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Original Article

Isolation, Molecular Characterization and Antibiotic Susceptibility Pattern of *Pasteurella multocida* Isolated from Cattle and Buffalo from Ahwaz, Iran

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ABSTRACT

Pasteurella multocida is a gram-negative, opportunistic pathogen and the common inhabitant of the upper respiratory tract of domesticated and wild animal species. It is a causative agent of numerous economically important diseases worldwide, such as enzootic bronchopneumonia in ruminants and haemorrhagic septicemia in cattle and buffalo. The present study was undertaken to determine the prevalence of *P. multocida* carriers, PCR-serogrouping and antibiotic susceptibility status of isolates detected in cattle and buffalo in Ahvaz, Iran. Nasopharyngeal and nasal swabs were collected from 227 cattle and 174 buffaloes. The swabs were streaked on MacConkey and sheep blood agar and incubated for 24-48 h at 37 °C. The presumptive *P. multocida* colonies were identified based on standard biochemical testing and further confirmed by PCR. A multiplex PCR was used to determine the five pathogen serogroups (i.e., A, B, D, E, and F). Antimicrobial susceptibility of *P. multocida* isolates was determined using the Kirby–Bauer disc diffusion method on Mueller-Hinton agar supplemented with 5% sheep blood. Out of 401 tested samples, *P. multocida* was recovered from 10/227 (4.4%) and 12/174 (6.89%) cattle and buffaloes, respectively. Fifteen isolates (68.19%) belonged to serogroup A, 5 (22.72%) to serogroup D, and 2 (9.09%) isolates were untypeable. No isolate belonged to B, E, and F serogroups. All *P. multocida* isolates were sensitive to nitrofurantoin, florfenicol, ciprofloxacin, enrofloxacin, trimethoprim-sulfamethoxazole, oxytetracycline, and ceftriaxone. The most common finding was resistance to tylosin (90.9%), followed by resistance to oxacillin (54.54%),.

Keywords: *Paseurella multocida*, Buffalo, Cattle, Capsular serogroup, Antibiotic sensitivity

L'isolement, l'identification moléculaire et profil de sensibilité aux antibiotiques en Pasteurella multocida isolé des vaches et des buffles à Ahvaz en Iran

Résumé: *Pasteurella multocida* est une bactérie gram-négative, opportuniste pathogène et flore normale des voies respiratoires supérieures des espèces animales domestiques et sauvages. Cette bactérie cause des maladies économiquement importantes partout dans le monde telles que bronchopneumonie enzootique chez les ruminants et la septicémie hémorragique chez les vaches et les buffles. L'objectif de cette étude est la détermination de la prévalence des porteurs de Pasteurella multocida chez les vaches et les buffles à Ahvaz, la détermination des sérogroupes capsulaires de bactéries et aussi l'évaluation de la sensibilité aux antibiotiques des isolats. Pour ce faire, les prélèvements nasal et rhinopharyngé ont été collectés de 227 vaches et 174 buffles.

Les écouvillons ont été mises en culture dans environnement Mac et Cancan et gélose au sang de mouton et mises à incuber pendant 24-48 heures à 37 C. Colonies soupçonnées à Pasteurella multocida ont été identifiées sur la base des tests biochimiques standards et confirmées par PCR. Les multiples réactions de PCR ont été utilisées pour déterminer les cinq sérogroupes capsulaires bactérien (A, B, D, E et F). L'évaluation de la sensibilité aux antibiotiques des isolats de Pasteurella multocida a été également mise en place en utilisant la méthode de diffusion sur disque (Kirby-Bauer) sur un milieu de gélose de Mueller Hinton additionné de 5% sang de mouton. Parmi 401 échantillons, Pasteurella multocida était trouvé respectivement de 10.227 (4.4%) et 12.174 (6.89%) des vaches et des buffles. 15 isolats (68.19%) appartenait au sérogroupe A, 5 isolats (22.72%) appartenait au sérogroupe D et 2 isolats (9.09%) étaient inclassable. Aucun isolats n'appartenait au sérogroupes B, E et F. Tous les isolats de Pasteurella multocida étaient sensibles à nitrofurantoïne, Florfenicol, ciprofloxacine, enrofloxacine,Triméthoprime sulfaméthoxazole , oxytétracycline, ceftriaxone. La plus grande résistance était à la tylosine (90.9%), ensuite à oxacilline (54.54%), streptomycine (45.45%), ampicilline (27.27%) , Erythromycin (13.63%) et enfin pénicilline (9.1%).

Mots clés: *Pasteurella multocida*, buffle, vache, sérogroupes, la sensibilité aux antibiotiques

INTRODUCTION

Pasteurella multocida (*P. multocida*) belongs to the family *Pasteurellaceae* and is a gram-negative bacterium responsible for a range of infections in many wild and domestic animals causing substantial economic losses (Dziva et al., 2008). *P. multocida* isolates are grouped serologically into serogroups A, B, D, E, and F based on their capsular antigens (Carter, 1955; Rimler and Rhoades, 1987). Each type gives rise to different kinds of illnesses among animals. *P. multocida* type A is the cause of fowl cholera in poultry and pneumonia in ruminants, meanwhile type D produces atrophic rhinitis in pigs and snuffles in rabbits. Capsular type F is predominantly associated with poultry disease, particularly turkeys, although it has occasionally been reported in ruminants. In cattle, capsular serotypes B and E are associated with hemorrhagic septicemia in the Asian (serotype B) and African (serotype E) countries (Boyce and Adler, 2000; Hunt et al., 2000). Capsular types of *P. multocida* (especially A, B, and E) may be isolated from healthy cattle and buffaloes being its carrier. These carriers may shed the organisms in their nasal secretion and provide a source of infection for the susceptible animals (Shayegh et al., 2010). Phenotypic techniques for characterization and serotyping of *P. multocida* isolates have low discriminatory power and are time-consuming (Garcia et al., 2011). Townsend et al. developed a polymerase chain reaction (PCR) assay for capsular typing of *P. multocida* strains based on identification and sequence analysis of the biosynthetic locus of the *P. multocida* capsule, which provided a greater understanding of the capsular polysaccharide composition and a genetic basis for the serologic differences. This assay represented a rapid and reproducible alternative to the serologic and non-serologic methods (Townsend et al., 2001). The prolonged and indiscriminate use of antibiotics resulted in the development of antibiotic resistance and even multi-drug resistant (MDR) forms of *P. multocida* (Shivachandra et al., 2004; Arora et al., 2005). Antimicrobial resistance of *Pasteurella* isolates varies according to the host animal species, time, geographical origin, and antimicrobial pretreatment of animals (Caprioli et al., 2000). The aim of the present study was to determine the prevalence of *P. multocida* carriers, PCR-serogrouping, and antibiotic susceptibility status of isolates detected in cattle and buffalo in Ahvaz, Iran.

MATERIALS AND METHODS

Specimen collection, culture, and bacterial identification. Nasopharyngeal and nasal swabs were collected from 227 cattle and 174 buffaloes. The age and sex of the animals were recorded. The swabs were transferred to the laboratory and were processed for isolation and identification of *P. multocida*. Briefly, the swabs were streaked onto MacConkey and sheep blood

agar and incubated for 24-48 h at 37 °C. The presumptive colonies were identified based on the standard morphological and biochemical tests (Markey et al., 2013).

***Pasteurella multocida*-specific polymerase chain reaction (PM-PCR).** In addition to the conventional methods of identification, all *P. multocida* isolates were subjected to PM-PCR using primer sets designed from the sequence of the *kmt1* described by Townsend et al. (1998). The sequence of oligonucleotide primers is described in Table 1. *P. multocida* type A was used as positive control. PCR was performed directly from single colonies grown on agar plates (Townsend et al., 2001). A pipette tip was lightly touched onto a colony, and the sample was then resolved in PCR amplification mixture (Amplicon-Denmark) containing 1.5 mM $MgCl_2$, 0.5 U of Taq DNA polymerase, 0.2 mM dNTPs, and primer (Bioneer South Korea) at the concentration of 3.2 µM. PCR was performed on an Eppendorf thermal cycler (Germany), with an initial denaturation at 95 °C for 4 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 9 min. The amplified products were separated by electrophoresis (1.5% agarose in 1X TAE) and visualized by Safe staining (Cinnagene Co.).

Capsular typing by multiplex PCR. Multiplex PCR was performed to detect capsular genes for all serogroups according to the method described by Townsend et al. (2001). The oligonucleotide sequences of primer sets specific for serogroups A, B, D, E, and F (Table 1) were synthesized by Bioneer (South Korea). *P. multocida* type A was used as positive control. The multiplex PCR mixture contained each primer within the six primer sets at a concentration of 3.2 mM, 1 U of Taq DNA polymerase, 1.5 mM $MgCl_2$, each deoxy-nucleoside triphosphate at a concentration of 200 mM, and 1X PCR buffer. For DNA, a pipette tip was lightly touched onto a single colony, and then was resolved in PCR amplification mixture (Townsend et al., 2001). PCR was performed with an initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation (95 °C, 45s), annealing (55 °C, 45s), extension (72 °C, 1 min), and a final extension step of 72 °C for 5 min. The amplified products were separated by electrophoresis (1.5% agarose in 1X TAE) and visualized by Safe staining (Cinnagene Co.).

TABLE 1. Sequences of the oligonucleotides used in the *P. multocida* specific polymerase chain reaction (PM-PCR) and multiplex capsular PCR typing assay

Serogroup	Sequence (5′ 3′)	Amplified size (bp)
All*(PM-PCR)*	F:ATCCGCTATTTACCCAGTGG R:GCTGTAAACGAACTCGCCAC	456
A	F:TGCCAAAATCGCAGTCAG R:TTGCCATCATTGTCAGTG	1044
B	F:CATTTATCCAAGCTCCACC R:GCCCGAGAGTTTCAATCC	760
D	F:TTACAAAAGAAAGACTAGGAGCCC R:CATCTACCCACTCAACCATATCAG	657
E	F:TCCGCAGAAAATTATTGACTC R:GCTTGCTGCTTGATTTTGTC	511
F	F:AATCGGAGAACGCAGAAATCAG R:TTCCGCCGTCAATTACTCTG	851

Antibiotic sensitivity assay. Twenty-two isolates of *P. multocida* were tested for antibiotic sensitivity against 15 different antibiotics such as penicillin, ampicillin, gentamicin, streptomycin, tetracycline, erythromycin, ciprofloxacin, enrofloxacin, nitrofurantoin, florfenicol, azithromycin, trimethoprim-sulfamethoxazole, tylosin, oxacillin, and ceftriaxone using the standard method proposed by National Committee for Clinical Laboratory Standards (NCCLS, 2008) and following the disc diffusion method (Baur et al., 1966). From an overnight culture of each isolate in blood agar, a bacterial suspension equal to a McFarland standard of 0.5 was prepared in 0.85% saline and was plated on Muller-Hinton agar medium enriched with 5% sheep blood. The culture was allowed to adsorb for 10 minutes, and then the antibiotic discs (Padtan Teb Co.) were placed on the plate at an appropriate distance from each other. The plates were incubated aerobically at 37 °C for 24 h. The diameters of inhibition zones surrounding the antibiotic discs were measured and subsequently matched with the standard inhibition zone diameters of the respective antibiotic discs. Based on the size of inhibition zones of various antibiotics, the isolates were classified as sensitive, intermediately

sensitive, or resistant according to the guidelines provided by the manufacturer of the antibiotic disk.

Statistical analysis. The results were analyzed using Chi-square and Fisher's exact tests with confidence level of 95%.

RESULTS

A total of 22 *P. multocida* isolates were recovered from the cultured samples. The *P. multocida* colonies on blood agar were small, glistening, non-haemolytic, and dewdrop-like in appearance with sweetish odor, whereas no growth was observed on McConkey agar. The isolates were gram-negative coccobacill. As regards biochemical characterization, all the isolates were positive for catalase, oxidase, and indole test, while no reaction was noted with citrate, urea, Methyl red (MR), Voges-Proskauer (VP), or gelatin liquefaction tests. The results of cultural and biochemical tests were in accordance with those of *P. multocida*. Suspected isolate to *P. multocida* in morphological and biochemical tests were confirmed for the species identification of *P. multocida* in PM-PCR assay and an amplicon of 456 bp was observed from all the isolates in agarose gel electrophoresis (Figure 1).

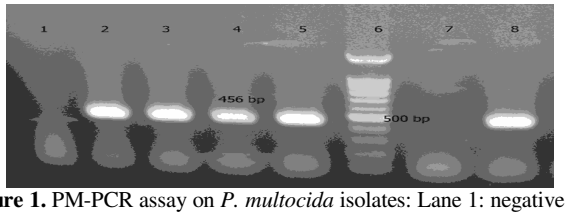

Figure 1. PM-PCR assay on *P. multocida* isolates: Lane 1: negative sample for *kmt1* gene, Lane 2, 3, 4, 5: 456 bp amplicon of *kmt1* gene, Lane 6: 100 bp ladder; lane 7: negative control, lane 8 positive control

The results showed that out of the 401 tested samples (127 from male cattle, 100 from female cattle, 122 from male buffaloes, and 52 from female buffaloes), which were studied during a 9-month period (from October 2014 until June 2015), *P. multocida* was recovered from 10/227 (4.4%) and 12/174 (6.89%) specimens from cattle and buffaloes, respectively. Thus, the carrier state of *P. multocida* in cattle and buffaloes was found to be 4.4% and 6.89%, respectively. Statistical analysis using Fisher's exact test did not show any significant association between sex, age, or season and the carrier state of *P. multocida* in cattle and buffalo (P>0.05). Table 2 shows the distribution of carrier state of *P. multocida* among different age groups of male and female cattle and buffaloes. In capsular PCR assay, out of 22 *P. multocida* isolates, 15 (68.19%) isolates belonged to serogroup A, 5 (22.72%) isolates to serogroup D, and two (9.09%) isolates were untypeable.

Table 2. Distribution of carrier state to *P. multocida* between age groups of male and female cattle and buffalo

Animal species	Sex	Age	No. positive (%)	No. negative (%)	Total
cattle	Male	<2/5	2(3.84)	50(96.16)	52
		>2/5	6(8)	69(92)	75
	Female	<2/5	0(0)	16(100)	16
		>2/5	2(2.38)	82(97.62)	84
	Total		10(4.4)	217(95.6)	227
Buffalo	Male	<2/5	4(8.33)	44(91.67)	48
		>2/5	4(5.4)	70(94.6)	74
	Female	<2/5	2(13.33)	13(86.67)	15
		>2/5	2(5.4)	35(94.6)	37
	Total		12(6.89)	162(93.2)	174
Total			22(5.48)	379(94.52)	401

No isolate belonged to B, E, and F serogroups. Seven (70%) isolates from cattle and 8 (66.67%) of buffalo, amplified a product of 1044 bp (type A), while 2 (20%) isolates of the cattle and 3 (25%) isolates of buffalo amplified a gene of 647 bp (type D) (Figure 2).

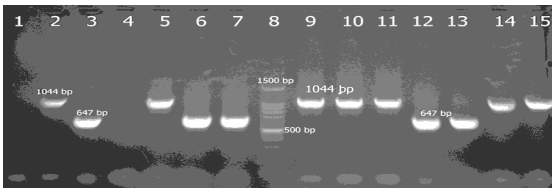

Figure 2. Multiplex capsular PCR assay on *P. multocida* isolates representing the two capsular types (A and D) of isolates detected: lane 2, 5, 9, 10, 11, 14: 1044 bp amplicon of type A. lane 3, 6, 7, 12, 13: 647 bp amplicon of type D. Lane 8: 100 bp ladder. Lane 1 negative control, lane 4: untypeable isolate. Lane 15: positive control type A.

One isolate in both cattle (10%) and buffaloes (8.33%) was untypeable. Table 3 shows the distribution of capsular serotypes among the isolates and animal species.

Table 3. Distribution of capsular serotypes among the isolates and animal species

Animal species	*P. multocida capsular* serogroups			Total (%)
	Type A (%)	Type D (%)	Untaypeable (%)	
Cattle	7(70)	2(20)	1(10)	10(100)
Buffalo	8(66.67)	3(25)	1(8.33)	12(100)
Total	15(68.19)	5(22.72)	2(9.09)	22(100)

Regarding antibiotic sensitivity testing, all *P. multocida* isolates were sensitive to nitrofurantoin, florfenicol, ciprofloxacin, enrofloxacin, trimethoprim-sulfamethoxazole, oxytetracycline, and ceftriaxone. The most common finding was resistance to tylosin (90.9%), followed by resistance to oxacillin (54.54%), streptomycin (45.45%), ampicillin (27.27%), erythromycin (13.63), and penicillin (9.1%). Nineteen isolates (36.86%) had intermediate sensitivity to erythromycin followed by ampicillin and streptomycin in 8 isolates (36.36%), gentamicin in 7 isolates (81.31%), oxacillin in 6 isolates (27.27%), as well as tylosin and azithromycin in 2 isolates (1.9%). The results of antibiotic sensitivity were interpreted and demonstrated in Table 4.

DISCUSSION

P. multocida is a commensal of the upper respiratory tract and tonsils and causes economically important diseases in ruminants such as cattle and buffaloes under extreme environmental conditions. Particular serotypes of *P. multocida* are responsible for some diseases in animals. Prevalence and distribution of serotypes vary in different areas over time. Identification of *P. multocida* serotypes is important considering the diversity and relationship of the diseases and capsular serogroups and serotypes. In addition, ongoing monitoring on outbreaks and emerging serotypes of *P. multocida* is essential for the development of effective vaccines for pasteurellosis in animals. The results of this study showed that 4.4% of cows and 6.89% of buffaloes were carriers for *P. multocida*. Haji Hajikolaei et al. (2006) showed 2.4% of examined cattle were nasopharyngeal carriers of the *P. multocida*.

Table4. Antimicrobial resistance profiles of P.multocida isolates against 15 antimicrobial agents

Antibiotic	Resistant	Sensitive	Intermediately sensitive
Nitrofurantoin	0	22(100)	0
Florfenicol	0	22(100)	0
Ciprofloxacin	0	22(100)	0
Ceftriaxone	0	22(100)	0
Enrofloxacin	0	22(100)	0
Oxytetracycline	0	22(100)	0
Trimethoprim sulfamethoxazole	0	22(100)	0
Azithromycin	0	20(90.9)	2(9.1)
Penicillin	2(9.1)	20(90.9)	0
Gentamicin	0	15(68.18)	7(31.81)
Tylosin	20(90.9)	0	2(9.1)
Oxacillin	12(54.54)	4(18.18)	6(27.27)
Streptomycin	10(45.45)	4(18.18)	8(36.36)
Ampicillin	7(31.81)	7(31.81)	8(36.36)
Erythromycin	3(13.63)	0(0)	19(86.36)

Shayegh et al. showed the prevalence of 13.25% in apparently healthy cattle and buffalo (Kaan et al. (2010); Shayegh et al., 2010; Khamesipour et al., 2014) presented the prevalence rates of 57.44% and 4.4% in cattle, respectively, and Imran et al. reported a prevalence of 3% in buffalo (Imran et al., 2007; Kaan et al., 2010; Khamesipour et al., 2014). Mustafa et al. (1978) reported subsequent haemorrhagic septicemia in cattle, they also found that the prevalence of carriers is high (44.4%), while in unrelated herds to the disease its rate was very low. Carriers of *P. multocida* in ruminant population fluctuate depending on factors such as population density, the number outbreaks of pasteurellosis, a recent exposure to the disease, and preventive measures. In addition, low prevalence of *P. multocida* carriers in this study or others could be due regular immunization programs, ruminant breeding system (indigenous or intensive systems), and stress management programs. The maximum number of carriers are found immediately after the outbreak of the disease and decreases afterwards. It is believed that stress plays an important role in activating the disease in carriers. In the diagnosis of *Pasteurella* infection, rapid confirmation of suspected cases of pasteurellosis

is a significant step, which is facilitated by species-specific PCR through eliminating the labour of intensive cultural and biochemical techniques for the diagnosis of pasteurellosis. The species-specific PCR technique can be applied for detection of *P. multocida* by using the template as a bacterial colony or by using the direct field samples such as the nasal swab, morbid materials like spleen, bone marrow, and heart blood. In this study, PM-PCR correctly identified all isolates as *P. multocida* strains by the presence of 460 bp band, which is consistent with other studies (Dutta et al., 2001; Dey et al., 2007; Arumugam et al., 2011). Certain capsular types are responsible for creating such conditions, and prevalence and distribution of each type vary from one area to another over time. Therefore, continuous monitoring of outbreak of emerging *P. multocida* serotypes is essential for developing effective vaccines to control pasteurellosis in animals. A multiplex PCR is a highly sensitive and rapid technique compared to the conventional capsular serotyping system. It is used for identification of capsular types. Capsular typing of *P. multocida* isolates by multiplex PCR indicates that cattle and buffalo isolates predominantly belong to capsular types A, accounting for 68.18% of them. Our observation is in agreement with findings of other studies, where capsular types A were detected at higher frequencies among ruminant-derived *P. multocida* isolates (Davies et al., 2004; Khamesipour et al., 2014). *P. multocida* serotype A is a common normal flora of the nasopharynx in ruminants, which can cause bronchopneumonia (Khamesipour et al., 2014). A small proportion (22.72%; 5/22) of *P. multocida* strains were type D, the presence of which in cattle and buffaloes is consistent with studies of other authors (Khamesipour et al., 2014). Two *P. multocida* isolates (9.09%; 2/30) were untypeable, which is in line with results of other studies (Dziva et al., 2004; Arumugam et al., 2011; Khamesipour et al., 2014). In this case, potential analysis could involve investigation of the presence of a non-capsulated strain by electron microscopy. In this study, the majority of *P. multocida* isolates were 100% sensitive to nitrofurantoin, florfenicol, ciprofloxacin, enrofloxacin, trimethoprim-sulfamethoxazole, oxytetracycline, and ceftriaxone. Similar results were reported for ciprofloxacin and trimethoprim-sulfamethoxazole (Hanan et al., 2000; Mohamed et al., 2012; Khamesipour et al., 2014) and enrofloxacin (Shayegh et al., 2009; Kroemer et al., 2012; Naz et al., 2012; Khamesipour et al., 2014). A study showed sensitivity of *P. multocida* to ceftiofur, enrofloxacin, florfenicol, and trimethoprim-sulfamethoxazole. The most resistance was to spectinomycin, tetracycline, erythromycin, and tilmicosin. Khamesipour et al. (2014) showed that *P. multocida* isolates were sensitive to ciprofloxacin, trimethoprim-sulfamethoxazole, doxycycline, enrofloxacin, nitrofurantoin, and tetracycline and had resistance to ampicillin, lincomycin, penicillin, rifampin, streptomycin, amoxicillin, erythromycin and florfenicol. Ampicillin, florfenicol, fluoroquinolones, and cephalosporins are the suggested antimicrobial agents against *P. multocida* (Markey et al., 2013). Considering the results of this study and those mentioned above, although isolates of *P. multocida* show resistance against many of the commonly used antibiotics, florfenicol and fluoroquinolones can therefore be used for prevention and treatment of bovine and buffalo *P. multocida* infections in the study area. Monitoring the antimicrobial susceptibility of *P. multocida* is important to determine resistance development and helping veterinarians to choose appropriate antibiotics against the diseases caused by this bacterium. However, antibiotic susceptibility studies should be renewed periodically given the possible resistance development among the *P. multocida* strains. Many studies showed the occurrence of resistance to antimicrobial agents among *P. multocida* isolates (Hunt et al., 2000; Davies et al., 2004; Arashima and Kumasaka, 2005). In summary, our results reflected that the capsular types A and D are the most common types in cattle and buffaloes, suggesting the important role of these types in the respiratory diseases of studied animals. Therefore, considerable attention should be paid to

these types in epidemiology of pasteurellosis and development of an effective vaccine. Additionally, according to the results of the antibiotic susceptibility test, fluoroquinolones, florfenicol, nitrofurantoin, trimethoprim-sulfamethoxazole, oxytetracycline and ceftriaxone are recommended for successful treatment of pasteurellosis and prevention of antibiotic resistance.

Ethics

I hereby declare all ethical standards have been respected in the preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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