Molecular studies on *Pasteurella species* isolated from ducks

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Abstract

Duck cholera is a fatal, contagious and septicemic disease of ducks caused by Pasteurella species. A total of 150 ducks were collected from ten farms in Kaliobia Governorate suspected to be suffering from Pasteurellosis that manifested by respiratory signs, sudden death, and nervous manifestation. Collected Samples from these ducks were liver, spleen, heart and lung which subjected for bacteriological examination. A total of 33 Pasteurella strains were isolated, 25 strain were Pasteurella multocida (recovered from liver samples) and 8 strain were Pasteurella pneumotropica (5 strains recovered from lung and 3 strain recovered from heart). Finding of antibiotic sensitivity test showed that Pastreulla isolates were sensitive to florofinicole (80%) and moderately sensitive to ciprofloxacine (60%), enrofloxacin (50%) and followed by tobramycin (40%). Amoxicillin, oxytetracycline and penicillin were less sensitive (30% each) while isolates showed absolute resistance to erythromycin (100%) followed by resistance to gentamycin (90%) and naldixic acid (80%) for both types of Pasteurella. PCR results showed that Cytotoxic protein (toxA) toxcin virulence gene was detected in 4 out of 10 studied strains and fimbrial protein (ptfA) virulence gene was detected in 4 out of 10 studied strains. Sequences of toxA and ptfA genes were submitted to Gen Bank and assigned accession numbers were MF167359 and MF382009, respectively.

Key words: *Pasteurella multocida- Pasteurella pneumotropica- toxA- ptfA -antibiotic sensitivity test- PCR- ducks.*

Introduction

Pasteurella multocida belonging to family *Pasteurellaceae* is a ubiquitous organism affecting many host species, thus causing several diseases like haemorrhagic septicaemia in cattle and buffalo, enzootic bronchopneumonia in cattle, sheep and goats, atrophic rhinitis in swine, fowl cholera in poultry and snuffles in rabbits (Harper *et al.,* 2006 and *Dziva et al.,* 2008). *P. multocida* is identified as a major threat for a poultry industry which hampers the profitable poultry production (Sellyei *et al.,* 2010). Clinically ducks associated with pasterullosis showed anorexia, fever, ruffled feathers, depression, mucus discharge from mouth and nostrils, increase respiratory rate and diarrhea. On postmortem examination: Petechial and ecchymotic hemorrhages were common, particularly in subepicardial (heart) and subserosal (liver) locations, hemorrhages on the coronary band of heart, hemorrhages on air sac membranes adjacent to lungs were evident. The liver was swollen accompanied with multiple, small, necrotic foci (Mohan and Pradeep Kumar, 2008).

Based on capsular antigens, *P. multocida* strains are differentiated into five serogroups. 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). Ewers *et al.* (2006) studied the virulence profiling of *P. multocida* isolates from different hosts and subsequently it has been used by many authors to understand the diversity of the pathogen recovered from different host origin (Bethe *et al.,* 2009; Tang *et al.,* 2009; Garc´ıa *et al.,* 2011; Ferreira *et al.,* 2012; Furianetal, 2013; Katsuda *et al.,* 2013 and Verma *et al.,* 2013).

Important pathogen factors include capsular and other virulence-associated genes (Katsuda *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). The main virulence factors of *Pasteurella was* Endotoxins (lipopolysaccharides, LPS) are particularly important in the septicaemic diseases such as fowl

cholera and bovine haemorrhagic septicaemia. *Pasteurella 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, a virulent PMT-positive strain and virulent PMT-negative strain have both been reported. However, PMT plays a role in atrophic rhinitis (mild to severe destruction of porcine nasal turbinate bones) and Filamentous hemagglutinins ($PfhB_1$ and $PfhB_2$), surface fibrils (Hsf_1 and Hfs_2), and fimbrial subunits (PtfA, FimA, Flp_1, Flp_2) are adhered to host cells, chemotaxis (Dashe *et al.,* 2015), the ptfA gene of which assemble to form type 4 fimbriae on the bacterial surface (Sellyei *et al.,* 2010).

P. pneumotropica is type of *pasteurella* that its main carriers are rat and mice but the clinical signs are seen if infected animals are stressed, nude mice may developed retrobulbar abcesses in lacrimal gland .*P. pneumotropica* has been associated with conjunctivitis, rhinitis, otitis and cervical lymphadenitis in mice and rat (Baker, 2003).

Aim of the work

Our objective from this study to investigate *Pasteurella* species that isolated from ducks in Egypt and determine the most sensitive antibiotic effective for these strains and throw spot light on the role of the duck in disease transmission as research papers reported. The disease in ducks is sporadic and scarce although *Pasteurella* species is one of important fatal infection in ducks.

Material and methods

Sample collection:

Samples collection

A total of 150 ducks of different ages and sexes were examined from 10 different duck farms at Kaliobia Governorate for bacteriological examination. Samples were taken from freshly dead ones (liver, heart blood, lung, kidney and spleen from each duck) from suspected clinically affected cases. Each examined organ was taken alone in sterile plastic bag, kept in icebox and transferred with minimum delay to the laboratory for bacteriological examination.

Phenotypic identification and genotypic determination of virulence factors of *Pasteurella* species:

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 (Das, 1958 and Melody *et al.,* 1994); Mac Conkey's agar; (All plates were incubated for 24 hours at 37ºC. The developed colonies were picked up and subculture 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).

In-Vitro anti-microbial sensitivity test:

The isolated *Pasteurella species* strains 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)

Detection of toxA and ptfA genes of Pasteurella multocida and pneumotropica by PCR:

PCR was applied on 10 selected *Pastereulla* isolates by using two sets of primers for detection of two virulence genes Cytotoxic protein (toxA) and fimbrial protein (ptfA) that may play a role in virulence of *Pasteurella spp.*

Polymerase chain reaction

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) and listed in Table (1).

PCR amplification: 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, µl of water, and 6 µl of DNA template. The reaction was performed in an Appliedbiosystem 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, 15 µl of the products was loaded in each gel slot. A gelpilot 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.

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	CTTAGATGAGCGACAAGG GAATGCCACACCTCTATAG	864	94˚C/ 5 min.	94˚C 30 sec.	48˚C 40 sec.	72˚C 50 sec.	72˚C 10 min.	**16**
ptfA	TGTGGAATTCAGCATTTTAGTGTGTC TCATGAATTCTTATGCGCAAAATCCTGCTGG	488	94˚C/ 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	

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.

2- Multiple sequence alignment was conducted using ClustalW application embedded in ***BIOEDIT version 7.0.4.1 software.***

3- Sequence editing, correction, frame adjustment, Amino acid alignment and allocation of antigenic sites were also conducted using different options of BIOEDIT version 7.0.4.1 software.

5- All finely adjusted sequences were exported from BIOEDIT version 7.0.4.1 software as separate FASTA files.

6- FASTA files were inserted into MEGA 5.05 DNA alignment tool and exported into MEGA format (*.meg).

7- MEGA file was used as a base for phylogenetic analysis using neighbor joining method.

8- One handered bootstrap replicates were conducted to assess the statistical support for the tree topology.

9- The resultant trees were saved as photos.

10- 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 TOXA and bankit2026599 for PTF gen of pasteurella multocida,

11- Sequence accession number was received 2 working days from date of submission.

Results and discussion

Clinical cases

The most common observed clinical signs showed by affected ducks were in the form of sudden death, greenish diarrhea, nervous manifestation, locomotory disturbance, depression and mucus discharge from mouth and nostrils. The most observed post mortem lesions were swollen liver with petechial hemorrhages and hemorrhages on heart. Similar clinical signs and postmortem picture were reported in ducks associated with pasterullosis by (Mohan and Pradeep Kumar, 2008).

Isolation and identification: A total of 33 isolates from 150 suspected birds collected from 10 farms (liver; heart blood; lung; kidney and spleen) were identified as *Pasteurella species* on the basis of the conventional bacteriological technique, from 33 isolates *Pasteurella multocida* represent the highest isolation of 76% (25/33) while the *Pasteurella pnemotropica* represent 24% (8/33). Isolated bacterial colonies on blood agar plates were small, glistening, mucoid and dew drop like, and appeared as Gram-negative coccobacilli when stained with Gram's stain and Leishman's staining technique revealed bipolar microrganisms. The isolates failed to grow on MacConkey agar and were found to be non-haemolytic on blood agar. These features were in agreament with previous researches (Akhtar, 2013 and Ievy *et al.,* 2013). Details of cultivation and biochemical tests were showed in Table (2). Similar findings were confirmatory with the findings of Belal (2013).

Table (2): Cultivation and biochemical tests for isolates

Feature	*p.multocida*	*p.pnemotropica*
Macckoncy agar	-ve	-ve
Haemolysis on blood agar	No	No
Catalase test	+ve	+ve
Indole test	+ve	+ve
Oxidase test	+ve	+ve
Urea hydrolysis	-ve	+ve
Growth on TSI	Yellow	Yellow
V.P test	-ve	-ve
Simmon citrate	-ve	-ve
Lysin decarboxylase	-ve	-ve

In the present study *P. multocida* were isolated from ducks by total percent of 22%, (33/150), these result were nearly to that reported by Sayedun *et al.* (2015) and Kumar *et al.* (2004) who isolated *P. multocida* with percentage of 11.42 , 34% and less than Kamruzzaman *et al.* (2016) who isolated *P. multocida* with percentage of 59.72%, respectively . Detection of *P. multocida* infection in ducks indicates its transmitting through nearly established poultry farms as reported by (Botzler, 1991). The present finding of *P. pneumotropica* infection in ducks is the first report in Egypt, *P. pneumotropica* was 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.

Antibiotic sensitivity test:

Our findings of antibiotic sensitivity for twenty *Pasteurella* isolates by disc diffusion method revealed that all isolates exhibited variable response to different antibiotics as shown in

Table (3). *Pasteurella* isolates were sensitive to florofinicol (80%) and were moderately sensitive to ciprofloxacin (60%) followed by enrofloxacin (50%), then tobramycin (40%). Amoxicillin, oxytetracyclin and penicillin were (30%) per each, then naldixic acid was 20%, gentamycin was 10%. Whereas, *Pasteurella* isolates exhibited absolute resistance to erythromycin (100%). The obtained results were not in accordance with (Kamruzzaman *et al.,* 2016) who detected that ciprofloxacin was the most effective antibiotic by 95% followed by gentamycin (85%), tetracycline and amoxicillin (75% per each). Also our finding 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.

Table (3): antibiotic sensitivity for twenty *Pasteurella* isolates by disc diffusion method:

Sensitivity / Antibiotics agent	sensitive	intermediate	Resistance	No. of isolates	Sensitivity (%)
Ciprofloxacin(10µg)	12	-	8	12/20	60%
Gentamycin(10µg)	2	-	18	2/20	10%
Tobramycin	8	-	12	8/20	40%
Amoxicillin(20µg)	6	-	14	6/20	30%
Erythromycin(10µg)	-	-	20	0/20	0.0%
Enrofloxacin(10µg)	10	3	7	10/20	50%
Oxytetracyclin(10µg)	6	-	14	6/20	30%
Pencillin	6	2	12	6/20	30%
Naldixic acid	4	-	16	4/20	20%
Florofinicol(30µg)	16	-	4	10/20	80%

Results of PCR:

In our study two virulence genes were detected by PCR test, *tox*A and *ptf*A genes by 40%, (4 out of 10 samples per each) (Table 4), the obtained results are similar to that obtained by Thales *et al.* (2016) who detected of *ptf*A, *tox*A and other genes in *Pasteurella* isolates.

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

Amplification of ptfA and toxA genes in pasteurella isolates:

The obtained results revealed that *ptfA* gene was detected in four out of 10 *Pasteurella* examined isolates and gave a characteristic band at 488bp (Fig. 1) whereas tox*A* gene was detected

only in 4 isolates out of 10 examined ones and gave positive amplification at 864bp as shown in (Fig. 2).

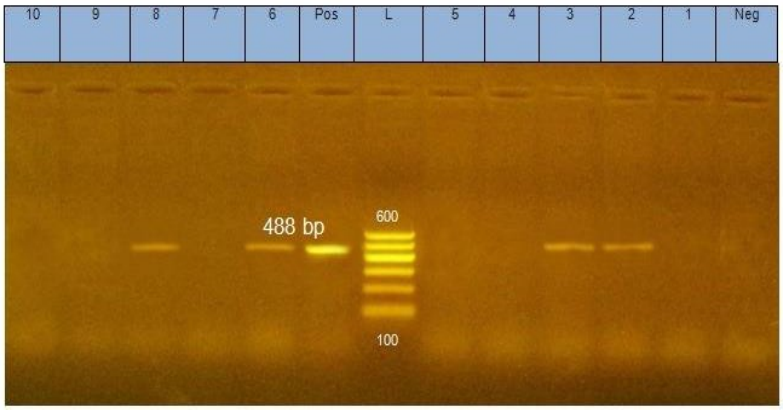


Fig. 1: Agarose gel electrophoresis of *ptf*A gene gene in 10 *Pasteurella* isolates, M: 100 bp DNA marker, lanes (2, 3, 6 and 8): positive amplification of *ptfA*gene at 488 bp, Positive control: standered strain from AHRI Dokki, Negative control.

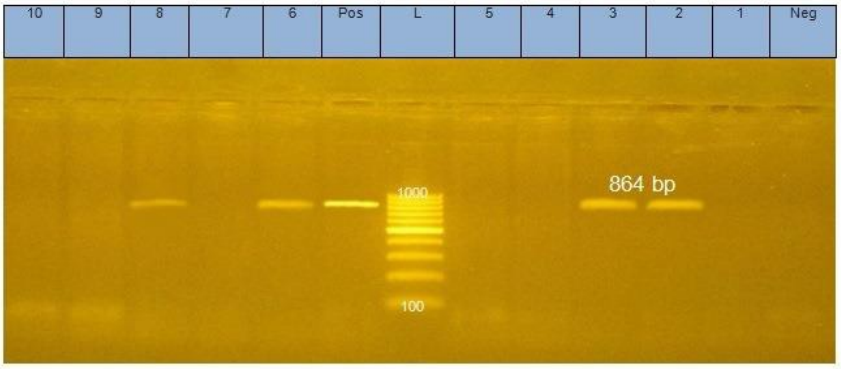
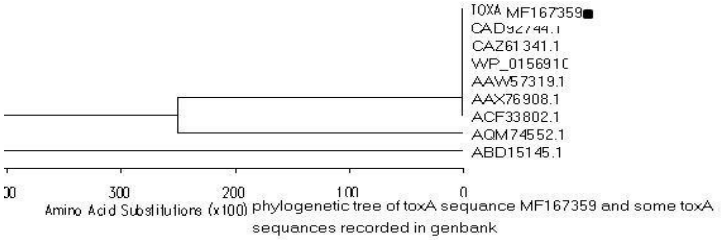


Fig. 2: Agarose gel electrophoresis of *tox* A gene in 10 *Pasteurella* isolates, M: 100 bp DNA marker, lanes (2, 3, 6 and 8): positive amplification of *tox*A gene at 864bp, Positive control: standered strain from AHRI Dokki, Negative control.

Nucleotide sequence accession number

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

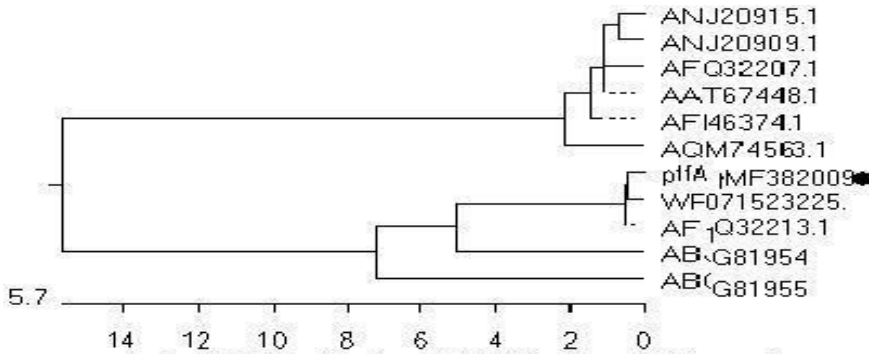


Percent Identity

	1	2	3	4	5	6	7	8	9	
1		98.9	98.9	98.9	98.9	98.9	98.6	3.5	20.0	1
2	0.0		100.0	100.0	100.0	100.0	99.6	4.3	5.0	2
3	0.0	0.0		100.0	100.0	100.0	99.6	4.3	5.0	3
4	0.0	0.0	0.0		100.0	100.0	99.6	4.3	5.0	4
5	0.0	0.0	0.0	0.0		100.0	99.6	4.3	5.0	5
6	0.0	0.0	0.0	0.0	0.0		99.6	4.3	5.0	6
7	0.4	0.4	0.4	0.4	0.4	0.4		4.3	5.0	7
8	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0		5.0	8
9	195.0	195.0	195.0	195.0	195.0	195.0	195.0	195.0		9
	1	2	3	4	5	6	7	8	9	

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

Precent identefy of pasteurella toxA MF167359 and some strains in gene bank



phylogenetic tree of ptfA accesion number MF382009 of our study and someot other accesion numbers recorded in genbank

Percent Identity

	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	

MF382009
ptfA
AFQ32213.1
WP_071523225.1
ABG81955.1
ABG81954.1
AQM74563.1
AAT67448.1
ANJ20915.1
AFI46374.1
AFQ32207.1
ANJ20909.1

Precent identfy between pasteurella ptfAgene (MF382009) and some strains in gene bank

Phylogenetic analysis and nucleotide comparison

The nucleotides sequences of toxA gene and ptfA gene showed percent identity with.1, EGP03065.1) which Submitted (22-JUN-2011). The obtained genetic the selected sequences published on gene bank ranged from 98%-100%. Most of the aligned sequences were isolated from chicken as AQM74552.1, which Submitted (27-AUG-2016) and AFQ32207.1which Submitted

(05-JUN-2012) while others were isolated from wild birds as Anand1_poultry （EGP02957data 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

We concluded from the present study pay attention of scientist to pasterullosis in ducks as the disease cause deaths in duck flocks and subsequently economic loss. *P. pneumotropica* was firstly isolated from duck in Egypt. Florofinicol is the drug of choice for treatment of *Pasteurella* in ducks.

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