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Original Article

Molecular evaluation of *Pasteurella multocida* in livestock, characterization of virulence genes, and pathogenic potential of the retrieved isolates in Iran

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Abstract

Background: Pasteurellosis remains one of the most economically important diseases in Asia. **Aims:** The present study aimed to isolate *Pasteurella multocida* from cattle, sheep, and goats, perform molecular identification and capsular typing, evaluate the presence of selected virulence genes, examine pathogenicity using minimum infectious dose (MID) assay, and determine antimicrobial susceptibility. **Methods:** The study was performed on 600 samples collected from ailing sheep, goats and cattle. Identification of *P. multocida* isolates using standard biochemical tests and *kmt*1 gene amplification, molecular capsular typing, and identification of 14 virulence genes were determined by PCR method. The pathogenicity of each isolate was assessed by injecting a minimum infectious dose (9×10^6 CFU/ml) of the isolate suspension into Balb/C mice. The susceptibility of isolates for 9 antibiotics was investigated using the disk diffusion method according to CLSI criteria. **Results:** A total number of 80 isolates were identified as *P. multocida* from 38 sheep, 28 goats and 14 cattle. Type A was the most prevalent capsular type (62.5%), while capsular type B was not detected. Among the virulence genes, *exb*D (97.5%), *plp*B (90%), *pm*HAS (82.5%), *exb*B (80%), and *tox*A (80%) were the most frequently observed. *hsf*2 was the only adhesion gene in cattle. MID results showed that 62 isolates infected mice. **Conclusion:** Our results indicate that the presence of a greater number of virulence factors does not correlate with increased pathogenicity. Pathogenesis of *tox*A gene is mostly dependent on the host and this gene alone cannot cause disease in sheep, goats and cattle. Among the studied antibiotics, enrofloxacin was preferred for the treatment of pasteurellosis.

Key words: Capsular typing, *kmt*1 gene, Minimum infectious dose, *Pasteurella multocida*, Virulence genes

Introduction

Pasteurella multocida is a Gram-negative coccobacillus and immobile pathogen belonging to the *Pasteurellaceae* family. It infects a wide range of animal hosts and is a normal inhabitant of the respiratory tract of healthy animals (Piorunek *et al*., 2023). This pathogen is associated with a variety of diseases in animals, including: hemorrhagic septicemia in cattle, fowl cholera in poultry, enzootic bronchopneumonia in cattle, sheep, and goats; and pneumonic and septicemic pasteurellosis in wild animals and human. *Pasteurella multocida* is known to be the cause of significant economic loss to the livestock industry in many countries, including the United States, Africa, Iran and Bangladesh (Bowles *et al*., 2000; Sarangi *et al*., 2014; Li *et al*., 2018; Mohammadpour *et al*., 2020). The climate of the region affects the prevalence of *P. multocida* (Gao *et al*., 2016). Pasteurellosis is more common during the rainy and humid season due to moist conditions and the presence of stressors for hosts (Jamali *et al*., 2014). The prevalence of *P. multocida* in the north of Iran, in Mazandaran province (a wet and rainy area), was 83%. On the other hand, the prevalence of this microorganism is lower in southern Iran (a hot and dry area) and was reported to be 16.6% (Tahamtan *et al*., 2014).

P. multocida can be identified using cultural and antigenic methods. However, conventional methods such as biochemical tests are time consuming, sometimes unreliable, and lack the sensitivity required to identify and differentiate pathogens. The use of polymerase chain reaction (PCR) and DNA-based methods have overcome the disadvantages and limitations of phenotypic diagnosis. For instance, analysis of nucleotide sequences

of the 16S rRNA and amplification of *kmt*1 genes are commonly employed methods (Khamesipour *et al*., 2014). The *kmt*1 gene is a unique DNA fragment used to differentiate *P. multocida* species (Mahboob *et al*., 2023). The primers used for *P. multocida* are based on the detection of the *kmt*1 gene, which encodes the outer membrane protein present in all *P. multocida* strains (Ferreira *et al*., 2015).

P. multocida pathogenicity has been associated with different virulence factors, including capsules (*hya*D-*hya*C, *bcb*D and *dcb*F), fimbriae and adhesins (*fim*A, *hsf*1 and *hsf*2), outer membrane proteins such as protectins (*omp*H, *oma*87 and *plp*B), iron-regulated and acquisition proteins (*exb*B and *exb*D), toxins (*tox*A), extracellular enzymes such as neuraminidase (*nan*B and *nan*H), hyaluronidase (*pm*HAS), superoxide dismutases (*sod*A and *sod*C), and lipopolysaccharides (LPS) (Ferreira *et al*., 2015; Cao *et al*., 2024).

Based on capsular antigens, *P. multocida* strains are classified into five serogroups of A, B, D, E and F. The capsule is an important virulence factor for *P. multocida*, with capsule-encoding genes located within a single region of the genome (Khamesipour *et al*., 2014; Furian et *al*., 2016; Duan *et al*., 2024).

Animal models are essential for investigating and identifying the pathogenesis of microorganisms. Mice are commonly used as an animal model to determine the virulence and mortality of *P. multocida*, as they are easy to handle and are a cost-effective research tool compared with other hosts. Furthermore, mice exhibit similar symptoms to those observed in goats, sheep and cattle (Weiser *et al*., 2003; He *et al*., 2019). The minimum infectious dose (MID) assay is a useful method for evaluating pathogenicity. In this method, the mortality time is measured by inoculating a certain dose of bacteria into the peritoneum of mice. Mortality within 24 h is indicative of an acute form of isolate (Tahamtan *et al*., 2014).

Antimicrobial resistance among bacterial pathogens is a major concern in both veterinary and human medicine. Antibiotics are frequently used for the treatment of pasteurellosis. However, the prolonged use of antibiotics has resulted in the emergence of multidrug resistant forms of *P. multocida* (Petrocchi-Rilo *et al*., 2020).

Currently, several methods are used to diagnosis *P. multocida* strains and study their virulence factors. It is possible that certain non-specific hosts exist for different serotypes of *P. multocida* or a virulence gene may not be functional in some serotypes. Conversely, there are a number of underestimated genes that are more important in pathology. These factors collectively could change our understanding of traditional defense strategies, such as vaccination, potentially leading to the development of a new specific gene of specific or different serotype.

The existing vaccine is expensive and may have limited efficacy; therefore, the development of a potent vaccine with cross protection is warranted (Zhao *et al*., 2022). Consequently, it is necessary to identify circulating strains in different species and evaluate genetic factors associated with virulence and pathogenesis. Despite the significant impact of *P. multocida* in Iran, there is limited information regarding the domestic animal isolates. Therefore, the present study aimed to:

1) Isolate *P. multocida* from livestock and determine the prevalence of infection
2) Identify molecular capsular types
3) Determine virulence factor genes
4) Evaluate pathogenesis of isolates in laboratory animals using MID test
5) Detect antibiotic resistance to suggest a sufficient therapeutic protocol in Fars province

Materials and Methods

Bacterial strains

This cross-sectional descriptive study was performed on six hundred sterile swab samples collected from the nasal cavity and tonsils of ailing sheep (200), goats (200) and cattle (200) from December 2019 to October 2020 in 15 farms of Fars province, Iran. Samples were collected from symptomatic animals including nasal discharge, loss of appetite, fever (41-42°C) and lethargy. The swabs were placed in Amies transport medium (Merck, Germany) and transported to the laboratory under refrigeration.

Biochemical identification

Swab samples were streaked on blood agar medium (Merck, Germany) containing 5% sheep blood and MacConkey agar (Merck, Germany), and incubated for 24 h at 37°C. Suspected isolate colonies were selected and identified using standard biochemical procedures, including the production of catalase, oxidase, indole, citrate utilization, methyl red (MR) and Vogaes Proskaure (VP) (Khamesipour *et al*., 2014; Ferreira *et al*., 2015).

Molecular identification

Bacteria were cultured overnight in brain hearth infusion (BHI) broth (Merck, Germany) at 37°C and 200 µL of bacterial suspension was submitted to the DNA extraction procedure described in previous study (Tahamtan *et al*., 2014).

To verify the accuracy of susceptible isolates, *kmt*1 gene was amplified using specific forward and reverse primers: 5′-ATC CGC GAT TTA CCC AGT GG-3′ and 5′-GCT GTA AAC GAA CTC GCC AC-3′ by PCR (Khamesipour *et al*., 2014; Ferreira *et al*., 2015). 460 bp fragments revealed that the isolates belong to *Pasteurlla* species (Mahboob *et al*., 2023).

PCR amplification was performed in 25 µL reaction mixtures containing: 1 µL of each DNA template, 0.4 µM of each primer, 2.5 mM $MgCl_2$, 0.2 mM of dNTPs, 10X PCR Buffer and 1.25 U *Taq* DNA polymerase. The PCR conditions were as follows: initial denaturation at 94°C for 5 min followed by 32 cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1

min, extension at 72°C for 1 min and final extension at 72°C for 8 min. PCR products were detected by agarose gel electrophoresis system (Analytikjena, Germany) on 1.5% agarose gels and were visualized under UV light by gel documentation system (Gel Doc, Kodak Company, USA) after staining with ethidium bromide. After identification, the isolates were inoculated into BHI broth containing 20% glycerol (Merck, Germany) and kept at -70°C for further use.

PCR of capsular genes and molecular serotyping

P. multocida strains were evaluated by PCR for capsular typing (by capsule biosynthesis genes) as described by Furian *et al*. (2013). Specific primers for amplification of *hya*D-*hya*C (capsular type A), *bcb*D (capsular type B), and *dcb*F (capsular type D) genes are described by Townsend *et al*. (2001). *P. multocida* isolates which did not yield bands on the basis of PCR capsular type were classified as untypeable strains. Fourteen virulence genes of the current isolates were detected by PCR. They included *fim*A, *hsf*1, *hsf*2, *tox*A, *exb*B, *exb*D, *nan*B, *nan*H, *pm*HAS, *omp*H, *oma*87, *plp*B, *sod*A and *sod*C. All primers were provided by Takapouzist Company, Tehran, Iran. Primers used for detection of virulence genes and gene functions in *P. multocida* isolates are described by Khamesipour *et al*. (2014). Amplified products were visualized with ethidium bromide on 1.3% agarose gel by gel documentation system (Gel Doc, Kodak Company, USA). PMSHI-9 (Genbank accessionNo.: JF694004.1) was used as positive control and master mix without any DNA was used as negative control.

Antimicrobial susceptibility test

Antimicrobial resistance profiles by nine antimicrobial agents were determined using Clinical and Laboratory Standards Institute (CLSI, 2018) procedures. Initially, the bacteria were cultivated in nutrient broth (Merck, Germany) and incubated at 37°C until the turbidity reached 0.5 McFarland standard (approximately 1.5×10^8 CFU/ml bacteria). Then, the grown bacteria were cultured on Mueller Hinton agar plates (Merck, Germany). Antibiotic discs, including penicillin (5 µg), amoxicillin (25 µg), ampicillin (10 µg), gentamicin (10 µg), erythromycin (15 µg), norfloxacin (10 µg), ciprofloxacin (5 µg), enrofloxacin (10 µg) and tetracycline (30 µg) were placed on the plates and incubated under aerobic condition at 37°C for 16-18 h. Susceptible and resistant isolates were determined by measuring the diameter of zone of inhibition (in mm), according to the manufacturer's (Padtan-Teb, Tehran, Iran) instructions.

Mice bioassay

Minimum infectious dose (MID)

To examine the pathogenesis of the 80 isolates, male Balb/C mice were assessed using MID method. Ten-week-old male mice weighing 18-20 g was selected and three mice were used for each isolate. A total of 240 mice were used in the experimental groups and three were used as controls (Jabbari *et al*., 2014; Tahamtan *et al*., 2014). All mice were received from the laboratory of Animal Resource Center of Razi Vaccine and Serum Research Institute, Shiraz, Iran. Animal care and experimental procedures were carried out according to the recommendations of the National Council on Animal Experimentation Control. Isolates were cultured in BHI broth. Mice were inoculated intraperitoneally (IP) with 0.5 ml containing 9×10^6 CFU/ml. The mice in the control group were injected with 0.5 ml of BHI broth. Finally, the mice in the control and test groups were monitored for 48 h after inoculation and MID for each isolate was calculated (Jabbari *et al*., 2014; Tahamtan *et al*., 2014; Devi *et al*., 2018).

Statistical analysis

Data were analyzed by SPSS version 16 (SPSS Inc., Chicago, IL, USA) and Fisher's exact test. P-value less than 0.05 was considered as statistically significant.

Results

Results of biochemical and molecular identification

From 600 nasal and tonsillar swab samples, 80 (13.33%) isolates were identified as *P. multocida*, of which 38 (19%) isolates were obtained from sheep, 28 (14%) from goats and 14 (7%) from cattle. The isolates were Gram-negative and coccobacilli, grown on blood agar without any hemolysis, but they did not grow on MacConkey agar. Their MR, VP and Citrate tests were negative but their Catalase, Oxidase and Indole tests were positive. The isolates were identified with *kmt1* specific primers, yielding 460 bp fragments by PCR technique (Fig. 1).

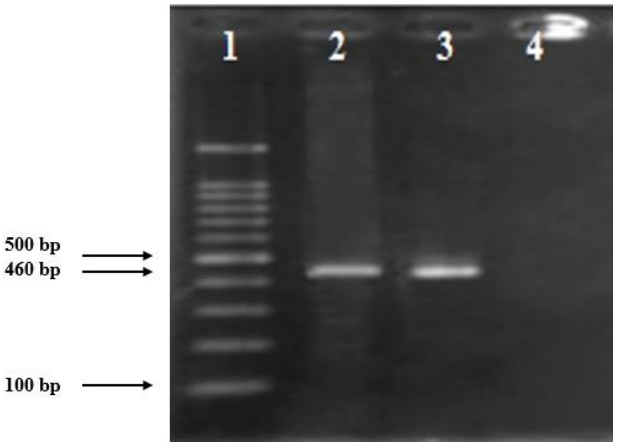

Fig. 1: 460 bp fragments from *kmt*1 gene amplification. Lane 1: Marker 100 bp, Lane 2: Positive control, Lane 3: Positive sample, and Lane 4: Negative control

Distribution of capsular genes in *P. multocida* isolates

Serogroups of *P. multocida* were determined using PCR of *hya*D-*hya*C and *dcb*F genes. Capsular types A and D were identified. The sizes of the amplicons were

1044 bp and 610 bp, respectively (Figs. 2 and 3).

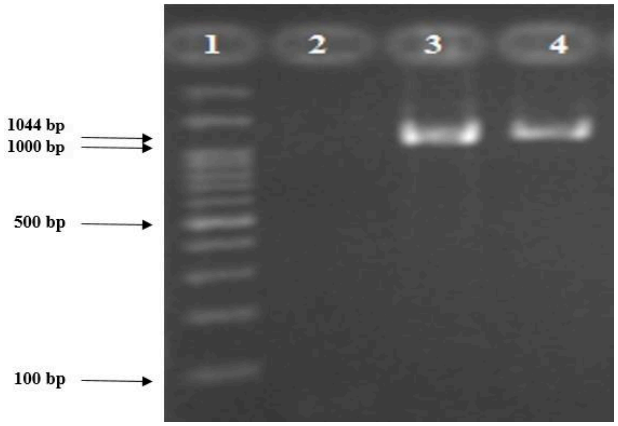

Fig. 2: 1044 bp fragments from *hya*D-*hya*C gene amplification. Lane 1: Marker 100 bp, Lane 2: Negative control, Lane 3: Positive control, and Lane 4: Positive sample

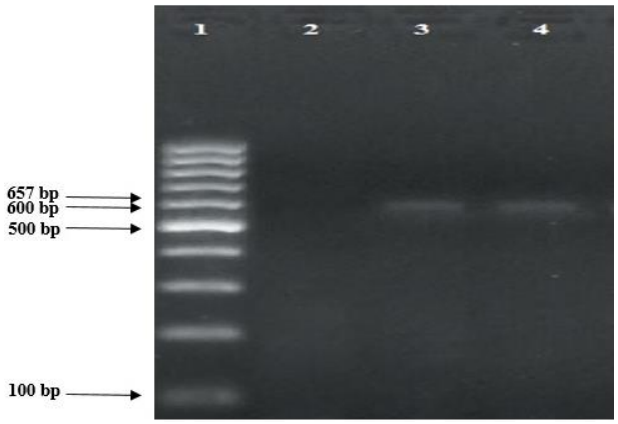

Fig. 3: 657 bp fragments from *dcb*F gene amplification. Lane 1: Marker100 bp, Lane 2: Negative control, Lane 3: Positive control, and Lane 4: P: Positive sample

Table 1 shows the capsular type distribution among different hosts. Only two types, A and D, were identified. Capsular type A was dominant and detected in 64 and 36% of sheep and goat isolates, respectively. Statistical analysis indicated a significant relation between capsular types and hosts (P<0.05).

Table 1: Distribution of capsular types among the hosts

Capsular types	Sheep (%)	Goats (%)	Cattle (%)	Total (%)
Type A	32 (64)	18 (36)	0 (0)	50 (62.5)
Type B	0 (0)	0 (0)	0 (0)	0 (0)
Type D	2 (33.3)	2 (33.3)	2 (33.3)	6 (7.5)
Untypeable	4 (16.4)	8 (33.3)	12 (50)	24 (30)
Total	38 (47.5)	28 (35)	14 (17.5)	80 (100)

Distribution of virulence factor genes in *P. multocida* isolates

Tables 2 and 3 show the distribution of virulence factors of *P. multocida* isolates characterized by capsular types and host species. The *hsf*1 and *bcb*D (encoding capsular type B) host genes were not detected in any of the isolates. In this study, *exb*D, *plp*B, *pm*HAS, *exb*B and *tox*A were frequently detected in the isolates. Among the protectin-encoding genes, *plp*B was more frequently observed than *omp*H and *oma*87 genes (90% versus

Table 2: Results of virulence factor genes for *P. multocida* isolates according to capsular type (n=80)

Virulence gene	Capsular type			Total
	Type A (n=50)	Type D (n=6)	Untypeable (n=24)	
Outer membrane protein				
*omp*H	38	4	8	50
*plp*B	50	4	18	72
*oma*87	36	2	6	44
Adherence factors				
*fim*A	32	2	6	40
*hsf*1	0	0	0	0
*hsf*2	42	6	10	58
Neuraminidase				
*nan*H	22	2	12	36
*nan*B	24	0	6	30
Superoxide dismutases				
*sod*A	36	4	4	44
*sod*C	38	2	10	50
Iron regulated-proteins				
*exb*B	46	4	14	64
*exb*D	48	6	24	78
Toxin				
*tox*A	34	6	24	64
Hyaluronidase				
*pm*HAS	46	0	20	66

Table 3: Distribution of virulence factor genes for *P. multocida* isolates according to hosts

Virulence gene	Host			Total
	Sheep (n=38)	Goats (n=28)	Cattle (n=14)	
Outer membrane protein				
omph	28	20	2	50
*plp*B	38	26	8	72
*oma*87	28	14	2	44
Adherence factors				
*fim*A	22	18	0	40
*hsf*1	0	0	0	0
*hsf*2	30	20	8	58
Neuraminidase				
*nan*H	18	10	8	36
*nan*B	18	10	2	30
Superoxide dismutases				
sodA	30	10	4	44
*sod*C	30	20	0	50
Iron regulated-proteins				
*exb*B	34	22	8	64
*exb*D	38	26	14	78
Toxin				
*tox*A	34	16	14	64
Hyaluronidase				
*pm*HAS	34	20	12	66

62.5% and 55%, respectively). Furthermore, *exb*D 78 (97.5%) and *exb*B 64 (80%), which encode iron-regulatory proteins, *pm*HAS 66 (82.5%) (encoded hyaloronidase) and *tox*A (encoded dermonecrotic toxin) 64 (80%), were more frequently observed in the isolates.

All capsular type A isolates had *plp*B gene. All type D isolates had *tox*A, *hsf*2 and *exb*D genes, but *pm*HAS and *nan*B genes were not observed among them.

*plp*B and *exb*D genes had the highest frequency in sheep and goat isolates. Although all cattle isolates have *tox*A and *exb*D genes, they do not have *fim*A and *sod*C genes. Statistical analysis showed significant association between these isolates and virulence factor genes (P≤0.05).

Antibiotic susceptibility test for *P. multocida* isolates

Antibiotic resistance patterns among the isolates were detected. Levels of antibiotic resistance in the isolates of the present study were: penicillin 52 (72.5%), amoxicillin 62 (77.5%), ampicillin 62 (77.5%), enrofloxacin 4 (5%), ciprofloxacin 20 (25%), norfloxacin 12 (15%), tetracycline 30 (37.5%), gentamicin 32 (40%), and erythromycin 40 (50%). The high and low frequency of antibiotic resistance were amoxycillin (77.5%), ampicillin (77.5%), and enrofloxacin (5%), respectively. According to results, 2.5% and 5% of isolates were susceptible and resistant to all antibiotics, respectively. But 92.5% were resistant to at least one antibiotic. For capsular type A, the high and low antibiotic resistance was allocated to ampicillin (76%) and enrofloxacin (4%), respectively. All capsular type D isolates were resistant to amoxycilin, ampicilin, gentamicin and erythromycin, but were susceptible to enrofloxacin, norfloxacin and ciprofloxacin. Statistical analysis showed that there is significant correlation between capsular types and antibiotic resistance (P≤0.05). All cattle isolates were resistant to amoxicillin and ampicillin and sensitive to enrofloxacin and norfloxacin. Sheep and goat isolates showed the lowest resistance to enrofloxacin and norfloxacin and the highest resistance to amoxycillin (P≤0.05).

Mice bioassay results

Table 4 showed the MID of isolates at different time intervals according to capsular type. In the mice bioassay, 62 of the 80 isolates (77.5%) resulted in mortality in the inoculated mice. Control mice were monitored for 48 h. In each MID group, the highest number of isolates had capsule type A, except in MID16. Type D isolates had only MID8 and MID12.

Table 4: MID in mice bioassay for *P. multocida* isolates according to capsular type

Capsular type	MID(4)*	MID(8)	MID(12)	MID(16)	MID(20)
Type A	2	8	22	4	2
Type D	0	2	4	0	0
Untypeable	2	2	6	8	0

* Minimum infectious dose after 4 h

Table 5 presents the MID results characterized by virulence factors genotype, for all *P. multocida* isolates. Our results confirm that the high frequency of virulence factors in *P. multocida* does not always induce death in mice, and the presence of more or special virulence factors does not correlate with increased pathogenicity.

Table 5: MID in mice bioassay for *P. multocida* isolates according to virulence factors genotype

Strain ID	MID	Virulence genes
1	Live	*plp*B, *fim*A, *pm*HAS, *tox*A, *exb*D
2	Live	*omp*H, *fim*A, *pm*HAS, *tox*A, *exb*D
3	Live	*plp*B, *fim*A, *pm*HAS, *tox*A, *exb*B, *exb*D
4	Live	*omp*H, *plp*B, *fim*A, *hsf*2, *nan*H, *sod*C, *tox*A, *exb*B, *exb*D
5	Live	*plp*B, *nan*H, *sod*A, *tox*A, *exb*B, *exb*D
6	Live	*plp*B, *nan*H, *sod*A, *tox*A, *exb*B, *exb*D
7	Live	*omp*H, *plp*B, *oma*87, *pm*HAS, *soda*
8	Live	*omp*H, *oma*87, *plp*B, *fim*A, *hsf*2, *sod*A, *sod*C, *tox*A, *exb*B, *exb*D
9	Live	*oma*87, *plp*B, *fim*A, *pm*HAS, *nan*H, *exb*D
10	Live	*plp*B, *hsf*2, *tox*A, *exb*D
11	Live	*omp*H, *plp*B, *fim*A, *hsf*2, *sod*C, *exb*B, *exb*D
12	Live	*omp*H, *plp*B, *fim*A, *hsf*2, *sod*C, *exb*B, *exb*D
13	Live	*plp*B, *pm*HAS, *nan*H, *sod*C, *tox*A, *exb*B, *exb*D
14	Live	*plp*B, *pm*HAS, *nan*H, *sod*C, *tox*A, *exb*B, *exb*D
15	Live	*oma*87, *plp*B, *fim*A, *hsf*2, *pm*HAS, *sod*A, *sod*C, *exb*B, *exb*D
16	Live	*oma*87, *plp*B, *hsf*2, *pm*HAS, *nan*B, *sod*C, *tox*A, *exb*B, *exb*D
17	Live	*omp*H, *oma*87, *plp*B, *pm*HAS, *nan*H, *sod*A, *tox*A, *exb*B, *exb*D
18	Live	*omp*H, *oma*87, *plp*B, *hsf*2, *pm*HAS, *nan*B, *sod*A, *sod*C, *exb*B, *exb*D
19	MID4*	*oma*87, *plp*B, *hsf*2, *omp*H, *pm*HAS, *sod*A, *tox*A, *exb*B, *exb*D
20	MID4	*omp*H, *oma*87, *plp*B, *fim*A, *hsf*2, *pm*HAS, *nan*H, *sod*C, *exb*B, *exb*D
21	MID4	*omp*H, *oma*87, *plp*B, *hsf*2, *pm*HAS, *nan*B, *sod*C, *tox*A, *exb*B, *exb*D
22	MID4	*oma87*, *plpB*, *fimA*, *hsf2*, *pmHAS*, *sodA*, *sodC*, *exbB*, *exbD*
23	MID8	*omp*H, *plp*B, *fim*A, *hsf*2, *nan*H, *sod*C, *tox*A, *exb*B, *exb*D
24	MID8	*plp*B, *fim*A, *pm*HAS, *tox*A, *exb*B, *exb*D
25	MID8	*omp*H, *oma*87, *plp*B, *fim*A, *hsf*2, *sod*A, *sod*C, *tox*A, *exb*B, *exb*D

26	MID8	*omp*H, *plp*B, *oma*87, *pm*HAS, *soda*
27	MID8	*omp*H, *oma*87, *plp*B, *hsf*2, *pm*HAS, *nan*B, *sod*A, *sod*C, *exb*B, *exb*D
28	MID8	*omp*H, *oma*87, *plp*B, *pm*HAS, *nan*H, *sod*A, *tox*A, *exb*B, *exb*D
29	MID8	*oma*87, *plp*B, *fim*A, *hsf*2, *pm*HAS, *nan*B, *sod*A, *sod*C, *tox*A, *exb*B, *exb*D
30	MID8	*omp*H, *oma*87, *plp*B, *fim*A, *hsf*2, *pm*HAS, *nan*B, *sod*A, *sod*C, *exb*B, *exb*D
31	MID8	*omp*H, *oma*87, *plp*B, *hsf*2, *pm*HAS, *nan*B, *sod*A, *sod*C, *exb*B, *exb*D
32	MID8	*oma*87, *plp*B, *hsf*2, *pm*HAS, *sod*A, *tox*A, *exb*D
33	MID8	*omp*H, *oma*87, *plp*B, *hsf*2, *pm*HAS, *sod*A, *sod*C, *tox*A, *exb*B, *exb*D
34	MID8	*omp*H, *plp*B, *hsf*2, *pm*HAS, *nan*H, *sod*A, *sod*C, *tox*A, *exb*B, *exb*D
35	MID12	*plp*B, *hsf*2, *tox*A, *exb*D
36	MID12	*oma*87, *plp*B, *fim*A, *pm*HAS, *nan*H, *exb*D
37	MID12	*pm*HAS, *nan*H, *tox*A, *exb*B, *exb*D
38	MID12	*pm*HAS, *nan*H, *tox*A, *exb*B, *exb*D
39	MID12	*Hsf*2, *pm*HAS, *nan*H, *tox*A, *exb*B, *exb*D
40	MID12	*Hsf*2, *pm*HAS, *nan*H, *tox*A, *exb*B, *exb*D
41	MID12	*omp*H, *plp*B, *fim*A, *hsf*2, *sod*C, *tox*A, *exb*D
42	MID12	*omp*H, *plp*B, *fim*A, *hsf*2, *sod*C, *tox*A, *exb*D
43	MID12	*oma*87, *plp*B, *hsf*2, *pm*HAS, *sod*A, *tox*A, *exb*D
44	MID12	*omp*H, *oma*87, *plp*B, *hsf*2, *pm*HAS, *nan*B, *sod*A, *sod*C, *exb*B, *exb*D
45	MID12	*omp*H, *hsf*2, *nan*H, *sod*A, *tox*A, *exb*B, *exb*D
46	MID12	*omp*H, *hsf*2, *nan*H, *sod*A, *tox*A, *exb*B, *exb*D
47	MID12	*omp*H, *oma*87, *plp*B, *fim*A, *hsf*2, *pm*HAS, *nan*H, *sod*C, *exb*B, *exb*D
48	MID12	*oma*87, *plp*B, *hsf*2, *pm*HAS, *sod*A, *tox*A, *exb*B, *exb*D
49	MID12	*omp*H, *plp*B, *hsf*2, *pm*HAS, *nan*B, *sod*A, *sod*C, *tox*A, *exb*B, *exb*D
50	MID12	*plp*B, *hsf*2, *pm*HAS, *nan*H, *nan*B, *tox*A, *exb*B, *exb*D
51	MID12	*omp*H, *plp*B, *hsf*2, *pm*HAS, *nan*H, *sod*A, *sod*C, *tox*A, *exb*B, *exb*D
52	MID12	*omp*H, *oma*87, *plp*B, *hsf*2, *pm*HAS, *sod*A, *sod*C, *tox*A, *exb*B, *exb*D
53	MID12	*plp*B, *fim*A, *hsf*2, *pm*HAS, *sod*C, *tox*A, *exb*B, *exb*D
54	MID12	*plp*B, *fim*A, *hsf*2, *pm*HAS, *sod*C, *tox*A, *exb*B, *exb*D
55	MID12	*omp*H, *oma*87, *plp*B, *fim*A, *hsf*2, *pm*HAS, *nan*B, *sod*A, *sod*C, *exb*B, *exb*D
56	MID12	*oma*87, *plp*B, *fim*A, *hsf*2, *pm*HAS, *nan*B, *sod*A, *sod*C, *tox*A, *exb*B, *exb*D
57	MID12	*omp*H, *plp*B, *oma*87, *hsf*2, *pm*HAS, *nan*B, *sod*A, *sod*C, *tox*A, *exb*B, *exb*D
58	MID12	*omp*H, *oma*87, *plp*B, *fim*A, *pm*HAS, *nan*B, *sod*A, *sod*C, *tox*A, *exb*B, *exb*D
59	MID12	*omp*H, *oma*87, *plp*B, *fim*A, *hsf*2, *pm*HAS, *nan*B, *nan*H, *sod*C, *tox*A, *exb*B, *exb*D
60	MID12	*omp*H, *oma*87, *plp*B, *fim*A, *hsf*2, *pm*HAS, *nan*B, *nan*H, *sod*C, *tox*A, *exb*B, *exb*D
61	MID12	*omp*H, *oma*87, *plp*B, *fim*A, *hsf*2, *pm*HAS, *nan*B, *nan*H, *sod*C, *tox*A, *exb*B, *exb*D
62	MID12	*omp*H, *oma*87, *plp*B, *fim*A, *hsf*2, *pm*HAS, *nan*B, *nan*H, *sod*C, *tox*A, *exb*B, *exb*D
63	MID12	*omp*H, *oma*87, *plp*B, *fim*A, *hsf*2, *pm*HAS, *nan*B, *nan*H, *sod*A, *sod*C, *tox*A, *exb*B, *exb*D
64	MID12	*omp*H, *oma*87, *plp*B, *fim*A, *hsf*2, *pm*HAS, *nan*B, *nan*H, *sod*A, *sod*C, *tox*A, *exb*B, *exb*D
65	MID12	*omp*H, *oma*87, *plp*B, *fim*A, *hsf*2, *pm*HAS, *nan*B, *nan*H, *sod*A, *sod*C, *tox*A, *exb*B, *exb*D
66	MID12	*omp*H, *oma*87, *plp*B, *fim*A, *hsf*2, *pm*HAS, *nan*B, *nan*H, *sod*A, *sod*C, *tox*A, *exb*B, *exb*D
67	MID16	*omp*H, *fim*A, *pm*HAS, *tox*A, *exb*D
68	MID16	*plp*B, *fim*A, *pm*HAS, *tox*A, *exb*D
69	MID16	*plp*B, *hsf*2, *pm*HAS, *sod*C, *tox*A, *exb*D
70	MID16	*plp*B, *hsf*2, *pm*HAS, *sod*C, *tox*A, *exb*D
71	MID16	*plp*B, *hsf*2, *pm*HAS, *nan*H, *nan*B, *tox*A, *exb*B, *exb*D
72	MID16	*omp*H, *plp*B, *hsf*2, *pm*HAS, *nan*B, *sod*A, *sod*C, *tox*A, *exb*B, *exb*D
73	MID16	*omp*H, *oma*87, *plp*B, *fim*A, *pm*HAS, *nan*B, *sod*A, *sod*C, *tox*A, *exb*B, *exb*D
74	MID16	*omp*H, *plp*B, *oma*87, *hsf*2, *pm*HAS, *nan*B, *sod*A, *sod*C, *tox*A, *exb*B, *exb*D
75	MID16	*omp*H, *plp*B, *fim*A, *hsf*2, *pm*HAS, *nan*B, *nan*H, *sod*A, *sod*C, *tox*A, *exb*B, *exb*D
76	MID16	*omp*H, *oma*87, *plp*B, *pm*HAS, *nan*H, *sod*A, *tox*A, *exb*B, *exb*D
77	MID16	*omp*H, *oma*87, *plp*B, *fim*A, *hsf*2, *pm*HAS, *nan*B, *nan*H, *sod*A, *sod*C, *tox*A, *exb*B, *exb*D
78	MID16	*omp*H, *oma*87, *plp*B, *fim*A, *hsf*2, *pm*HAS, *nan*B, *nan*H, *sod*A, *sod*C, *tox*A, *exb*B, *exb*D
79	MID20	*omp*H, *oma*87, *plp*B, *pm*HAS, *nan*H, *sod*A, *tox*A, *exb*B, *exb*D
80	MID20	*omp*H, *plp*B, *fim*A, *hsf*2, *pm*HAS, *nan*B, *nan*H, *sod*A, *sod*C, *tox*A, *exb*B, *exb*D

* Minimum infectious dose 4 h

Discussion

Pasteurellosis is a zoonotic disease transmitted to humans primarily through contact with nasal secretions and animal bites, leading to cellulitis and edema (Wilson and Ho, 2013; Gharibi *et al*., 2017; Piorunek *et al*., 2023). The predominant syndrome of pasteurellosis in animals is upper and lower respiratory disease, in the form of rhinitis and pneumonia, respectively. *P. multocida* is a significant infectious agent in domestic animals in Iran, resulting in substantial economic loss. The present study represents the first large-scale report evaluating the pathogenesis of *P. multocida* isolates from different livestock species in this region. Furthermore, the effect of virulence genes on pathogenicity in laboratory animals was investigated using MID results.

These findings may provide valuable information for further study.

In this study, we detected several virulence genes in *P. multocida* isolated from pneumonic hosts. Virulence factors play important roles in infection and pathogenicity; however, the contribution of an individual gene remains unclear (Nanduri *et al*., 2009).

P. multocida capsule serotypes exhibit distinct pathogenicity in animal species. For example, serotype A is the most common cause of pneumonia in goats and sheep, while serotype B can cause hemorrhagic septicemia in cattle, often leading to high mortality (Tabatabaei and Abdolahi, 2023). Atrophic rhinitis in pig is often associated with toxigenic strains of *P. multocida* serogroup D (Cao *et al*., 2024). In our current isolates, capsular type B was not detected. Only type D capsule was identified in cattle isolates, potentially due to livestock being kept in close proximity, or location, facilitating horizontal transmission (Dabo *et al*., 2007).

The attachment of pathogens to host cells is a prerequisite for bacteria to effectively employ their virulence factors and cause infection (Vu-Khac *et al*., 2020). *fim*A and *hsf*2 encoded proteins are similar to fimbriae in *P. multocida* that are adhesion molecules and play a crucial role in attachment to cell surfaces, host invasion and colonization (Harper *et al*., 2006). While Tang *et al*. (2009) and Gharibi *et al*. (2017) demonstrated that, in different hosts, *fim*A, *hsf*1 and *hsf*2 were present in all pathogenic *P. multocida* isolates, our results showed that *hsf*1 were not detected in any of the isolates. *fim*A and *hsf*2 were detected in 40 (50%) and 58 (72.5%) of pneumonic isolates, respectively. Therefore, the high prevalence of the *hsf*2 gene may be a crucial factor in bacterial attachment to the epithelial cells of hosts. Our results on adhesion genes indicate that *hsf*2 plays a crucial role in attachment and invasion in cattle, as cattle isolates contained only this gene.

In the present study, the *tox*A gene (encoded dermonecrotic toxin) was identified in 64 (80%) of the pneumonic isolates. Our results indicated this gene was present 100% of the cattle isolates, 80% in goat isolates and 42.5% in sheep isolates. While Tang *et al*. (2009), Khamesipour *et al*. (2014), and Gharibi *et al*. (2017) reported a low frequency in the *tox*A gene, particularly in cattle strains, Shayegh *et al*. (2008) reported a high frequency of this gene in sheep strains.

The *pm*HAS gene encodes hyaluronan synthase PmHAS, an enzyme that has an important role in hyaluronic acid formation, which is the main component in capsular type A structure (Furian *et al*., 2016). In ourstudy, *pm*HAS was present in 92% (46 out of 50 isolates) of the capsular type A isolates, but was not detected in capsular type D isolates. Further, it exhibited the highest frequency in sheep isolates among the hosts examined. These results are consistent with the results of Tang *et al*. (2009), who reported *pm*HAS gene was significantly associated with capsular type A.

The *nan*B and *nan*H encoded sialidases remove sialic acid conjugated to glycolipids and glycoproteins of eukaryotic cells. Sialidase has important roles in bacterial adherence, colonization and persistence (Dabo *et al*., 2007; Hatfaludi *et al*., 2010). *P. multocida* uses sialidase to obtain carbon sources (by hydrolyzing sialic acid) (Nugroho *et al*., 2022). In the current study, *nan*H and *nan*B were identified in 36 (45%) and 30 (37.5%) of pneumonic isolates, respectively. They were detected less in pneumonic cattle isolates than sheep and goat isolates. The frequency of these genes differs from the report of Khamesipour *et al*. (2014), who detected *nan*B and *nan*H genes in 83.3% and 80% of isolates, respectively. Jamali *et al*. (2014) reported the frequency of these genes to be 74%. These differences can be may be attributed to the climatic conditions of the region.

P. multocida outer membrane proteins (OmpH, Oma87, and PlpB) are protective immunogens called protectins. Similar to our results, they are usually found in capsular type A strains (Harper *et al*., 2006; Shayegh *et al*., 2008; Tahamtan and Hayati, 2014). Among three protectins tested in this study, *plp*B gene (encoded *Pasteurella* lipoprotein B) had the highest frequency (90%). PlpB is an ABC transport protein required for the uptake of methionine into the cell, and plays a key role in the pathogenesis (Merlin *et al*., 2002). The *omp*H and *oma*87 genes were detected in 50 (62.5%) and 44 (55%) of the isolates, respectively. Other studies detected these genes with high frequency (more than 80%) in their isolates (Tang *et al*., 2009; Khamesipour *et al*., 2014; Gharibi *et al*., 2017).

*sod*C and *sod*A genes encode superoxide dismutase. This enzyme has antioxidant functions (May *et al*., 2001). Our results indicate that *sod*C and *sod*A were detected in only 50 (62.5%) and 44 (55%) of isolates, respectively. These results are inconsistent with Gharibi *et al*. (2017) and D'Amico *et al*. (2024), who found the *sod*C gene in 100% of *P. multocida* isolates, and Furian *et al*. (2013) and Khamesipour *et al*. (2014), who reported a high prevalence (more than 80%) of *sod*A gene.

In contrast to Khamesipour *et al*.'s study (2014), our results showed that *P. multocida* strains had moderate resistance against gentamicin, erythromycin and tetracycline. A comparison of these results showed the increase of antimicrobial resistance from 2014 to 2020. Similar to other studies, current isolates had the highest and lowest resistance to beta-lactams (penicillin, amoxycillin and ampicillin) and quinolones (enrofloxacin, ciprofloxacin, and norfloxacin), respectively (Tang *et al*., 2009; Jamali *et al*., 2014; Khamesipour *et al*., 2014).

Generally, a high frequency of virulence factors in *P. multocida* is believed to be directly associated with pathogenicity and mortality. Khamesipour *et al*. (2014) reported that a higher frequency of the virulence factors among isolates from symptomatic animals may suggest their role in pathogenesis of *P. multocida*-associated bovine respiratory disease. However, our MID results did not confirm this hypothesis. The results indicated that differences in the frequency of virulence genes in *P. multocida* caused different mortality rates in mice. For example, Table 5 shows strain 8, which has many

virulence genes, did not cause mortality in mice, while strain 24 (with 6 virulence factors) was able to kill mice with MID8. In addition, isolates with similar virulence factors were found to have different MIDs (strains 17, 28, 76, and 79). This result indicates that other unknown factors may also be involved in the pathogenicity of *P. multocida*.

These analyses on *P. multocida* are extremely difficult due to the following reasons:

1) Diversity and interference of virulence factors in *P. multocida.* The high number of virulence genes in *P. multocida* and the unclear role of individual genes in causing the disease.

2) Virulence genes present in asymptomatic hosts may only cause infection, but not necessarily pathogenicity (Gharibi *et al*., 2017).

3) Pathogenesis of virulence genes is often host-dependent. For example, while the *tox*A gene is responsible for atrophic rhinitis disease and mortality in swine (Peng *et al*., 2019), our results indicated that the pathogenesis of the *tox*A gene is mostly host-dependent, and *tox*A alone is not related to the disease in sheep, goats and cattle as no symptoms of atrophic rhinitis were observed in these hosts.

This study evaluated the frequency of *P. multocida* and its virulence factors in ailing cattle, sheep and goats. Given the constantly changing nature of circulating strains, it is necessary to periodically evaluate the phenotypic and genotypic characteristics of this bacterium. Our results provide new insights into the epidemiology of *P. multocida* and its virulence factors. In this study, *exb*D, *plp*B, *pm*HAS, *exb*B, and *tox*A exhibited high frequency in the isolates, respectively. All cattle isolates had type D capsules and among the adhesin genes, only *hsf*2 gene was detected in cattle isolates. MID results indicated having more or specific virulence factors is not a reason for its pathogenicity and the pathogenesis of the *tox*A gene is mostly host-dependent. Furthermore, among the antibiotics tested in the present study, quinolones, especially enrofloxacin, appear to be a preferred and appropriate drug for treatment and prevention of pasteurellosis. However, due to the widespread use of antibiotics in farms, the risk of emergence of quinolone-resistant isolates is very high. Therefore, the regional pattern of antimicrobial resistance on therapeutic regimes must be taken into consideration. The results of this study can be useful for selecting appropriate strains for effective vaccines.

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Conflict of interest

The authors declare no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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