Molecular identification by multiplex polymerase chain reaction of *Pasteurella multocida* in cattle and buffaloes in Baghdad

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Summary

The aim of this study was to identify pasteurella multocida and their types by PCR in cattle's and buffaloesi bagdad from March to August 2012 on 204 animals , including 102 cattle and 102 buffaloes at slaughter houses from Baghdad .Blood samples and nasal swaps were collected , before slaughtering and lung tissues of slaughtered animal , and from 54 clinically suspected cases of pasteurellosis , including 27 bovines ,and 27 buffaloes the samples taken included blood and nasal swabs . *Pasteurellamultocida* were isolated from 94 animals include 49 cattle 45 buffaloes. The typing of the isolates by multiplex PCR for genotyping *Pasteuerllamultocida* revealed 93 isolates of type B , 31 from cattle and 62 from buffaloes ,and 81 isolates of type A , 55 from cattle and 26 from buffaloes .

Keywords: *Pasteurella multocida* , Multiplex PCR , Cattle, Buffaloes .

Introduction

Pasteurellosis is a major disease of cattle and buffaloes. The peracute form of the disease in cattle and buffaloes caused by *Pasteurella multocida* is known as haemorrhagic septicaemia (HS). *Pasteurella multocida* is an important pathogen causing number of diseases in various domestic and wild animals (1). The most important diseases are HS, pneumonia and septicaemic pasteurellosis in cattle, buffaloes, sheep and goats, pneumonia, atrophic rhinitis and septicaemia in pigs and fowl cholera or avian cholera in poultry resulting in to heavy economic losses. The organism is also responsible for pneumonic pasteurellosis in small ruminants (2 - 4). Diagnosis of the Pasteurella infections has been traditionally based on the clinical signs and isolation of the organism, which are very tedious and time consuming practices (5 and 6). In recently, nucleic acid based techniques have been reported to be rapid and sensitive means of diagnosis particularly in case of outbreaks (3, 4 and 7). Use of antibiotics has widely been reported for the treatment of various diseases caused by *P multocida*. However, the prolonged and indiscriminate use of antibiotics has resulted in the development of resistance among various strains of the organism (8) and even multi drug resistant (MDR) forms of *P.multocida* have emerged (9 and 10).

This study aimed to identify *Pasteurella multocida* and their types by multiplex PCR in cattle and buffaloes in Baghdad.

Materials and Methods

Whole blood , nasal swabs were collected from 54 suspected clinical cases of the 27 buffaloes and 27 cows; while from slaughter houses blood, and nasal swabs were collected before slaughtering and lung samples after slaughter from 204 slaughtered animals. To identification of *P.multocida*, Brain heart broth, Blood agar (BA) and MacConkey agar (MCA) were used as primary culture media for preliminary isolation of organisms from the samples according to methods described by Quinn (11and12).

P.multocida isolates were identified by using gram and methylene blue stains and biochemical tests, Oxidase, Catalase, Indole, Citrate utilization, Nitrate reduction and fermentation of sugars, glucose, sucrose, lactose (12).

Antibiotic susceptibility of the above isolates was tested for sensitivity against 7 different antibiotics. tetracycline, gentamicin, chloramphenicol, ampicillin, Cefotaxime, Nalidixicacide and Trimethoprime sulphonethozol following the disc diffusion method (9 and 12).

In College of Veterinary Medicine Al-Diwania University the isolates were subjected

to PCR technique (conventional PCR technique) using a set of primers as reported by (6). Amplification of 460bp band in PCR has been reported to be confirmatory for the species identification of *P. multocida*; Forward and reverse primers had the sequences 5′-ATCCCG CTA TTT ACC CAG TGC-3′ and 5′-GCTGTA AAC GAA CTC GCC AC-3′, respectively. The capsular genotyping of these isolates was carried out by multiplex PCR using five sets of primers as described by (3, 6 and7). The sequences of the primers used in the capsular PCR were as follows:

Name Sequence (5′ to 3′)
CapA-F TGCCAAAATCGCAGTCAG
CapA-R TTGCCATCATTGTCAGTG
CapB-F CATTTATCCAAGCTCCACC
CapB-R GCCCGAGAGTTTCAATCC
CapE-F TCCGCAGAAAATTATTGACTC
CapE-R GCTTGCTGCTTGATTTTGTC

Size of the multiplex PCR amplicons are as follows:

Amplicon size	Capsular type
1044 bp	A
760 bp	B
511 bp	E

The whole bacteria from single colonies grown on blood agar were transferred to brain heart infusion broth after that used in the amplification reaction and the concentrations of all PCR ingredients and cycling conditions for PCR as well as capsular PCR were same as (6). The amplified gene products were subjected to agarose gel electrophoresis using 1.5% agarose and then visualised by UVgel documentation system (4 and 7).

Results and Discussion

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

The samples of buffaloes and cattle isolates which showed the presence of bipolar organisms in smears of *P.multocida* are in agreement with (1and13).

During the present study *P.multocida* was isolated from blood of many buffaloes and cattle(fig.1). Different researchers also isolated *P.multocida* at different places from the outbreaks of HS in different animals such as from cattle and buffaloes in India (1), Pakistan (14), and Malaysia (3), from bovines and buffaloes in Nigeria (15), and from cattle, buffaloes, sheep and poultry from Punjab (16).

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 (1 and 12).

The *P.multocida* isolates were identified by various biochemical tests all the isolates were positive for indole test, oxidase, catalase and nitrate reduction but citrate was utilized by none of the isolates. All the isolates fermented glucose, sucrose. Similar findings have been reported by (17 - 19).

The results of present study indicated all the isolates failed to ferment lactose. This is in agreement with the findings of (3 and 18).

Distribution of HS during the period of the study showed in (Table, 1) throughout 6 months and the peak was observed in March and April, *i.e.* during the rainy season and declined during the months of June until July. Similar findings were also observed by (20 and 21) as they reported positive correlation of HS outbreaks with high rainfall, humidity and low temperature.

Sex of the animals with pasteurellosis during the period of the study is shown in (Table, 2) the highest incidence was observed in the females under 1 year of age. This result is in agreement with (22) found in their research that young animal were more susceptible than adult, and agree with (23) who found that *Pasteurella multocida* were the main isolated bacterial cause of pneumonia in young cow and among the cow and buffaloes they found that female buffaloes most commonly susceptible.

Table, 1: Numbersof infected animals with HS according to month of the study

Animals			March	April	may	June	July	August
cattle	male	Under 1 year	1	2	1	0	0	0
		Adult	1	1	4	4	2	1
	Female	Under 1 year	4	3	5	5	0	1
		Adult	2	2	4	5	1	0
Buffalo	male	Under 1 year	4	4	1	2	0	0
		Adult	1	3	2	0	0	0
	female	Under 1 year	7	5	2	0	0	1
		Adult	5	2	3	2	0	1

Table, 2: Numbers of clinical cases infected with HS from slaughter house according to sex of animals.

Animals			Clinical cases	Slaughter house
Cattle	Male	Under 1 year	0	4
		Adult	1	12
	Female	Under 1 year	2	16
		Adult	3	11
Buffalo	Male	Under 1 year	2	9
		Adult	1	5
	Female	Under 1 year	3	12
		Adult	3	10

In PCR technique(fig,2) an amplicon of 460 bp was observed from all the isolates in agarose gel electrophoresis. In conventional and multiplex PCR technique, 15(16%) cases of bovines and 31(33%) cases of buffalo origin amplified a product of 760 bp(fig.3) which revealed serotype (B) while rest of the 34(36%) bovines and 14 (15%) buffalo isolates amplified a gene fragment of 1044 bp which reveal serotype (A) and there is no record that the two types founded in the same animal (Table, 3). The species specific PCR technique can be applied for detection of *Pasteurella multocida* by using template as bacterial colony or by using the direct field samples such as nasal swab, morbid materials like spleen, one marrow, and heart blood. A multiplex PCR is a highly sensitive and susceptible rapid technique to the conventional capsular serotyping system and used to identification of capsular types. The serotyping specific primers used in this technique were designed following sequence identification and analysis of the capsular biosynthetic section of each capsular group (7).The development of molecular tools has paved the way for rapid and specific identification of infectious agents, thus colony PCR was conducted for detection of *P.multocida* (1). These findings confirmed the results obtained by (5, 6, 9 and 19).Also, (4 and 7) reported that the same primer pair gave amplification from all strains of *P.multocida* (serotypes A, B, D, E and F). Other studies (3, 4 and 24) they found that using conventional and multiplex molecular methods for rapid detection and differentiation of *Pasteurella multocida* serogroups A, B and E isolates involved an outbreak of haemorrhagic septicaemia affecting buffaloes and cattle. It is evident from present results and previous reports, that PCR technique provides rapid identification of *P.multocida* isolates and can be used as rapid diagnostic method specific for detection of *P.multocida* infection.while multiplex PCR technique provides rapid way to serotyping *P.multocida* isolates.

Table(4) showed the types of samples taken from cattle and the highest percentage of isolates taken from lung samples (38.2%) followed by nasal swabs samples (33.33%) and the lowest were blood samples (6.8%) in slaughter house cases, while in clinical cases the percentage of isolates from nasal swabs samples was (22.22%), and non from blood samples, and these differences (P> 0.05) were significant.

Table, 3: Shows types of pasteurella detected by PCR assay

Animals			*Pasteuralla multocida* serotype A	*Pasteuralla multocida* serotype B
Cattle	Male	Under 1 year	3	1
		Adult	9	4
	Female	Under 1 year	11	7
		Adult	11	3
Buffalo	Male	Under 1 year	2	9
		Adult	3	3
	Female	Under 1 year	1	14
		Adult	8	5

Table, 4: Shows types of samples taken from cattle and the positive cases infected with HS.

Place	Samples of cattle	NO. of Identification of samples	Positive cases	Serotype A	Serotype B	%
Slaughter house	Nasal swabs	102	34	22	12	33.33%
	lung	102	39	29	10	38.2%
	Blood	102	7	0	7	6.8%
Clinic	Nasal swabs	27	6	4	2	22.22%
	Blood	27	0	0	0	0%
Total		360	86			23.8%

Table(5) showed the types of samples taken from buffalo and its explain that the highest percentage of isolates taken from lung samples (33.3%) followed by nasal swabs samples (27.4%) and the lowest were blood samples (13.7%) in slaughter house cases, while in clinical cases nasal the percentage of isolates from swabs samples was (25.9%), and from blood samples was (18.5%), and these differences were significant (P>0.05). These result agree with (23) found that slaughter house was most reliable to isolate *Pasteurella multocida* pathogen for that its better than farm cases as lung specimens added to the other specimens blood and nasal swabs. During the present study was found that the infected lung samples recorded the highest number of isolates followed by the nasal swabs samples and the blood recorded the lowest number of isolates. Similar findings have also been reported by (21) they found that the lung sample the highest number of isolates of *P.multocida* and agree with (25) who found that the lung samples recorded the high percentage of isolate followed by nasal swabs.

Table, 5: Shows types of samples taken from buffaloes and the positive cases HS.

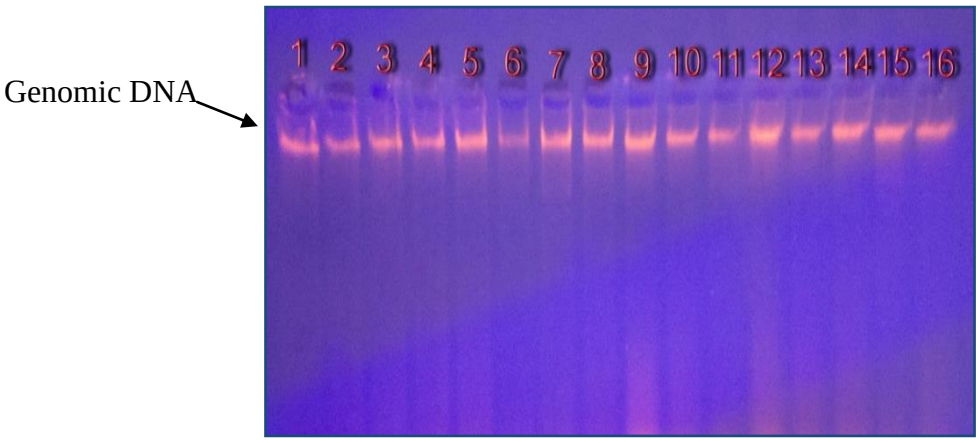
Place	Samples of Buffalo	NO. of Identification of samples	Positive	Serotype A	serotype B	%
Slaughter house	Nasal swabs	102	28	12	16	27.4%
	lung	102	34	11	23	33.3%
	Blood	102	14	0	14	13.7%
clinic	Nasal swabs	27	7	3	4	25.9%
	Blood	27	5	0	5	18.5%
total		360	88			24.4%

Antibiotic susceptibility of *P.multocida* isolates are important in determining appropriate therapy against bacterial pathogens.The bacterial organisms over a period of time change their antibiogram patterns and develop resistance against commonly used chemotherapeutic agents (1 and 10). In the present study *P.multocida*

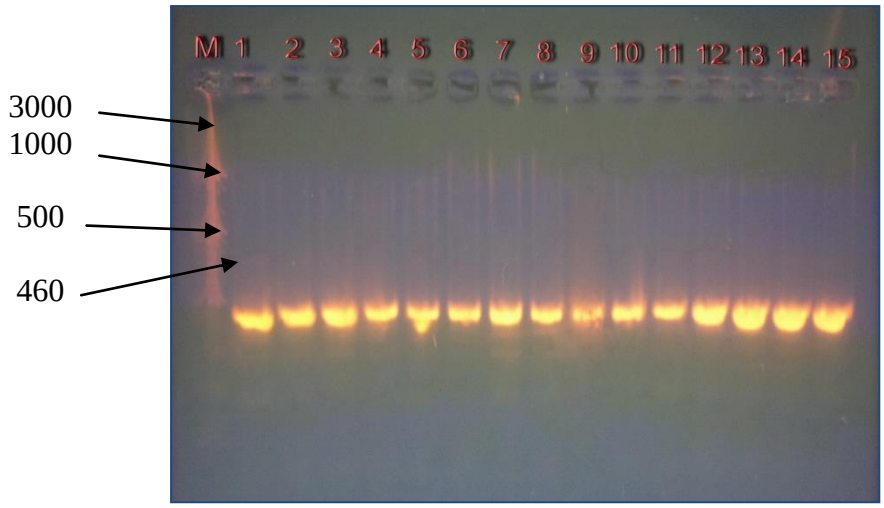
isolates were tested for their antibiotic susceptibilty pattern against seven commonly used antibiotics (Table 6). Present results (Table 6) were in accordance with the findings of (26 and 27). Chloromphinocolas 100% of serotype B isolates were susceptible to it and 91.3% of serotype A isolates were susceptible to it. However (1) found all the isolates were found sensitive to chlorampenicol, while (28) found 90% of the isolates sensitive to chloramphenicol and (26) found 63.46% of the isolates sensitive to chloramphenicol. Gentamicin was found effective on 61% for Serotype B isolates 64% for Serotype A isolates which is similar to the findings of (25) Sharma found the majority of isolates sensitive to gentamicin and moderate sensitivity to gentamicin was reported by (27). In the present study Cefotaxime was found effective on 100% of the isolates. It is in accordance with the findings of (9) while (27) found 90 % of isolates sensitive to Cefotaxime. The susceptibility of isolates to Tetracycline were 39% of serotype B isolates and 30% of serotype A isolates while (14 and 29) found all the isolates were sensitive to tetracycline. Rajini (23) reported 100 %resistant to tetracycline. Ampicillin found effective on 34% serotype of B isolates and on 35% of serotype A isolates, while (21) observed 85.7 %isolates sensitive but (28) observed 100% of the isolates were sensitive to ampicillin. In this study Nalidixic acid was effective on 92% of serotype B isolates and 77% of serotype A isolates. The same results were found by (27). Trimthprime Sulphonethozol was found effective on 79% of Serotype B isolates and 82% of serotype A isolates, while (27) found 90% of isolates sensitive to Trimthprime sulphonethozol .

Table, 6:A ntibacterial susceptibility of isolates *Pasteurella multocida* .

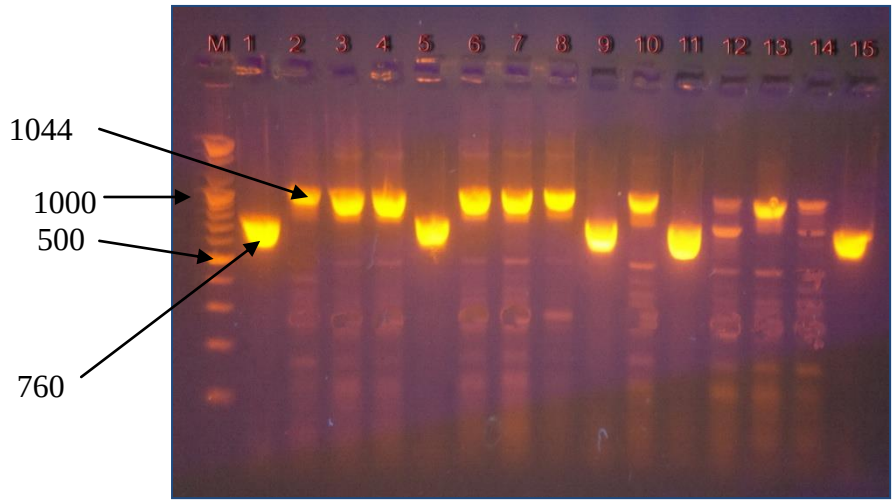
Anti bacterial agents	code	Serotype B			Serotype A		
		S	I	R	S	I	R
Tetracycline	TE	37 39.7%	42 45.1%	14 15%	25 30.8%	39 48.1%	17 20%
Gentamycine	CN	57 61.2%	29 31.1%	7 7.5%	52 64. 1%	14 17.2%	15 18.5%
Ampicilline	AM	32 34.4%	43 46.2%	27 29%	29 35.8%	33 40.7%	19 23.4%
Cefotaxime	CTX	93 100%	0 0%	0 0%	81 100%	0 0%	0 0%
Trimthprime Sulphonethozol	SXT	74 79.5%	16 17.5%	3 3.5%	67 82.7%	9 11.1%	0 0%
Chloromphinocol	C	93 100%	0 0%	0 0%	74 91.3%	7 8.6%	00%
Nalidixicacide	NA	86 92.4%	7 7.2%	0 0%	63 77.7%	18 22.2%	0 0%



Figure, 1: Shows Genomic DNA of pasteurella multocida (1-16) positive isolate which isolates from cattle and buffalo as appear in agarose 2% by using electrophoresis in PCR technique.



Figure, 2: PCR amplification of *Pasteurella multocida* DNA using PCR 2% agarose (460 bp) (M) represent DNA ladder(1-15) represent positive samples (460bp).



Figure, 3: Multiplex capsular PCR assay on *Pasteurella multocida* isolates representing the two types (A, B) of isolates detected (M) represent ladder DNA (2,3,4,6,7,8,10,13) represent type A (1044 bp) (1,5,9,11,15) represent type B (760 bp.

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التحري الجزيئي عن الباستوريلا ملتسدا باستخدام تفاعل تسلسل البلمرة في الابقار والجاموس في بغداد

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الخلاصة

ان الهدف من هذه الدراسة هو التحري عن الباستوريلاملتسدا اجريت هذه التجربة للفترة من اذار الى آب 2012 على 204 حيوان من المجازر في بغداد ، تضمنت 102 ابقار و 102 جاموس . جمعت عينات من الدم ومسحات انفية قبل الذبح وكذلك جمعت عينات الدم والمسحات الانفية وقطع من الرئة بعد الذبح وتم جمع 54 حالة سريرية مشكوك بها للإصابة بجراثيم الباستوريلا ,تضمنت 27 حالة من الابقار و27 حالة من الجاموس والنماذج التي اخذت منها كانت الدم و مسحات انفية .تم عزل جراثيم الباستوريلا ملتسدا من 94 حيوان ,كان منها 49 من الابقار و45 من الجاموس . تم تشخيص وتنميط جراثيم الباستوريلا بواسطة تفاعل تسلسل البلمرة حيث اظهرت 93 عزلة من نوع B , منها 31 عزلة من الابقار و62 عزلة من الجاموس ، وكذلك 81 عزلة من نوع A , منها 55 عزلة من الابقار و26 عزلة من الجاموس.

الكلمات المفتاحية: الباستوريلا القاتلة، سلسلة تفاعل البلمرة المتعدد , الأبقار , الجاموس.