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DETECTION AND PATHOGENICITY OF *PASTEURELLA MULTOCIDA* ISOLATED FROM LAYER FARMS IN EGYPT

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ABSTRACT

In this study 300 samples were taken from different layer farms in Egypt (El-Sharqia, Elmina, Assiut and Sohag) showing remarkable signs for fowl cholera and examined for detection of *P. multocida*. Isolation of *P. multocida* was from liver, lung and trachea. The targeted bacteria were isolated, identified and molecularly characterized. *P. multocida* were recovered from 8 cases (2.6%) and confirmed using phenotypic characterization. By multiplex PCR assay which considered a rapid diagnostic method for fowl Cholera confirmatory recognition regardless to serotypes, isolates were *P. multocida* serotype (A) at expected size; 1044 bp. Antimicrobial susceptibility test used to determine the minimal inhibitory concentration to all isolates resulted in high susceptibility to amoxicillin and doxycycline and with variable pattern of sensitivity to the other antibiotic. Studying pathogenicity of *P.multocida* capsular type A was carried out in Ross broiler chickens aged 21 days old through oropharyngeal inoculation (0.5 ml) brain heart infusion broth containing $2.93x10^8$ CFU. Characteristic mild respiratory signs were observed within 48 h and persisted for 9 day with 8% mortality. Mild septicemic lesions comprising of white necrotic foci and pinpoint hemorrhages in the coronary fat of the heart, liver and sever inflammation in pancreas were observed. This study has documented the incidence of fowl cholera in broiler chickens to some extent mild to moderate degree of the disease.

Key words: *Pasteurella maltocida*, Multiplex PCR, Pathogenicity, Broiler chickens.

INTRODUCTION

Fowl Cholera is considered as a main problem of avian species worldwide as it is a highly contagious bacterial disease of wild and domesticated birds (Singh *et al*., 2014), associated with severe economic crisis for commercial laying hens due to poultry production loss, cost of treatment, and mortality (Marza *et al*., 2015).

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Pasteurella multocida is a gram-negative, non-motile and non-spore forming, bacillus. Serologic indicators in the gel diffusion test grouped *P. mulocida* into five capsular serotypes (A, B, D, E, and F) and each is generally concomitant with a specific host (Akhtar *et al.*, 2016), serotype (A) is supposed to cause the most of fowl cholera cases (Marza *et al*., 2015) and 16 serotypes according to the spreading of somatic antigens (Wilson *et al*., 1993). Molecular techniques expanded more prominence in serotyping *P. multocida* as a consequence of the primitive limitations of bio-typing

that relied on biochemical and serological tests.

Laying flocks, worldwide particularly in countries having hot humid environment, are more susceptible to Fowl Cholera as compared with younger chickens (Wang *et al.*, 2009). However, *P. multocida* is normally inhabitant in the respiratory tract of avian species and disease emerged when the birds are stressed (Harper *et al.*, 2006). The main route of infection is not certainly well-known; whereas, P. multocida may attain the body via the digestive tract or the respiratory one, that supposed to be the important site (Wilkie *et al*., 2012). The clinical picture course of Fowl cholera in avian species is from a few hours to numerous days as in per-acute cases the first sign report is sudden death. In many cases, the disease extended for prolonged course. Signs that often occur are nasal discharge, congestion of comb and wattles, facial edema, ataxia, nervous manifestation, fever, loss of appetite, depression accompanied with high morbidity rate reached 50% (Shah *et al*., 2008). After infection birds remain carrier up to 9weeks. All ages are susceptible to infection with FC, but rarely arise in saleable poultry of less than 8weeks of age (Rimler *et al*., 1998).

Antimicrobials are widely used in treatment of FC on the basis of selection to the most efficacious antibiotic with varying success depending mainly on the kind of drug used (Rimler and Glisson, 1997). However, long administration and misuse of antibacterial resulted in the emergence of multidrug resistance (MDR) among *P. multocida* strains which in turn led to a decline in the antimicrobials efficacy against diseased birds. Moemen *et al*. (2012)

Pathogenicity of *P.multocida* was detected by inoculation of animals including rodents, chicks and embryonated eggs (Mohamed *et al*., 2012). Moreover, mice inoculation suspected to cause disease with consequent isolation of pure bacterial growth and is considered a main diagnostic tool for FC (Dziva *et al.*, 2008), but it is not satisfactory to appraise the pathogenicity (Schivachandra *et al*., 2006). The present communication deals with isolation, identification, antimicrobial sensitivity against *P. multocida* isolated from layer farms in Egypt and detection of its pathogenicity in Ross broiler chickens.

MATERIALS AND METHODS

Sampling:

Three hundred cases collected from different layer farms in Egypt (El-Sharqia, Elmina, Assiut and Sohag) showing signs of septicemia (congestion and cyanosis of comb and wattles), nasal and ocular discharge, conjunctivitis, greenish diarrhea, and increased mortality (5-10%). Bacterial cultures were from liver, heart, trachea and lung of freshly dead birds using brain heart infusion (BHI) broth, incubated at 37ºC for 24h then sub-cultured on sheep blood agar and concurrent biochemical reactions were assessed for isolation and identification of *P. multocida* (Blackall and Miflin, 2000). Suspected isolates were preserved at -80°C for further studies.

Pathogenicity assay in mice:

P. multocida isolates were grown for 18h at 37ºC in BHI broth. About 0.2 ml of each culture containing 2.4×10^8 colony forming units (cfu)/ml was inoculated into each of three mice by sub-cutaneous route and observed for 72h, to study the mortality pattern. Bacteria was re-isolated on a blood agar plate using heart blood collected from dead mice, and an impression smear from the liver was prepared on microscopic slides for bacterial observation, using Giemsa stain (Moemen *et al.*, 2012).

Molecular detection of *P.multocida*:

DNA of the suspected bacteria was extracted according to QIAamp DNA mini kit instructions and amplified via multiplex-polymerase-chain reaction assay using *P.*

multocida`s universal and capsular serotype specific primers (Table 1) according to OIE (2012).

The assay reaction was accomplished in Biometra thermocycler (Germany). Precisely, an initial 95ºC heating start for 5min. followed by 30cyles of 95ºC, 55ºC, 72ºC for 1min each and a final extension step at 72ºC for 6min. were done.

The reaction products (5ul) were visualized by UV after electrophoresis in gel documentation system (Syn-gene, Gene Genius Bio Imaging System, UK) (Bhimani *et al*., 2014). A 100-1000bp DNA-ladder (Gene ruler, Fermentas) was used as a DNA-molecular weight marker.

Table1: Sequence of universal and capsular serotype specific primers and applied for detection of capsular-type genes in *Pasteurella multocida* strains

Target agent	Target gene	Primers sequences	Amplified segment (bp)	Reference
P. multocida	*Kmt1*	ATC-CGC-TAT-TTA-CCC-AGT-GG GCT-GTA-AAC-GAA-CTC-GCC-AC	460	OIE (2012)
Serogroup A		TGC-CAA-AAT-CGC-AGT-GAG TTG-CCA-TCA-TTG-TCA-GTG	1044	
Serogroup D		TA-CAA-AAG-AAA-GAC-TAG-GAG-CCC CAT-CTA-CCC-ACT-CAA-CCA-TAT-CAG	657	

Antimicrobial susceptibility testing:
P. multocida strains were tested for their susceptibility to amoxicillin, florfenicol, tetracycline, trimethoprim-sulfamethoxazole, Erythromycin, streptomycin and doxycycline. The antibiogram was determined using broth micro-dilution method (Tang *et al.*, 2009). Evaluation of the MICs was done using an interpretive criterion from National Committee for Clinical Laboratory Standards (NCCLS, 2018).

Pathogenicity of *P. multocida* in broiler chickens:
This was carried out on 50 Commercial 1-day-old chicks Ross broiler breed (El-Waddi Co, Egypt) were housed until the age of infection on experimental poultry shed in the Department of Avian and Rabbit diseases under strict hygienic conditions in separate rooms used for both pathogenicity and protection studies. At 21day old chickens were divided into 2 groups (25/each). The first group was inoculated oropharyngeally with 0.5 ml brain heart infusion broth containing $2.93x10^8$ CFU *P. multocida* serotype (A) (Akhtar, 2013). The second one was kept as a Control negative.

Morbidity and mortality were recorded for a period of 14 dpi and bacterial re-isolation was verified. Concurrently, liver and heart were collected from dead and scarified birds and cultured in 5 mL of BHI, as described by Christensen and Bisgaard (2006).

RESULTS

Incidence of *P.multocida*: *P. multocida* was detected in 8 cases studied (2.6%). Isolates were established as *P. multocida* upon extended phenotypic identification showing small mucoid dew- drops like colonies were observed on BHI agar and non-haemolytic on blood agar. Moreover,

there was no growth on MacConkey agar (fig.1) and typical Gram-negative, bipolar coccobacilli was revealed by Gram and Methylene blue stains from recent cultures, blood films and tissue smear prepared from positive sample.

Fig.1: Dew drop like, mucoid colonies on Brain heart infusion agar (left) and no growth on MacConkey agar (right)

Pathogenicity assay mice:
Inoculated mice showed moralities within 48h. Heart blood, liver and lung impression Giemsa stained smears revealed characteristic bipolarity. Re-isolation showed typical culture for *P. multocida***.**

Capsular genotyping: multiplex PCR revealed that all isolates belonged to *P. multocida* serotype (A) within the expected size; 1044 bp. (Fig. 2). No amplicons specific for other capsular serotypes were reported.

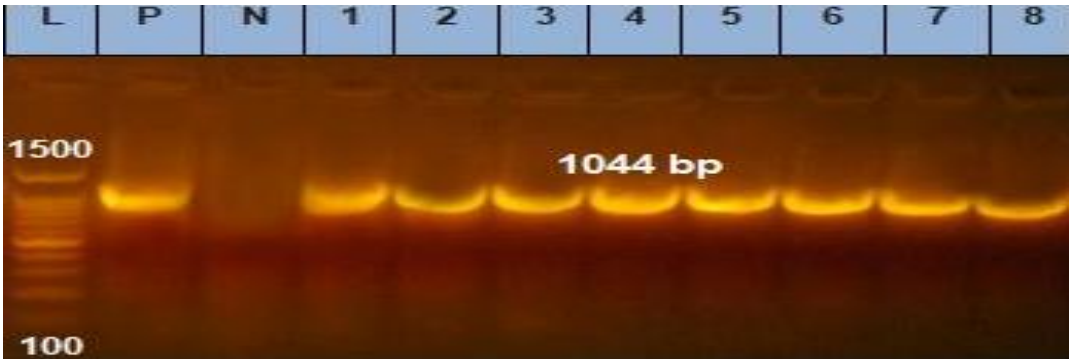

Fig.2: Gel profiling of *P. multocida* capsular gene. Lane P= +ve control, Lane N= -ve control, Lane 1-8= *P.multocida* isolates, L=100-bp DNA ladder

Antimicrobial susceptibility: According to MIC test results shows that all isolates were (100%) resistant to Lincomycin, erythromycin, sulphaquinxaline, Spectinomycin, 87.5% were resistant to streptomycin, Cephradine, florphenicol. while they were (100%) sensitive to amoxicillin, (12.5%) were sensitive to doxycycline and (75%) were sensitive to gentamicin.Different variations of MICs were detected for resistant strains, whereas the MICs of streptomycin and erythromycin were distinctly higher revealed128 µg/ml, while doxycycline was16µg/ml with 50% and was (8 µg/ml) with 37.5% for all isolates (table .2).

Table 2: Minimum inhibitory concentrations of anti-microbial agents against examined *P. multocida* isolates

Antimicrobial agents	<2	4	8	16	32	64	128	>256	Resistance breakpoints	Resistance %
Amoxicillin	8	-	-	-	-	-	-	-	>64	0%
Doxycycline	-	-	3	4	-	1	-	-	>32	12.5%
Gentamicin	1	-	1	-	2	2	2	-	>16	75%
Florphenicol	-	1	-	-	-	5	2	-	>8	87.5%
Streptomycin	1	-	-	-	-	-	4	3	>4	87.5%
Cephradine	-	1	-	-	1	2	4	-	>8	87.5%
Erythromycin	-	-	-	-	-	-	5	3	>8	100%
Lincomycin	-	-	-	-	-	-	4	4	>16	100%
Spectinomycin	-	-	-	-	-	-	5	3	>4	100%
Sulphaquinxaline	-	-	-	-	-	-	-	8	>256	100%

Pathogenicity of *P.multocida* in broiler chickens:

P. multocida type (A) in chickens produced characteristic mild clinical picture for respiratory system affection consisted of coughing, sneezing and frothy eyes within 48 h and persisted for 9day post infection with 8% mortality. Mild septicemic lesions including: white necrotic foci and pinpoint hemorrhages in heart, liver and sever inflammation in pancreas were observed (Fig.3), Heart blood and liver impressions represented characteristic bipolarity of *p. multocida* stained with Giemsa and typical growth of dew drop, mucoid, non-haemolytic colonies in sheep blood agar resulted from re-isolation, there were neither signs nor gross pathological lesions in the control group.

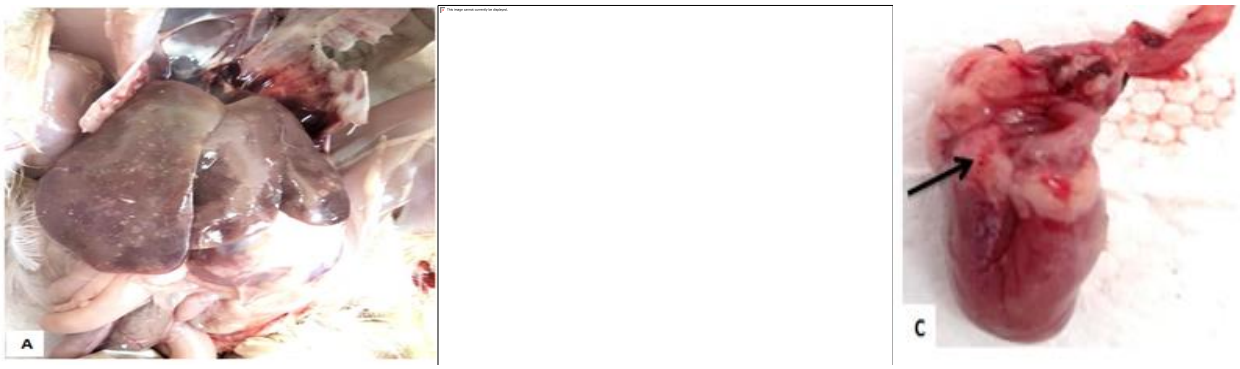

Fig.3: Representing post-mortem lesions of *p.multocida* type A after 48h post oropharyngeal infection, (A): showing small necrotic foci and subscapular pinpoint ecchymotic haemorrhages in liver (B) sever pancreatitis with pinpoint haemorrhages and (C): Ecchymotic haemorrhages in the coronary fat of the heart.

DISCUSSION

The prevalence of FC isolated from different layer farms in this work was (2.6%) in Egypt compared with Hasan *et al.* (2010) who reported incidence of FC 4.25% in broiler chicken and 12.05% in layer flocks, while Hossain *et al.* (2013) recorded13.04% prevalence in chickens and this may be attributed to differences in breeds, age of the chickens and/or the resistance power of the commercial chicken because of improved management and biosecurity. Moreover, wet lands, overcrowded rearing of birds in hot humid environment, stresses and age of birds

could be improvement factors for high incidence of pasteurellosis in poultry farms (Akhtar *et al*., 2016).

Pasteurella spp. isolates were positive for *P. multocida* serotype (A) representing amplification of 1044bp through multiplex PCR which is a consistent technique for capsular serotyping of FC and this data is similar to previous reports which confirmed that serotype (A) considered as the main cause of fowl cholera, while other serotypes B, D and F are less commonly attendant with the infection Moemen *et al*. (2012).

P. multocida strains differs in their activities to current available chemotherapeutics as there was high resistance to lincomycin, streptomycin and erythromycin in which conformer with those findings who mentioned that the aminoglycosides usually represented poor efficacy against FC (Gutiérrez and Rodríguez, 1993), while the more active drugs were amoxicillin and doxycycline in contrast to Moemen *et al*. (2012) who reported high resistance rate to tetracycline and amoxycillin (100%), and this variation may be due to over or limited previous exposure and/or indiscriminate use of antibiotics for prevention and control of the disease (Kamruzzaman *et al*., 2016).

Mortality rate in the pathogenicity test in broiler chickens was 8% during the experiments, although moderate clinical signs of fowl cholera were perceived during the 14dpi with *P. multocida* Type (A), and variable lesions were found in dead and scarified birds as mentioned by (Christensen and Bisgaard, 2000 and Glisson *et al*., 2003) who observe the clinical manifestations of fowl cholera mostly from the first to fifth dpi with intermediate to chronic signs in chickens.

Affections of *P. multocida* among poultry species and different ages are of variable degrees within a type (Mbuthia *et al*., 2008); and this recorded variations of our study and other ones could be due to the virulence of *P. multocida* serotypes used, chicken breeds and age susceptibility. From this work we concluded that Fowl cholera could induce mild clinical view in broiler chickens with low mortality percentage.

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الكشف عن والتاثير الممرض للباستيريلا ملتوسيدا المعزولة من مزارع دجاج البياض في مصر

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تم اجراء هذه الدراسة علي ٣٠٠ حالة من مزارع بياض مختلفة في مصر(الشرقية , المنيا , اسيوط وسوهاج) ، تتراوح اعمارها من ٣٠-٧٠ اسبوع بها علامات ملحوظة لكوليرا الطيور (احتقان عام وشديد في العرف والدلايات , افرازات من الأنف والعين مع وجود إسهالات) مصاحبة بزيادة معدل الوفيات (٥-١٠٪). ومن ثم تم تجميع عينات من الكبد والرئة والقصبة الهوائية لعزل الباستيريلا ملتوسيدا علي المستنبتات الصناعية المخصصة والتي اسفرت عن وجود ميكروب الباستيريلا مالتوسيدا في ٨ حالات (٢,٦٪) وتم تأكيدها باستخدام التوصيف المظهرى للخلايا والمستعمرات البكتيرية . وباستخدام تفاعل البلمرة المتسلسل المتعدد تم تمييزها جزيئيًا و تم تأكيد العزلات أنها الباستيريلا ملتوسيدا من النوع A عند الوزن ١٠٤٤ زوج قاعدي. تم إخضاع العزلات لقابلية مضادات الميكروبات لتحديد التركيز المثبط الأدنى و نتج عنه حساسية عالية لجميع العزلات للأموكسيللين والدوكسيسيكلين (١٠٠%) وبدرجات متفاوتة من الحساسية للمضادات الحيوية الاخري. اجريت تجربة للكشف عن إمراضية الباستيريلا مالتوسيدا النوع A علي ٥٠ كتكوت تسمين سلالة الروس من خلال تقسيم الكتاكيت الي مجموعتين (٢٥ بكل مجموعة)عند عمر ٢١ يوم : المجموعة الاولي تم اعدائها عن طريق الفم (٠,٥ مل يحتوي علىCFU $x10^8$ ٢,٩٣) بينما المجموعة الاخري ظابطة غير معدية وتمت ملاحظة الكتاكيت ١٤ يوم بعد العدوي. لوحظت علامات تنفسية خفيفة مميزة خلال ٤٨ ساعة واستمرت لمدة ٩ أيام مع نسبة وفيات ٨٪. ومن ثم وجود آفات تشريحية ملحوظة تكونت من بؤر نخرية بيضاء علي الكبد ,انزفة في الدهن التاجي للقلب والكبد والتهاب شديد في البنكرياس وقد وثقت هذه الدراسة حدوث كوليرا الطيور في الدجاج اللاحم إلى حد ما بدرجة خفيفة إلى متوسطة من المرض .