Animal Health Research Journal Vol. 6, No 4, December 2018 pp. 97-113

ISSN: 2356-7767

Studies on *Pseudomonas aeruginosa* infection in hatcheries and chicken

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Received in 15/11/2018
Accepted in 18/12/2018

Abstract

The aim of this work was to spot light on the presence of *Pseudomonas aeruginosa* (*P. aeruginosa*) strains in hatcheries and dead in shell embryos, identify the presence of some virulent genes in these bacteria, demonstrate the susceptibility of the organism to different antimicrobials as well as detect the pathogenicity in newly hatched chicks. A total of 406 samples representing 200 and 206 swabs from hatcheries environment and yolk sacs of late dead in shell embryos were collected from 8 hatcheries in Damietta governorate, Egypt. *P. aeruginosa* was isolated and identified morphologically, microscopically, biochemically and serologically. Some virulent genes (*tox*A, *psI*A and *fli*C) of *P. aeruginosa* were detected using polymerase chain reaction (PCR). The antimicrobial susceptibility of *P. aeruginosa* was tested *in vitro*. Day and 11 days old broiler chicks were challenged with *P. aeruginosa* to determine the pathogenicity of the isolated strains. The results showed that *P. aeruginosa* was recovered from 16 (8%) out of 200 hatcheries and from 17 (8.25%) out of 206 chicken embryos samples. Isolated strains of *P. aeruginosa* showed presence of *tox*A, *psI*A and *fli*C virulent genes. *P. aeruginosa* strains were resistant (100%) to amoxycillin/clavulanic acid, doxycycline and erythromycin. The pathogenicity test of a day and 11 days old chicks revealed that *P. aeruginosa* was highly pathogenic induced mortality rates of 72 and 40%, respectively. Septicaemia with congestion and haemorrhages of internal organs, unabsorbed yolk sacs, pneumonia, greenish exudates in the abdominal cavity and peticheal haemorrhage on liver were the predominant lesions. Histopathological changes supported the previous lesions. In conclusion, *P. aeruginosa* is a great importance pathogen of newly hatched chicks based on presence of virulent genes as well as *in vivo* pathogenicity study.

Keywords: *Antimicrobials, chickens, virulence genes, P. aeruginosa, pathogenicity, PCR.*

Introduction

Pseudomonas species are ubiquitous organisms and usually present in soil, humid areas and water (**Barnes, 2003**). Infection of chickens especially young's with *Pseudomonas aeruginosa* (*P. aeruginosa*) induces septicaemia, mortalities, respiratory manifestations and diarrhea or localized infections in different organs (**Walker *et al.,* 2002**). High rates of late embryonic deaths and loses of newly hatched chicks were recorded after infection with *P. aeruginosa* (**Fekadu, 2010**). Conventional methods for isolation and identification of *P. aeruginosa* faces some difficulties in accurate detection of the bacteria due to misidentification with closely related Gram-negative bacilli (**Al-Ahmadi and Roodsari, 2016**). So, the use of molecular techniques as polymerase chain reaction (PCR) improves accurate and rapid identification of *P. aeruginosa* (**Anuj *et al.,* 2009**). *P. aeruginosa* possess different virulent factors inducing toxicity and pathogenicity (**Fadhil *et al.,* 2016**). Potential virulence factors secreted by *P. aeruginosa* that are im-

portant in its pathogenicity include exotoxin A (*tox*A), which 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**). Other genes like (*pslA*) is responsible for biofilm formation (**Ghadaksaz *et al.,* 2015**). Flagellar gene (*fliC*) plays important roles in tissue penetration (**Ertugrul *et al.,* 2018**).Treatment of *P. aeruginosa* is difficult as it resists antibiotics due to presence of intrinsic and acquired antibiotic resistance mechanisms (**Lister *et al.,* 2009**). The opportunistic pathogenic nature of *P. aeruginosa*, biofilm formation, induction of chronic infections are factors responsible for high level of multiple drug resistance (**Wei and Ma, 2013 and Rasamiravaka *et al.,* 2015**).

Therefore, this study was designed to identify *P. aeruginosa* strains from hatcheries and dead in shell embryos, detection of some virulent genes (*tox*A*, psI*A and *fli*C), test the susceptibility of the isolated strains to various antimicrobials *in vitro* and finally examine their pathogenicity in newly hatched chicks.

Materials and Methods

Samples collection

Samples were taken from 8 hatcheries in Damietta governorate, Egypt during the period from March to August, 2018. A total of 406 samples representing 200 swabs from hatcheries environment and 206 swabs from yolk sacs of late dead in shell embryos (Table 1). Samples were collected in sterile plastic containers, kept in ice box and transported as soon as possible to the laboratory.

Isolation and identification of Pseudomonas isolates

Isolation of Pseudomonas isolates was done according to **Quinn *et al.* (2002)**. 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. The purified colonies were examined for their colonial morphology, pigment production and odour. Identification was carried out through Gram staining and biochemical reactions including; oxidase, catalase, urea, citrate utilization, gelatin hydrolysis, indole, methyl red, Voges Proskauer, and sugar fermentation of glucose, mannose and xylose, sucrose, lactose and maltose.

Serological identification of *P. aeruginosa* isolates

Serological identification was done in Animal Health Research Institute, Dokki, Egypt using antisera from Denka Seiken Co. Ltd, Tokyo, Japan. It was carried out for detection of somatic antigen "O" using *P. aeruginosa*antisera according to the method of **Homma (1980)**. Agglutination kit contained polyvalent (I, II and III) and monovalent (A, B, C, D, E, F, G, H, I, J, K, L, M and N) antisera. Agglutination was described as positive if it caused a positive slide agglutination reaction.

Molecular detection of *P. aeruginosa* virulent genes

Presence of specific virulent genes of *P. aeruginosa* (*tox*A*, psI*A and *fli*C) was examined using PCR. DNA extraction from *P. aeruginosa* isolates 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°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. Oligonucleotide primers were supplied from Metabion (Germany) are listed in Table (2).For PCR amplification, primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp

Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler. The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the products was loaded in each gel slot. A gel pilot 100 bp DNA Ladder (Qiagen, Germany, GmbH) and generuler 100 bp ladder (Fermentas, Germany) were 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.

In vitro*-antibiotic susceptibility test of *P. aeruginosa

The used chemotherapeutic agents discs (Oxoid) and the inhibition zones (susceptible, intermediate susceptibility and resistant) are illustrated in Table (3). *In vitro* antibiotic susceptibility test of *P. aeruginosa* strains was carried out using disc diffusion method (**Clinical Laboratory and Standards Institute, 2015**). Culture with 0.5 McFarland density was distributed onto the Müller-Hinton agar. The antibiotic discs were distributed on the agar surfaces and the zones of inhibition were measured after 24 hr.

Table (1). The number of the examined samples distributed in Damietta governorate, Egypt

Locality	Number of samples	
	Hatcheries	Chicken embryos
Om El-Reda	25	28
Farskour	25	29
Zarka	25	22
Kafer-Saad	50	58
Cinania	25	15
Kafer El-Batekh	50	54
Total	200	206

Table (2). Virulence target genes,oligonucleotide primers, amplicon sizes and cycling conditions for *P. aeruginosa*

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
***tox*A**	GACAACGCCCTCAGCATCACCAGC CGCTGGCCCATTCGCTCCAGCGCT	396	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 7 min.	**Matar *et al.*, (2002)**
***psl*A**	TCCCTACCTCAGCAGCAAGC TGTTGTAGCCGTAGCGTTTCTG	656	94˚C 5 min.	94˚C 30 sec.	60˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Ghadaksaz *et al.*, (2015)**
***fli*C**	TGAACGTGGCTACCAAGAACG TCTGCAGTTGCTTCACTTCGC	180	94˚C 5 min.	94˚C 30 sec.	56.2˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	

Table (3). The interpretation of *P. aeruginosa* antibiogram pattern

Antibiotic Disc (Code)	Disc content/ µg	Interpretation (Diameter of the zone/ mm)		
		Susceptible ≥	Intermediate Susceptibility	Resistant ≤
Amoxycillin/Clavulanic acid (AMC)	20/10	18	14-17	13
Colistinsulphate (CT)	10	11	9-10	8
Doxycycline (Do)	30	16	13-15	12
Erythromycin (E)	15	21	16-20	15
Nalidixic acid (NA)	30	19	14-18	13
Streptomycin (S)	10	15	12-14	11

Pathogenicity test of *P. aeruginosa* in chickens

The experiment was done according to the National Regulations on Animal Welfare and Institutional Animal Ethical Committee (IAEC). A total of 80, day-old Cobb chicks was obtained from local hatcheries and 5 birds were subjected for bacteriological examination to confirm absence of *P. aeruginosa*. 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*. Chicks were divided into 3 equal groups, each containing 25 birds. Chicks of group (1) were challenged at day old, while chicks of group (2) were challenged at 11 days old. Chicks in group (3) were kept as blank control negative non challenged group. Each chick in groups (1) and (2) 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 and Walker *et al.*, 2002**), while those in group (3) were inoculated with sterile phosphate buffer saline. All chickens were kept under observation for 10 days to detect clinical signs, mortalities and post-mortem lesions. Samples were collected from dead birds for re-isolation of *P. aeruginosa*. At the end of the study, samples including; liver, spleen and heart were collected from sacrificed birds for re-isolation of *P. aeruginosa*.

Histopathological examination:

Tissue specimens from lung, heart and liver were collected from each group at the end of experiment, fixed in 10% neutral buffered formaline, routinely processed by standard paraffin embedding technique, sectioned at 4 micron and finally stained with Hematoxylin and Eosin (H&E) (**Bancroft and Gamble, 2002**).

Results

Based on cultural, morphological and biochemical characteristics of the isolates, a total of (16,8%) and (17, 8.25%) *P. aeruginosa* isolates were recovered from 200 hatchery and 206 chicken embryos samples, respectively (Table 4).

Morphologically, *P. aeruginosa* is aerobic β haemolytic colonies on blood agar, on MacConkey agar; it has pale colonies of non-lactose fermenter. The plates containing characteristic colonies of *P. aeruginosa* (large, irregular, translucent and produced a greenish diffusible pigment and characterized by fruity smell). Microscopically, the organism is Gram-negative short rod. Biochemically, it is positive oxidase, catalase, urea, citrate utilization and gelatin hydrolysis, negative for indole, methyl red and Voges Proskauer. It shows sugar fermentation of glucose, mannose and xylose, but not sucrose, lactose and maltose.

Serological identification of *P. aeruginosa* revealed that all of isolates were belonged to types G and M (Table 4).

Molecular detection of virulence genes in isolated *P. aeruginosa* showed presence of *tox*A and *psI*A genes in all strains and *fli*C in almost strains (Figures 1, 2 and 3).

Strains of *P. aeruginosa* showed *in vitro* susceptibility (48.48%) to streptomycin and colistinsulphate (36.36%) but resistant (100%) to amoxycillin/clavulanic acid, doxycycline and erythromycin and (78.78%) to nalidixic acid and (78.78%) to nalidixic acid (Table 5 and Figure 4).

Clinical signs of *P. aeruginosa* challenged chicks were depression, off food, reluctance, sleepy appearance, respiratory signs and greenish diarrhea. Mortalities began on day old *P. aeruginosa* challenged chicks 12-16 hr post challenge (PC) with mortality rate of (18/25, 72%), while appeared on 11 days old chicks 24 hr PC with mortality rate of (10/25, 40%). The post-mortem examination of the dead chick's revealed septicemia, congestion with peticheal haemorrhages on liver, spleen and lung, distention of gall-bladder, unabsorbed yolk sacs, greenish exudates in the abdominal cavity and hyperemia in intestine. Gross lesions of sacrificed birds showed congestion of the liver, spleen and kidneys and lung, liver necrosis and unabsorbed yolk sacs. *P. aeruginosa* organism was re-isolated from dead and sacrificed birds. No clinical signs or mortalities was recorded on phosphate buffer saline inoculated chicks.

The results of histopathological examination of dead and sacrificed chicks that challenged at one day old are collected in plate (1).The lung had severe interstitial pneumonia with highly edematous thickening of alveolar wall, highly congested alveolar capillaries as well as mononuclear inflammatory cell infiltration with some heterophiles throughout the interstitial tissue (Figure A). The bronchi and bronchioles had hyperplastic proliferation of their epithelial lining especially goblet cells were had marked hyperplastic proliferation. Mononuclear inflammatory cell infiltration with some heterophiles around bronchi and bronchioles could be detected. Sub pelural edema could be detected. The heart showed marked subepicardial edema with dilatation of sub epicardial blood vessels (Figure B). The liver showed highly congested hepatic sinusoids with disarrangement of hepatic rosettes as well as hepatic cell degeneration and necrosis with infiltration of mononuclear inflammatory cells with some heterophils (Figure C). Wide patches of hepatic cells showed coagulative necrosis with infiltration of mononuclear inflammatory cells and some heterophils (Figure D) as well as Kupffer cell activation could be detected.

The histopathological lesions of dead and sacrificed chicks that challenged at 11 days old are represented in plate (2). The lungs revealed bronchopneumonia with marked hyperplastic proliferation of its epithelial lining as well as highly hyperplastic proliferated goblet cells (Figure E). The bronchial lumen filled with exudates consisted of desquamated epithelial cells, mononuclear inflammatory cells, heterophils and red blood cells. Marked peribroncheal heterophils and mononuclear cell infiltration could be observed (Figure E). The lung showed patches of consolidated pneumonia and the alveolar lumen was filled with fibrinopurulent exudates (Figure F) with desquamated epithelial cells with necrosis in alveolar wall and heterophils detection in alveolar lumen. Other patches of pulmonary tissue had haemorrhagic pneumonia. Moreover, the lung had pulmonary blood vessel with large red attached thrombus consisted of fibrin, mononuclear cells and red blood cells (Figure G).Other blood vessels had perivascular mononuclear inflammatory cell infiltration with many heterophils and myriads of bacteria. The heart had marked myocarditis, edema and some hemorrhage between cardiomyocytes. (Figure H). Zenker's necrosis of most cardiomyocytes could be detected. The wall of most interstitial blood vessels showed degenerative

and necrotic changes (Figure H). The liver had dilated hepatic sinusoids with dissociated hepatic rosettes, vacuolar degeneration of many hepatocytes, other hepatocytes had pyknotic nuclei (Figure I). Multiple foci of degenerated and necrotic hepatocytes were seen around central veins which replaced by mononuclear inflammatory cell infiltration and heterophils. (Figure I). Kupffer cell activation could be detected. Portal area revealed proliferation of bile duct and highly dilated portal blood vessel. Mononuclear inflammatory cells and hetrophils infiltration could be detected around portal area (Figure J). Other patches of hepatic tissue had highly dilated portal blood vessel surrounded by dissociated, degenerative, necrotic hepatocytes and marked large haemorrhgic area (Figure K).

Table (4). The incidence and the predominant serotypes of *P. aeruginosa* in Damietta governorate, Egypt

Locality	Hatcheries				Chicken embryos			
	Number of samples	Number of positive	% positive	Serotype	Number of samples	Number of positive	% positive	Serotype
Om El-Reda	25	6	24	M	28	5	17.85	G and M
Farskour	25	4	16	M	29	3	10.3	M
Zarka	25	0	0		22	0	0	
Kafer-Saad	50	3	6	M	58	5	8.6	G and M
Cinania	25	0	0		15	0	0	
Kafer El-Batekh	50	3	6	G	54	4	7.4	G
Total	200	16	8		206	17	8.25	

Table (5). Results of antibiogram test against *P. aeruginosa*

Antibiotic Disc (Code)	Disc content/ µg	Antimicrobial efficacy (%) against 33 strain of *P. aeruginosa*		
		Susceptible	Intermediate Susceptibility	Resistant
Amoxycillin/Clavulanic acid (AMC)	30	0	0	33 (100%)
Colistinsulphate (CT)	10	11 (33.3%)	12 (36.36%)	10 (30.3%)
Doxycycline (Do)	30	0	0	33 (100%)
Erythromycin (E)	15	0	0	33 (100%)
Nalidixic acid (NA)	30	0	7 (21.2%)	26 (78.78%)
Streptomycin (S)	10	16 (48.48%)	9 (27.27%)	8 (24.2%)

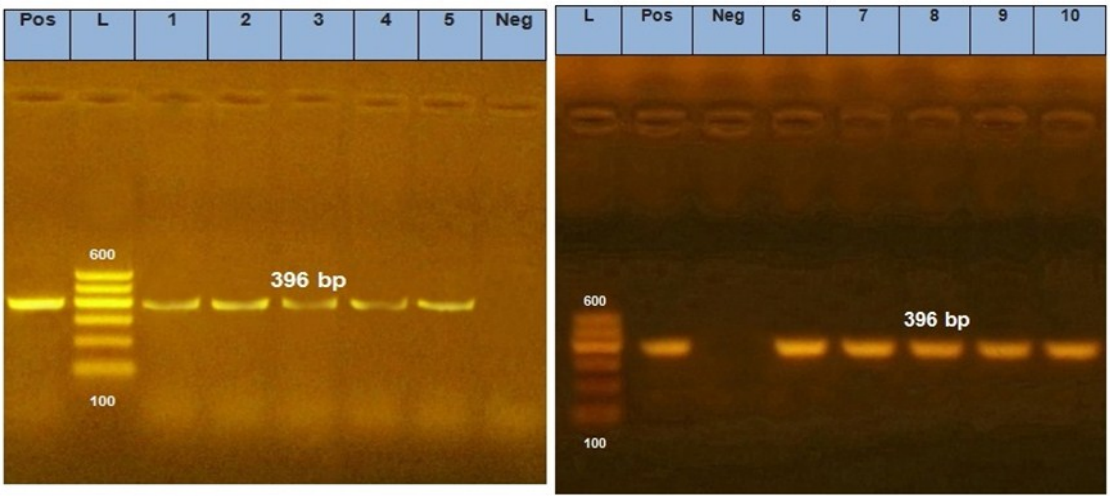


Figure (1). PCR amplification using Pseudomonas genus-specific primers (*tox*A gene), Pos= positive control, L= ladder, lines 1-10 = clinical isolates of *Pseudomonas genus*, Neg= negative control

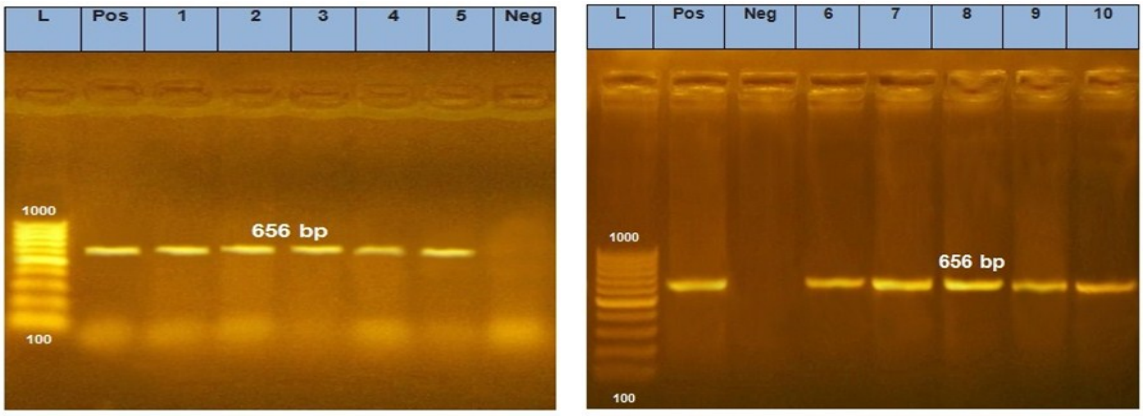


Figure (2). PCR amplification using Pseudomonas genus-specific primers (*psI*A gene), Pos= positive control, L= ladder, lines 1-10 = clinical isolates of *Pseudomonas genus*, Neg= negative control

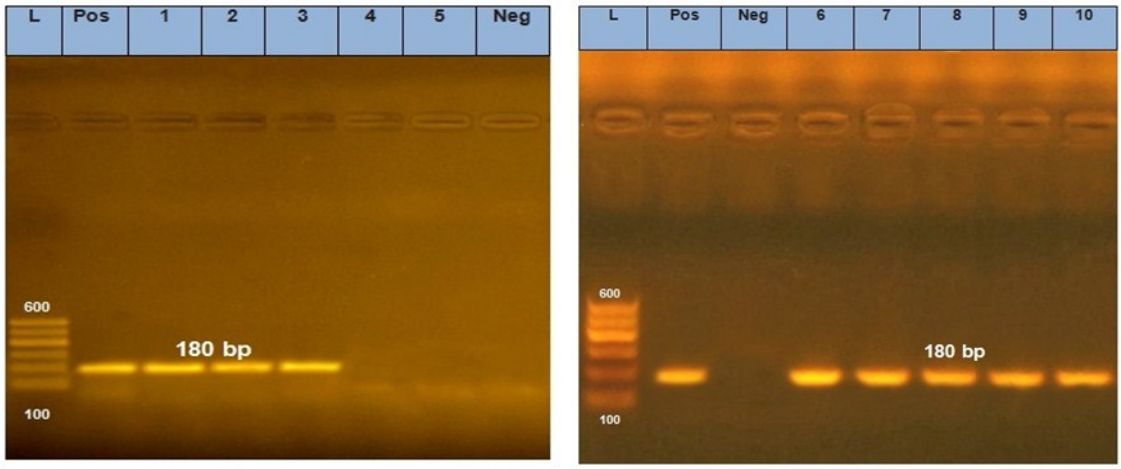


Figure (3). PCR amplification using Pseudomonas genus-specific primers (*fil*C gene), Pos= positive control, L= ladder, lines 1, 2, 3, 4, 6-10 = clinical isolates of *Pseudomonas genus*, Neg= negative control

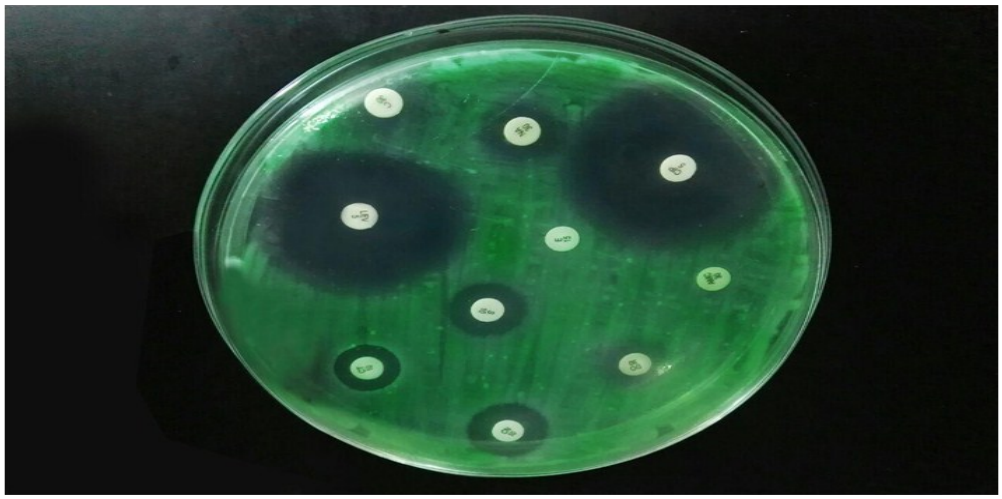


Figure (4). Results of antibiogram test against *P. aeruginosa*

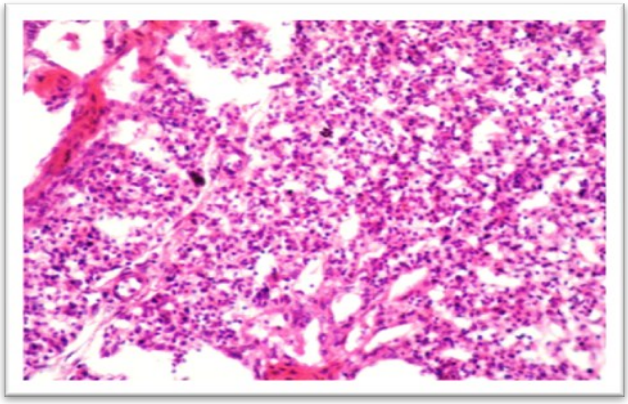


Plate 1:
Fig. (A) Lung showed severe interstititial pneumonia with highly edematous thickening alveolar wall, highly congested alveolar capillaries as well as mononuclear inflammatory cell infiltration with heterophils through the interstitial tissue. H&E X200

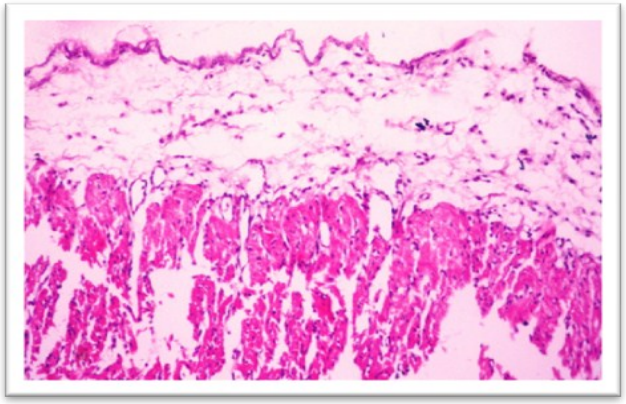


Fig. (B) Heart showed marked subepicardial edema with dilatation of sub epicardial blood vessels. Marked edema could be detected between cardiac muscle fibers accompanied by some degenerative changes of cardimyocytes. H&E X200

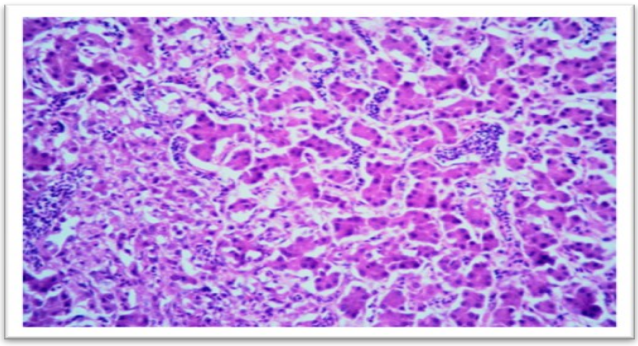


Fig. (C). Liver showed highly congested hepatic sinusoids with disarrangement of hepatic rosettes as well as patches of hepatic cell necrosis with infiltration of mononuclear inflammatory cells with some heterophils. Kupffer cell activation could be observed. H&E X400

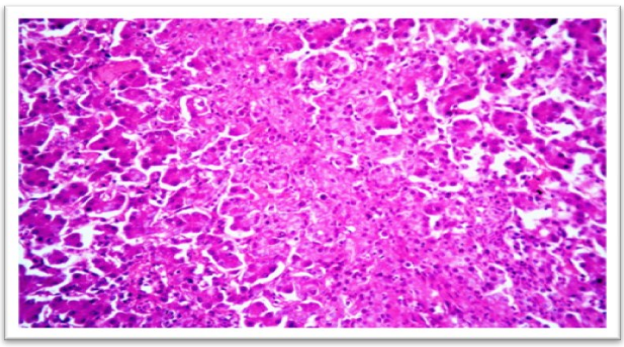


Fig. (D). Liver showed highly congested hepatic sinusoids with disarrangement of hepatic rosettes as well as large esinophilic structurless patches of hepatic cells coagulative necrosis. H&E X400

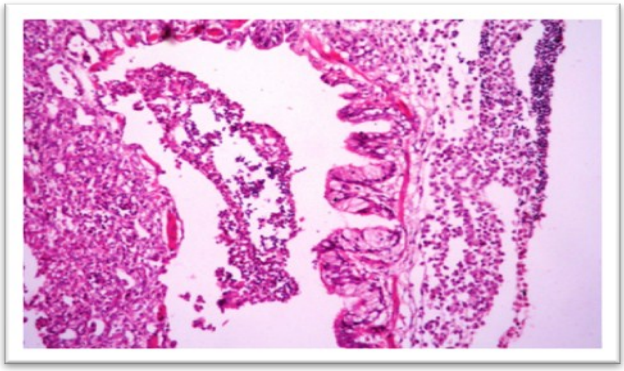


Fig. (E): Lung showed bronchus with marked hyperplastic proliferation of its epithelial lining as well as highly hyperplastic proliferated goblet cells. The bronchial lumen had exudates with desquamated epithelial cells, mononuclear inflammatory cells, heterophils and red blood cells. Marked aggregation of peribroncheal heterophils and mononuclear inflammatory cell could be seen. H&E X200

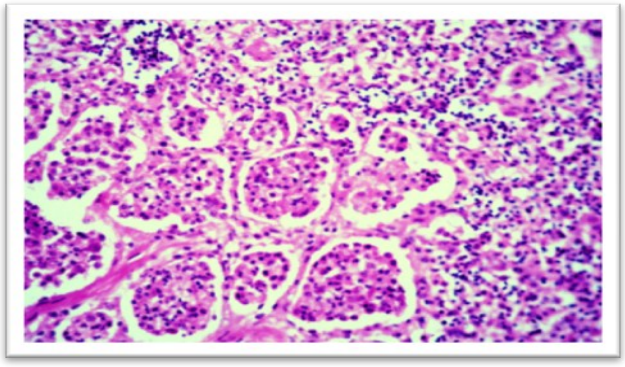


Fig. (F): lung showed patches of consolidated pneumonia, alveolar lumen filled with fibrinopurulent exudates with desquamated epithelial cells, as well as other patches of haemorrhagic pneumonia could be seen. H&E X400

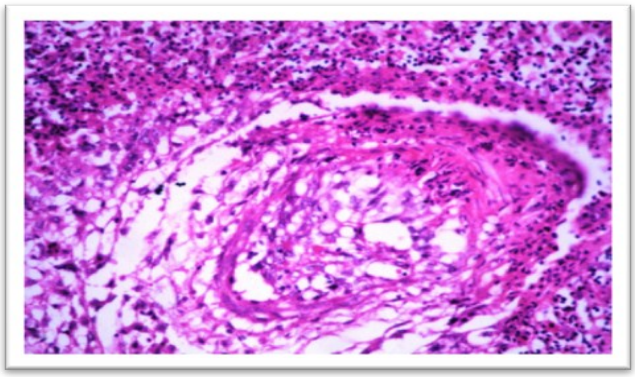


Fig. (G). lung showed pulmonary blood vessel with large red attached thrombus consisted of fibrin, mononuclear cells and red blood cells. Haemorrhgic pneumonia could be seen around thrombosed blood vessel. H&E X400

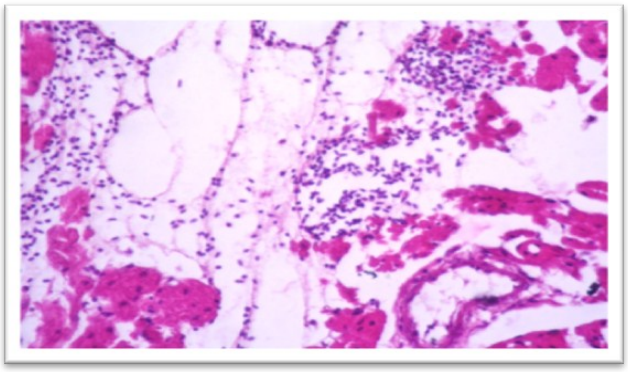
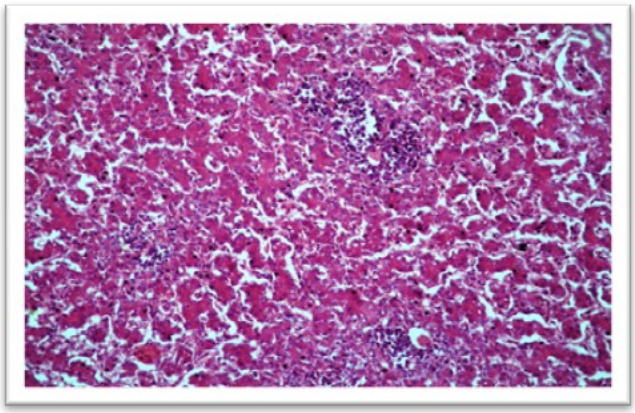


Fig. (H). Heart showed marked interstitial edema and some hemorrhage between cardiac muscle fibers. Zenker's necrosis of most myocardial fibers could be seen as well as degenerative and necrotic changes through the wall of most interstitial blood vessels. H&E X400



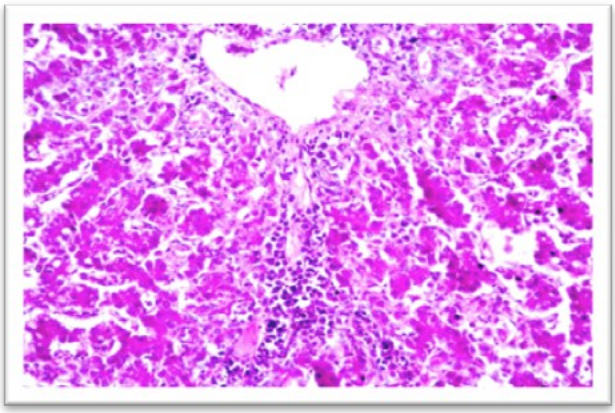


Plate 2:

Fig. (I); liver showed dilated hepatic sinusoids with vacuolar degeneration of many hepatocytes, other hepatocytes had pyknotic nuclei. Multiple foci of degenerated and necrotic hepatocytes were seen around central veins which replaced by mononuclear inflammatory cell infilteration and heterophils. Kupffer cell activation could be seen. H&E X200

Fig. (J); liver showed portal area with hyperplastic proliferation of bile duct and highly dilated portal blood vessel. Mononuclear inflammatory cell and hetrophils infiltration could be detected around portal area. Most hepatocytes had degenerative and necrotic changes. H&E X200

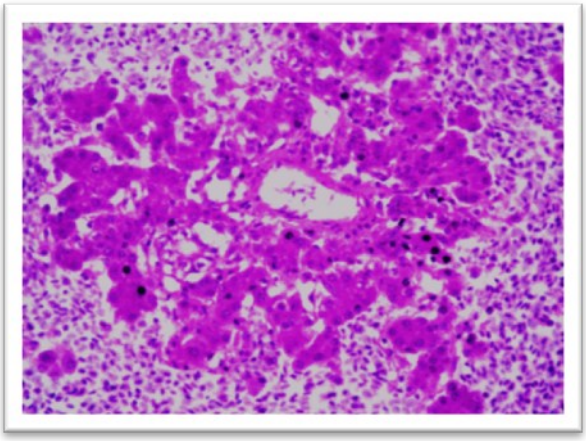


Fig. (K): liver showed highly dilated portal blood vessel, surrounded with degenerative and necrotic hepatocytes. Marked large haemorrhgic areas near necrotic hepatocytes could be seen. H&E X400

Discussion

An opportunistic *P. aeruginosa* infection is common in poultry where infection in eggs through yolk kills embryos (**Devriese *et al.*, 1975**). The most important source of *P. aeruginosa* was environment contamination (**Walker *et al.*, 2002**). *P. aeruginosa* causes serious infection in young and growing chickens and invade fertile eggs causing death of embryos and newly hatched chicks (**Saad *et al.*, 1981 and Mohamed, 2004**). The disease may be systemic, affecting multiple organs or localized in tissues as infraorbital sinus or air sacs producing swelling of the head, wattles, sinuses and joints of birds (**Pattison *et al.*, 2008**).Globally, outbreaks of *P. aeruginosa* infection with a mortality rate that may reach 90% among chicks have been reported (**Narula and Kuppuswamy, 1969**).

Preliminary identification tests including; cultural, microscopical and biochemical identifications of *P. aeruginosa* showed that the organism was recovered from16 (8%) out of 200 hatcheries swabs and from 17 (8.25%) out of 206 chicken embryos samples. Similar findings were observed by **Chakrabarty *et al.* (1980)** and **Betty *et al.* (2007)** who isolated *P. aeruginosa* with an incidence 8% from 100 chicken suffering from respiratory symptoms. In Egypt, **Farghaly *et al.* (2017)** detected of *P. aeruginosa* in in 42 samples after examination of 480 ones with a percentage of 8.75.Lower isolation percentages were recorded by **Awaad *et al.* (1981)**, **Mrden *et al.* (1988)**, **Younes *et al.* (1990) Choudhury *et al.* (1993)**, **Hayford (2017)** and **Hussein *et al.* (2008)** who isolated *P. aeruginosa* from chickens samples in rates of 2.9, 3.6, 4.9,4.75, 1.8 and 2.6%, respectively. However, higher rates of *P. aeruginosa* recovery were detected by **Muschin and Ziv (1973)** and **Nashed (1981)** who found *P. aeruginosa* in percentages of 15% and 14.1%; respectively from unhatched chicken eggs. **El-Bakry, (1983)** and **Hassan, (2013)** *were isolated P. aeruginosa* at the rate of 20% at Quena Governorate, Egypt. **Kamel *et al.* (2011)** isolated *P. aeruginosa* from chicken's respiratory tract by percentage 31% (28/90). **Elsayed *et al.* (2016)** isolated *P. aeruginosa* with percentage 22.9% (38 /166) and a high isolation rate from dead-in-shell embryos yolk sac 26/50 (52%) but the liver samples2-40 days old diseased and freshly dead were 12/100 (12%). **Mohamed (2004)** recorded that *P. aeruginosa* was found in rates of 21% of dead in shell chicken embryos, 17.6% in baby chicks and 3.3% in dead broilers.

Serological examination of *P. aeruginosa* is very important for epidemiological studies as serotyping facilitates the detection of the prevalent serotypes and locating sources of infection (**Nedeljković *et al.*, 2015**). In this study,

serological identification of *P. aeruginosa* isolates revealed that the most prevalent serotypes were G and M. Egyptian studies by **El-Gohary (2004)** cleared that isolated *P. aeruginosa* strains from poultry ration and water were belonged to serotypes H and B, while **El-Gohary *et al.* (2012)** demonstrated that the predominant serotypes were A, B, D, F, H, K, L and M. In ostriches, **Ali and Yousef (2003)** serogrouped 19 isolates of *P. aeruginosa* as H, H, K, L and M, while **Ali and Ibrahim (2004)** detected 9 "O" serogroups (A, B, F, I, H, J, K, L and N).

Due to difficulties and inaccuracy of phenotypic characterization of *P. aeruginosa*, PCR amplification and sequencing for detection of species specific conserved genes (*tox*A, *psI*A and *fli*C,) in the genome of *P. aeruginosa* can be utilized in its identification and classification (**Kidd *et al.,* 2011**).The ubiquity of *tox*A gene among *P. aeruginosa* clinical isolates is consistent with an important role for these virulence factor in chicken respiratory disease (**Tartor and El-Naenaeey, 2016**).The toxA gene, an inherent genetic sequence located on *P. aeruginosa* chromosome and regulating the synthesis of exotoxin A (**Xu *et al.,* 2004**)

Exotoxin A is an important virulence factor of *P. aeruginosa* that inhibits protein biosynthesis leading to great tissue and organ damage (**Jenkins *et al.,* 2004**). *PslA* polysacchride virulent gene present *P. aeruginosa* is responsible for initial attachment and adhesion (**Ma *et al.,* 2006 and 2007**), guide of exploration and microcolony formation (**Zhao *et al.,* 2013**), primary biofilm scaffold (**Ma *et al.,* 2009**), signaling molecule to stimulate biofilm formation (**Irie *et al.,* 2012**) and antibiotics resistance (**Yang *et al.,* 2011**). *P. aeruginosa* has polar flagella that composed of protein units (flagellin), which encoded by the *fliC* gene. Flagella are responsible for the pathogenicity, chemotaxis, and colonization of bacteria to the host cells (**Moosavian *et al.,* 2018**).

The most worrying feature that presents a huge difficulty during therapy is the intrinsic resistance of *P. aeruginosa* (**Seol *et al.,* 2002 and Pool *et al.,* 2011**). The presence of chromosomally encoded penicillinases and cephalosporinases, coupled with efficient efflux porins and external biofilm matrix has made most strains of *P. aeruginosa* naturally resistant to a wide range of antibiotics (**Ferguson, 2007**). *P. aeruginosa* is also known to harbor antibiotic resistant plasmids, integrons and transposons and is able to transfer these genes to other species (**Opal and Pop -vicas, 2004**).

The results of the antibiogram of *P. aeruginosa* in this work revealed susceptibility (48.48%) to streptomycin but resistance (100%) to amoxycillin/clavulanic acid, doxycycline and erythromycin and (78.78%) nalidixic acid. Intermediate susceptibility was seen for colistinsulphate (36.36%). Nearly similar finding with this study was observed by **Farghaly *et al.* (2017)** who demonstrated that 42*P. Aeruginosa* chicken's isolates showed high sensitivity for norfloxacin, ciprofloxacin, and levofloxacin with the percentage of 80.9, 76.2 and 73.8, respectively, 76.2 % to colistin sulfate and gentamicin and 66.7 to streptomycin; however, high resistance (100%) to ampicillin, nalidixic acid and lincomycin followed by trimethoprimsulfamethoxazole with percentage78.6. As well, **Elsayed *et al.* (2016)** recorded sensitivityof*P. Aeruginosa* strains to ciprofloxacin and gentamycin. **Al-Adl (2014)** mentioned that strains of *P. aeruginosa* were highly susceptible to colistin sulfate and norfloxacin, intermediately sensitive to gentamycin and ciprofloxacin and resistant to lincomycin, nalidixic acid, streptomycin, chloramphenicol and doxycycline. However, **Kilonzo-Nthenge *et al.* (2008)** demonstrated that *P. aeruginosa* isolated from chicken was sensitive to tetracycline, ampicillin, streptomycin, kanamycin, nalidixic acid, gentamicin, erythromycin, ciprofloxacin, cefoxitin and colistin. Higher sensitivity of *P. aeruginosa* organism to chloramphenical and streptomycin was observed by **Sadasivan *et al.* (1977)** and **Mohamed (2004)**. *P. aeruginosa* avian isolates were determined to be susceptible to gentamicin in the study of **Koncicki and Szubstarska, (1988)** and **Walker *et al.* (2002)**. Contrary results were recorded by **Hayford**

(2017) who found resistanceof *P. aeruginosa* to gentamicin and ciprofloxacin.

Clinical signs of *P. aeruginosa* challenged chicks were depression, off food, reluctance, sleepy appearance, respiratory signs and greenish diarrhea. Mortalities began on day old *P. aeruginosa* challenged chicks 12-16 hr post challenge (PC) with mortality rate of (18/25, 72%), while appeared on 11 days old chicks 24 hr PC with mortality rate of (10/25, 40%). The post-mortem examination of the dead chick's revealed septicemia, congestion with peticheal haemorrhages on liver, spleen and lung, distention of gall-bladder, unabsorbed yolk sacs, greenish exudates in the abdominal cavity and congested intestine. Gross lesions of sacrificed birds showed congestion of the liver, spleen and kidneys, lung, liver necrosis and unabsorbed yolk sacs. *P. aeruginosa* organism was re-isolated from dead and sacrificed birds. No clinical signs or mortalities was recorded on phosphate buffer saline inoculated chicks. Nearly similar results were demonstrated by **Mohamed (2004)** who found that subcutaneous inoculation of 3 days old chicks with *P. aeruginosa* induced mortality reached 80% with sleepy appearance, closed eyes, sitting on hocks and diarrhea while the gross lesions revealed congestion of all internal organs, peticheal haemorrhages on liver and spleen, congested lungs, swollen of kidneys with deposition of ureats in the ureters, enteritis, enlargement of the gall-bladder and unabsorbed congested yolk sacs. *P. aeruginosa* produced mortality, dyspnea, septicaemia with congestion of internal organs and perihepatitis and pericarditis (**KheirEldin and Awaad, 1985**). **Awaad *et al.* (1981)** observed 100% mortalities after subcutaneous inoculation with *P. aeruginosa*, however, oral inoculation induced 6.6% mortalities. **Satish and Priti (2015)** found that *P. aeruginosa* induced 100% mortality when inoculated intramuscularly in 7 days old chicks where dead birds showed congested liver with petechial haemorrhages and distended dark gall bladder, congested heart, lungs and kidneys, enlarged yolk sac and haemorrhagic intestine. **Walker *et al.* (2002)** and **Joh *et al.* (2005)** reported that the mortality rate was 95% in chick's inoculated *P. aeruginosa* through yolk where dead birds showed congestion and enlargement of liver, peritoneal fluid in the abdomen, epicarditis, omphalitis, and some solidified yolks.

Severe interstitial pneumonia of one day old chicks led to increase thickening of alveolar wall and capillary membrane and decrease the number of ventilated alveoli resulting in hypoxia. **Van Delden (2004)** discussed that elastin protein of connective tissues and its degradation which carried out by LasB 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 haemorrhagic pneumonia, also explains the destruction of hepatic sinsoides which led to haemorrhage (wide patches of accumulated red blood cells) in hepatic tissue. **Rejmanj *et al.* (2007)** stated that *P. aeruginosa* infection alters the permeability function of the epithelial and endothelial junction and that clarifies pulmonary blood vessels thrombosis. Most histopathological changes of lung, heart and liver were inflammatory in nature that come in agree with **Timurkaan *et al.* (2008)** who found inflammatory reaction through heart and lung especially in young birds. **Genovese *et al.* (2000)** & **Ferro *et al.* (2004)** who stated that heterophils functions in 1-14 days old chickens were found inefficient compared to older birds, that inefficiency in function continued until 21 days of age and that was correlated with an increase in susceptibility to bacterial infection. Kupffer cells activation which detected in liver of all infected chicken comes in agree with **Katja *et al.* (2015)** who described that Kupffer cells are important cells for filtration of *P. aeruginosa* during systemic infection and that in corporation with heterophils which are essential for bacterial control. Most of liver diseases in chicken were associated with liver failure which was explained by **Supertika *et al.* (2006)** who found degeneration and necrosis of hepatic tissue in infected 10 days old chickens. The prominent microscopic lesions of the dead

broilers with *P. aeruginosa* were multiple foci of coagulative necrosis with intra lesion bacteria and heterophilic infiltration in the liver and the serosal surface was covered with fibrinopurulent exudates, diffuse fibrinous exudates with bacterial colonization in the epicardium and decreased lymphocytes and moderately increased reticulocytes in the spleen (**Joh *et al*., 2005**).

In conclusion, *P. aeruginosa* is of great importance pathogen of newly hatched chicks (one day old chicken) based on presence of virulent genes as well as *in vivo* pathogenicity study. Therefore, good hygiene especially in hatcheries is fundamental to Ps. control. The farm management should take stringent measures against all the possible sources of infection. The measures may include, the farm workers should be trained on how to avoid environmental associated infectious diseases and change disinfectants periodically in hatcheries, incubators and house environment. The use of suitable antibiotic in the day – old chicks could have helped reduce flock mortality. Generally, hygienic mea-sures are necessary to be applied to restrict the continuous abuse of antibiotics, especially in poultry farms. Further studies are in need to study the effect of this pathogen on Egyptian poultry industry.

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