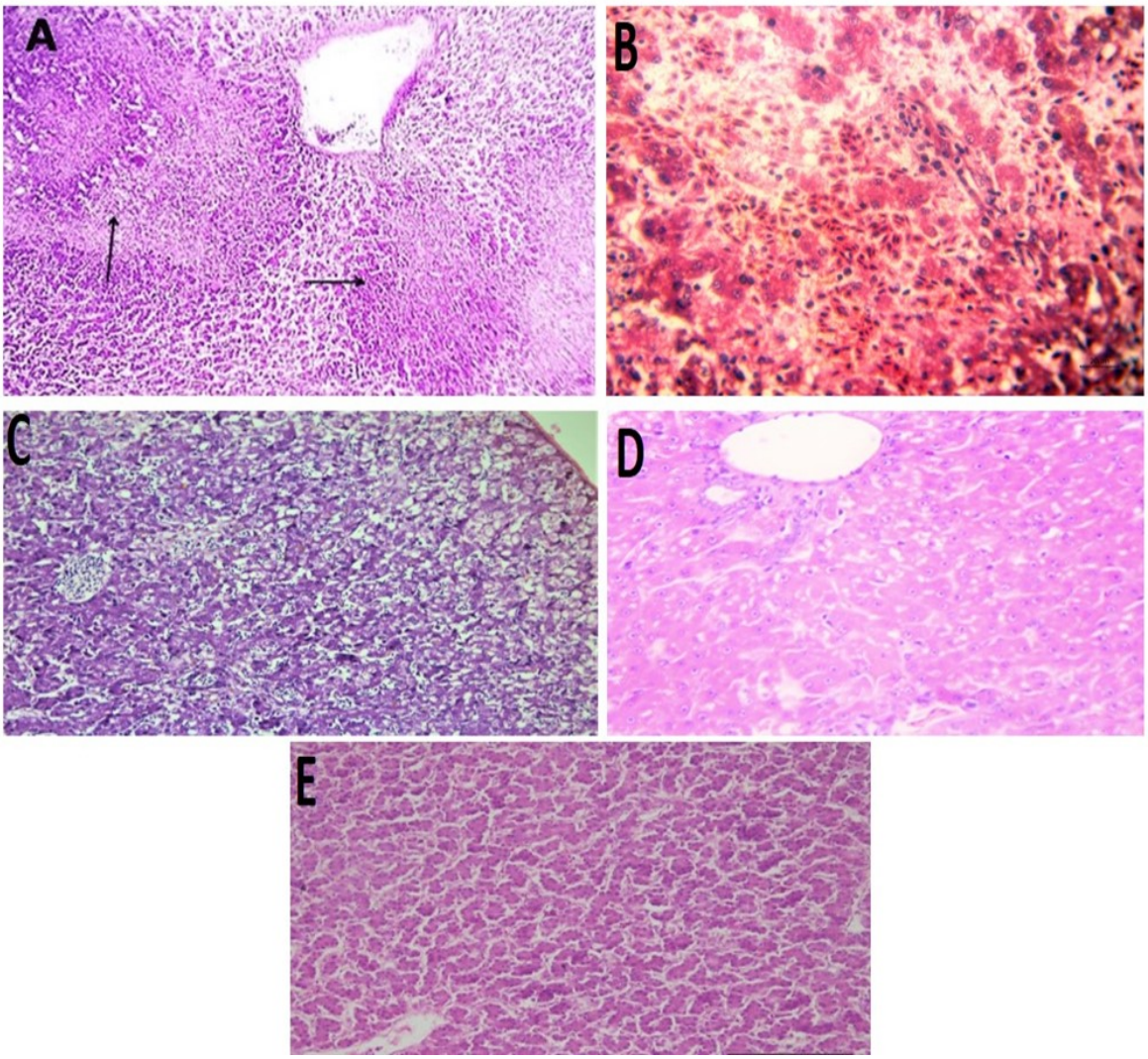

Fig (10) Photomicrograph of sections from liver stained with H&E.
(A) Liver of infected chick with pseudomonas at first week showing multifocal areas of coagulative necrosis (arrows) X 100.
(B) Liver of infected chick with pseudomonas at first week showing focal necrotic area containing numerous erythrocytes, edema and a few inflammatory cells X100.
(C) Liver of infected chick with pseudomonas at 2nd week showing vacuolar degeneration of hepatocytes and dilated sinusoids X 200.
D) Liver of treated chick at first week showing mild vacuolation of a few hepatic cells X400.
(E) Liver of treated chick at 2nd week showing marked improvement histological structure of hepatic tissue X100.

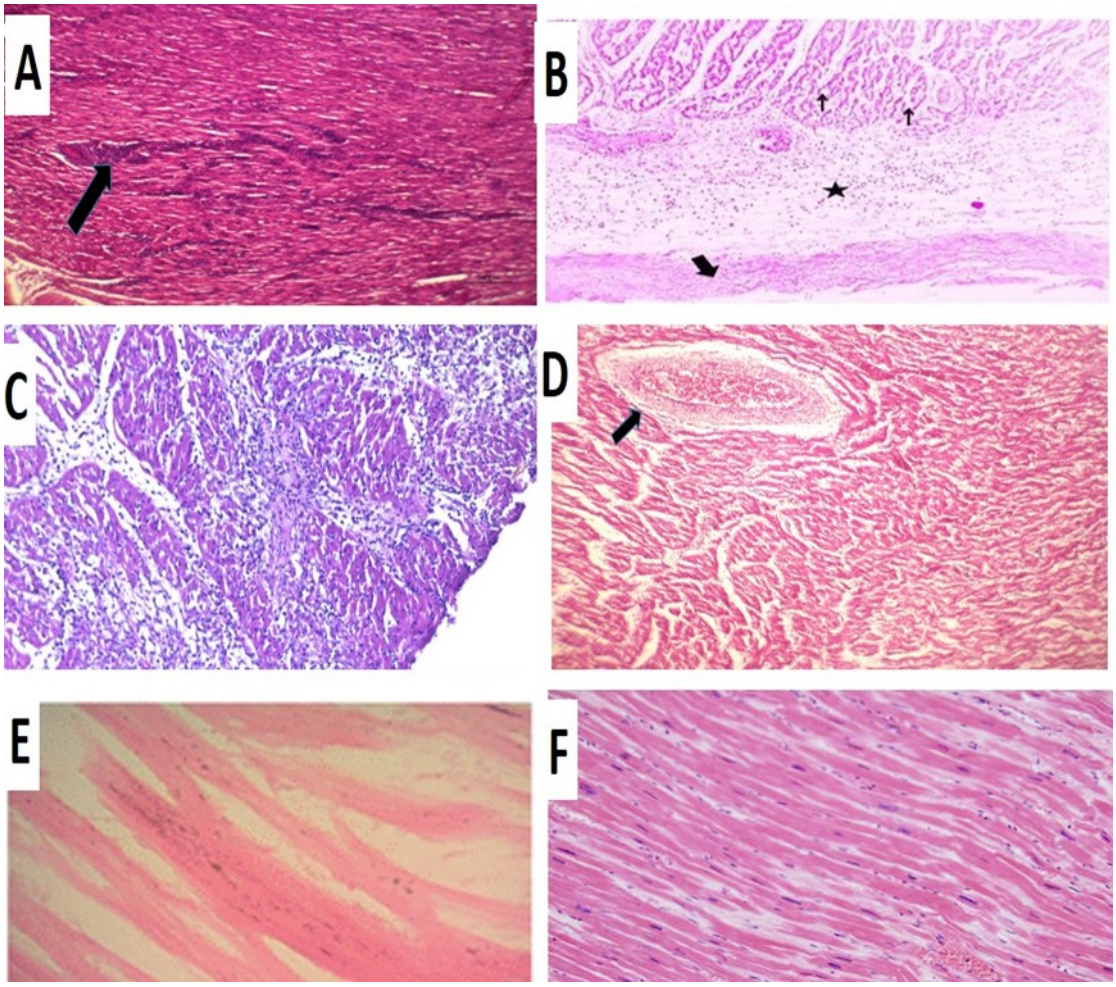

Fig (11) Photomicrograph of sections from heart stained with H&E.
(A) Heart of infected chick with pseudomonas at first week showing inflammatory cells aggregation (thick arrow) among degenerated muscle fibers. X: 100
(B) Heart of infected chicken with pseudomonas at first week showing fibrinopurulent pericarditis characterized by marked edema (asterisk), numerous heterophils and deposition of pale eosinophilic fibrin threads (thick arrow), besides congested capillaries and severe vacuolation in cardiac muscle fibers (thin arrows). X: 100
(C) Heart of infected chicken with pseudomonas at 2nd week showing focal proliferation of connective fibrous tissues with diffuse interstitial infiltration with mononuclear cells, some neutrophils and edemaX: 200.
(D) Heart of treated chick at first week showing little interstial and perivascular edema (thick arrow). X: 200
(E) Heart of treated chick at first week showing mild cardiac myo-degeneration. H&E, X: 200.
(F) Heart of treated chick at 2nd week showing apparently normal cardiac muscle. X: 200

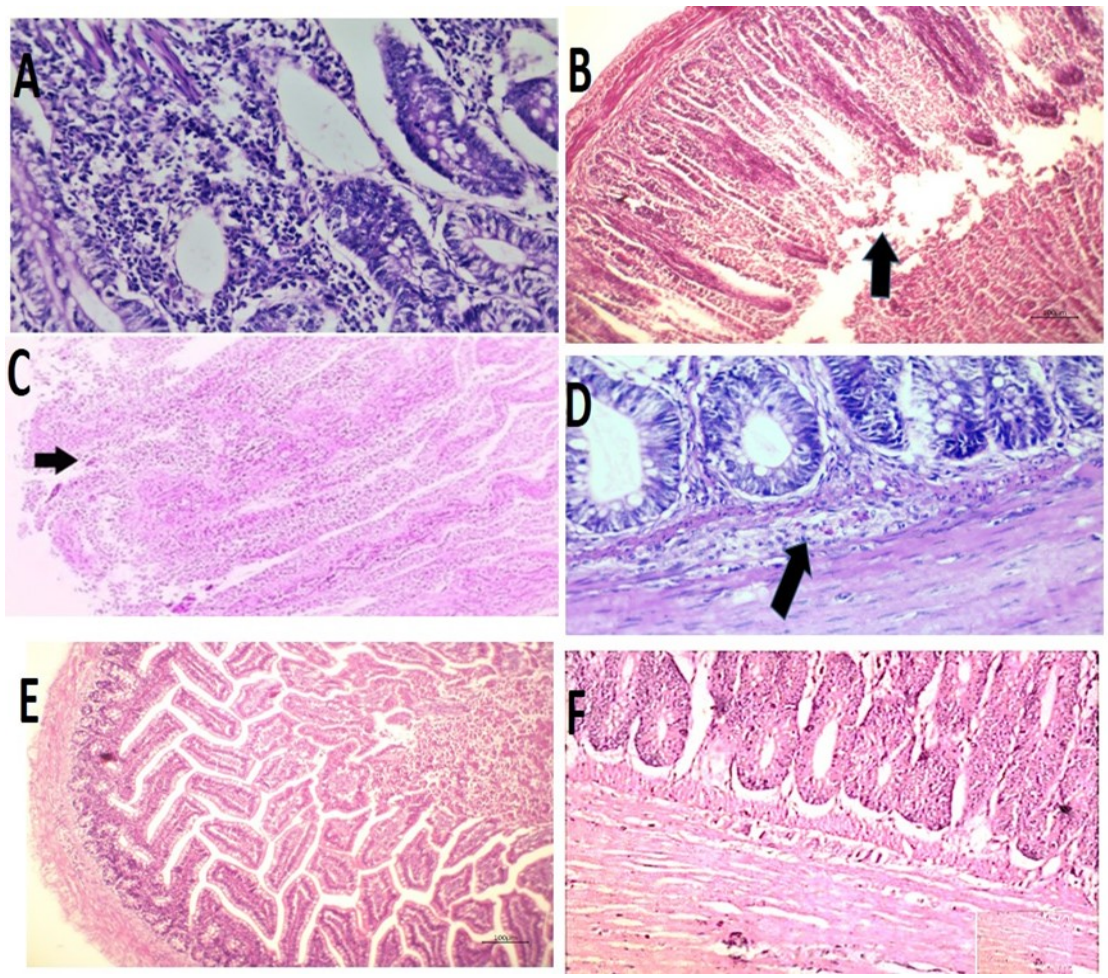

Fig (12): Photomicrograph of sections from intestine stained with H&E.
(A) Intestine of infected chicks with pseudomonas at first week showing some crypts are dilated and denuded of epithelial cells and lamina propria mildly expanded with mononuclear cells and few heterophils infiltrate. X400.
(B) Intestine of infected chicks with pseudomonas at first week showing focal destruction of villus tips and desquamation of enterocytes (thick arrow). X: 200
(C) Intestine of infected chicks with pseudomonas at 2nd week showing fusion of intestinal villi (thick arrow). X: 100
(D) Intestine of treated chick at first week showing mild degeneration of epithelium and crypts, few granulocytes, heterophils in submucosa (thick arrow) X400.
(E) Intestine of treated chick at first week showing goblet cell metaplasia in villus enterocytes and crypts beside mild exudate within the lumen. X 200.
(F) Intestine of treated chick at 2nd week showing apparently normal intestinal tissue. X200

DISSCUSION

In the present study, a total of 55 *Pseudomonas* isolates (12.2%) were recovered from 450 collected samples collected from infertile egg, dead in shell embryos, baby chicks with omphalitis and hatcheries environment. *P. aeruginosa* was isolated from young chickens with high mortalities and late dead in shell embryos **(Kebede 2010).**

The isolation rate of *P. aeruginosa* was (12%) from dead in shell embryo. Nearly similar results were recorded by **Rezk (2010)** who isolated P. aeruginosa from dead in shell embryo with total recovery rate (15.9%). Higher recovery rates were recorded in a previous study carried out by **Mohamed (2004), Elsayed et al. (2016); Shahat et al. (2019)** who isolated *P.aeruginosa* from dead in shell embryo with incidence rates of 52%, 21%and 19% respectively. On the other hand, lower incidence of *P. aeruginosa* 1.2% was mentioned by **Amer et al.(2017) .**

In the present study, the incidence of *P. aeruginosa* in baby chicks was 15%. Similarly, **Mohamed (2004)** and **Farghal et al. (2017)** isolated *P.aeruginosa* from one-day old chicks with a percentage of 17.6% and13% respectively. Higher result were obtained by **Shahat et al. (2019)** who isolated *P.aeruginosa* from young chicks with incidence rates of 42%. However, lower isolation rates 2.5% and 2.83% were reported by **Abd El-Tawab et al. (2014); Amer et al. (2017).** The isolation rate from hatcheries environment (6.7%) were similar to findings of **Eraky et al. (2020)** who isolated *P. aeruginosa* with an incidence of 8% from hatcheries .

Beta-lactams are a significant antibiotic group used to control and prevent bacterial infections in humans and animals ***(*Hawkey and Jones 2009*).*** Their uses have always been followed by the development of resistance, most commonly caused by beta-lactamases **(Bush and Bradford 2016)** . ESBLs vary in their hydrolysis of cephalosporins. Therefore, resistance to at least one of them should be considered as positive in the screening test **(Drieux et al. 2008; CLSI 2014).** Twenty five out of 55 screened *Pseudomonas* isolates were found to be resistant to one or more of the screening agents, with an overall incidence of 45.45% (25/55) and were designated as (suspect ESBL producers). Incidence of resistance to ceftazidime , cefotaxime, ceftriaxone, and aztreonam was found to be 45.4 5% (25/55), 41.82 (23/55), 29.1% (16/55), and34.55% (19/55), respectively. In a study on antimicrobial sensitivity of 39 *P. aeruginosa* strains isolated from broilers infections in Egypt , resistance to ceftriaxone, cefotaxime and aztreonam were 74.5%,70.2% and 44.7% respectively .On the other hand, all isolates were sensitive for ceftazidime **(Ismail 2016).**

Since ESBLs are inhibited by clavulanic acid/sulbactam, tests that use these inhibitors to reverse ESBL resistance were used commonly for phenotypic confirmation of ESBL production **(Drieux et al. 2008, CLSI 2014).** Of the 25 'suspect ESBL producers', ESBL production was confirmed in 8 isolates by double disk diffusion test. These results were conceded with **Ismail (2016)** who reported that ESBLs was confirmed in 6/47 out of the investigated *P. aeruginosa* isolated from chickens. We were unable to confirm ESBL production in the remaining 17 isolates. These results might be due to concurrent production of other resistance mechanism like ACBLs and metallo beta-lactamases (**Al-Ouqaili 2019).**

The obtained result revealed that *oprL* gene was amplified in all the eight studied strains .These results drew near the other previous investigations Hassan **(2013; Shahat et al. (2019).** It considers 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) .**There is a strong relationship between the detection of *oprL* and phenotypic antibiotic resistance due to its implication in efflux transport systems affecting cell permeability **(Qin et al. 2003, Lavenir et al. 2007).** Also, Exotoxin A (*toxA*) was detected in all studied strains. This result came in accordance with those recorded by **Elsayed et al. (2016), Shahat et al. (2019), Eraky et al. (2020).** Exotoxin A (*toxA*), is the most toxic

virulence factor detected in this organism (**Dong et al. 2015).** It inhibits protein biosynthesis, it has a necrotizing activity on tissues causing cell death and contributes to the colonization process **(Michalska and Wolf 2015).**

Resistance to -lactams in Gram-negative bacteria is primarily mediated by -lactamases. Many types of -lactamases have been described, but TEM-, SHV, OXA, CMY, and CTX-M-type -lactamases are the most common in Gram-negative bacteria (**Bradford 2001).** Regarding to the occurrence of Beta-lactamase genes in *P. aeruginosa* isolates, the obtained result revealed that- *bla*SHV and *bla*TEM genes were detected in (8/8) *and* (7/8) of studied isolates respectively. whereas none of the isolates harbored, *bla*OXA and *bla*CTX-M genes. Similarly, previous studies detected TEM-and SHV in *P. aeruginosa* **(Elhariri et al. 2017 Abd El-Tawab et al. 2018).**

ESBL *P. aeruginosa* was found to be resistant to ampicillin, chloramphenicol and trimethoprim/ sulphamethoxazole, tetracycline and streptomycin. While, it was found to be sensitive to Norfloxacin, Ciprofloxacin and colistin sulphate. These results came in accordance with **Abd El-Tawab et al. (2014), Farghal et al. (2017), Shahat et al. (2019)**. Our results detected high multi resistance among isolates which may be due to the production of hydrolytic enzymes and the acquisition of resistance mechanisms by *P. aeruginosa* strains (**Rostamzadeh et al. 2016).**

Our experimental study was conducted to confirm pathogenicity of isolated virulent field strain of ESBL *producing P. aeruginosa* and to assess the efficacy of norfloxacin in controlling infection. Chicks in experimentally infected non treated group showed sleepy appearance, closed eyes, diarrhea. While the gross lesions revealed congestion of all internal organs, petechial hemorrhages on liver and spleen, pericarditis, and enteritis. Our results were parallel to the results of **(Mohamed (2004) Eraky et al. (2020).** Mortality rate was 43.3% in infected untreated group .Higher mortality rate (70%) were recorded by **Eraky et al. (2020).**

On the other hand, groups treated with norfloxacin in the drinking water at dose level of 10mg/Kg live body weight led to a great reduction in clinical signs, PM lesions, the mortality rates (20%), decrease in the number of pseudomonas positive birds and lowered the reisolation rate from different organs**.** Our results is similar to that reported by **Badr et al.(2006); Fatma (2013).** Norfloxacin therapy has high therapeutic concentration in serum and tissues after the oral administration (Goossens et al. 1985)and accompanied with a prolonged excretion period, so the duration of the antibacterial activity was effectively prolongs (**Avoire et al. 1990 Pecquet et al. 1990**) and this explains the significant reduction in the reisolation rate among norfloxacin treated group.

The histopathological examination of lung in infected groups in first week showed congested pulmonary blood vessel with edema and hyperplasia of the lining epithelium of secondary bronchiole similar finding reported by **Bakheet and Torra (2020)** who reported that lung chick showed interstitial pneumonia with multiple thrombi in pulmonary blood vessels and hyperplasia in bronchial epithelium. At 2nd week perivascular fibrosis with leucocytic cells infiltration and congestion of pulmonary blood vessels, these finding were supported by **Shukla Satish (2016) Mowafy and El Oksh (2017).**The liver in infected group showed multiple areas of coagulative necrosis, focal replacement by erythrocytes and edema in 1st week .while in 2nd week, showed vacuolar degeneration of hepatocytes and dilated sinusoids . **Joh et al. (2005)** reported that the prominent microscopic lesions in liver of the broilers infected with P. aeruginosa were multiple foci of coagulative necrosis with heterophilic infiltration. Also, **Eraky et al. (2020)** stated that the liver of chicks experimentally infected with pseudomonas had dilated hepatic sinusoids, vacuolar degeneration of many hepatocytes, ,mononuclear inflammatory cell infiltration and heterophil. **van Delden (2004)** reported that elastin protein of connective tissues and its degradation was carried out by Las B enzyme which is an important enzyme of elastolytic activity that produced by P. aeruginosa. That explains the destruction in alveolar wall and blood vessel wall which led to

hemorrhagic pneumonia, and destruction of hepatic sinusoids which led to hemorrhage (wide patches of accumulated red blood cells) in hepatic tissue.

The histopathological finding in the heart after 1st week from infection in infected group were edema, degenerated myocardial muscles, fibrinopurulent pericarditis. At 2nd week the cardiac muscles showed focal proliferation of connective fibrous tissues with diffuse interstitial infiltration with mononuclear cells, some heterophiles and edema. These findings were in accordance with a previous reported by **Joh et al. (2005)** who found the serosal surface was covered with fibrino-purulent exudates, diffuse fibrinous exudates with bacterial colonization in the pericardium. Most histopathological changes of heart and liver were inflammatory in nature that come in agree with **Timurkaan et al. (2008)** who found inflammatory reaction through heart especially in young birds. Inflammatory cells infiltration mainly lymphocytes and heterophils in the different organs might be due to the antigenic stimulation of pseudomonas (**Coles 1986 Hafez et al. 1987)**

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The histopathological finding in the intestine in infected group showed destruction of superficial villus, edema with inflammatory cells infiltration. Similarly **Badr et al. (2006**) reported that intestine in chicks infected with pseudomonas showed epithelial hyperplasia of mucosa with cystic formation of goblet cells .

The histopathological lesions in lung, heart, liver and intestine in infected treated group were less severe as compared to infected groups. fluoroquinolone therapy has the advantages of low minimal inhibitory concentration (MIC) and high therapeutic concentration in serum, tissues and feces after the oral administration (**Goossens et al. 1985)** accompanied with a prolonged excretion period which may persist for several days after therapy cessation, effectively prolongs the duration of the antibacterial activity **(Pecquet et al. 1990).** These results was matched with **Badr et al. (2006)** who reported that treatment of quail experimentally infected with pseudomonas with fluoroquinolone (Ciprofloxacin, Enrofloxacin, and Ofloxacin) reduced severity of lesions found that Liver of ciprofloxacin treated quail showing mild degenerative changes.

Conclusion

This study established the presence of ESBL-*P. aeruginosa* in infertile egg ,dead in shell embryo and baby chicks . These alarms the public health impact as one day old chicks may serve as vector for transmission of ESBL-*P. aeruginosa* to human. In addition, *P. aeruginosa* isolates showed marked resistance to several antibiotics that commonly used in the poultry production in Egypt. Hence, more attention should be paid to risk of bacterial contamination and the possible related effect on hatchability and health of produced one day old chicks. Subsequently, it will reduce the spread of antibiotic resistance to drugs of clinical interest.

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