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Virulence gene profiles of *Pasteurella multocida* strains isolated from cattle and buffalo

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ABSTRACT

Pasteurella multocida is responsible for numerous economically relevant diseases in domestic animals worldwide. In cattle and buffaloes the organism is associated with hemorrhagic septicemia (HS) and bovine respiratory disease (BRD). The aim of this study was to investigate twelve virulence associated genes in 22 strains of *P. multocida* isolated from slaughtered cattle and buffaloes. The most frequently detected genes among bovine isolates were *ptfA, nanH, exbBD-tonB* and *oma87*; whereas *hgbB* and *toxA* genes occurred less frequently. Some of the adhesions, sialidases, iron acquisition and protectin proteins occurred at considerably ($P<0.05$) higher frequencies in bovine isolates. The prevalence of oma87, *exbBD-tonB* and *hgbA* genes from buffaloes was significant ($P<0.05$), whereas the prevalence of *hgbB*, *ompH*, *pfhA* and *toxA* genes was much lower. All tested strains of *P. multocida* contained the *sodC* gene and only 22.7% of them had *sodA*. By using the virulence gene profiles, 12 and 21 different gene combinations were identified among the strains isolated from cattle and buffaloes, respectively, of which Profile C1 was the most common, with all strains possessing toxA. Our results indicate the presence of virulence factors (VFs) in *P. multocida* strains isolated from the tested cattle and buffaloes. The occurrence of these factors in apparently healthy animals could possibly indicate early infection or a contained infection which did not lead to disease. Moreover, differences in the frequency of these factors may indicate variations in the pathogenicity of the organism.

Key words: virulence gene profile, *Pasteurella multocida*, buffalo, cattle

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Introduction

Respiratory infections cause considerable economic loss in sheep, cattle, buffalo, and other production animals. They often result from viral and bacterial infection interactions. One of the major bacterial etiologic agents in respiratory infections in farm animals is *Pasteurella multocida* (AHMAD et al., 2014). This organism is commonly found in the upper respiratory tract of humans and many animal species. It is responsible for numerous economically relevant diseases throughout the world, including hemorrhagic septicemia (HS) in cattle and buffaloes, bovine respiratory disease (BRD), fowl cholera (FC) in poultry, atrophic rhinitis (AR) in porcines, and snuffles in rabbits (GYLES et al., 2010). In humans, infection with *P. multocida* is usually associated with contact with animals, most usually through bites and scratches by pet animals, resulting in edema, cellulitis and bloody purulent exudate at the wound site (TAMASKAR and RAVAKHAH, 2004; ABRAHAMIAN and GOLDSTEIN, 2011). In severe cases, *Pasteurella* infection can rapidly progress to complications such as endocarditis, osteomyelitis and meningitis. Respiratory infection in humans is relatively uncommon, but may occur in patients with chronic pulmonary disease (BROOK, 2009; HEY et al., 2012).

HS is a serious acute and highly fatal disease affecting cattle and buffalo, caused by two specific serotypes of *P. multocida*. It is considered to be the economically most important disease in tropical regions of the world (RIMLER and WILSON, 1994; DABO et al., 2007). *P. multocida* serotype B:2 causes the disease in Asia, southern Europe and the Middle East, while serotype E:2 is the most common cause in Africa (TAYLOR et al., 1996). The disease is characterized by abrupt onset of high fever, lethargy, nasal discharge, respiratory distress, a rapid course of edematous swelling in the throat and brisket region, swollen and hemorrhagic lymph nodes, and widespread hemorrhaging (SHIVACHANDRA et al., 2011). In Iran the disease has an enzootic nature and has been associated with bovine death. It is endemic in Khouzestan, Mazandaran, Guilan, and western and eastern Azarbaijan (BAHARSEFAT and FIROUZI, 1977). In cattle and other ungulates, along with *Mannheimia haemolytica*, *Trueperella pyogenes, Mycoplasma bovis*, and *Histophilus somni*, *P. multocida* is implicated as a common pathogen related to BRD, or shipping fever (nonsepticemic pneumonia) (CONFER, 2009). The principal serotype of *P. multocida* isolated from most cases of shipping fever in cattle is A:3, manifesting usually as chronic bovine fibrinopurulent bronchopneumonia, pleurisy, and occasionally with fibrinonecrosis. BRD is a significant cause of morbidity and mortality in the cattle industry throughout the world (DABO et al., 2007; CONFER, 2009). Moreover, *P. multocida* may occasionally cause localized infections, abortion and mastitis in cattle (WELSH et al., 2004).

The pathogenesis of *P. multocida* is a result of complex interactions between special host factors and specific bacterial virulence factors (VFs). The major VFs that have been identified to date include lipopolysaccharide (LPS) and capsule protein (HARPER et al.,

2006). However, many other virulence associated genes may be related, including genes encoding structures such as: iron acquisition related factors (*hgbA*, *hgbB*, *exbBD-tonB* and *tbpA*), bacterial adhesions and colonization factors (*hsf-1*, *hsf-2*, *tadD*, *pfhA*, *ptfA* and *fimA*), outer membrane proteins (*oma87*, *psl*, *ompA*, *ompH* and *plpB*), extracellular enzymes, such as superoxide dismutases (*sodA*, *sodC* and *tbpA*) and neuraminidase (*nanB* and *nanH*), and toxins (*toxA*) (HATFALUDI et al., 2010; KATOCH et al., 2014). The VFs of *P. multocida* may lead to a better understanding of the epidemiology, pathogenesis, protective immunity and vaccine development against *P. multocida* infections in hosts (TOMICH et al., 2007; HATFALUDI et al., 2010). There is now clear evidence that some *P. multocida* products are critical for virulence in some but not all hosts. Recently, EWERS et al. (2006) showed that *toxA* alone is related to the disease status in swine, and *tbpA* and *pfha1* are associated with bovine diseases. Molecular techniques have been developed to identify genes associated with virulence in *P. multocida*, but the frequency of these genes in various hosts has not been clearly determined (GUENTHER et al., 2008; BETHE et al., 2009). The aim of this study was to investigate the presence of the virulence associated genes in strains of *P. multocida* isolated from cattle and buffalo in Khouzestan province, in south-west Iran.

Materials and methods

Bacterial strains. A total of 22 strains of *P. multocida* were used in this study. These organisms were isolated from 401 nasopharyngeal and nasal swab samples, randomly collected from slaughtered cattle and buffaloes (10 (4.4%) strains out of 227 cattle swab samples and 12 (6.8%) strains out of 174 buffalo swab samples) at the Ahvaz industrial abattoir in Khouzestan province, south-west Iran, during the period from October 2014 to June 2015. All the isolates had been already identified as *P. multocida* based on conventional cultural and biochemical tests, confirmed using polymerase chain reaction (PCR) technique, and by application of specific primers to kmt1. Also, capsular typing was performed by multiplex PCR using specific primers for the capsule biosynthesis genes. All samples were stored in sheep blood at a temperature of -80 °C.

DNA extraction. All isolates were initially cultured on 5% sheep blood agar (Merck, Germany) plates and incubated at 37 °C for 24 hrs. Individual colonies were inoculated into test tubes containing 2 mL Tryptic Soy Broth (TSB) (Merck, Germany) and incubated at 37 °C for 18 to 24 hrs. An aliquot of 500 µL of a TSB culture of each sample was separated for DNA extraction and heated at 100 °C for 10 min, and then centrifuged for 10 min at 8500 g. 200 µL of supernatant fluid was used for molecular tests and frozen at -20 °C until further use. Prior to investigating virulence gene prevalence, a PCR protocol for species-specific amplification of the *kmt1* gene was performed (BOOM et al., 1990; EWERS et al., 2006).

Table 1. The virulence genes selected, their enzymatic function or process, the primer sequences and the size of the amplicons

Gene function and gene	Process or enzyme	Primer sequence (5'-3')	Amplicon size (bp)	References
Adhesins				
ptfA	type IV fimbriae	TGTGGAATTCAGCATTTTAGTGTGTC TCATGAATTCTTATGCGCAAAATCCTGCTGG	488	Doughty et al. (2000)
pfhA	hemagglutinin	AGCTGATCAAGTGGTGAAC TGGTACATTGGTGAATGCTG	275	Ewers et al. (2006)
Sialidases				
nanB	sialidase	GTCCTATAAAGTGACGCCGA ACAGCAAAGGAAGACTGTCC	554	Ewers et al. (2006)
nanH	sialidase	GAATATTTGGGCGGCAACA TTCTCGCCCTGTCATCACT	360	Ewers et al. (2006)
Protectins				
ompH	porin	CGCGTATGAAGGTTTAGGT TTTAGATTGTGCGTAGTCAAC	438	Ewers et al. (2006)
oma87	porin	ATGAAAAAACTTTTAATTGCGAGC TGACTTGCGCAGTTGCATAAC	948	Ewers et al. (2006)
Iron acquisition				
exBD-tonB	iron metabolism	GGTGGTGATATTGATGCGGC GCATCATGCGTGCACGGTT	1144	Ewers et al. (2006)
hgbA	iron uptake	TGGCGGATAGTCATCAAG CCAAAGAACCACTACCCA	419	Ewers et al. (2006)
hgbB	iron uptake	ACCGCGTTGGAATTATGATTG CATTGAGTACGGCTTGACAT	788	Ewers et al. (2006)
Toxins				
toxA	dermonecrotic toxin	CTTAGATGAGCGACAAGGTT GGAATGCCACACCTCTATA	865	Ewers et al. (2006)
Superoxide dismutase				
sodA	superoxide dismutase	TACCAGAATTAGGCTACGC GAAACGGGTTGCTGCCGCT	361	Ewers et al. (2006)
sodC	superoxide dismutase	AGTTAGTAGCGGGGTTGGCA TGGTGCTGGGTGATCATCATG	235	Ewers et al. (2006)

Detection of virulence genes. P. multocida strains were evaluated for the presence of twelve virulence related genes, including *oma87, ompH, ptfA, pfhA, nanB, nanH, exBD-tonB, hgbA, hgbB, sodA, sodC* and *toxA,* using multiplex PCR in the presence of specific oligonucleotide primers. The sequence of each primer pair, the function of each one of the studied genes, and the predicted sizes of the amplified products for the specific primers used in this study are shown in Table 1.

In this study, three multiplex-PCR protocols were used (Table 2) in order to allow the simultaneous detection of selected virulence genes as described by EWERS et al (2006).

Table 2. Multiplex-PCR protocols: virulence associated genes and PCR conditions

Multiplex	Virulence genes	Step	Function	Temperature (°C)	Time	Number of cycles
Multiplex 1	*sodA, hgbA, ptfA, pfhA*	First step	Initial denaturation	95	5 min	1
		Second step	Denaturation	94	30 sec	25
			Annealing	55	30 sec	25
			Extension	72	1 min	25
		Third step	Final extension	72	5 min	1
Multiplex 2	*ompH, exBD-tonB, nanH, toxA*	First step	Initial denaturation	95	5 min	1
		Second step	Denaturation	94	30 sec	25
			Annealing	55	30 sec	25
			Extension	72	1 min	25
		Third step	Final extension	72	5 min	1
Multiplex 3	*oma87, sodC, hgbB, nanB*	First step	Initial denaturation	95	5 min	1
		Second step	Denaturation	94	30 sec	25
			Annealing	55	30 sec	25
			Extension	72	1 min	25
		Third step	Final extension	72	5 min	1

For all reactions, 5 μL of the DNA template was added to the 20 μL mixture, containing 0.5 μL of each primer (10 picomol), 1.5 μL of 2.5 mM $MgCl_2$, 0.5 μL of 10 mM dNTPs, 2U of Taq DNA polymerase, 2.5 μL of 10x PCR buffer, (all reagents made from SinaClon Bioscience Co**.,** Iran), and sterile distilled water. The amplification reactions were performed in a thermocycler (Eppendorf, mastercycler gradient, Germany) according to the reaction conditions described in Table 2. 10 μL of PCR products were separated by electrophoresis (100 volts for 1 hour) in a 1% agarose gel, stained with 0.5 μg/mL safe stain. DNA fragments were visualized by UV transillumination (UVitec, the United Kingdom). The molecular size of the PCR products were compared with a 100 bp DNA ladder (EWERS et al., 2006; FURIAN et al., 2013). In the present study, positive control strains of *P. multocida* were obtained from Aerobic Bacterial Vaccines Department, Razi

Vaccine and Serum Research Institute, Karaj, Iran, and sterile water was the negative control.

Statistical analysis. The results were analyzed statistically using SPSS software version 19.0 (SPSS Inc., Chicago, IL). Descriptive statistics were computed to determine the proportions of the different VFs among the isolates. Chi square or Fisher's-exact tests were used for determination of the statistical significance of differences between the proportions, and p-values of <0.05 were considered statistically significant.

Results

All isolates were screened for the presence of twelve different genes coding for VFs, and the results are given in Table 3. Among the 10 bovine *P. multocida* isolates, the twelve virulence gene prevalences ranged from 20% (*toxA*) to 100% (*sodC*). Some virulence genes, such as *ptfA, nanH, oma87* and *exbBD-tonB,* were each found to occur in 90% of the strains. Our data show that these genes occur at significantly greater ($P<0.05$) frequency in bovine isolates of *P. multocida*. Of the adhesin-encoding genes studied, *ptfA* (90%) was more prevalent than *pfhA* (60%; ($P<0.05$), and of the sialidase-encoding genes, *nanH* (90%) was more prevalent than *nanB* (60%; ($P<0.05$). Also, the results showed that only 20% of the tested bovine isolates had the *toxA* gene and 40% of them had the *hgbB* gene (Fig.1). Similarly, among the strains isolated from buffaloes, the virulence genes ranged in prevalence from 25% (*toxA*) to 100% (*sodC*) (Table 3).

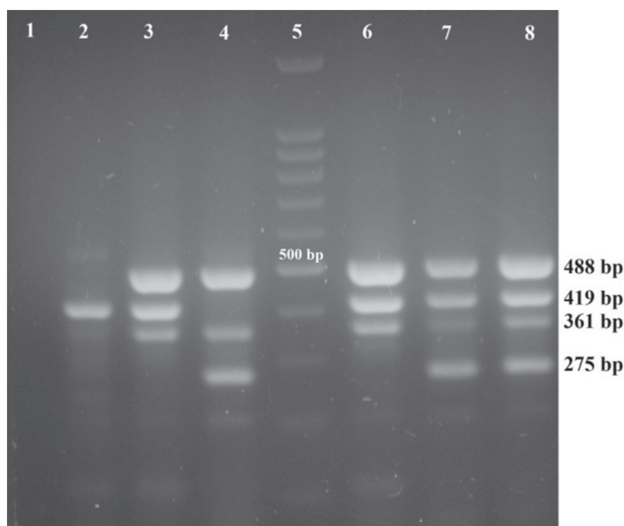

Fig. 1. Electrophoresis on 1% agarose gel stained with safe stain and the amplification products associated with the genes studied (multiplex-PCR 1): *pfhA* (275 bp), *sodA* (361 bp), *hgbA* (419 bp) and *ptfA* (488 bp). Legend: 5 = Marker (100 bp), 1 = Negative control, 2-4 and 6-7 = Samples, 8 = Positive control.

Most of the strains presented the *oma87* and *exbBD-tonB* (91.6%), and *hgbA* (83.3%) genes (P<0.05). The type 4 fimbrial subunit encoding *ptfA* and the sialidase-encoding *nanH* genes were present in 75%, and autotransporter protein *nanB* and superoxide dismutase *sodA* genes were present in 66.6% of the *P. multocida* strains isolated from buffaloes. With respect to the genes encoding hemoglobin-binding proteins, the most frequent was *hgbA* (83.3%), followed by the *hgbB* gene (41.6%; P<0.05) and among the genes encoding outer membrane proteins, the *oma87* (91.6%) gene was more frequent than the *ompH* (41.6%; P<0.05) gene. According to our results, all tested strains of *P. multocida* contained the *sodC* gene and only 22.7% possessed soda.

Table 3. Frequency of virulence genes in 22 strains of *Pasteurella multocida* isolated from cattle and buffalo

Virulence genes	Positive strains (Cattle)		Positive strains (Buffalo)		Total positive strains	
	Total (n = 10)	Total (%)	Total (n = 12)	Total (%)	Total (n = 22)	Total (%)
Adhesins						
ptfA	9	90	9	75	18	81.8
pfhA	6	60	4	33.3	10	45.4
Sialidases						
nanB	6	60	8	66.6	14	63.6
nanH	9	90	9	75	18	81.8
Protectins						
ompH	7	70	5	41.6	12	54.5
oma87	9	90	11	91.6	20	90.9
Iron acquisition						
ex*BD-tonB*	9	90	11	91.6	20	90.9
hgbA	7	70	10	83.3	17	77.2
hgbB	4	40	5	41.6	9	40.9
Toxins						
toxA	2	20	3	25	5	22.7
Superoxidedismutase						
sodA	6	60	8	66.6	14	63.6
sodC	10	100	12	100	22	100

In the present study, by using gene prevalence data, 12 and 21 different gene profile patterns (C1-12 and C1-21) were identified among *P. multocida* strains isolated from cattle and buffalo respectively, with collection C1 detecting all genes except toxA, and the most common in both groups of collections (Table 4).

Table 4. Distribution of gene combinations in 22 strains of *P. multocida* isolated from cattle and buffalo

Collections in 10 strains isolated from cattle			Collections in 12 strains isolated from buffalo		
Genetic collection	Number of strains	Absent genes	Genetic collection	Number of strains	Absent genes
C1	8	*toxA*	C1	9	*toxA*
C2	6	*hgbB*	C2	8	*pfhA*
C3	5	*toxA, hgbB*	C3	7	*ompH*
C4	4	*nanB*	C4	7	*hgbB*
C5	4	*pfhA*	C5	6	*toxA, hgbB*
C6	3	*toxA, hgbA*	C6	6	*ompH, pfhA*
C7	3	*ompH, sodA*	C7	5	*sodA*
C8	2	*nanB, pfhA*	C8	5	*hgbB, pfhA*
C9	2	*ompH, sodA, pfhA*	C9	4	*nanB*
C10	1	*ptfA*	C10	4	*toxA, ompH*
C11	1	*oma87*	C11	4	*pfhA, ptfA*
C12	1	ex*BD-tonB*	C12	3	*nanH*
-	-	-	C13	3	*ptfA*
-	-	-	C14	3	*ompH, nanH*
-	-	-	C15	3	*ompH, nanH, pfhA, ptfA*
-	-	-	C16	3	*ompH, toxA, hgbB*
-	-	-	C17	2	*hgbA*
-	-	-	C18	2	*sodA*
-	-	-	C19	2	*hgbA, hgbB*
-	-	-	C20	1	ex*BD-tonB*
-	-	-	C21	1	*oma87*

Discussion

Despite the high importance of infections caused by *P. multocida*, the pathogenicity and host specificity of the organism is not well understood (HARPER et al., 2006). There are only a few studies that have determined the frequency of VFs correlated with pathogenic mechanisms (EWERS et al., 2006; BETHE et al., 2009; TANG et al., 2009; FERREIRA et al., 2012; FURIAN et al., 2013; KHAMESIPOUR et al., 2014). VFs play a key

role in disease production by bacterial pathogens, and their major functions include competence, adherence, synthesis and export of capsules, and evasion of host immune responses (NANDURI et al., 2009). The present study has provided novel information on the prevalence and distribution of the various VFs of strains of *P. multocida,* isolated from cattle and buffalo.

The presence of adhesion proteins on the bacterial surface is usually associated with virulence, since they are known to play a crucial role in promoting host invasion and bacterial colonization (KLINE et al., 2009). Therefore, the related genes involved in this step are frequent targets for analysis of virulence genotypes in *P. multocida* (HATFALUDI et al., 2010). Studies by EWERS et al. (2006), using various clinically healthy and diseased hosts, and TANG et al. (2009) from clinical respiratory disease in pigs, have demonstrated that among the adhesins identified in samples of *P. multocida,* including *pfhA*, *tad*, *ptfA*, *fimA*, and *hsf-1,2*, only *fimA* and hsf-1,2 were present in all pathogenic strains. The *ptfA* gene, which encodes a subunit of type IV fimbriae, was described in 81.8% of the isolates tested in the current study, a result similar to that observed in other studies (TANG et al., 2009; FERREIRA et al., 2012; FURIAN et al., 2013; KHAMESIPOUR et al., 2014). The high prevalence of the *ptfA* gene was expected since it has been identified as a key factor in bacterial attachment to the surface of the epithelial cells of hosts (EWERS et al., 2006). In contrast to the above mentioned adhesion related gene, the filamentous hemagglutinin gene *pfhA* had a rather low prevalence (45.4%) in *P. multocida* strains. This lower frequency was also found in other studies. EWERS et al. (2006) detected *pfhA* in 46.2% of 104 bovine samples analyzed. Similarly, SHAYEGH et al. (2008) reported a low percentage of *pfhA* in clinically healthy and diseased sheep, and observed a correlation between the presence of the gene and disease in ovines. Additionally, TANG et al. (2009) and FURIAN et al. (2013) detected 15% and 60% of phfA in associated strains of *P. multocida* isolated from swine with clinical respiratory disease, and fowl cholera, respectively.

The sialidases are enzymes that remove sialic acid conjugated to glycolipids and glycoproteins of eukaryotic cells (HATFALUDI et al., 2010). In the present study, two genes that encode sialidases, *nanB* and *nanH*, were identified in 63.6% and 81.8% of isolates, respectively. The *nanB* gene was detected in all samples in other studies (EWERS et al., 2006; FURIAN et al., 2013), but was observed in 81.5% and 83.3% strains of *P. multocida* from clinical respiratory disease in pigs, and pneumonic and apparently healthy slaughter cattle, tested by TANG et al. (2009) and KHAMESIPOUR et al. (2014), respectively. The frequency of *nanH* was similar to that cited by KHAMESIPOUR et al. (2014), but it differs from EWERS et al. (2006), TANG et al. (2009), FERREIRA et al. (2012) and FURIAN et al. (2013) who detected this gene in 88.5%, 97%, 67.3% and 96% of samples, respectively.

The *ompH* and *oma87* genes, which encode porins of *P. multocida*, were detected in 54.5% and 90.9% of the analyzed samples, respectively. *ompH* and *oma87* genes were identified in all samples in other studies (DAVIES et al., 2004; EWERS et al., 2006; BETHE et al., 2009; FURIAN et al., 2013) and were the most frequently detected genes in isolates in

other studies (TANG et al., 2009; FERREIRA et al., 2012; KHAMESIPOUR et al., 2014). OmpH is a major outer membrane porin that forms a homotrimeric channel, and has shown some potential as a protective antigen (CHEVALIER et al., 1993). Oma87 is an 87-kDa outer membrane protein of all *P. multocida* strains that is expressed *in vivo* (RUFFOLO and ADLER, 1996). OmpH and *Pasteurella* lipoprotein E (PlpE) are protective surface antigens associated with *P. multocida* serotype A: 1, A: 3, and A: 4 strains isolated from cattle with shipping fever (WU et al., 2007; OKAY et al., 2012), and from birds with fowl cholera (HATFALUDI et al., 2012). Porins such as OmpH are candidates for heterologous vaccine development because they are generally conserved among species, and are highly immunogenic (LEE et al., 2007).

In this study, three genes involved in iron metabolism, *exBD-tonB*, *hgbA* and *hgbB*, were detected in 90.9%, 77.2% and 40.9% of isolates, respectively. The high prevalence of the *exBD-tonB* and *hgbA* genes was similar to that cited by other researchers (EWERS et al., 2006; BETHE et al., 2009; TANG et al., 2009; FURIAN et al., 2013; KHAMESIPOUR et al., 2014). In contrast, the low frequency of the *hgbB* gene was also found in other studies. EWERS et al. (2006) detected *hgbB* in 57.7% of bovine samples analyzed. FERREIRA et al., (2012) obtained similar results, detecting *hgbB* less frequently (30.4%) in strains of *P. multocida* isolated from rabbits. However, FURIAN et al. (2013) and KHAMESIPOUR et al. (2014) identified the *hgbB* gene from 100% and 93.3% of poultry and bovine strains, respectively. The TonB complex, composed of three proteins, is responsible for the proton motive force required to internalize iron into the periplasmic space (KREWULAK and VOGEL 2008). The energy generated by the complex is required for different mechanisms of iron uptake (HATFALUDI et al., 2010). hgbA is an example of a protein present in the outer bacterial membrane that binds to host glycoproteins that contain iron, in this case hemoglobin (BOYCE et al., 2010).

All strains of *P. multocida* were positive for sodC, but 63.6% of them had *sodA*. Our findings about the high frequency of *sodC* are similar to those found in other studies, but in contrast to the results of the present study, other researchers have also reported the high prevalence of *soda* (EWERS et al., 2006; FERREIRA et al., 2012; FURIAN et al., 2013; KHAMESIPOUR et al., 2014). The enzymes encoded by these genes have antioxidant functions, and they were only described after genome sequencing of the strain Pm70 in 2001 (MAY et al., 2001).

The *toxA* gene was only described in 22.7% of the isolates tested in the current study. The low prevalence of this gene may be related to the fact that the gene encoding dermonecrotic toxin is more frequent in atrophic rhinitis in swine. EWERS et al. (2006) also showed a low frequency of the gene, which was detected in only 5.8% of the strains isolated from cattle. Similarly, TANG et al. (2009) and KHAMESIPOUR et al. (2014) identified it in 4.7% and 10% of swine and bovine strains, respectively. Moreover, FERREIRA et al. (2012) and FURIAN et al. (2013) reported that none of the tested strains contained the *toxA* gene. However, SHAYEGH et al. (2008) detected the gene in 70% of strains isolated from diseased sheep. Some authors have reported that this gene is not

carried on the chromosome, but is carried on a lysogenic bacteriophage that infects the host (PULLINGER et al., 2004).

The generated virulence gene profiles of *P. multocida* were compared, and according to the presence or absence of the tested genes the strains isolated from cattle and buffaloes were clustered in 12 and 21 gene combinations, respectively. We performed analysis of the distribution of the genetic profiles in our samples, evaluating only the presence or absence of the genes among the samples. Acquiring strains from different origins and studying groups of samples isolated from healthy animals may reveal a possible epidemiological link between a specific group of genes, and shows the distribution of the carrier state to the organism between the tested animals. The virulence gene profile was found to be a good tool for differentiation of *P. multocida* strains.

In conclusion, our results reveal the presence of VFs in *P. multocida* strains isolated from tested cattle and buffaloes, and new epidemiological information on the prevalence of these various virulence factors. The occurrence of these virulence factors in apparently healthy animals could possibly indicate early infection, subclinical disease, or contained infection which did not lead to disease. Additionally, it is possible that the occurrence of these virulence factors without disease may be due to the fact that these factors are quiescent or the quantity of them is below a disease causing threshold. Also, since *P. multocida* is a facultative anaerobic bacterium that is commonly found in clinically healthy calves, isolation of this bacterium from the upper respiratory tract should not be neglected. In contrast, the pathogenicity of *P. multocida* depended on various virulence factors, and the number and differences in the frequency of these factors may indicate variations in the pathogenicity of the organism. Knowledge of the distribution patterns of *P. multocida* virulence factors will help to develop a suitable homologous vaccine candidate that can elicit protective immunity against all serotypes. However, further study is required to clarify the pathogenesis and the role of *P. multocida* virulence factors in immunity.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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SAŽETAK

Pasteurella multocida odgovorna je za mnoge gospodarski važne bolesti domaćih životinja diljem svijeta. U goveda i bivola ta je bakterija povezana s pojavom hemoragijske septikemije (HS) i respiratorne bolesti. Cilj je ovog rada bio istražiti prisutnost 12 gena odgovornih za virulenciju u 22 izolata bakterije *P. multocida* izdvojena iz zaklanih goveda i bivola. Najčešće dokazani geni iz goveđih izolata bili su *ptfA, nanH, exbBD-tonB* i *oma87*, dok su geni *hgbB* i *toxA* bili rjeđe dokazani. Neki od adhezina, sijalidaza, proteina koji na sebe vežu slobodno željezo i zaštitnih proteina dokazani su sa znatno većom učestalošću (P<0,05) u goveđih izolata. Prevalencija gena *oma87*, *exbBD-tonB* i *hgbA* bila je značajno viša (P<0,05) dok je prevalencija gena *hgbB*, *ompH*, *pfhA* i *toxA* bila niža u bivoljih izolata. Svi pretraženi izolati bakterije *P. multocida* sadržavali su gen *sodC,* a samo 22,7% njih i gen *sodA*. S obzirom na profil gena za virulenciju, 12 različitih kombinacija ustanovljeno je među izolatima iz goveda, a 21 kombinacija među izolatima iz bivola od kojih je profil C1 bio najčešći u izolata koji su posjedovali *toxA*. Rezultati naznačuju prisutnost čimbenika virulencije u izolata bakterije *P. multocida* izdvojenih iz pretraženih goveda i bivola. Pojava tih čimbenika virulencije u klinički zdravih životinja mogla bi značiti ranu infekciju ili infekciju koja se neće klinički očitovati. Razlika u učestalosti spomenutih čimbenika također upućuje na različitost u patogenosti izolata.

Ključne riječi: virulencija, genski profil, *Pasteurella multocida*, bivol, govedo

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