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BACTERIOLOGICAL STUDIES ON THE CAPSULE OF *PASTEURELLA MULTOCIDA* ISOLATED FROM POULTRY IN BENI SUEF GOVERNORATE

(With 4 Tables and 6 Figures)

By

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(Received at 3/9/2011)

دراسات عن الغلاف الخارجى لميكروبات الباستيريلا ملتوسيدا المعزوله من الدواجن فى محافظة بنى سويف

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عند اجراء الفحص البكتريولوجى لعدد (320) عينه مأخوذه من طيور نافقه ومريضه تم عزل ميكروب الباستيريلا مولتوسيدا (180) بنسبة (56.25%) وتم تعيين الخصائص الشكليه والبيوكميائيه للميكروبات المعزوله باستخدام الميكروسكوب العادى والأختبارات البيوكيميائيه وعند فحص خمس معزولات من هذه الميكروبات على أساس درجة ما أحدثته هذه المعزولات من نسبة نفوق واعراض مرضيه عند العدوى الطبيعيه بالميكروسكوب الألكترونى تم تحديد سمك الغلاف الخارجى لهذه المعزولات وكانت كالتالى : 122 nm, 118 nm, 98 nm, 83nm, 81 nm, وبمقارنة نتائج الفحص المعملى لضراوة و مقاومة هذه المعزولات للسيروم كومبليمنت وقدرتها على أفراز السموم تبين ان هناك علاقه طرديه بين سمك الغلاف الخارجى لميكروبات الباستيريلا مولتوسيدا المعزوله من الداواجن المصابه بكوليرا الطيور وضرواتها و قدرتها على مقاومة السيرم كومبليمنت وقدرتها على أفراز السموم.

SUMMERY

Bacteriological examination of 320 dead and diseased bird revealed isolation of 180 isolates of *P. multocida* with an incidence of 56.25%. The morphological and biochemical characters of the isolated bacteria were determined by ordinary microscope and biochemical reactions. Five isolates of *P. multocida* were visualizated by electron microscope on the bases of difrrence in mortality rate, symptoms and severity of the disease in normal infection, and the capsular thickness of these isolates were determined. The capsule thickness of the examined isolates were

122 nm, 118 nm, 98 nm, 83nm, 81 nm, the virulence,resistance to serum complement and toxigenicity of these isolates were compared experamintaly in checkins.The laboratory examination of five isolates of *P. multocida* with different thickness revealed that there was a correlation between the capsule thickness and the pathogenecity and toxigenicity of the examined isolates.

Key words: *P.multocida, poultry, electron microscope.*

INTRODUCTION

Pasteurella multocida is a gram-negative coccobacillus that is the causative agent of a wide range of diseases in animals and birds, including fowl cholera, a disease of poultry with worldwide economic importance (Carpenter *et al.,* 1988). The organism can occur as a commensally in the naso-pharyngeal region of apparently healthy animals and it can be a primary or secondary pathogen in the disease process of birds (Tatum *et al.,* 2005).

Kardos and Kiss (2005) cleared that fowl cholera was responsible for significant losses in poultry husbandry and Stokholm *et al.* (2010) reported that the mortality rate in commercial free-range organic layer flocks in Denmark affected by erysipelas and fowl cholera ranged from approximately 2% to 91%, with a mean of 20.8%. The pathogenesis of fowl cholera is not well understood at the molecular level, but it is likely that susceptible birds are colonized via the trachea and/or lungs, and once bacteria penetrate to the bloodstream, they multiply rapidly in the liver and spleen (Boyce *et al.,* 2004). Toward the end stages of the disease, high levels of bacteremia often occur (Boyce *et al.,* 2002).

Relatively little information is available on *P.multocida* virulence factors involved in producing fowl cholera. Because of the complex nature of bacterial pathogenesis (Rhoades and Rimler 1993). Survival of the bacteria in the blood is critical for pathogenesis, and the *P. multocida* capsule has been identified as the major virulence determinant that allows the bacteria to survive complement-mediated killing and to evade phagocytosis (Boyce and Adler 2000; Chung *et al.,* 2001).

Capsules are highly hydrated polysaccharides located external and adherent to the bacterial cell wall, the location of extracellular

polysaccharides at the outermost surface of the cell is important because they are the first portal of entry and the last barrier to excretion of substances in and out of the cell. Various hypotheses have been postulated about the function of the bacterial capsule, these include protection against desiccation in the environment (Ophir, and Gutnick 1994) phagocytosis and the bactericidal activity of serum complement (Benchetrit *et al.,* 1977; Boyce and Adler 2000). Boyce and Adler (2000) used electron microscope and P.C.R. to clear that capsules from a range of pathogenic bacteria are key virulence determinants, and the capsule has been implicated in virulence in *P. multocida*. Borrathybay *et al.* (2003) showed that the capsule thickness of avian strains of *P. multocida* was correlated with their pathogenicity for chickens. Saif *et al.* (2003) stated that endotoxins are produced by all *P. multocida*, both virulent and nonvirulent, they may contribute to virulence; however invasion and multiplication of a strain are necessary for the production of sufficient quantities of endotoxin in vivo to contribute to pathologic processes

The aim of the present work was to study the role of capsule in the pathogenesis of fowl cholera and the toxin produced by field isolates of *P. multocida* as virulence markers of *P. multocida*.

MATERIALS and METHODS

Samples:

A total number of 320 freshly dead and diseased chickens showing diarrhea and respiratory disorders were collected from different private farms at Beni-Suef Governorate from March 2007 till October 2010. The birds were submitted to bacteriological examinations.

Isolation and identification.

Isolation and indentification of *P. multocida* was carried out according to CruickShank *et al.* (1975); Collee *et al.* (1996).

The internal organs of examined birds (liver, heart, spleen, lungs and trachea as well as nasal and eye discharges) were inoculated onto broth media and incubated for 18–24 h then streaked onto solid media, dextrose starch agar, blood agar, trypticase soya agar and incubated at 37°C for 24 h. Differentiation of suspected colonies were done through colonial morphology hemolysis, staining reaction, cellular morphology,

biochemical reactions specially carbohydrate fermentation, indol production, motility, catalase and oxidase tests.

Bacterial strains and isolates:-

Five strains and isolates (two strains were identified by PCR in previous study in 2008 and three field isolates) of *P. multocida* were used to study the role of capsule in the pathogenesis of *P. multocida*. The strains and isolates were chosen on the bases of variation in mortality rate, symptoms, and severity of the disease in normal infection.

Electron microscopy techniques:-

Bacteria were grown in tryptic soya broth at 37°C for 18 h before growth on blood agar (tryptic soya agar containing 5% calf blood) for 18 to 24 h and prepared for electron microscopy as described by Jacques and Graham (1989)

Assessment of virulence of *P. multocida* for chickens:-

Sixty apparently healthy Balady chickens 4 weeks old were used to assess the virulence of *P. multocida* isolates for chickens, it was proved that they were free from *P. multocida* through cultural and serological examination, the chickens were divided into 6 groups (ten birds each) 5 groups were used to assess the virulence of five isolates of *P.multocida* which isolated from naturally infected chickens and group 6, (ten bird) were kept in parallel as uninfected controls. The five groups of chicken were inoculated separately intravenous with 0.1 ml of overnight broth containing 10^9 CFU/ml of *P. multocida* according to Chung *et al.* (2001)

Serum sensitivity assays:-

The sensitivity of *P. multocida* strains and isolates to the bactericidal complement activity of chicken serum was determined by the addition of 10^5 CFU to chicken serum, (serum was used at 90%) and subsequent incubation for 3 h at 41°C with aeration according to Chung *et al.* (2001), viable counts were determined at 0 and 3 h. Complement activity was inactivated in control samples by heating at 60°C for 30 min.

Assessment of toxin production:- according to Rhoados and Rimler (1990).

a- Preparation of endotoxin:-

Bacteria were grown in tryptic soya broth at 37°C for 18 h before growth on blood agar (tryptic soya agar containing 5% calf blood) for 18 to 24 h then inoculated into 50-ml volumes of brain heart infusion broth in 250-ml conical shake flasks and incubated at 37°C.

Culture supernates, as a source of endotoxin, were prepared from different isolates, the broth cultures were centrifuged at 7000 g for 10 min at 4°C. The resulting supernates were passed through 0.45-pm membrane filters.

b- Experimental infection:-

Thirty (one week old) balady chickens were used to assess the toxin production by *P. multocida* isolates, it was proved that the chickens were free from *P. multocida* through cultural and serological examination, the chickens were divided into 6 groups (five birds each) 5 groups were used to assess the toxin production of five isolates of *P.multocida* which isolated from naturally infected chickens and group 6, (five bird) were kept in parallel as controls. The five groups of chicken were inoculated separately intraperitonealy with 0.5 ml of culture supernates of *P. multocida* isolates.

RESULTS

Bacteriological examination of 320 dead and diseased bird revealed isolation of 180 isolates of *P. multocida* with an incidence of (56.25%). The morphological and biochemical characters of the isolated bacteria was determined by ordinary microscope and biochemical reactions.

Electron microscopy techniques:-

Visualization of five isolates by electron microscope as shown in Fig. (1-6) revealed that,the capsule thickness (2-6) of *P. multocida* isolates were 122 nm, 118 nm, 98 nm, 83nm, 81 nm, respectively.

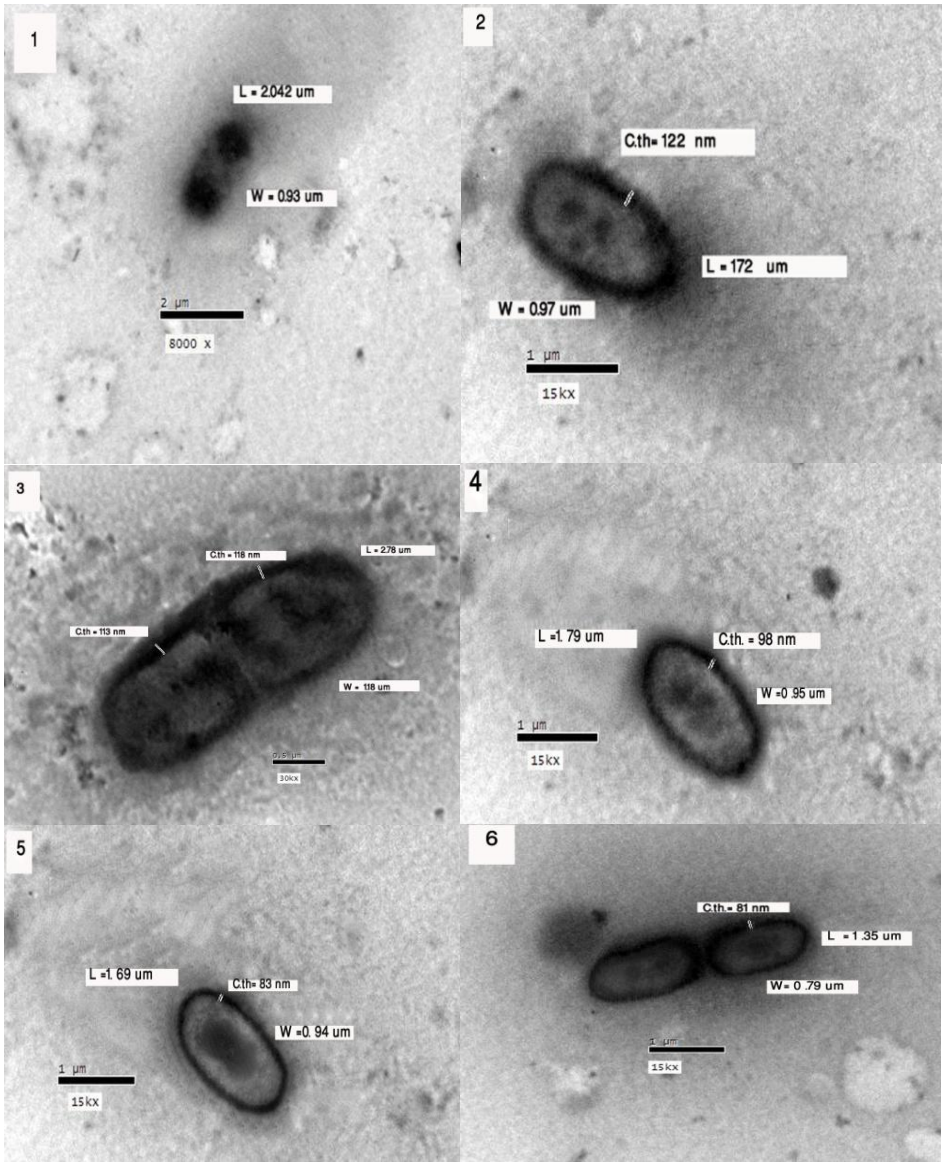

Fig.1. Electron micrographs of *P. multocida* (8000X) notice the bipolarity
Fig.2. The capsule thickness 122 nm (15 KX)
Fig.3. The capsule thickness 118 nm (30 KX)
Fig.4. The capsule thickness 98 nm (15 KX)
Fig.5. The capsule thickness 83 nm (15 KX)
Fig.6. The capsule thickness 81 nm (15 KX)

Assessment of virulence of *P. multocida* for chickens:-

Table 1: Survival of chickens infected with *P. multocida* strains and isolates

Strain or isolate	Capsular thickness	No. of survivors/group size	%
1	122 nm	0/10	0
2	118 nm	3/10	30
3	98 nm	2/10	20
4	83 nm	5/10	50
5	81 nm	5/10	50
6 contal	-		

Serum sensitivity assays:-

Table 2: The sensitivity of *P. multocida* strains and isolates to the bactericidal complement activity of unheated chicken serum

Strain or isolate	Capsular thickness	No of cfu at 0h	No of cfu at 3h	Survival ratio
1	122 nm	10^5	$1.2\ 10^7$	1.2 %
2	118 nm	10^5	$9.0\ 10^6$	0.9 %
3	98 nm	10^5	$5.0\ 10^6$	0.5 %
4	83nm	10^5	$4.0\ 10^6$	0.4 %
5	81nm	10^5	$5.0\ 10^6$	0.5 %

Survival rate = (CFU/ml at t = 3h)/ (CFU/ml at t = 0h).

Table 3: The sensitivity of *P. multocida* strains and isolates to the bactericidal complement activity of heated chicken serum

Strain and isolates	Capsular thickness	No of cfu at 0h	No of cfu at 3h	Survival ratio
1	122 nm	10^5	1.9 10^7	1.9 %
2	118 nm	10^5	2.1 10^7	2.1 %
3	98 nm	10^5	2.0 10^7	2 %
4	83nm	10^5	1.8 10^7	1.8 %
5	81 nm	10^5	1.5 10^7	1.5 %

Survival rate = (CFU/ml at t = 3h)/ (CFU/ml at t = 0h).

Assessment of toxin production:-

Table 4: Survival of chickens injected with culture supernates of *P. multocida* strains and isolates

Strain and isolates	Capsular thickness	No. of died /group size	Survival ratio
1	122 nm	5/5	0
2	118 nm	4/5	20
3	98 nm	3/5	40
4	83nm	2/5	60
5	81 nm	3/5	40
6 control	-		

DISCUSSION

P. multocida is a gram-negative coccobacillus that is the causative agent of a wide range of diseases in animals and birds, including fowl cholera, that affects 100 wild avian species (Botzler, 1991). In the present study *P. multocida* was isolated from (56.25%) of dead and diseased chickens showing symptoms of fowl cholera Woo and Kim (2006) isolated *P. multocida* from two outbreaks of fowl cholera in Korea and Stokholm *et al.* (2010) reported that *P. multocida* is one of two microorganisms which produce mortalities in commercial free-range organic layer flocks in Denmark ranged from approximately 2% to 91%, with a mean of 20.8%.

Many strains of *P. multocida* express a capsule on their surfaces (Rimler and Rhoades. 1987). Capsules are highly hydrated polysaccharides located external and adherent to the bacterial cell wall (Sutherland, 1977). The location of extracellular polysaccharides at the outermost surface of the cell is important because they are the first portal of entry and the last barrier to excretion of substances in and out of the cell (Cheng, and Costerton. 1975). Also Snipes and Hirsh (1986), Hansen and Hirsh (1989) and *Jaques et al.* (1993), showed that there is a correlation between the capsule and the virulence of *P. multocida*. In the present study capsule of some *P. multocida* isolates was visualized by electron microscope and the capsular thickness of these isolates were determined and compared with their pathogenicity for chickens. The capsule thickness of *P. multocida* isolates as shown in Fig. (2-6) were 122 nm, 118 nm, 98 nm, 83nm, 81 nm, respectively.

Table (1) cleared that the increase of capsular thickness of *P. multocida* isolates the more virulence of the isolate for chicken similar result was recorded by Borrathybay *et al.* (2003). Regarding to table(2&3) we shown that the all tested strains were resistant to unheated serum at different levels but the survival ratio was high in heated serum the results agreed with Diallio and Frost (2000) and Chung *et al.* (2001), also the tables cleared that the more capsular thickness,the more virulence and the highest survival ratio, this agreed with Muhairwa *et al.* (2002) who stated that the most severe lesions in experimentally infected chickens were produced by a serum-resistant strain. Table (3) clearing the toxigenic activities of the examined isolates, the five isolates were toxigenic for chickens these may attributed to that the bacteria were isolated from diseased birds this result agreed with Nielsen *et al.* (1986); Rhoades and Rimler (1990) while Saif *et al.* (2003) stated that

endotoxins are produced by all *P. multocida*, both virulent and nonvirulent, they may contribute to virulence; however invasion and multiplication of a strain are necessary for the production of sufficient quantities of endotoxin in vivo to contribute to pathologic processes. In conclusion capsule of *P. multocida* is a major virulence determinant that allows the bacteria to survive complement-mediated killing and to evade phagocytosis which leading to multiplication of the bacteria and consequently produce the endotoxin, it is an essential virulence factor.

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