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Molecular Studies on *Pasteurella multocida* in Ducks

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ABSTRACT

Pasteurella multocida is the common cause of Duck septicemia (Pasteurellosis) which affects ducks leading to high economic losses to duck producers. *Pasteurella multocida* infections are associated with severe, life-threatening systemic disease involving both hemorrhagic pneumonia and septicemia. A cross-sectional study was carried out on 220 samples collected from different breeds of ducks (Mallard, Muscovy and Baladi) from Minufiya governorate, Egypt. Only 16 cases were positive for *Pasteurella multocida* infection (7.3%). These isolates were confirmed microscopically, biochemically and rapidly by using Vitek2 compact system. The application of PMT-ELISA and mouse lethality test for the 16 isolates of *P.multocida* serotypes for differentiation between toxigenic and non toxigenic isolates. All tested of 16 isolates showed to be toxigenic by using PMT-ELISA and mouse lethality test. The application of PCR and multiplex PCR for the detection of *tox* A gene and capsular serotyping of toxigenic isolates of *P.multocida* respectively. All 16 *P.multocida* isolates were positive to *tox* A and capsular type A (100%). This study concluded that *P. multocida* serve as a major cause of Duck septicemia (Pasteurellosis) which affects ducks leading to high economic losses in poultry industry. Also indicated that the majority of *P. multocida* serotypes are toxigenic.

Keywords: *P. multocida*, Ducks, *PMT-ELISA*, *tox*A gene.

INTRODUCTION

Among poultry, ducks are considered as a second widespread species following the chickens in Egypt and are being considered as an asset to the poor farmers (Ramachandran and Ramakrisnan, 1982). Duck cholera is a fatal, contagious and septicemic disease of ducks caused by *P. multocida*—a bacterium with a broad host range and ubiquitous distribution (Ramachandranpillai *et al*., 2012). It also causes severe economic losses not only in terms of mortality but also in terms of low productivity of egg and meat (Baki *et al*., 1991).

P. multocida is a Gram negative, non-motile, non-spore forming rod shaped organism occurs in single or in pairs or occasionally as chain forms or filament. A capsule can be demonstrated using indirect method of staining of freshly cultured bacteria by Hiss's stain (Calnek *et al*., 1997). The organisms show bipolar character in Leishman's stain (Buxton and Fraser, 1977). *P. multocida* pathogen consists of five capsular type A, B, D, E, and F and there is relationship exist between the capsular type and disease predilection (Boyce and Adler, 2001).

P. multocida classification recently based on combination of capsular typing with Heddleston somatic typing for example A: 1 indicates a strain that is its capsular group is A and lipopolysaccharide 1, the serogroup is generally related to the diseases (Rimler *et al.*, 1984)

The technique of VITEK2 has improved the field of bacterial screening by providing a much faster, more reliable cheaper and highly sensitive technique for bacterial identification. (Wallet *et al.,* 2005)

Differentiation between toxigenic and nontoxigenic strains of *Pasteurella multocida* is essential for the control and diagnosis of the diseases caused by the microorganism. In addition, health monitoring programmes should be based on laboratory tests for determination of toxigenic strains of *Pasteurella multocida* combined with clinical inspections of flocks. Previous methods of differentiation have relied on the biological activities of *Pasteurella multocida* toxin, as lethality in mice, dermonecrotic effect in guinea pigs and cytopathic effect on embryonic bovine lung (Eamen *et al.,* 1988)

Enzyme linked immunosorbent assay (ELISA) is an important diagnostic serological assay. It is simple, speed, efficient, cost-effective of procedures as a cell culture is not need. One laboratory worker can perform it for several hundred samples besides being highly sensitive and specific (Takada-Iwao *et al.,* 2007). Detection of *Pasteurella multocida* toxins using ELISA was employed by different authors (Alt *et al.,* 1993, Weber *et al.,* 1993 and Matschullat *et al.,* 1994). They reported a high percentage for *Pasteurella multocida* toxin using ELISA rather than other methods. Moreover, (El-Eragi *et al.,* 2001) and (Takada- Iwoa *et al.,* 2007) declared that ELISA had sensitivity at least 86% and 99% specificity. On the other hand, (Filion *et al.,* 1985) detected 100% sensitivity and specificity using ELISA.

Polymerase chain reaction (PCR) has been developed for rapid, sensitive and specific detection of *Pasteurella multocida* within 3-4 hours (Miflin and Blackall, 2001).

MATERIALS AND METHODS

Sample collection and processing

A total of 220 samples collected from ducks of different breeds (33 Mallard, 136 Muscovy and 51 Baladi (native breed)) showing diarrhea, respiratory disorders and mortalities, samples were collected from Minufiya governorate, Egypt. The obtained samples were bone marrow and brain from 75 freshly dead birds. Lung, liver, heart blood, spleen, joints and pericardial exudates from 45 live birds and 100 nasal swabs from live birds.

Nasal swabs were collected by means of sterile cotton swabs then the collected swabs were placed in sterile tubes containing brain heart infusion broth and transported in ice box, while specimens from aseptically collected tissue (livers, spleens, heart blood, lungs, pericardial exudates, joints, bone marrow and brains) were obtained using sterile wire loop through a seared surface.

Samples were collected aseptically and transferred for further bacteriological examination at the bacteriology labs, Faculty of Veterinary Medicine, University of Sadat City, Minufiya, Egypt. The collected samples were streaked directly onto the surface of blood agar and on MacConkey agar media then incubated at 37˚C in a candle jar for 24-48 hours as described by (Glisson *et al.,* 2008).

The suspected isolates (n=36) were identified morphologically by Gram staining according to (Cruickshank *et al*., 1975) and biochemically following the standard methods as described by (Glisson *et al*., 2008) and (OIE, 2008).

Typical *P. multocida* colonies were examined for their size, color, consistency, shape and microscopic examination after Gram's staining (bipolarity). For the conformation of *P. multocida* , biochemical reactions including Oxidase, Catalase, Indole, Citrate utilization, urease, Triple sugar iron agar medium (TSI) and Sugar fermentation test.

Identification of *P. multocida* isolates by Vitek2 compact system (BioMe'rieux, 2006).

Inoculum Preparation: From the isolated colonies grown on the Mueller Hinton agar 24 h at 37˚C, a bacterial suspension was prepared in 3 mL of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) in a 12x75 mm clear plastic (polystyrene) test tube. The turbidity of the suspension was adjusted to a McFarland standard of 0.5 with the help of a VITEK-2 DensiCheck instrument. The time between the preparation of inoculum and filling of the card was always less than 30 min. Identification with the VITEK-2 system was performed using a Gram Negative (GN) card according to the Manufacturer's instructions. The culture suspension was inoculated into the GN Card with the help of a vacuum device inside the filling chamber. The cards were later transferred into the loading

chamber where the cards were sealed and were incubated in a rotating carousel at 37˚C. Each loaded card was removed from the carousel for every 15 minutes, transported to the optical system for reaction readings and the returned to the carousel incubator until the next read time. Data was collected at 15-minute intervals during the entire incubation period.

Mouse lethality bioassay

P. multocida isolates were tested for their ability of toxin production. Each isolate of *P. multocida* strain was cultured on blood agar and incubated for 24 hrs at 37°C. All mice were injected intraperitoneally (I/P) by 0.1 ml of bacterial suspension of (1.0 x 10^3 CFU). The mortality rates and post mortem changes were recorded from heart blood of dead mice, re-isolation of inoculated strains was carried out and blood films were prepared and stained with Leishman's stain following the standard methods described by (Ozbey and Muz, 2006).

One step immunoassaey (PMT)

All suspected colonies on the agar plate were harvested after overnight incubation at 37 ˚C for detection of *P. multocida* toxin by one step immunoassaey (PMT) (Foged *et al*., 1987)

Molecular characterization of *P. multocida* isolates

Extraction and purification of DNA

Two hundred 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 provided in the kit. Oligonucleotide Primer that used were supplied from Metabion (Germany) are listed in table (1). PCR ampilification cycling of the gene was applied with the temperature and time conditions of primer during PCR that are shown in (Table 2).

The amplification For *tox* A gene PCR, primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp GT 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, However in serotyping by multiplex PCR, 11 µl of DNA template. The reactions were performed in an Applied biosystem 2720 thermal cycler as listed in table (2).

Analysis of the PCR Products: (Sambrook, *et al.*, 1989)

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. Gelpilot 100 bp and 100 bp plus DNA Ladders (Qiagen, Germany, GmbH) 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 : Oligonucleotide primers sequences and amplified PCR product for *P. multocida* genes used in PCR.

Target gene	Sequence	Amplified product	Reference
tox A	ATC-CGC-TAT-TTA-CCC-AGT-GG	460	OIE (2012)
	GCT-GTA-AAC-GAA-CTC-GCC-AC		
Serogroup A	GC-CAA-AAT-CGC-AGT-GAG	1044	
	TTG-CCA-TCA-TTG-TCA-GTG		
Serogroup B	CAT-TTA-TCC-AAG-CTC-CAC-C	760	
	GCC-CGA-GAG-TTT-CAA-TCC		
Serogroup D	TA-CAA-AAG-AAA-GAC-TAG-GAG-CCC	657	
	CAT-CTA-CCC-ACT-CAA-CCA-TAT-CAG		
Serogroup E	TCC-GCA-GAA-AAT-TAT-TGA-CTC	511	
	GCT-TGC-TGC-TTG-ATT-TTG-TC		
Serogroup F	AAT-CGG-AGA-ACG-CAG-AAA-TCA-G	851	
	TTC-CGC-CGT-CAA-TTA-CTC-TG		

Table 2 : Cycling conditions of the primer during PCR

Target agent	Primary denaturation	Secondary denaturation	Annealing	Extension	No of cycles	Final extension
P. multocida	94˚C / 10 min.	94˚C / 1 min.	55˚C / 1 min.	72˚C / 1 min.	35	72˚C / 10 min.

RESULTS

Prevalence of *P. multocida* in ducks suffer from duck septicemia.

The results revealed that the prevalence of *P. multocida* in ducks suffer from duck septicemia was higher in Muscovy ducks 136 (4.5%), then Baladi (native breed) 51 (1.8%) and mallard ducks 33 (0.9%). While the overall prevalence rate from all collected samples (220) was 7.3% as showed in table (3).

Table 3 : Overall prevalence rate of *P. multocida* from different cases.

Affected bird	Samples type	*P. multocida* colonies		Ducks					
		No	%	Mallard		Muscovy		Baladi	
				No.	%	No.	%	No.	%
				1	25	2	14.2	2	22.2
Freshly dead "5"birds, 6.6%	Bone marrow	2	2.6	-	-	1	7.1	1	11.1
	Brain	5	6.6	1	25	2	14.2	2	22.2
	Total	7	4.6	1	12.5	3	10.7	3	16.6
Severly disease "4" birds, 8.8%				1	33.3	2	20	1	33.3
	Lung	4	8.8	1	33.3	2	33.3	1	33.3
	Liver	2	4.4	-	-	1	-	1	33.3
	Spleen	4	8.8	1	33.3	2	33.3	1	33.3
	Heart blood	4	8.8	1	33.3	2	33.3	1	33.3
	Joints	4	8.8	1	33.3	2	33.3	1	33.3
	Pericardial exudate	4	8.8	1	33.3	2	33.3	1	33.3
	Total	22	8.1	5	27.7	11	18.3	6	33.3
Mild disease "7"birds,7%				-	-	6	22.2	1	16.6
	Nasal swabs	7	7	-	-	6	22.2	1	16.6
	Total	7	7	-	-	6	22.2	1	16.6
Total"16" 7.3%	Total samples	36	6.9	6	19.4	20	17.4	10	23.8

Phenotypic and Biochemical identification of *P. multocida* obtained from infected ducks.

Suspected colonies were identified by Gram staining which appeared as Gram negative, capsulated, bipolar, non-motile, non-spore forming, also represented the same biochemical profile of *P.multocida* oxidase positive, production of indol, MR and VP negative, urease negative, citrate utilization negative, no production of hydrogen sulfide, and TSI (A/A).

Both Gram-negative coccobacilli and oxidase- positive isolates were subculture onto blood agar medium, at which *P. multocida* colonies were small non-haemolytic, dew drop-like, non-mucoid and iridescent colonies that are smooth and no pigmented. Regarding to the biochemical identification of *P. multocida* All tested isolates were confirmed using VITEK2 compact system by using specific Gram negative card and the result revealed that excellent identification with 98% probability of *P.multocida*.

Differentiation between toxigenic and nontoxigenic isolates of *P. multocida* isolates from infected ducks:

One step enzyme immunoassay for detection of *Pasteurella multocida* toxin

All suspected *P.multocida* isolates are tested for detection of toxin by one step enzyme immunoassay for detection of *Pasteurella multocida* toxin (PMT), which resulted that all isolates are toxigenic as showed in table (4) and fig (1).

Table (4): optical density value of *P.multocida toxin isolates by PMT-ELISA* (one step enzyme immunoassay)

Health status	Number of examined samples	Number of positive cases	OD readings of control -ve samples	OD readings of control +ve samples	Range OD readings of +ve samples	Toxigenic *P.multocida* isolates	%
Mild disease	100	7	0.135	0.53	0.72 to 1.3	7	100%
Severly disease	45	4				4	100%
Freshly dead	75	5				5	100%
Total	220	16				16	100%

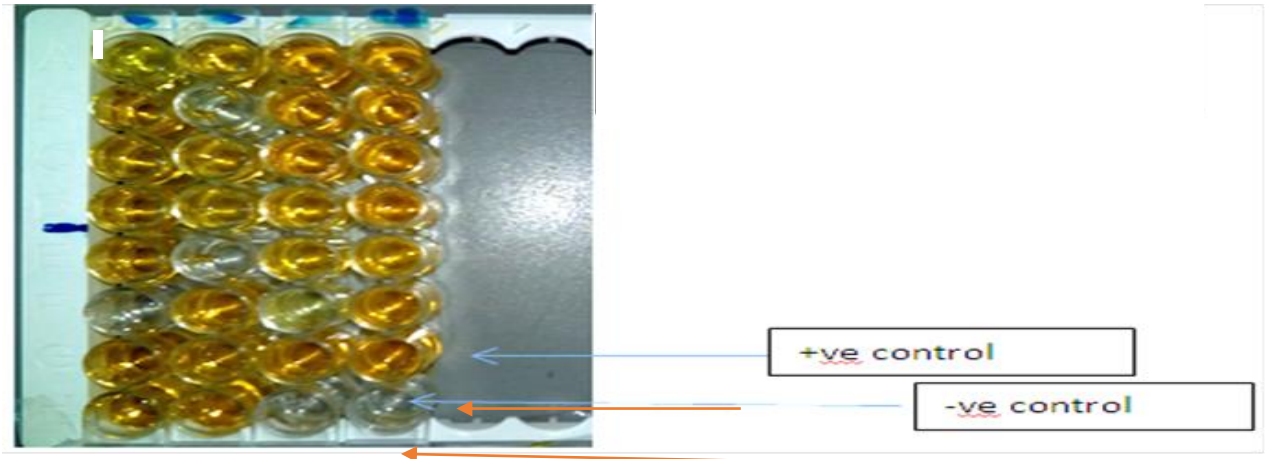

Fig. (1) ELISA plate showing toxigenic isolates of *P.multocida*.

- **Mouth lethality test for identification of toxigenic *P.multocida*:**

All mice inoculated with the suspected *P. multocida* isolates were died within 24-72 hours, showing generalized septicemia with highly congested trachea, lungs and enlarged spleen as showed in fig. (2).

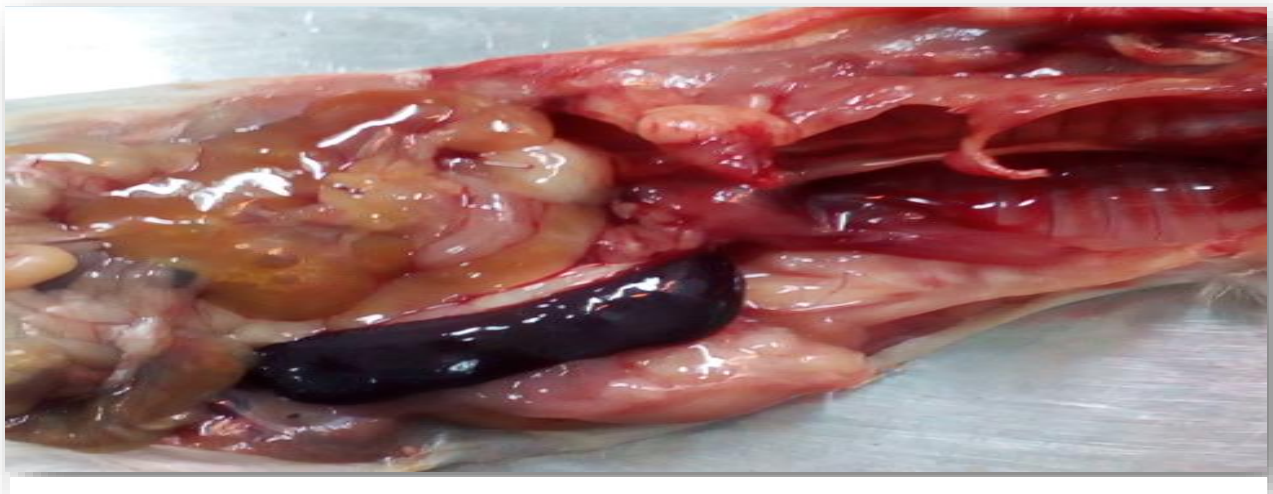

Fig. (2) Dead mice showing septicemia and splenomegaly

Pure cultures of inoculated isolates were obtained from the heart blood of the dead mice. Stained smear from the heart blood demonstrated a large number of bipolar organisms when stained with Leishman's and Giemsa stain as showed in fig. (3).

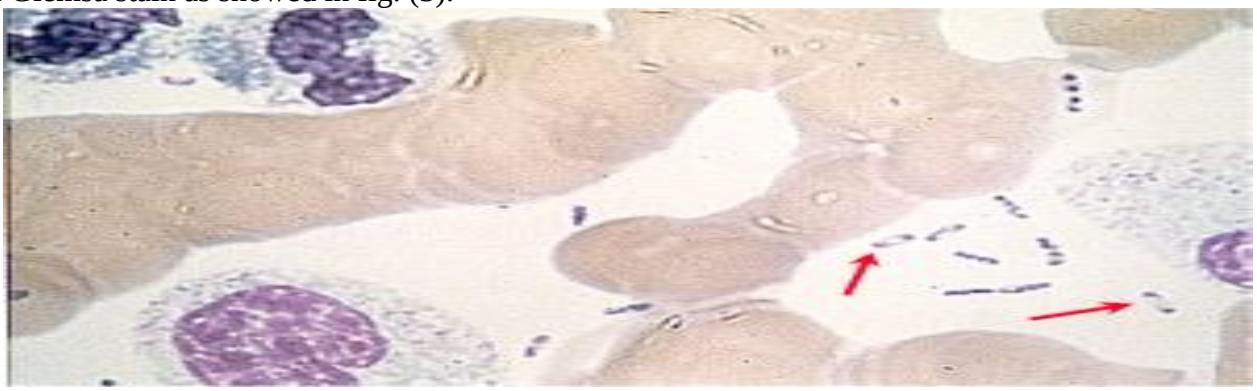

Fig. (3) Impression smear from lung tissue stained by Leishman stain showing bipolarity of *Pasteurella multocida* isolates.

Molecular detection of *Pasteurella multocida* isolates:

- **PCR for confirmation of toxigenic *P. multocida* isolates using *tox* A gene:**

The results revealed that *tox* A gene was detected in all 16 isolates (100 %) of tested *P. multocida* isolates by PCR reaction with amplified fragment 864 pb as showed in fig (4).

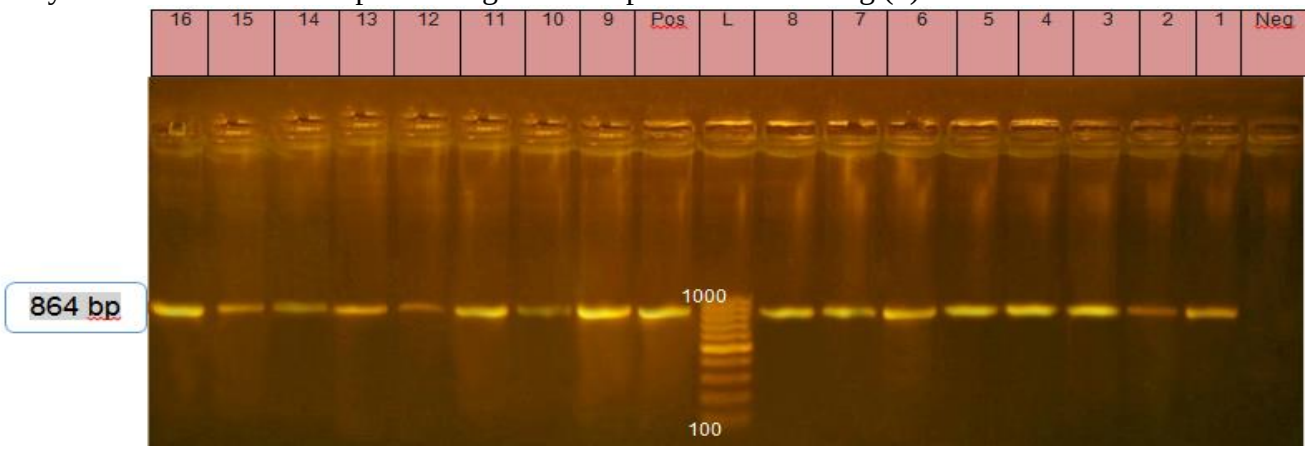

Fig. 4: 1.5% Agarose gel electrophoresis of *PCR* product of *tox* A gene at 864 pb of *P. multocida*. P: for positive control, "Neg"; Negative control; Lane L (100-1000 bp marker); Lanes 1 to 16 (Positive *P. multocida tox*A gene) at (864 bp).

Capsular serotyping of toxigenic *P. multocida* isolates:

All 16 *P.multocida* isolates which were positive *tox* A gene, were submitted for capsular typing by multiplex PCR and found that all isolates were positive to capsular type A (100%) with amplified fragment 1044 pb as showed in fig (5).

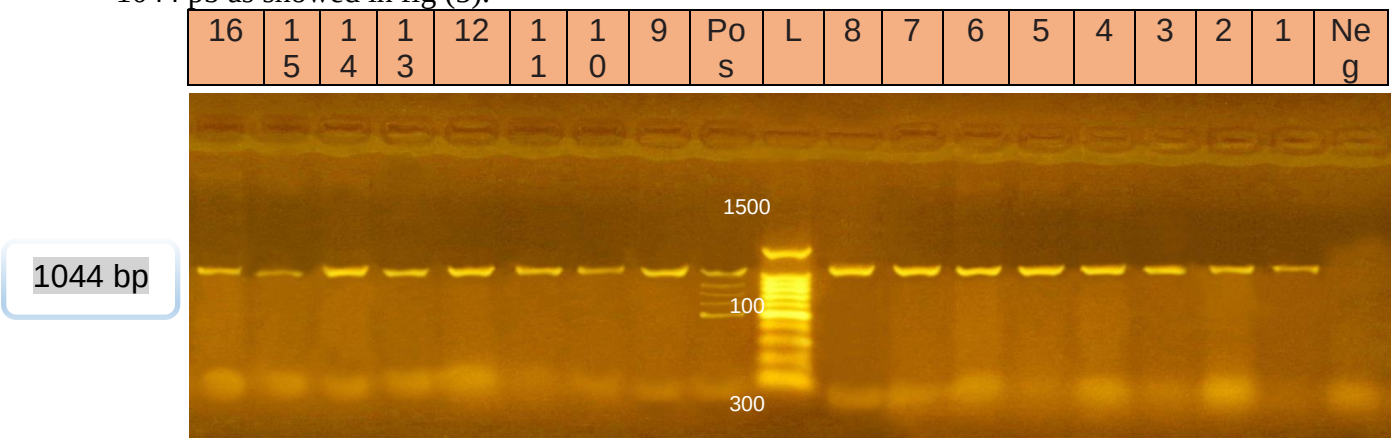

Fig. 5: 1.5% Agarose gel electrophoresis of *multiplex* PCR for molecular typing of toxigenic *P. multocida* isolates. at 1044pb. P: for positive control, "Neg"; Negative control;
Lane L (100-1500 bp marker); Lanes 1 to 16 (Positive *P. multocida* capsular type A gene) at (1044 bp).

DISCUSSION

Pasteurella multocida is the common cause of duck septicemia (Pasteurellosis) which affects ducks leading to high economic losses in poultry industry. *Pasteurella multocida* infections are associated with severe, life-threatening systemic disease involving both hemorrhagic pneumonia and septicemia (Kamruzzaman *et al.,* 2016).

P. multocida causes severe clinical manifestations in ducks including exhaustion, drowsiness, loss of appetite, ruffled feathers, swimming in circles, lameness, laboured breathing, cyanosis, watery green-yellowish diarrhea and mucous discharge from the mouth. As for postmortem lesions, they include: haemorrhages in the heart, liver, gizzard and intestines besides white spots and necrotic foci in the liver and spleen as described by Abd-El-Rahman *et al.,* (2009).

In the present study, out of 220 ducks which suffer from duck septicemia examined, sixteen samples were positive to *P. multocida* (7.3 %) as obtained with Eid *et al.,* (2019) who isolated *P. multocida* from ducks with proportion of (7.3%), but it is lower than the level reported by Zhangcheng *et al.,* (2018) who isolated (13.4%) of *P. multocida* from ducks in china, (Bhattacharya, 2005) who recorded 18 isolates of *P. multocida* with a high percentage (77.77%) of *P. multocida* isolates that were recovered from ducks, Walaa and Lamyaa (2016) who isolated (63%) of *P. multocida* in duck flocks and Kamruzzaman *et al.,* (2016) who isolated (34%) *P. multocida* from sick ducks.

These results are higher than the level reported by (Mbuthia *et al.,* 2008) who recorded an isolation rate of 6.2% and (Muhairwa et al., 2001) who recorded that The prevalence of *P. multocida* was 7%. However All isolates were investigated using conventional (biochemical) methods and reinvestigated using VITEK2 compact system. All suspected colonies were oxidase, catalase positive and urease, methyl red, Voges-Proskauer negative. Our results were in agreement with different authors who found Vitek give reliable, rapid and higher correct identification results (Funke *et al.,* 1998, Gavin *et al.*, 2002, Ling *et al.*, 2001 and Chatzigeorgiou *et al.,* 2011). All biochemical results of *P. multocida* isolates in the present study agree with the biochemical findings of Karaivanov (1984).

All *P. multocida* strains were investigated for toxin production by mouse lethality test and PMT ELISA (one-step enzyme immunoassay). All positive *P.multocida* strains, were proved to be toxigenic strains in incidence of 100% using mouse lethality and one-step enzyme-linked immunoasorbent assay. These results were in agreement with El-Eragi *et al.,* (2001) and Takadaiwao *et al.,* (2007) who revealed that the sensitivity and specificity of ELISA technique is 86% and 99% respectively. On the other hand, Filion *et al.,* (1985) detected 100% sensitivity and specificity of ELISA technique, similar to Khalid *et al.,* (2017) who showed that all the recovered strains of *P. multocida* killed all the inoculated mice within 24–72 hr.

In this study All 16 positive *P. multocida* isolates with PMT-ELISA were submitted for identification of *tox* A gene by PCR and found that all isolates were positive to *tox* A gene with amplified fragment 864 pb. These findings were similar to Many authors; Ewers *et al.,* (2006), Shayegh *et al.,* (2010) and Khamesipour *et al.,* (2014) who recorded *tox* A genes in *P. multocida* isolates.

Then all 16 *P. multocida* isolates that were applied for *tox* A gene detection were submitted for capsular typing by multiplex PCR and found that all isolates were positive to capsular type A with amplified fragment 1044 pb, these results were in agreement with Zhangcheng *et al.,* (2018) who recorded that the capsular types of *P. multocida* were confirmed by multiplex PCR. As the results of multiplex PCR specific for capsular antigens of *P. multocida*, 22 strains (95.7%) were found type A with a 1044 bp amplified fragment, whereas 1 strain (4.3%) was type F with 851 bp length fragment. Zhong *et al.,* (2018) concluded that capsular typing strategy identified two categories of capsular genotypes (A and F) for the 16 avian *P. multocida* isolates, type A was the most common capsular genotype for the avian *P. multocida* isolates 93.75% .

CONCLUSION

Incidence of *P. multocida* in Muscovy breed is higher than that is found in Baladi and Mallard breeds. Concerning to the confirmatory identification by Vitek2 compact system using Gram negative card is based on established biochemical methods and the system identify an organism by using a methodology based on the

characteristics of the data have been collected from known strains to estimate the typical reactions of the claimed species to set of discriminating.

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