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CHARACTERIZATION OF *PASTEURELLA MULTOCIDA* IN DIFFERENT EGYPTIAN CHICKEN FLOCKS

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ABSTRACT

In the present study, phenotypic and genotypic characterization of *Pasteurella multocida* (*P. multocida*) strains from different Egyptian layers and breeders chicken flocks was carried out. Organs were collected from 55 chicken's flocks represented 39 layers and 16 broilers and layers breeders in Egypt during the period 2016-2017. Six birds were taken from each suspected flock with a total number 330 birds that representing 234 and 96 freshly dead layers and breeders chickens, respectively. Suspected organs containing *P. multocida* were subjected for isolation and identification of the organism, as well as testing of pathogenicity in mice. Moreover, genotypic characterization of *P. multocida* isolate was done using Polymerase Chain Reaction (PCR). Capsular and somatic typing was done. The phenotypic and genotypic characterization results revealed that 6 flocks were positive for *P. multocida* out of 55 ones (10.9%). Out of 330 dead chickens, 36 (10.9%) were positive for *P. multocida*. All *P. multocida* strains were pathogenic for mice. Capsular typing of *P. multocida* using multiplex PCR showed that all strains were type A, while somatic typing revealed presence of types 1 and 3. It could be concluded that *P. multocida* is circulating in layers and breeder chicken flocks in Egypt creating severe economic losses.

Keywords: *Pasteurella,* PCR, Layers and Breeders, Egypt.

INTRODUCTION

Fowl cholera (FC) is one of the most important contagious world-wide diseases adversely threatens poultry production (OIE, 2008 and Xiao *et al.*, 2015). In chickens, FC is an acute septicaemic disease causing high morbidity and mortality or chronic infections (Glisson *et al*., 2008 and Furian, *et al*., 2016). The disease severely affects layers and breeders flocks (Kumar *et al*., 2004 and Wang *et al*., 2009). FC is caused by *Pasteurella multocida* (*P. multocida)* bacteri which belongs to Family *Pasteurellaceae*. *P. multocida* is Gram-negative coccobacilli, non-motile and non-spore former capsulated microorganism (Eigaard *et al*., 2006). Avian *P. multocida* capsular sero-group are A, B, D and F, while somatic serotypes are 1 to 16. Serotypes A:1, A:3 A:3,4 and D are considered the most common cause of outbreaks of FC in most countries (Ranjan *et al*., 2011, Zahoor *et al*., 2014 and Hossain *et al*., 2018). Diagnosis of FC is still depends on clinical and pathological conditions and traditional identification of *P. multocida* morphologically, biochemically (Mutters *et al*., 1985) and serologically (Carter, 1955 and Rimler and Rhoades, 1987). Conventional diagnostic methods for *P. multocida* detection is not effective in all cases since it is time consuming and less sensitive as compared to molecular technique like, polymerase chain reaction (PCR) (Kamp *et al*., 1996). For more specific and rapid detection of *P. multocida*, PCR is used (Townsend *et al*., 1998, Sellyei *et al*., 2008, Panna *et al*., 2015 and Li *et al*., 2018).

In spite of medication and vaccination, the incidence of *P. multocida* infection increases (Jonas *et al*., 2001). So, the aim of this work was to phenotypically and genotypically characterize *P. multocida* in layers and breeders chicken flocks in different Egyptian governorates.

MATERIALS AND METHODS

Samples: Organs were collected from 55 chicken's flocks represented 39 layers and 16 broilers and layers breeders in El-Sharqia, El-Gharbia, El-Qalubia and El-Minofia governorates, Egypt during the period September 2016 till August 2017. The history of suspected flocks is presented in Table (1). The total number of examined flocks distributed in different Egyptian governorates is illustrated in Table (2). The suspected flocks were suffered from mortalities (10-15%) and respiratory manifestations. Post-mortem lesions were septicaemia, congestion of all internal organs, pin headed necrotic foci with congestion of the liver, severe spleen congestion and pneumonia. Samples including; liver, heart and spleen were taken from freshly dead birds. The samples were uniquely identified, transported in sterile plastic bags to the laboratory and kept in refrigerator at 2-5 °C till examined.

Table 1. History of the suspected *P. multocida* infected Egyptian flocks.

Farm No.	Governorate	Type of production	Age / weeks	Number of chickens	*Vaccination
1	El-Sharqia	Layer	25	7000	-
2	El-Sharqia	Layer	28	15500	-
3	El-Sharqia	Layer	38	10300	-
4	El-Sharqia	Layer	39	9000	+
5	El-Sharqia	Layer	40	13245	-
6	El-Sharqia	Layer	31	5320	-
7	El-Sharqia	Layer	26	4000	+
8	El-Sharqia	Layer	32	15000	+
9	El-Sharqia	Layer	90	19000	+
10	El-Sharqia	Layer	43	19000	-
11	El-Sharqia	Layer	40	15000	+
12	El-Sharqia	Layer	46	17200	-
13	El-Sharqia	Layer	25	14400	-
14	El-Sharqia	Layer	26	14000	-
15	El-Sharqia	Layer	43	17000	+
16	El-Sharqia	Breeders	28	14000	-
17	El-Sharqia	Breeders	48	11000	+
18	El-Sharqia	Breeders	32	50001	+
19	El-Sharqia	Breeders	64	13600	+
20	El-Sharqia	Breeders	29	19000	-
21	El-Sharqia	Breeders	21	10000	+
22	El-Sharqia	Breeders	33	17500	-
23	El-Gharbia	Layers	20	18000	+
24	El-Gharbia	Layers	21	17500	-
25	El-Gharbia	Layers	40	17500	-
26	El-Gharbia	Layers	16	16000	+
27	El-Gharbia	Layers	29	16000	+
28	El-Gharbia	Layers	62	16500	-
29	El-Gharbia	Layers	18	12000	+
30	El-Gharbia	Layers	18	20000	-
31	El-Gharbia	Layers	24	15000	+
32	El-Gharbia	Layers	25	16000	-
33	El-Gharbia	Breeders	38	17000	+
34	El-Gharbia	Breeders	35	15600	+
35	El-Gharbia	Breeders	47	16500	+
36	El-Gharbia	Breeders	50	13000	+
37	El-Qalubia	Layer	79	15000	+
38	El-Qalubia	Layer	10	10000	-
39	El-Qalubia	Layer	53	15000	+
40	El-Qalubia	Layer	21	12000	+
41	El-Qalubia	Layer	117	17000	+
42	El-Qalubia	Layer	28	14000	-
43	El-Qalubia	Layer	9	16000	-
44	El-Qalubia	Layer	24	19000	-
45	El-Qalubia	Breeder	24	14000	+
46	El-Gharbia	Breeder	43	23000	+
47	El-Qalubia	Breeder	26	18500	+
48	El-Minofia	Layer	27	16000	+
49	El-Minofia	Layer	10	18500	-
50	El-Minofia	Layer	31	18000	+
51	El-Minofia	Layer	28	19000	+
52	El-Minofia	Layer	22	16000	-

53	El-Minofia	Layer	28	14400	+
54	El-Minofia	Breeder	98	19000	+
55	El-Minofia	Breeder	39	16500	+

- = Not vaccinated + = Vaccinated

*Vaccination against FC was done using inactivated oil adjuvant bacterine prepared from local *P. multocida* serotypes A: 1, 3 and 4 strains. Chickens were vaccinated at 8-10 weeks old and boostered at 16-17 weeks old using intramuscular route.

Table 2. Total number of examined flocks distributed in different Egyptian governorates.

Governorate	Type of production		Total
	Layers	Breeders	
El-Sharqia	15	7	22
El-Gharbia	10	4	14
El-Qalubia	8	3	11
El-Minofia	6	2	8
Total	39	16	55

Phenotypic characterization of *P. multocida*

Isolation and identification: Six chickens were taken from each suspected flock with a total number 330 birds that representing 234 and 96 freshly dead layers and breeders chickens; respectively. From each bird; liver, heart and spleen samples were collected and inoculated under aseptic condition into brain heart broth and incubated at 37°C for 18-24 hrs as described by Cowan (1985). Subsequent selective subculture *P. multocida* isolates was done on sheep blood agar, modified Das media and MacConkey agar under aerobic conditions at 37°C for 28 hrs to obtain pure cultures. The agar plates were checked every day for suspected *P. multocida* colonies. Pure cultures were subjected for Gram staining for morphological identification of the bacteria (Cheesbrough, 2006). Biochemical identification was done using catalase, oxidase, Indol production, nitrate reduction, H_2S production, methyl red, Voge's proskaur, urease activity, gelatin liquefaction and sugar fermentation tests (Blackall and Miflin, 2000). All isolates were maintained freeze-dried and kept at –80 °C till using.

Mouse inoculation: For detection of pahogenicity of *P. multocida* strains, identified strains were incubated in brain heart infusion broth for 24 hrs and then the turbidity was adjusted at McFarland tube Number 4. From the tube, 0.2 ml containing 2.4×10^8 colony forming unit (CFU)/ml of growing *P. multocida* culture was inoculated intraperitoneal into each of three mice. Inoculated mice were observed for 3 days for mortalities. From dead mice, heart blood was collected and streaked on blood agar and liver impression smear was taken and stained for microscopical examination of *P. multocida* organism (Curtis *et al*., 1980 and Wilson *et al*., 1993).

Genotypic characterization and typing of *P. multocida*

Polymerase Chain Reaction (PCR): Molecular characterization of *P. multocida* isolates from layers and breeders chicken was done using PCR as described in OIE Manual (2008). Extraction of DNA was done according to G-spin™ genomic DNA kit instructions. Specific oligonucleotide primers sequences and amplified specific products are seen in Table (3). Cycling conditions of the primers during PCR (temperature and time conditions) are shown in Table (4). The electrophoresis and staining process were carried out as Sambrook *et al.* (1989).

Table 3. Oligonucleotide primers sequences of *P. multocida* (OIE, 2008).

Target gene	Primers sequences	Amplified Segment (bp)
KMT1T7 KMT1SP6	ATC-CGC-TAT-TTA-CCC-AGT-GG GCT-GTA-AAG-GAA-CTC-GCC-AC	460
Serogroup (A)	TGC- CAA- AAT-CGC-AGT-GAG TTG-CCA-TCA-TTG-TCA-GTG	1044
Serogroup (D)	TTA-CAA-AAG-AAA-GAC-TAG-GAG-CCC CAT-CTA-CCC-ACT-CAA-CCA TAT-CAG	657

Table 4. Cycling conditions of the primers of *P. multocida* during PCR.

Target gene	Primary denaturation	Secondary denaturation	Annealing	Extension	Final Extension
KMT1	94 C° 5 min.	94 C° 1 min.	55 C° 1 min.	72 C° 1 min.	72 C° 10 min.

Typing: Capsular typing of *P. multocida* isolates was done using multiplex PCR (OIE, 2008), while somatic serotyping was carried out as Heddleston *et al*. (1972) using gel diffusion precipitation test.

RESULTS AND DISCUSSION

Phenotypic characterization of *P. multocida* isolates: Fowl cholera, caused by *P. multocida,* is a bacterial disease affecting domestic and wild birds inducing devastating economic hazards (Christensen and Bisgaard, 2000, Aye *et al*., 2001, Pedersen *et al*., 2003, Biswas *et al*., 2005, Chrzastek *et al*., 2012 and Singh *et al*., 2014). All ages are susceptible to *P. multocida* infection; however, death losses in chickens usually occur in laying flocks (Rhoades and Rimler, 1991 and Muhairwa *et al*., 2001). Diagnosis of FC depends on identification of the causative agent, following isolation from suspected birds showing signs and lesions consistent with this disease (Anonymous, 2000).

In this study, the percentage of *P. multocida* in different examined flocks in different Egyptian governorates is illustrated in Table (5) where the highest incidence of *P. multocida* isolation was present in El-Sharqia governorate. The total number of positive *P. multocida* chicken flocks was 6 out of 55 layers and breeders chicken flocks (10.9%) that representing 5 out of 39 (12.8%) and 1 out of 16 (6.25%) from layers an breeders flocks; respectively. However, out of 330 examined layers and breeders dead chicken, 36 (10.9%) *P. multocida* strains were detected that representing 30 out of 234 (12.8%) from layers and 6 out of 96 (6.25%) from breeders. Similarly, Arshed *et al*. (2003) isolated *P. multocida* from 16 layer flocks aged from 18-24 weeks in and around Faisalabad, Mehmood *et al*. (2016) confirmed presence of different *P. multocida* serotype A isolates in 5 commercial layers flocks of Karachi, Lahore, Vehari and Toba Tek Singh (Pakistan). In India, Pillai *et al*. (2013) isolated 10 isolates of *P. multocida* serotype A:1 from 155 samples of poultry and deer. In Bangladesh, several studies were done on the prevalence of FC where Hasan *et al*. (2010), Belal (2013) and Hossain *et al*. (2013) found the disease incidence 12.50% in layers and 4.25% in broilers, 59.72% in backyard poultry and 13.04% in chicken; respectively. Balakrishnan and Roy (2012) identified 8 *P. multocida* isolates from chickens and turkeys, in Chennai, on the basis of biochemical characteristics, pathogenicity studies in mice and PCR.

These differences in isolation rates may be due to the number of samples, method of isolation, presence of stress and age of birds sampled.

This study showed that isolation of *P. multocida* from liver, heart and spleen on specific media revealed presence of grayish and small mucoid dew drop like colonies on DAS medium, non-haemolytic colonies on blood agar after 48 hrs incubation at 37°C and there was no growth on MacConkey agar. *P. multocida* has been detected in liver, spleen, blood and lungs of infected birds (Hunter and Wobeser, 1980). For isolation of *P. multocida*, selective enrichment along with inoculation of blood agar increases the isolation rate (Moore *et al*., 1994). The colonies characteristics of *P. multocida* organisms are in support of the findings of Divivedi and Sodhi, (1989), Shivachandra *et al*. (2005) and Christensen and Bisgaard (2006).

Gram's stained smears from suspected *P. multocida* colonies showed Gram negative coccobacilli. These findings are in accordance with Purushothaman *et al*. (2008).

All suspected *P. multocid*a isolates were positive for catalase, oxidase, Indol production, nitrate reduction and H_2S production tests, while negative for methyl red, Voge's proskaur, urease activity and gelatin liquefaction tests. Moreover, they fermented glucose, fructose, mannose, mannitol, sucrose, sorbitol and xylose without gas production but not ferment arabinose, inositol, lactose, maltose, salicin, dulcitol and raffinose. These findings of biochemical reactions for *P. multocida* are parallel to with the findings of Christensen *et al*. (2014).

Mouse assay is an efficient and widely accepted method for detection of *P. multocida* (Baldrias *et al*., 1988). The isolated *P. multocida* strains in this study were pathogenic for mice, where mice died within 24-48 hrs after inoculation. Congestion and septicaemia of the internal organs were seen. Dew drop, mucoid and non-haemolytic colonies characteristic for *P. multocida* were seen on blood agar media. These results are in agreement with the findings of Balakrishnan and Mini (2001).

Genotyping and typing characterization of *P. multocida* isolates: Molecular detection of *P. multocida* using PCR was carried out; where *P. multocida* strains isolated from layers and breeders chicken exhibited amplification of PCR amplicons of 460 bp (Figure 1) as reported by Ranjan *et al*. (2011) and Akhtar (2013). Cultivation and identification of *P. multocida* by standard bacteriological

methods can be ambiguous because of V-factor requirements or non-typeable strains, including cross-reaction in serotyping and viable but non-culturable cells (Krause *et al*., 1993). Townsend *et al*. (2000) indicated that detection of *P. multocida* from direct culture was less efficient than either mouse inoculation or PCR, as well as detection of *P. multocida* by PCR was particularly successful as some isolates were shown to be non-pathogenic for mice. PCR technique is used usefully for detection of DNA of *P. multocida* (Townsend *et al*., 1998 and Purushothaman *et al*., 2008).

Here, somatic serotyping of the isolated strains of *P. multocida* revealed that they were serotypes 1 and 3, while capsular typing of *P. multocida* strains were confirmed to be type A using multiplex PCR, indicated by the amplification of 1044 bp size (Figure 2). Similar results were reported by Kwaga *et al*. (2013). In Egypt, Moemen *et al*. (2012) demonstrated that *P. multocida* serotype A:1 strains caused 80% mortality, however, type D strains caused 20% mortality in chickens. Capsular serotypes of *P. multocida* including; A, B, D, E, and F found in chickens, as serotype A causes FC in avian species (Harper *et al*., 2006), other serotypes as B and E do not cause infection in avian species (Shivachandra *et al*., 2006 and Glisson *et al*., 2008). In Bangladesh, Panna *et al*. (2015) confirmed presence of *Pasteurella* spp. type A in chicken by PCR. Zhang *et al*. (2004) reported that most *P. multocida* isolates were serotype A:1 (57.14%) which is considered the main cause of acute forms of FC . Ninety-two out of 123 *P. multocida* strains belonged to serotype A:1 was recorded by Shivachandra *et al*. (2006). In Asian countries, common serotype of *P. multocida* associated with FC is A:1 after characterization with PCR (Gunawardana *et al*., 2000, Townsend *et al*., 2000, Kumar *et al*., 2004 and Dziva *et al*., 2008).

From the above-mentioned results, it could be concluded that *P. multocida* strains are circulating in Egyptian layers and breeders chicken flocks in spite of vaccination and medication. So, continuous surveillance studies are the must to detect the presence of the organism that induces severe losses in the field.

Table 5. Percentage of *P. multocida* in different examined flocks in different Egyptian governorates.

Governorate	Layers				Breeders			
	No. of Flocks	Positive flocks (%)	No. of examined birds	Positive (%)	No. of Flocks	Positive flocks (%)	No. of examined birds	Positive (%)
El-Sharqia	15	2 (13.3)	90	12 (13.3)	7	1 (14.28)	42	6 (14.28)
El-Gharbia	10	1 (10)	60	6 (10)	4	0 (0)	24	0 (0)
El-Qalubia	8	1 (12.5)	48	6 (12.5)	3	0 (0)	18	0 (0)
El-Minofia	6	1 (16.67)	36	6 (16.67)	2	0 (0)	12	0 (0)

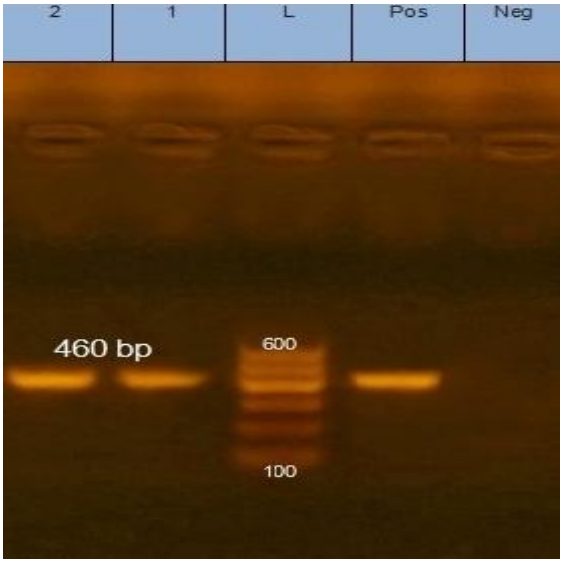

Fig. 1. Polymerase chain reaction (PCR) of *P. multocida*
Lane L: Marker, 100 bp DNA ladder, Lanes 1 and 2: amplification of 460 bp fragments specific for *P. multocida*

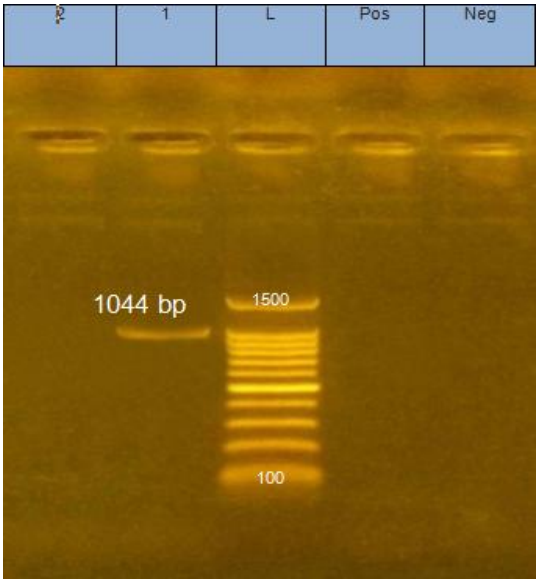

Figure (2): Multiplex capsular polymerase chain reaction (PCR) typing of *P. multocida*
Lane L: Marker, 100 bp DNA ladder, Lane 1: strain of *P. multocida* belongs to capsular A type (1044bp)

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