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MOLECULAR STUDIES ON *PASTEURELLA SPECIES* ISOLATED FROM DUCKS

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ABSTRACT

The present study was conducted on 150 ducks collected from ten farms in Kaliobia Governorate suspected to be suffering from Pasteurellosis that is manifested by respiratory signs, sudden death, and nervous manifestation. Samples were taken from the liver, spleen, heart and lung and examined bacteriologically. A total of 33 Pasteurella isolates were isolated, 25 isolates were *Pasteurella multocida* and 8 isolates were *Pasteurella pneumotropica*. The invitro-antibiotic sensetivity test showed that Pasteurella isolates were highly sensitive to florofinicole followed by ciprofloxacine and enrofloxacin. Meanwhile, the isolates showed absolute resistance to erythromycin followed by gentamycin, amoxicillin, oxytetracycline, penicillin, tobramycin and naldixic acid for both types of Pasteurella. PCR results showed that cytotoxic protein (*tox*A) gene and fimbrial protein (*ptf*A) gene were detected in 4 out of 10 studied strains for both genes. Sequences of *tox*A and *ptf*A genes were submitted to Gen Bank and assigned accession numbers were MF167359 and MF382009, respectively.

Key words: *Pasteurella multocida*- *Pasteurella pneumotropica*- *tox*A- *ptf*A -antibiotic sensitivity test- PCR- ducks.

INTRODUCTION

Pasteurella multocida belonging to family Pasteurellaceae is a ubiquitous organism affecting multi host species, thus causing several diseases like fowl cholera in poultry, snuffles in rabbits, haemorrhagic septicaemia in cattle and buffalo, enzootic bronchopneumonia in cattle, sheep, and goats and atrophic rhinitis in swine, (Harper *et al.*, 2006, Dziva *et al.*, 2008 and Markey *et al.*, 2013). *Past. multocida* is identified as a major threat for a poultry industry which hamper the profitable poultry production (Sellyei *et al.*, 2010).

Clinically ducks associated with pasterullosis showed anorexia, depression and respiratory manifestation (Eldin and Reda, 2016), lameness and corneal turbidity (Takahashi *et al.*, 1996). On postmortem, petechial and ecchymotic hemorrhages were common, particularly in subepicardial (heart) and subserosal (liver) locations. The liver was swollen accompanied with multiple, small, necrotic foci. Pneumonia and air sacculitis were commonly seen (Fouad and Hebat Allah, 2008 and Mohan and Pradeep Kumar, 2008).

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Based on capsular antigens, *P. multocida* strains are differentiated into five serogroups i.e., type A causing fowl cholera pathogen and bovine shipping fever, type B causing hemorrhagic fever in ungulates, type D causing atrophic rhinitis in swine, type E, an African serotype, infecting cattle and buffalo; and type F also causing fowl cholera (Carter, 1955 and Rimler *et al.*, 1987). Several studies detected the virulence profiling of *P. multocida* isolates from different hosts (Ferreira *et al.*, 2012; Furian *et al*, 2013; Katsuda *et al.*, 2013 and Verma *et al.*, 2013). These virulence factors (VFs) and outer membrane proteins are important for pathogenesis, functionality, protective immunity and vaccine development against *P. multocida* infections (Hatfaludi *et al.*, 2010 and Katsuda *et al.*, 2013). The main virulence factors of Pasteurella *were* Endotoxins (lipopolysaccharides, LPS) that are particularly important in the septicaemic diseases such as fowl cholera and bovine haemorrhagic septicaemia. *P. multocida* serotyes A and D can produce a cytotoxic protein named *P. multocida* toxin (PMT), which stimulates cellular cytoskeletal rearrangements and growth of fibroblasts. Interestingly, avirulent PMT-positive strains and virulent PMT-negative strains have both been reported. However, PMT plays a role in atrophic rhinitis (mild to severe destruction of porcine nasal turbinate bones) and Filamentous hemagglutinins (*Pfh*B1 and *Pfh*B2), surface fibrils (*Hsf*_1 and *Hfs*_2), and fimbrial subunits (*Ptf*A, *Fim*A, *Flp*_1, *Flp*_2) are Adhesion to host cells, chemotaxis (Dashe *et al.*,

2015), the *ptf*A gene of which assemble to form type 4 fimbriae on the bacterial surface (Sellyei *et al.,* 2010).

Past. pneumotropica was found to be latent in laboratory rats, mice, guinea pigs and hamsters (Baker, 2003). Naturally occurring disease which has been recorded in laboratory mice include pneumonia, conjunctivitis, otitis, abortion and abscesses (Charles, 2009 and Justin *et al.,* 2014). As duck cholera is contiguous, septicemic and fatal disease for ducks and little studies were investigated with the virulence of this pathogen so the present study was conducted to isolate pasteurella species from ducks and study their virulence factors.

MATERIALS AND METHODS

Sample collection:

2.1. Samples collection

A total of 150 diseased pekin ducks of different ages (3 weeks-7weeks) were collected from 10 different duck farms at Kaliobia Governorate. The collected ducks showed mortalities ranged from 30-40%. No previous history for vaccination of collected ducks against duck cholera. Samples were taken from liver, heart, lung, kidney and spleen of each duck for bacteriological examination.

2.2. Studies the bacteriological character and their virulence factor

The surface of organs was seared by hot spatula, and then a sterilized loopfuls were inoculated onto Tryptone soya broth and incubated aerobically at 37ºC for 24 hours. A loopful from incubated Tryptone soya broth was streaked onto sheep blood agar, Baird Parker agar with 1ml of 0.1% of crystal violet as Pasteurella has ability to grow in presence of 0.1 % crystal violet and egg yolk tollurite (Melody *et al.,* 1994); Mac Conkey's agar, all plates were incubated for 24 hours at 37ºC. The developed colonies were picked up and subcultured for purification. The purified colonies were morphologically identified by Gram stain and Leishman's staining technique and biochemical tests (Carter, 1984 and Markey *et al.,* 2013).

2.3. In-Vitro antibiotic sensitivity test:

The Pasteurella isolates were subjected to the sensitivity test against different antibiotics, using the disc and agar diffusion method (Finegold and Martin, 1982) for their susceptibility against 10 anti microbial agents representing classes of different antimicrobial agents (ciprofloxacin, gentamycin, tobramycin, amoxicillin, erythromycin, enrofloxacin, oxytetracycline, penicillin, naldixic acid and florofinicol)

2.4. Detection of *tox*A and *ptf*A genes of Pasteurella multocida by PCR according to (Sambrook *et al.*, 1989):

PCR was applied on random10 selected *Pastereulla multocida* isolates by using two sets of primers for detection of two virulence genes Cytotoxic protein (*tox*A) and fimbrial protein (*ptf*A)

DNA extraction:

Using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations.

Oligonucleotide Primer: Primers used were supplied from Metabion (Germany) and listed in Table (1).

PCR amplification: Primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1

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.

Table 1: Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary Denaturation	Amplification (35 cycles)			Final extn.	Ref.
				Secondary denaturation	Annealing	Extension		
toxA	CTTAGATGAGC GACAAGG	864	94˚C/ 5 min.	94˚C 30 sec.	48˚C 40 sec.	72˚C 50 sec.	72˚C 10 min.	**Tang *et aL.*, 2009**
	GAATGCCACAC CTCTATAG							
ptfA	TGTGGAATTCA GCATTTTAGTGT GTC	488	94˚C/ 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	
	TCATGAATTCTT ATGCGCAAAAT CCTGCTGG							

Sequencing protocol: By Dye termination method (Sanger *et al.,* 1977).

Steps of sequence analysis:

1- The received sequence was imported into alignment window with the downloaded highly similar sequences into BIOEDIT version 7.0.4.1 software. And MEGA 5.05 DNA alignment tool

2- Sequence submission was conducted following the instructions offered by the web tool Bankit of GenBank http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank with the following numbers: bankit2012800 seq for *tox*A and bankit2026599 for *ptf*A gene of *pasteurella multocida*.

RESULTS

Clinical cases:
The most common observed clinical signs showed by affected ducks were sudden death, greenish diarrhea, nervous manifestation, locomotory disturbance, depression and mucus discharge from mouth and nostrils. The post mortem lesions showed swollen liver with necrotic foci, hemorrhages on heart, pneumonia and airsacculitis.

Bacteriological examination:
The bacteriological examination of samples collected from 150 diseased ducks revealed the recovery of 33 pasteurella isolates, where 25 isolates (76%) were identified as *Pasteurella multocida* and 8 isolates (24%) were identified as *Pasteurella pnemotropica*. the isolated bacterial colonies on blood agar plates were small, glistening, mucoid, dew drop like and non-haemolytic, and appeared as Gram-negative coccobacilli when stained with Gram's stain. Leishman's staining technique revealed bipolar microrganisms. Moreover, the isolates failed to grow on MacConkey agar.

Antibiotic sensitivity test:
The results of invitro- antibiotic sensitivity test (Table, 2) revealed that the isolated Pasteurella species were highly sensitive to florofinicole (84.8%) followed by ciprofloxacine (60.6%) and enrofloxacin (51.5%). Meanwhile they were highly resistance to erythromycin (100%), followed by gentamycin (84.8%); amoxicillin, oxytetracycline and penicillin (69.7% per each); tobramycin (66.7) and naldixic acid (63.6%) for both types of Pasteurella.

Table 2: Antibiotic sensitivity for 33 Pasteurella species isolates by disc diffusion method:

Sensitivity	Sensitive		Intermediate		Resistance		A.A
Antibiotics agent	**No.**	**%***	**No**	**%***	**No**	**%***	
Ciprofloxacin (10µg)	20	60.6	0	0.0	13	39.4	S
Gentamycin (10µg)	5	15.1	0	0.0	28	84.8	R
Tobramycin	11	33.3	0	0.0	22	66.7	R
Amoxicillin (20µg)	10	30.3	0	0.0	23	69.7	R
Erythromycin (10µg)	-	-	0	0.0	33	100	R
Enrofloxacin (10µg)	17	51.5	3	9.1	16	48.4	S
Oxytetracyclin (10µg)	10	30.3	0	0	23	69.7	R
Pencillin 10 units	10	30.3	2	6.0	23	69.7	R
Naldixic acid (30µg)	12	36.4	0	0.0	21	63.6	R
Florofinicol (30µg)	28	84.8	0	0.0	5	15.1	S

%* in relation to total number of pasteurella isolates (33), A.A Antibiogram Activity,
S sensitive, R resistance

PCR results:
The results of PCR (Table, 3) showed that, *tox*A and *ptf*A genes were detected in 4 strains of 10 examined ones for each.

Table 3: Virulence gene *tox*A and *ptf*A detected by PCR test in *P.multocida*

Sample	Results	
	toxA	***ptfA***
1	-	-
2	+	+
3	+	+
4	-	-
5	-	-
6	+	+
7	-	-
8	+	+
9	-	-
10	-	-

A set of primers were designed for amplification of *tox*A and *ptf*A genes in 10 *pasteurella multocida* isolates to be used in a PCR. Analysis of the PCR products on agrose gel electrophoresis revealed successful amplification of *ptf*A gene at 488bp (Fig. 1) and *tox*A gene at 864bp (Fig. 2).

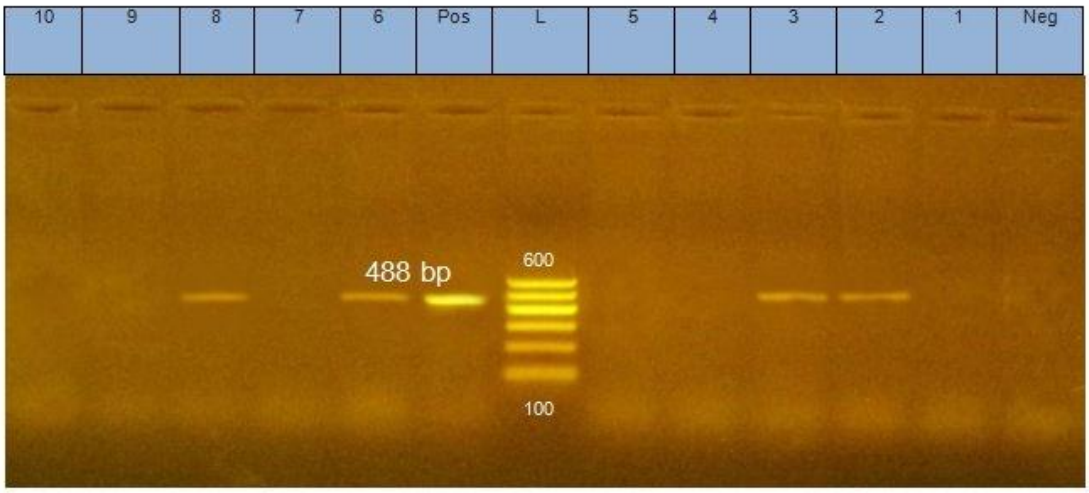


Fig. 1: Agarose gel electrophoresis of *ptf*A gene gene in 10 *Pasteurella multocida* isolates, M: 100 bp DNA marker, lanes (2, 3, 6 and 8): positive amplification of *ptf*Agene at 488 bp, Positive control: standered strain from AHRI Dokki, Negative control.

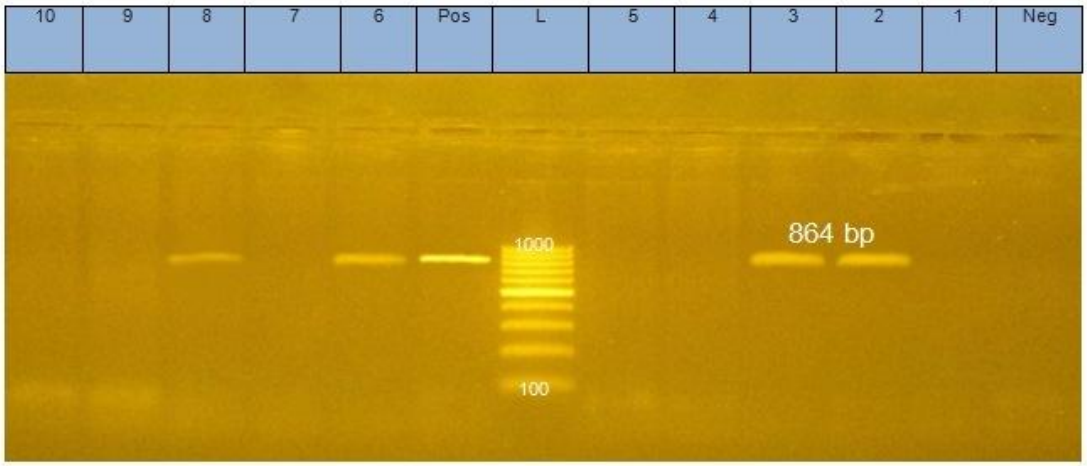


Fig. 2: Agarose gel electrophoresis of *tox*A gene in 10 *Pasteurella multocida* isolates, M: 100 bp DNA marker, lanes (2, 3, 6 and 8): positive amplification of *tox*A gene at 864bp, Positive control: standered strain from AHRI Dokki, Negative control.

Nucleotide sequence accession number

Partial gene sequence of *tox*A and *ptf*A of *Pasteurella multocida* isolate was submitted to Gen Bank and assigned accession number were MF167359 and MF382009, respectively.

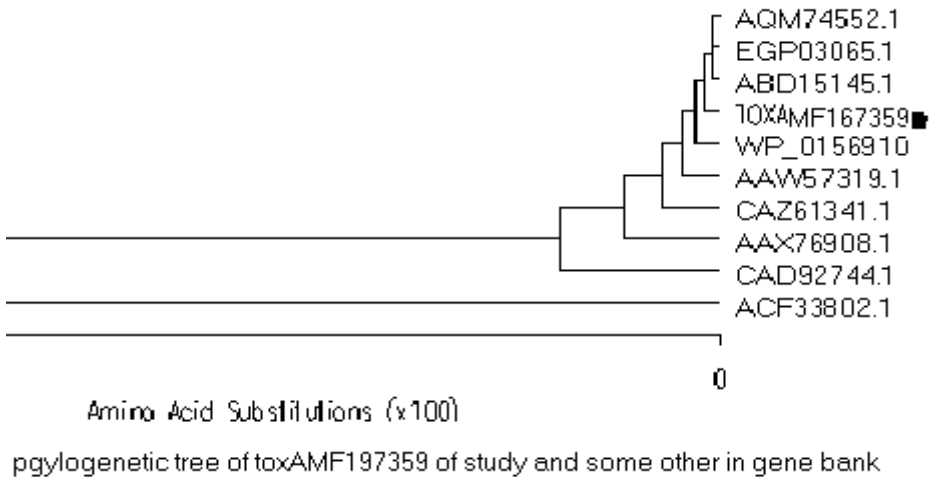


Fig. 3: Phylogenetic analysis of nucleotide of *tox*A MF167359 in comparison with other selected strain form gene bank

Percent Identity

	1	2	3	4	5	6	7	8	9	10	
1		98.9	98.9	98.9	98.9	98.9	98.6	98.5	98.5	96.7	1
2	0.0		100.0	100.0	100.0	100.0	99.6	100.0	100.0	100.0	2
3	0.0	0.0		100.0	100.0	100.0	99.6	100.0	100.0	100.0	3
4	0.0	0.0	0.0		100.0	100.0	99.6	100.0	100.0	100.0	4
5	0.0	0.0	0.0	0.0		100.0	99.6	100.0	100.0	100.0	5
6	0.0	0.0	0.0	0.0	0.0		99.6	100.0	100.0	100.0	6
7	0.4	0.4	0.4	0.4	0.4	0.4		99.5	99.5	98.9	7
8	0.0	0.0	0.0	0.0	0.0	0.0	0.5		100.0	100.0	8
9	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0		100.0	9
10	0.0	0.0	0.0	0.0	0.0	0.0	1.1	0.0	0.0		10
	1	2	3	4	5	6	7	8	9	10	

TOXA MF167359
CAD92744.1
AAX76908.1
CAZ61341.1
AAW57319.1
WP_015691094.1
ACF33802.1
ABD15145.1
AQM74552.1
EGP03065.1

Fig. 4: Nucleotide identities and divergence of *tox*A MF167359 compared to other selected strains.

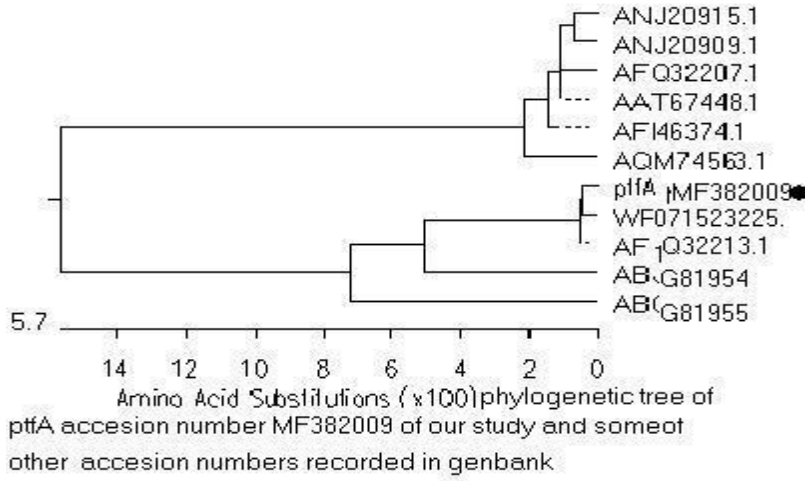
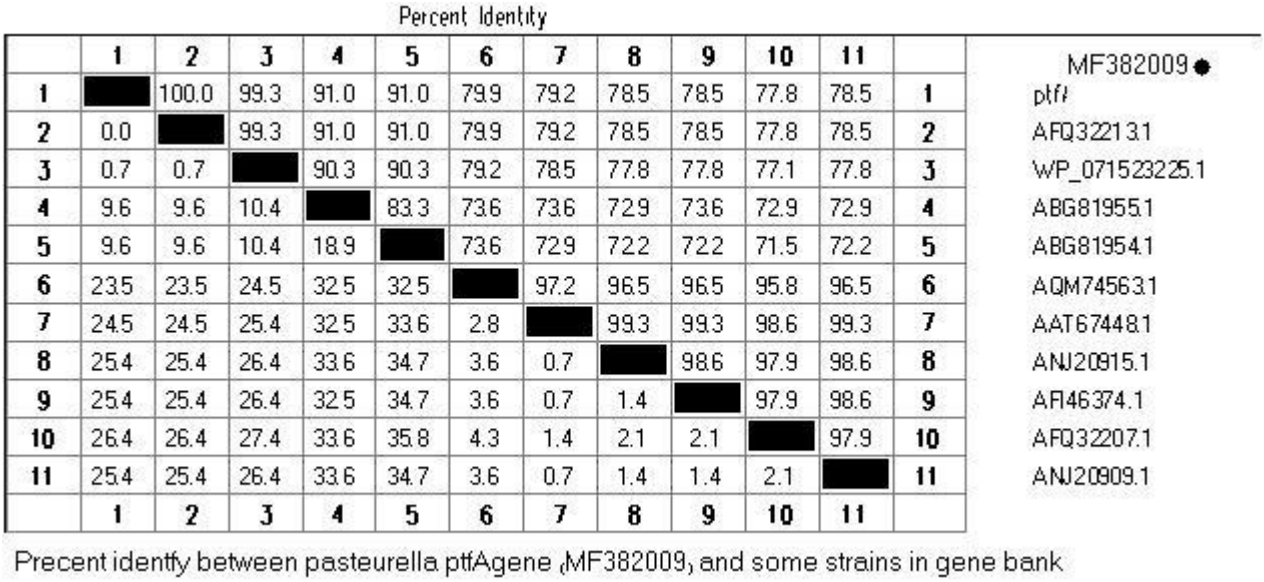


Fig. 5: Phylogenetic analysis of nucleotides of *ptf*A MF382009 in comparison with other selected strain form gene bank



	1	2	3	4	5	6	7	8	9	10	11	
1		100.0	99.3	91.0	91.0	79.9	79.2	78.5	78.5	77.8	78.5	1
2	0.0		99.3	91.0	91.0	79.9	79.2	78.5	78.5	77.8	78.5	2
3	0.7	0.7		90.3	90.3	79.2	78.5	77.8	77.8	77.1	77.8	3
4	9.6	9.6	10.4		83.3	73.6	73.6	72.9	73.6	72.9	72.9	4
5	9.6	9.6	10.4	18.9		73.6	72.9	72.2	72.2	71.5	72.2	5
6	23.5	23.5	24.5	32.5	32.5		97.2	96.5	96.5	95.8	96.5	6
7	24.5	24.5	25.4	32.5	33.6	2.8		99.3	99.3	98.6	99.3	7
8	25.4	25.4	26.4	33.6	34.7	3.6	0.7		98.6	97.9	98.6	8
9	25.4	25.4	26.4	32.5	34.7	3.6	0.7	1.4		97.9	98.6	9
10	26.4	26.4	27.4	33.6	35.8	4.3	1.4	2.1	2.1		97.9	10
11	25.4	25.4	26.4	33.6	34.7	3.6	0.7	1.4	1.4	2.1		11
	1	2	3	4	5	6	7	8	9	10	11	

Fig. 6: Nucleotide identities and divergence of *ptf*A MF382009 compared to other selected strains.

Phylogenetic analysis and nucleotide comparison
The nucleotides sequences of *tox*A MF167359 gene and *ptf*A MF382009 gene showed percent identity with the selected *pasteurella multocida* strains published on gene bank ranged from 96.7 to 100% (fig 3-fig4) and 71.5 to 100% (fig5-fig6), respectively. The selected sequences were isolated from chicken and wild birds.

DISCUSSION

Duck cholera is one of the important disease affecting ducks causing severe economic losses; therefore our investigation was carried out to isolate pasteurella species from ducks collected from ten farms in Kaliobia Governorate. In our study the affected ducks showed sudden death (30-40%), greenish diarrhea, nervous and respiratory manifestation. The post mortem lesions showed swollen liver with necrotic foci, hemorrhages on heart, pneumonia and airsacculitis. Similar clinical signs and postmortem picture were reported in ducks associated with pasterullosis by Fouad and Hebat Allah, 2008; Mohan and Pradeep Kumar, 2008 and Eldin and Reda, 2016.

The bacteriological examination of samples collected from 150 diseased ducks revealed isolation of 33 pasteurella isolates. The isolated bacterial colonies on blood agar plates were small, glistening, mucoid, dew drop like and non-haemolytic. They appeared as Gram-negative coccobacilli when stained with Gram's stain. Leishman's staining technique revealed bipolar microrganisms. These features were in agreement with previous researches (Akhtar, 2013; Belal, 2013 and Ievy *et al.*, 2013).

In the present study *P. multocida* were isolated from ducks by total percent of 16.7%, (25/150). Nearly similar results were recorded by Kumar *et al.* (2004) and Sayedun *et al.* (2015) who isolated *P. multocida* from avian species including ducks with percentage of 34% and 11.42, respectively but disagreed with Kamruzzaman *et al.* (2016) who isolated *P. multocida* from ducks with higher percentage of 59.72%. Moreover 5.3% (8/33) *P. pneumotropica* strains were isolated. Theses isolates were currently isolated from rat or guinea pig bite wound (Anne-Lise *et al.*, 2005), its occurrence in ducks indicate the role of rodent as reservoir for transmission of the disease to other susceptible flocks and pay attention to application of biosecurity in ducks farms.

The results of invitro- antibiotic sensitivity test (Table, 2) revealed that, the isolated pasteurella species were highly sensitive to florofinicole (84.8%) followed by ciprofloxacine (60.6%) and enrofloxacin (51.5%). Meanwhile they were highly resistance to erythromycin (100%), followed by gentamycin (84.8%); amoxicillin, oxytetracycline and penicillin (69.7% per each); tobramycin (66.7) and naldixic acid (63.6%) for both types of Pasteurella. The obtained results are not in agreement with (Kamruzzaman *et al.*, 2016) who cited that ciprofloxacin was the most effective antibiotic by 95% followed by gentamycin (85%), tetracycline and amoxicillin (75% per each). Also our results differed from that obtained by Dashe *et al.* (2015) who showed that ciprofloxacin, streptomycin and gentamycin were highly effective against *P. multocida*. On the other hand, Maity *et al.* (2012) reported that *P. multocida* was sensitive to amoxiclav, chloramphenicol, and moderately sensitive to amikacin, cefotaxime, neomycin and norfloxacin but resistant to ciprofloxacin and lomefloxacin. The variation in the sensitivity grade among various studies may be due to over or limited previous exposure and indiscriminate use of

antibiotics as feed additives and/or preventive or curative agents.

In our study *tox*A and *ptf*A genes were detected in 4 strains of 10 examined ones for each. Similar results were recorded by Thales *et al.* (2016) who detected *tox*A and *ptf*A in Pasteurella isolates. Strains *tox*A MF167359 and *ptf*A MF382009 in our study shared nucleotides identities 96.7 to 100% and 71.5 to 100%, respectively with selected *pasteurella multocida* sequences published on gene bank. Most of the aligned sequences were isolated from chicken as AQM74552.1, which Submitted (27-AUG-2016) while others were isolated from wild birds as EGP03065.1 which Submitted (22-JUN-2011). Data indicated that application of strategies to control the access of wild birds to duck farms where they act as reservoir for the pasterullosis, also the data revealed cross infection between ducks and chicken which give great attention to avoid multi species breading.

CONCLUSION

Finally the present study concluded that pasterullosis (duck cholera) is a serious disease in duck farms with economic importance. Application of good biosecurity in duck farms to avoid transmission of *p. pneumotropica* from rodent to duck is needed important, the use of effective vaccination against duck cholera to control the disease in ducks; Moreover Florofinicol is the drug of choice for treatment of Pasteurella species in ducks.

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دراسات جزيئية على ميكروبات الباستيرلا المعزولة من البط

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الدراسة المقدمة أجريت على عدد ١٥٠ بطة جمعت من عدد ١٠ مزارع بالقليوبية مشتبه بإصابتها بمرض كوليرا البط كانت تعانى من أعراض تنفسية وموت مفاجيء وأعراض عصبية. أخذت العينات من الكبد والطحال والقلب والرئة لفحصها معمليا. أوضحت الاختبارات المعملية واختبارات الكيمياء الحيوية عن وجود ٣٣ معزولة (باستيرلا ٢٥ باستيرلا مولتوسيدا و ٨ باستيرلا نيموتروبيكا) وقد تم عمل اختبار حساسية للمعزولات ووجد ان المعزولات كانت عالية الحساسة بنسبة ٨٤% للفلورفينيكول وللسبروفلوكساسين و للانروفلوكساسين بينما أظهرت المعزولات مقاومة لكل من الاثرومايسين والجينتاميسن والأموكسلين والأوكسي تتراسيكلين والبنسلين وتوبراميسين وللنالدكسيك اسيد. تم عمل اختبار تفاعل البلمرة المتسلسل لجينين من جينات الضراوة للباستيرلا وهما *ptf*A , *tox*A. وكانت نسبة وجودهما ٤٠% وتم تسجيل كل من جينات الضراوة *ptf*A , *tox*A فى بنك العترات الدولى بكود MF167359, MF382009.