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Original Article

Prevalence of adhesionVirulence factor genes, antibiogram, and pathogenicity of avian *Pasteurella multocida* isolate from Iran

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ABSTRACT

Pasteurella multocida possesses various virulence factors, including capsule, lipopolysaccharide, fimbriae, toxins, outer membrane proteins, and adhesions. Adhesins have a crucial role in mediating colonization and invasion of the host. The aim of the present study was to identify the prevalence of adhesion factor genes and resistance/sensitivity patterns among the avian *P. multocida* isolates from Iran. A total of 30 isolates of *P. multocida* were used for this study. All isolates were obtained from the poultry cases dead from fowl cholera in the northern parts of Iran. The results of the polymerase chain reaction analysis for the frequency of virulence-associated genes showed that the genes encoding adhesins (i.e., *ptfA*, *fimA*, *hsf-1*, *pfhA,* and *ompH*) were found in all (100%) of the isolates. However, the frequency of two genes including *tadD* and *toxA* were 50% and 70%, respectively. Thegenotyping patterns were classified into four groups according to the virulence factors in *P. multocida* isolates. Genotype pattern I, which included the isolates harbouring all of the examined virulence factor genes showed the highest frequency (43.3%). Pathogenicity test showed that all of the isolates classified as genotype I were pathogen or highly pathogen in the mice model. The sensitivity of the isolates to penicillin, ampicillin, lincospectin, florfenicol, tylosin, and tiamulin was 100%. However, the sensitivity rates to flumequin, enrofloxacine, and nalidixicacid were 96.6% and 80%, respectively. The findings of the current study will be helpful to elucidate the disease process and develop an efficient multivalent local vaccine.

Keywords: *Pasteurella multocida*, Adhesion factors, Antibiogram, Pathogenicity, Fowl cholera

La prévalence des gènes de facteurs de Virulence de facteur d'adhérence, antibiogramme et pathogénicité dans les isolats des oiseaux de *Pasteurella multocida* en Iran

Résumé: Les adhésions ont un rôle très important dans la création de la colonisation et l'attaque à l'hôte. En outre, leur présence au niveaux de bactéries sont souvent associées à la virulence. Les adhésions étaient le but des études récentes de génotypage virulence. Le but de la présente étude est d'identifier la prévalence des gènes de facteurs de virulence et les modèles de résistance/sensibilité dans les isolats des oiseaux de Pasteurella multocida en Iran. Un total de 30 isolats de Pasteurella multocida ont été utilisés dans la présente étude. Tous les isolats proviennent des oiseaux morts de choléra aviaire dans les régions du Nord de l'Iran. Les résultats de l'analyse PRC pour déterminer la fréquence des gènes associés à la virulence ont montré que le gènes codant des adhésions ptfA, fimA, hsf-1, pfhA et ompH ont été trouvés (100%) dans tous les isolats. En revanche, la fréquence des deux gènes de tadD et toxA était respectivement 50% et 70%. La fréquence des modèles de génotype sont classées en quatre groupes (I-IV) selon les facteurs de virulence des isolats Pasteurella multocida. Génotype modèle I comprend les isolats qui contiennent tous les gènes de facteurs de virulence testés.

Ce modèle a montré la plus haute fréquence (43.3%). Des tests de pathogéncité ont montré que tous les isolats classés comme génotype I étaient aiguë ou suraiguë dans un modèle de souris. La sensibilité des isolats à Pénicilline, ampicilline, lincoseptin, florfenicol, Tiamuline et tylosine était 100% et la sensibilité envers Flemequine, Enrofloxacin et Nalidixic acid était respectivement 96% et 80%. Les résultats de cette recherche seront utile pour clarifier le processus de maladie et aussi pour produire un vaccin multivalent en utilisant des souches régionales.

***Mots clés*:** Pasteurella multocida, facteurs d'adhésion, antibiogramme, pathogénicité, choléra aviaire

INTRODUCTION

Pasteurella multocida is a gram-negative bacteriumincluding fowl cholera in avian species, hemorrhagic septicemia in ungulates, shipping fever, pneumonia in cattle, atrophic rhinitis in swines, and snuffles in rabbits. This bacterium also causes infection in humans primarily through dog and cat bites (Blackall et al., 1997). Avian pasteurellosis, also known as fowl cholera is a highly contagious, systemic, and severe disease affecting the wild and domestic birds frequently resulting in high mortality and morbidity. This disease affects over 100 avian species and is of major economic significance throughout the world in the areas of domestic and commercial poultry production (Christensen et al., 2004; Harper et al., 2006). Avian pasteurellosis can occur in peracute/acute and subacute/chronic forms. All types of poultry are susceptible to this disease. However, the turkeys, pheasants, and partridges are highly susceptible to peracute/acute forms of this disease. In the chickens, the most common forms of the disease are acute and chronic types. The peracute/acute form of pasteurellosis leads to a death due to the end-stage bacteremia and endotoxic shock. The signs of acute cholera have been reproduced by the injection of endotoxin from *P. multocida* (Fegan et al., 1995; Wilkie et al., 2000; Varga et al., 2013). *P. multocida* has been classified by serological methods into five capsular serogroups (i.e., A, B, D, E, and F) and 16 serotypes, which can be distinguished by the analysis of heat-stable precipitin antigens (Christensen et al., 2004; Davies, 2004). *P. multocida* possesses various virulence factors, including capsule, lipopolysaccharide, fimbriae, toxins, iron-regulated and iron acquisition proteins, sialic acid-binding proteins, hyaluronidase, outer membrane proteins, and adhesins (Ewers et al., 2006; Harper et al., 2006). Adhesins have a crucial role in mediating colonization and invasion of the host. Therefore, their presence on the bacterial surfaces is usually correlated with virulence. Adhesins have been targeted in the recent virulence genotyping studies, which demonstrated that *fimA* (fimbriae), *hsf*-1, 2 (autotransporter adhesins), *pfhA* (filamentous hemagglutinin), *tad* (nonspecific tight adherence protein), and *ptfA* (subunit of type 4 fimbriae) are present in the pathogenic isolates of *P. multocida* (Tang et al., 2009). Despite the economic importance of the infections caused by *P. multocida*, the mechanisms by which this agent causes different diseases are still poorly understood (Harper et al., 2006). The high antigenic variability as well as the different hosts and courses of infection complicate the establishment of an efficient vaccine (Davies et al., 2003). There are only a few studies, which detected or determined the frequency of virulence gene patterns (Ewers et al., 2006; Shayegh et al., 2008; Atashpaz et al., 2009; Bethe et al., 2009; Tang et al., 2009). There are even fewer data available examining the strains of avian origin. Regarding this, the aim of the present study was to identify the prevalence of adhesion factor genes and resistance/sensitivity patterns among avian *P. multocida* isolates in Iran.

MATERIALS AND METHODS

Bacterial isolates. For the purpose of the study, a total of 30 isolates of *P. multocida* were collected (Table1). All isolates were obtained from the poultry cases dying due to fowl cholera in the northern

provinces of Iran (i.e., Gilan and Mazandaran). The isolates were kept at -70 ºC as the original isolation or freeze-dried cultures. The ampoules containing freeze-dried cultures were opened under sterile conditions, and the content was dissolved in brain heart infusion (BHI) broth (Difco). Subsequently, they were inoculated on blood agar (containing 5% sheep blood) and incubated at 37 ºC for 24 h to ensure that they represented pure cultures. They were identified as *P. multocida* through using the biochemical procedures, including catalase, oxidase, indol production, urease activity, ornithine decarboxylase, and carbohydrate fermentation.

Phenotypic characterization. Antimicrobial usceptibility test: The determination of the susceptibility profile was performed using dilution and disc diffusion methods according to the standardized protocol of the document M31-A3, issued by the Clinical and Laboratory Standards Institute. The antimicrobial agents tested in this study were penicillin, nitrofurantoein, streptomycin, sulphamethoxazole-trimethoprim, florfenicol, lincospectin, tylosin, oxytetracycline, flumequine, enrofloxacin, chloramphenicol, and nalidixic acid (Padtanteb). Furthermore, such reference strains as *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as quality control organisms in all antimicrobial susceptibility tests (Ferreira et al., 2012).

Pathogenicity test: The pathogenicity of each isolate was tested in groups of six-week BALB/c mice. Each group of mice (i.e., three mice in each group) was inoculated intraperitoneally with 0.1 ml of culture containing 0.3×10^8 organisms per ml. The control group of mice was injected with a fresh BHI broth. All mice were kept under observation for 72 h, and their mortality was recorded. The blood smears were prepared from the heart blood of the dead mice and stained with Giemsa stain. Subsequently, the liver and lung samples of the dead mice were streaked onto 10% sheep blood agar and incubated at 37 ºC for 24 h. The aspirated heart blood was also inoculated in the BHI broth and incubated at 37 ºC for 18 h. Then, the broth culture was streaked onto the blood agar and MacConkey agar.

DNA extraction. The BHI overnight culture of each sample was used for the extraction of DNA by heat treatment. One ml aliquot of each culture was centrifuged (13000 g×15 min) and the resultant pellets were washed twice and resuspended in 200 µl of HPLC-grade water. After boiling for 20 min and centrifugation (13000 g 5 min), 3 µl of supernatant was used as DNA template for polymerase chain reaction (PCR) assay. The concentration of the DNA template was determined using a spectrophotometer at OD 260-280 nm (Eppendorf, Germany).

Molecular identification through PM-PCR and cap-PCR: The *PM*-PCR was used for the detection of *kmt1* species specific gene fragment as described previously (Townsend et al., 1998; Ewers et al., 2006; Tang et al., 2009). The capsular type of the isolates was determined using the cap-PCR as designated by Townsend et al. (2001). Furthermore, the somatic serotype of the isolates was previously identified by agar gel diffusion according to the Hedleston method. The *PMI31* (capsular serogroup A) and *PMI32* (capsular serogroup B) were used as the reference strains of *P. multocida*, which were obtained from the Department of Aerobic Bacterial Vaccines, Razi Institute, Karaj, Iran.

PCR detection of adhesin-related genes: All isolates were analyzed using the PCR for the presence of the adhesin-related genes. The PCR analysis of adhesin-associated genes (i.e., *ptfA*, *fimA*, *hsf-1*, *pfhA*, *tadD,* and *ompH*) was conducted as described previously (Townsend et al., 1998; Ewers et al., 2006; Tang et al., 2009). The details of the adhesin genes, sequences of the oligonucleotide primers, and the expected size of each amplicon are described in Table 1.

The PCR reactions were performed in a final volume of 25 µl at the following reagent concentrations: 10 mM Tris-Hcl, pH of 8.3, 50 mM KCI, 200 µM dNTP, 0.5 µM of each primer, 1.5 mM MgCl2, 2.5 U Taq DNA polymerase, and 1 µl of template DNA. The amplification mixtures were submitted to 35 cycles of 94ºC for 1 min, 68ºC for 1 min, 72 ºC for 1 min, and

Table 1. Details of primers and citations used for the detection of adhesion-related genes in avian *Pasteurella multocida* isolates

Genes	Description	Primer name	Nucleotide sequence	Amplicon size	References
PM (*kmt1*)	*Pasteurella multocida* (all)	*Kmt1* *Sp6*	5-'ATCCGCTATTTACCCAGTGG-3' 5'-GCTGTAAACGAACTCGCCAC-3'	460	Townsend et al., 1998
hyaD	Capsule type A	*hyaD-F* *hyaD-R*	5-'TGCCAAAATCGCAGTCAG-3' 5'-TTGCCATCATTGTCAGTG-3'	1044	Townsend et al., 2001
ompH	Outer membrane protein H	*ompH-F* *ompH-R*	5-'ACTATGAAAAAGACAATGGTAG-3' 5'-GATCCATTCCTTGCAACATATT-3'	1100	Luo et al., 1997
fimA	Fimbriae	*fimA-F* *fimA-R*	5-'CCATCGGATCTAAACGACCTA-3' 5'-AGTATTAGTTCCTGCGGGTG-3'	866	Tang et al., 2009
ptfA	Type IV fimbriae	*ptfA-F* *ptf-R*	5-'TGTGGAATTCAGCATTTTAGTGTGTC-3' 5'T CATGAATTCTTATGCGCAAAATCCTGCTGG-3'	488	Ewers et al., 2006
hsf1	Autotransporter adhesin	*haf1-F* *hsf1-R*	5-'TTGAGTCGGCTGTAGAGTTCG-3' 5'-ACTCTTTAGCAGTGGGGACAACCTC-3'	654	Tang et al., 2009
pfhA	Filamentous hemagglutinin	*pfhA-F* *pfhA-R*	5'-TTCAGAGGGATCAATCTTCG-3' 5'-AACTCCAGT TGGTTTGTCG-3'	286	Tang et al., 2009
tadD	Putative nonspecific tight adherence protein D	*tadD-F* *tadD-R*	5-'TCTACCCATTCTCAGCAAGGC-3' 5'-ATCATTTCGGGCATTCACC-3'	4416	Tang et al., 2009

Table 2. Frequency of antimicrobial susceptibility of *P. multocida* isolated from poultry in Iran

Antibiotics	Sensitivity No (%)	Intermediate No (%)	Resistance No (%)
Penicillin	30 (100)	-	-
Ampicillin	30 (100)	-	-
Flumequin	29 (96.6)	-	1 (3.3)
Lincospectine	30 (100)	-	-
Florfenicol	30 (100)	-	-
Nalidixic acid	24 (80)	5 (16.6)	1 (3.3)
Enrofloxacin	29 (96.6)	1 (3.3)	-
Tylosin	30 (100)	-	-
Tiamulin	30 (100)	-	-
Tetracycline	28 (93.3)	-	2 (6.6)
Bacitracin	-	-	30 (100)
Cloxacillin	-	-	30 (100)
Furazolidon	1 (3.3)	4 (13.3)	25 (83.3)

final extension at 72ºC for 7 min. The PCR products were detected by horizontal gel electrophoresis of 10µl aliquot in a 1.5% agarose gel (Invitrogen Ultrapure™ Agarose®-Carlsbad, USA.) in TAE buffer, stained with ethidium bromide and visualized under the UV light. In addition, the molecular sizes were determined based on a 100 bp ladder molecular weight marker.

Sequencing: The PCR products was purified for sequencing using the PCR product purification kit (Roche, Germany). All purified PCR products were sequenced by Invitrogen Laboratory, South Korea. The alignments of the Iranian isolates were compared both to each other and to adhesion sequences of the reference strains, which were available in the GenBank databases.

RESULTS

The species-specific PCR confirmed all the isolates as *P. multocida*. The prevalent capsule biosynthesis gene detected in the 30 *P. multocida* isolates was found to be *capA*. None of the isolates harboured the *capB*, *capE,* and *capF* genes. The results of the antibiogram

regarding the 13 antimicrobial agents of the *P. multocida* isolates are illustrated in Table 2. The analysis of sensitivity patterns demonstrated relative and complete sensitivity in all the tested samples. Sensitivity of the isolates to penicillin, ampicillin, lincospectin, florfenicol, tylosin, and tiamulin was 100%. In addition, the sensitivity rates to enrofloxacine, and nalidixicacid were 96.6% and 80%, respectively. Out of the 30 tested strains, 3.4 % (1/30) were resistant to at least four tested drugs. The resistance to cloxacillin and bacitracin was more frequent (100%), followed by furazolidon (83.3%). The results of the PCR analysis for the frequency of virulence-related genes (i.e., *ptfA*, *fimA*, *hsf-1*, *pfhA*, *tadD*, *ompH* and *toxA*) are shown in tables 3 and 4. Genes encoding adhesins (i.e., *ptfA*, *fimA*, *hsf-1*, *pfhA,* and *ompH*) were found in all of the isolates (100%). Nevertheless, the frequency of two genes (i.e., *tadD* and *toxA*) were 50% and 70%, respectively. The genotyping patterns were classified into four groups according to the virulence factors in *P .multocida* isolates. As indicated in Table 4, the genotype pattern I, which included the isolates harbouring all of the examined virulence factor genes, showed the highest frequency (43.3%). Furthermore, the pathogenicity test revealed that all of the isolates classified as genotype I were pathogen or highly pathogen in the mice model.

Table 3 . Frequency of adhesin-coding genes among avian *P . multocida* isolates

Genes	Adhesin factors	No. of positives (%)
capA	Capsule type A	30 (100)
ompH	Outer membrane protein H	30 (100)
hsf1	Autotransporter adhesin	30 (100)
ptfA	Type IV fimbriae	30 (100)
pfhA	Filamentous hemagglutini	30 (100)
tadD	Putative nonspecific tight adherence protein D	15 (50)
fimA	Fimbriae A	30 (100)
toxA	Dermonecrotic toxin	21 (70)

DISCUSSION

P. multocida possesses various virulence factors, including capsule, lipopolysaccharide, fimbriae, adhesins, toxins, iron-regulated and iron acquisition proteins, sialic acid metabolism proteins, hyaluronidase, and outer membrane proteins (Ewers et al., 2006; Harper et al., 2006). These virulence factors play important roles in the pathogenesis of *P. multocida*. In the present study, the *P. multocida* isolates recovered from the avian pasteurellosis in the northern and central parts of Iran were tested for adhesin genes carriage and antibiotic susceptibility patterns. Among the genes encoding adhesin proteins, the *ptfA*, *fimA*, *hsf-1*, *pfhA,* and *ompH* genes were present in 100% of the isolates. However, two genes, namely *tadD* and *toxA,* were observed to have lower prevalence among the isolates with 50% and 70% frequency, respectively (Table 3). These virulence factors facilitate the colonization and invasion of the host, the avoidance or disruption of the host defense mechanisms, injury to host tissues, and/or stimulation of a noxious host inflammatory response (Harper et al., 2006). The profile of adhesion genes has been studied as a typing method for the characterization of bacterial pathogens including *P. multocida* (Ewers et al., 2006; Atashpaz et al., 2009; Tang et al., 2009; Ferreira et al., 2012). In a study carried out by Sarangi et al. (2015), the frequency rates of *pfhA*, *ptfA,* and *hsf1* among the ovine *P. multocida* isolates were reported to be 95.4%, 95.5%, and 49.2%, respectively. Verma et al. (2013) found that the presence of *ptfA* gene had a positive association with the disease outcomes in cattle. They concluded that this could be an important epidemiological marker gene for characterizing the *P. multocida* isolates. Furthermore, they identified the *pfhA* gene among all (100%) capsular types A and B isolates of cattle and buffalo origin from India. In another study, the feline *P. multocida* isolates were positive for the presence of such adhesin genes as *fimA*, *hsf1*, *ptfA,* and *tadD* with frequency range of 12.1-63.4%. However, the *pfhA* gene was not detected in these isolates (Ferreira et al., 2012). The high frequency of adhesin-encoding genes (i.e., *ptfA*, *fimA*, *hsf-1*, *pfhA,* and *ompH*), obtained in the present study, is relevant to the invasion potential of the avian strains in Iran. Filamentous haemagglutinin gene (*pfhA*) is an

important epidemiological marker, the presence of which has been correlated with the occurrence of disease in the farm animals (Ewers et al., 2006; Shayegh et al., 2008; Bethe et al., 2009; Katsuda et al., 2013; Verma et al., 2013). The frequency of *pfhA* gene has been reported to be 60% in the avian *P. multocida* isolates by Furian et al. (2013). However, Shayegh et al. (2008) demonstrated that there was a correlation between the presence of the gene and disease in sheep. Ewers et al. (2006)reported different frequencies of *pfhA* gene according to the species studied. In addition, they revealed a correlation between the presence of the gene and the pasteurellosis incidence in the cattle. Sarangi et al. (2014) reported considerably high *pfhA* gene frequency (i.e., 85.3% and 100% in the pig and avian isolates, respectively). Regarding this, they suggested that the *pfhA* gene might be providing survival advantage to the bacterium in the host. Furthermore, they concluded that the occurrence of horizontal gene transfer led to such high *pfhA* gene prevalence among the isolates. In the present study, fimbriae type IV protein gene (*ptfA)* was found in all the avian *P. multocida* isolates. This finding is in line with those of the previous studies (Ewers et al., 2006; Bethe et al., 2009; Tang et al., 2009). In a recent study, Sellyei et al. (2010) identified two major alleles of *ptfA* among the avian strains. Type IV fimbriae is encoded by a highly conserved N-terminal nucleotide sequence (Hatfaludi et al., 2010). Despite the variations in immunogenicity among the isolates of *P. multocida* reported previously (Doughty et al., 2000), this gene is a candidate for heterologous vaccine development in the avian hosts (Ewers et al., 2006; Furian et al., 2013). Furthermore, there was an obvious correlation between some virulence factors and capsular serogroups. For example, *hsf-1*, which has been described to be an autotransporter adhesin, was more frequently seen in serogroup D, whereas *tadD* and *pfhA* genes were associated with serogroup A (May et al., 2001; Tang et al., 2009). According to the findings of the present study, fluoroquinolones and florfenicol were the most efficient drugs to be used against *P. multocida*. In conclusion, the *P. multocaida* isolates collected from the chickens showed resistance to such antimicrobial agents as cloxacillin, bacitracin, and furazolidon. The investigation of the antimicrobial susceptibility trends of *P. multocida* provides applied information for the veterinarians in selecting the most efficacious therapeutic agents. These findings have been also supported by the previous studies carried out in Brazil, France, North America, and Japan (Kehrenberg and Schwarz, 2001; Yoshimura et al., 2001; Portis et al., 2012; Rigobelo et al., 2013). However, continuous monitoring of antimicrobial susceptibility is essential to determine the current susceptibility status of *P. multocida* isolates. Currently, many key virulence factors of *P. multocida* are gradually being identified. It seems that different virulence factors have independently entered the *P. multocida* strains at different times in the evolutionary history of the species and at multiple positions within the phylogenetic tree. Since pathogenic behavior is predicted by both virulence factor repertoire and serogroup (Harper et al., 2006), the clonal associations of virulence factors must be evaluated. These distribution patterns would support some lines of evidence suggesting that factors involved in cross-protection may potentially serve as vaccine candidates that can elicit homologous protective immunity against all *P. multocida* subtypes (Vasfi Marandi and Mittal, 1997; Harper et al., 2006). The pathogenicity test of the isolates in mice showed that more than 70% of the highly pathogenic isolates harboured all the examined adhesin genes. However, the exact relationship of these genes with pathogenicity in chickens as the main host remains to be investigated. All of the isolates, which were resistant or moderately sensitive to nalidixic acid belonged to group II of virulence factor pattern. Furthermore, the relationship between the resistance to one antibiotic (i.e., nalidixic acid) and the virulence pattern among the *P. multocida* isolates is a subject for a distinctive investigation. Further studies are suggested to investigate the mechanisms of pathogenesis and determine unequivocally the role of these factors in immunity to

Pasteurella infections. Therefore, a detailed study on the presence of virulence-related genes recovered from different host species will be helpful to elucidate the disease process and develop disease control measures in the future.

Ethics

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

Conflict of Interest

The authors declare that they have no conflict of interest.

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