

PHENOTYPIC AND GENOTYPING CHARACTERIZATION OF *PASTEURELLA MULTOCIDA* IN FARM ANIMALS

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ABSTRACT

Pasteurella multocida which is the common cause of respiratory disease in cattle causing high economic losses. To achieve this objective, this study succeeded in isolating a total of 27 of *P. multocida* isolates from 280 samples (9.6%). Lung tissues showed the highest percentage of *P. multocida* isolation (13.1%), followed by Nasal Swabs (5%). These isolates were confirmed microscopically to be *P. multocida* by staining with Gram's and Leishman's stains, then biochemically by traditional test like indole, catalase, oxidase, urease, citrate utilization, sugar fermentation and gelatin liquefaction tests. Also, the results were confirmed using commercial test API 20E and PCR. For common gene and capsular serotyping of *P. multocida* by using multiplex capsular PCR typing system, a total of 9 representative isolates were tested and 5 isolates were positive for common gene of *P. multocida* and positive for capsular type E. Positive *P. multocida* isolates were tested for presence of

virulence factors by multiplex PCR for virulence genotyping which represented by virulence genes (TbpA, nanH, ompH and ptfA) which revealed 100% percentages of TbpA and nanH in lung tissues and nasal swabs in cattle and buffaloes isolates, the presence of ptfA gene in high percentages in lung tissues in buffalo more than in nasal swabs.

Keywords: *Pasteurella multocida*, Phenotypic, Genotyping, Farm animals.

INTRODUCTION

P. multocida is named in honour of Louis Pasteur who in classic experiment in the early 1880s attenuated the agent and thus produced the first deliberately developed vaccine (**Rimler and Glison, 1997**). It is one of the most fascinating gram-negative bacteria and is a commensal of the upper respiratory tract of many animal species. However, under predisposing circumstances the organism is the etiological agent of a wide range of economically important diseases, including fowl cholera in poultry, haemorrhagic septicaemia in cattle and buffalo, atrophic rhinitis in swine and snuffles in rabbits. The organism is also known to be the causative agent of Pasteurellosis in American bison, yak, deer, elephants, camels, horses, elk and other wild animals (**De Alwis, 1996**). The 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**).

The bacterial outer membrane forms the interface between host and gram negative bacterial pathogen. The constituents of the membrane are proteins, lipopolysaccharides and polysaccharides. Further proteins have been reported to be immunologically dominant (**Mukkur, 1978**) and

OMPs can play important pathogenic roles such as expression of virulence factor, adhesions and surface antigens. Also selection of *P. multocida* strains with high OMP production under modified cultural conditions could enhance vaccine potency (*Srivastava, 1998*).

Phenotyping methods, mainly biotyping and serotyping have been widely used in the taxonomy and epidemiological studies of this species (*Mohan et al., 1994*). Due to shortfalls associated with phenotypic techniques, genotyping techniques have been used extensively to differentiate epidemiologically significant strains of *P. multocida* (*Lainson et al., 2002*). RAPD is receiving more attention and has been applied for distinction of strains belonging to same or different species. This method has been used in a variety of bacteria (*Dziva et al., 2001*; *Chaslus-Dancla et al., 1996*).

VFs play a key role in disease production by bacterial pathogens (*Nanduri et al., 2009*). Among others, their functions include competence, adherence, synthesis, and export of capsules; and evasion of host immune responses (*Nanduri et al., 2009*). In the present study the factors have been detected in *P. multocida* isolated from the lungs of slaughter cattle. The higher frequency of the factors among isolates from pneumonic lungs suggests the role of these factors in disease occurrence. It was pointed out that virulence gene occurrence in *P. multocida* has a strong positive association with the outcome of infection with the organism in cattle (*Katsuda et al., 2013*). On the other hand occurrence of the factors in apparently healthy lungs could possibly indicate early infection or contained infection which couldn't lead to disease. It was previously reported that this facultative anaerobic bacterium is commonly found in clinically healthy calves (*Lainson et al., 2013*).

MATERIAL AND METHODS

1. Sampling:

A total of 120 nasal swabs collected from diseased cattle and buffaloes of different sex, age and localities in Gharbia Governorate and a number of 160 lung pieces collected from diseased cattle and buffaloes from abattoirs in Gharbia Governorate.

2. Methods:

2.1. Phenotyping characterization:

isolation of organisms from the samples according to methods described by *Cruickshank et al., (1975)* and *Quinn et al., (1994)*.

Identification of *P. multocida* Isolates by biochemical tests according to methods described by *Cruickshank et al. (1975)* and *Mackie and MacCarteny (1996)*.

Pathogenicity test was done according to *Ali (1991)*.

2.2. Genotyping characterization of *Pasteurella multocida*:

2.2.1. Capsular serotyping of *Pasteurella multocida* by Multiplex Capsular PCR:

It was done according to *Townsend et al., (2001)*.

2.2.2. Virulence genotyping of *Pasteurella multocida* by Multiplex PCR:

It was done for detecting virulence genes (ptfA, nanH, ompH and TbpA) according to *Ewers et al., (2006)*.

Table (1): Primer sequences, and PCR product size for Multiplex capsular PCR:

serogroup	gene	Name	Sequences	size
All	KMT1	KMT1T7	ATCCGCTATTTACCCAGTGG	460
		KMT1SP6	GCTGTAAACGAACTCGCCAC	
A	<i>hyaD-hyaC</i>	CAPA-FWD	TGCCAAAATCGCAGTCAG	1044
		CAPA-REV	TTGCCATCATTGTCAGTG	
B	<i>bcbD</i>	CAPB-FWD	CATTATCCAAGCTCCACC	760
		CAPB-REV	GCCCGAGAGTTTCAATCC	
D	<i>dcbF</i>	CAPD-FWD	TTACAAAAGAAAGACTAGGAGCCC	756
		CAPD-REV	CATCTACCCACTCAACCATATCAG	
E	<i>ecbJ</i>	CAPE-FWD	TCCGCAGAAAATTATTGACTC	511
		CAPE-REV	GCTTGCTGCTTGATTTTGTGTC	
F	<i>fcbD</i>	CAPF-FWD	AATCGGAGAACGCAGAAATCAG	851
		CAPF-REV	TTCCGCCGTCAATTACTCTG	

Table (2): Primer sequences, and PCR product size for Multiplex virulence PCR:

gene	Name	Sequences	size
TbpA	KMT1T7	TTGGTTGGAAACGGTAAAGC	728
	KMT1SP6	TAACGTGTACGGAAAAGCCC	
nanH	CAPA-FWD	CACTGCCTTATAGCCGTATTCC	964
	CAPA-REV	AGCACTGTTACCCGAACCC	
ompH	CAPB-FWD	CGCGTATGAAGGTTTAGGT	438
	CAPB-REV	TTTAGATTGTGCGTAGTCAAC	
ptfA	CAPD-FWD	TGTGGAATTCAGCATTTTAGTGTGTC	488
	CAPD-REV	TCATGAATTCTTATGCGCAAAATCCTGCTGG	

RESULTS

Table (3): Number of suspected isolates of *P. multocida* from nasal swabs and lung tissues:

Type of samples	Number	Number of suspected isolates of <i>P. multocida</i> from nasal swabs and lung tissues	
		+ ve	%
Nasal swabs	120	6	5.0%
Pneumonic lung tissues	160	21	13.1%
Total	280	27	9.6%

Table (4): Total number of suspected *P. multocida* from nasal swabs and lung tissues according to animal:

Type of sample	Animal	Number of examined sample	Number of positive cases	Incidence percentage
Nasal swabs (120)	cattle	45	1	2.2%
	buffaloes	75	5	6.6%
Lung tissues(160)	cattle	50	8	16%
	buffaloes	110	13	11.8%
Total		280	27	9.6%

Identification of *pasteurella multocida* and capsular serotyping using Multiplex Capsular PCR Typing System:

The *Pasteurella multocida* multiplex PCR assay based on KMT1T7 and KMT1SP6 primers gives a positive signal by amplicon of approximately 460bp with five *Pasteurella multocida* isoates and also based on CAPA-FWD, CAPA-REV, CAPB-FWD, CAPB-REV, CAPD-FWD, CAPD-REV, CAPE-FWD, CAPE-REV, CAPF-FWD, CAPF-REV which give a positive signal by amplicon of approximately 511bp with five *Pasteurella multocida* isolates as in Fig. (1).

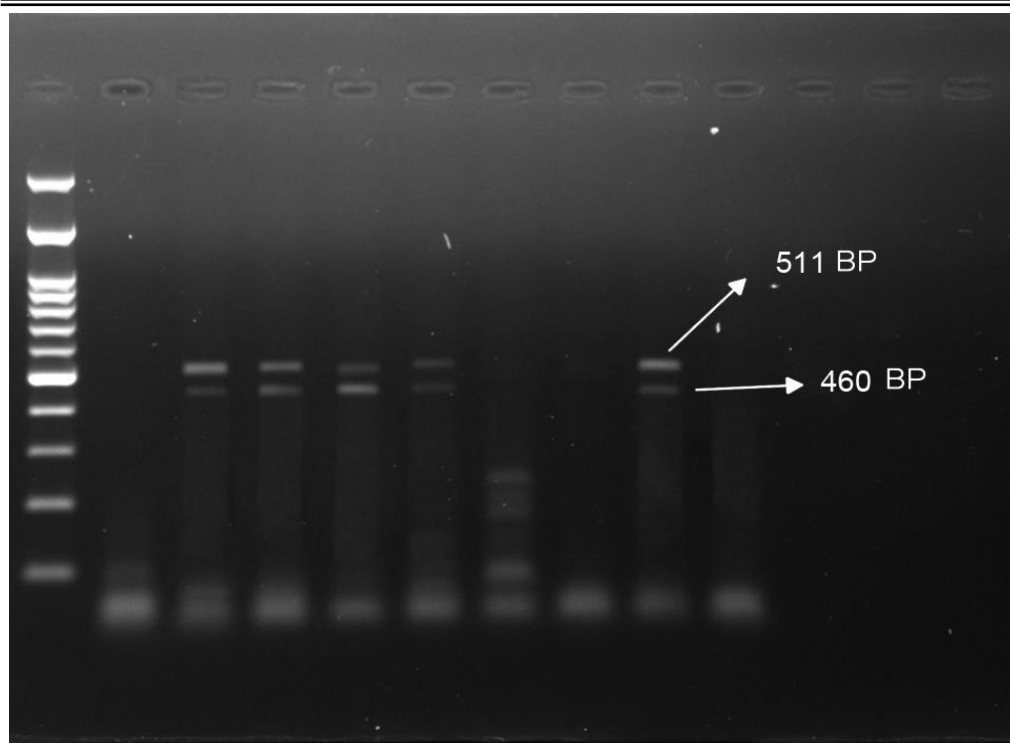


Fig. (1): Agarose gel electrophoresis PCR amplified products of *P. multocida* field isolates. Lane 1 DNA molecular size marker, Lane 3, 4, 5, 6, 9 are multiplex PCR products from *Pasteurella multocida* isolates, Lane 2, 7, 8, 10 are negative samples.

Identification of *pasteurella multocida* virulence genes using Multiplex PCR:

The *Pasteurella multocida* virulence genes based on OmpH, NanH, TbpA, Fim4 primers which revealed to OmpH, NanH, TbpA and ptfA virulence genes of *Pasteurella multocida* which give a positive signal of amplicon size of 488 bp for ptfA gene and 964 bp for NanH gene and 728 bp for TbpA gene.



Fig. (2): Agarose gel electrophoresis PCR amplified products of *P. multocida* field isolates. Lane 1 DNA molecular size marker, Lane 2: OmpH gene negative result, Lane 3: Fim4 gene positive result, Lane 4: NanH gene positive result, Lane 5: TbpA gene positive result.

Percent distribution of virulence associated genes in *P. multocida* according to samples type:

Type of Samples	TbpA Gene	NanH Gene	ptfA Gene	OmpH Gene
Swabs	33.3%	33.3%	0%	0%
Lung tissues	67.7%	67.7%	33.3%	0%
Total	100%	100%	33.3%	0%

DISCUSSION

The present study was performed to isolation and identification of *P. multocida* from suspected cases of pasteurellosis in cattle and buffaloes at Gharbia governorate.

The samples of buffaloes and cattle isolates which showed the presence of bipolar organisms in smears of *P. multocida* are in agreement with (*Asma et al., 2011*).

The bacterial colonies of *P. multocida* isolates on blood agar were small, non-haemolytic, round, smooth. All the isolates failed to grow on MacConkey agar. On the Gram staining the isolates were found to be Gram negative coccobacillary rods while on methylene blue stain showed the bipolarity of the bacteria. These results are similar to the findings of (*Quinn et al., 1994*).

In the present study, 280 samples were examined for presence of *pasteurella multocida* which revealed 27 positive samples with incidence 9.6%, and this results are nearly similar to *Sedeek and Thabat (2001)* isolated *Pasteurella multocida* with frequency (8.3%) from infected bovines and *Shayegh et al., (2010)* with frequency (6.02%) from cattle and buffaloes and this results are less than results of *Naz et al., (2012)* reported that *Pasteurella multocida* was most frequent (80%) isolated organism from infected lungs.

Although the molecular basis of the pathogenicity and host specificity of *P. multocida* is not well understood, the organism is known to possess a number of virulence factors which have integrated role in pathogenesis (*Hunt et al., 2000a and Harper et al., 2006*). The present study was thus carried out to study the prevalence of virulence associated

genes in the bovine isolates of *P. multocida*. The prevalence of 4 virulence associated genes which included genes coding for iron acquisition factors (tbpA), adhesion related genes (ptfA, nanH), outer membrane and porin proteins (ompH).

The prevalence of virulence associated genes was found to vary among *P. multocida* isolates recovered from various host species. Significant association between toxA and nanH genes with host origin was also observed. Dermonecrotxin gene was found to be positively associated with porcine isolates, whereas nanH gene was found to be positively associated with large ruminant isolates, more specifically with cattle isolates, which agrees well with the findings of (*Ewers et al., 2006*).

The type 4 fimbria (ptfA gene) was described in 33.3% of the isolates tested in the current study. The gene plays a key role of fixing bacterial pathogens on the surface of the epithelial cells of hosts, a phenomenon which is more common in pneumonic lungs in buffaloes (*Ewers et al., 2006*).

Presence of adhesins on the bacterial surface is usually linked to virulence as these proteins are known to play a crucial role in facilitating host invasion and colonization (*Kline et al., 2009*). Studies by (*Ewers et al., 2000*) and (*Tang et al., 2009*) have demonstrated that, of the adhesins; fimA, hsf-2, and ptfA are of frequent occurrence among pathogenic isolates of *P. multocida*.

The tbpA and nanH encoding genes presented by 100% of common occurrence among ruminant *P. multocida* strains and this results are similar to (*Ewers et al., 2006; Atashpaz et al., 2009*).

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التوصيف الظاهري والوراثي للباستيريلا مالتوسيدا في حيوانات المزارع
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ميكروب الباستيريلا مالتوسيدا هو المسبب الشائع لأمراض الجهاز التنفسي بالماشية التي تسبب خسائر اقتصادية عالية والعزل الأولي للميكروب وإجراء الاختبارات الكيميائية عليه وعزله باستخدام تقنية البى سى ار وعزل جينات الضراوة من الميكروب باستخدام تقنية البى سى ار. ولتحقيق هذه الأهداف تم عزل 27 عترة للباستيريلا مالتوسيدا من 280 عينة حقلية تم تجميعها بنسبة (9.6%). ومن نتائج العزل اتضح أن أعلى نسبة لعزل الباستيريلا مالتوسيدا كانت من عينات الرئة للحيوانات المذبوحة بنسبة (13.1%) ثم يليها عينات المسحات الأنفية بنسبة (5%). وقد تم التأكد من هذه المعزولات أنها الباستيريلا مالتوسيدا مجهريا بواسطة صبغها بصبغى الجرام والليشمان, وبيوكيميائيا بواسطة الاختبارات التقليدية وبواسطة اختبار تجارى API 20E وأيضا باستخدام تفاعل البلمرة المتسلسل (Polymerase Chain Reaction). ولعزل الجين الرئيسى للباستيريلا مالتوسيدا وجين الكبسول الخاص بالميكروب تم استخدام تقنية البى سى ار المتعددة وكانت النتيجة ايجابية لخمس معزولات للميكروب من أصل تسعة معزولات ممثلة للميكروب وأيضا تم عزل جين الكبسول E لخمس معزولات من أصل تسعة معزولات ممثلة للميكروب. وقد تم عمل اختبار البى سى ار المتعدد للمعزولات الايجابية لعزل جينات الضراوة المتمثلة في أربع جينات ضراوة (TbpA, nanH, ompH and ptfA) التي نتج عنها نسب عالية (100%) للجينين TbpA, nanH في عينات الرئة وعينات المسح الأنفي من معزولات الأبقار والجاموس ونتج أيضا نسبة عالية لجين ptfA فى عينات الرئة بالجاموس عن عينات المسح الأنفي.