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Identification, capsular typing and virulence factors of *Pasteurella multocida* isolates from Merino lambs in Extremadura (Southwestern Spain)

S. Fernandez[1]*, J. Galapero[1], L. Gomez[1], C.J. Perez[1], A. Ramos[1], D. Cid[2], A. Garcia[2], J. Rey[1]

[1]Faculty of Veterinary Medicine, University of Extremadura, Caceres, Spain
[2]Faculty of Veterinary Medicine, Complutense University, Madrid, Spain

*Corresponding author: safernandezd@unex.es

ABSTRACT: This report describes the prevalence, capsular type and virulence factors of *Pasteurella multocida* isolated from the respiratory tracts of healthy and diseased lambs. For this study, five hundred and ninety-eight fattening lambs from different feedlots of the community of Extremadura were analysed. Isolation and identification of *P. multocida* were performed using conventional bacteriological and biochemical techniques, while confirmation of *P. multocida* identification, capsular type and virulence factors was done using the polymerase chain reaction technique (PCR). Of 598 studied lambs (410 clinically healthy and 188 diseased sheep), *P. multocida* was isolated from 37 animals (6.2%), with prevalence rates of 0.49% (2/410) in healthy lambs and 18.62% (35/188) in diseased lambs confirming a close relationship between the presence of bacteria and disease. Capsular typing of *P. multocida* isolates demonstrated two capsular types: A (15) and D (22), with general prevalence rates of 40.5% and 59.5%, respectively, and with the exclusive presence of type D in healthy animals (100%) and types D and A among diseased animals (42.9% and 57.1%, respectively). Among four virulence genes investigated (*pfhA*, *hgbB*, *tbpA* and *toxA*), we found a remarkable high prevalence of *tbpA* (100%) (37/37) genes in *P. multocida*; *toxA* was only detected in some diseased lambs (11/37), while the rest of the studied genes were not detected. The high prevalence of *toxA* among isolates from diseased animals may imply an important role of this gene in the virulence of *P. multocida* isolates in sheep, especially in diseased lambs.

Keywords: *Pasteurellosis*; disease; bacteria; sheep; Extremadura; pneumonia

P. multocida comprises a diverse group of Gram-negative bacteria which are responsible for a wide range of economically important infections of animals, including domestic sheep (Davies et al. 2003). *P. multocida* is a bacterial pathogen associated with a variety of diseases in animals and causes pneumonic and septicaemic pasteurellosis in sheep (Soriano-Vargas et al. 2012).

As a pathogen, *P. multocida* causes different manifestations in various hosts. *Pasteurella* spp. are highly prevalent among animal populations, where they are often found as part of the normal microbiota of the oral cavity, nasopharyngeal and upper respiratory tracts. Many *Pasteurella* species are opportunistic pathogens that can cause endemic disease, and they are increasingly associated with epizootic outbreaks. Pneumonic pasteurellosis is a disease that mainly occurs in animals with compromised pulmonary defence mechanisms. Sheep are rather susceptible and can contract the disease

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if exposed to physical stress or unfavourable environmental conditions. Lambs are more susceptible and develop more severe infections. Infected sheep develop high fever, dyspnoea, cough, froth at the mouth and nasal discharges (Mohamed and Abdelsalam 2008). According to their capsular polysaccharide, isolates can be serologically differentiated into serogroups A, B, D, E and F (Chung et al. 1998). The infectious serogroups A and D of *P. multocida* associated with outbreaks of pneumonic pasteurellosis in sheep and goats have been implicated as both primary and secondary agents of pneumonia (Chandrasekaran et al. 1991; Zamrisaad et al. 1996). However, some strains of *P. multocida* are non-toxigenic and non-virulent and can be part of the normal respiratory flora in sheep and goats (Lichtensteiger et al. 1996).

Apart from outer membrane proteins and capsular antigens, virulence-associated genes (*tbpA*, *pfhA*, *toxA*, *hgbB*, *hgbA*, *nanH*, *nanB*, *sodA*, *sodC*, *oma87* and *ptfA*) play an important role in the pathogenesis of *P. multocida* (Ewers et al. 2006). Various studies have shown that the *pfhA* gene encoding filamentous haemagglutinin, the *hgbB* and *tbpA* genes encoding outer membrane proteins involved in the iron acquisition (Bosch et al. 2002; Cox et al. 2003) and the *toxA* gene encoding a dermonecrotic toxin are important epidemiological markers in *P. multocida* field isolates (Ewers et al. 2006).

To the best of the authors' knowledge, a specific study of *P. multocida* in sheep in Extremadura (Southwestern Spain) has never been done before. In particular, we sought to determine the prevalence, capsular type and virulence factors associated with *P. multocida* from healthy and diseased lambs in several feedlots of Extremadura to determine if there exists a relationship between their presence and the development of disease.

MATERIAL AND METHODS

Sampling. A total of 598 samples were collected from pneumonic lungs of diseased lambs (188) and clinically healthy lambs (410), from February to November 2012. Collected samples were classified into two groups: group A, samples from clinically healthy lambs; and group B, those collected from pneumonic lungs of slaughtered lambs. The tested samples were obtained from Merino lambs in Extremadura (Southwestern Spain). The experiment was performed with the permission of the local bioethics committee (approval No. 19/2014, date 13/02/2014).

Bacterial isolation and identification. All samples were plated onto 10% sheep blood agar and incubated at 37 °C overnight. Small, glistening, mucoid, dewdrop-like colonies could suggest the growth of *P. multocida*. Microscopic analysis revealed that all isolates were Gram-negative coccobacilli and biochemical tests were positive for indole, nitrate reduction, oxidase and catalase, while methyl red (MR), Voges-Proskauer (VP) and Simmons' citrate tests were negative. Biochemical tests were carried out using the Phoenix® automated identification system (Becton Dickinson Diagnostics, Franklin Lakes, USA) according to the manufacturer's instructions. Colonies identified as *P. multocida* were subcultured in blood agar to obtain a pure culture.

Molecular confirmation. Cells from blood agar medium were collected by centrifugation for 1 min at 13 000 *g* and washed once in sterile distilled H_2O. DNA was prepared with the GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich) according to the manufacturer's instructions and stored at –20 °C.

Molecular identification of *P. multocida* was carried out using the PCR technique to amplify a specific fragment of the *kmt1* gene of *P. multocida* (PM-PCR) using the primers KMT1SP6 and KMT1T7 described by Townsend et al. (1998) (Table 1). PCR conditions are described in Table 2.

AmpliTaq Gold DNA Polymerase 5 U/μl with gold buffer (Applied Biosystems) and the GeneAmp dNTP blend (Applied Biosystems) were used in 25-μl reactions containing 18 μl sterile ultrapure miliQ water, 2.5 μl PCR + $MgCl_2$ 1 ×/2 mM, 1 μl dNTPs 10 mM, 0.5 μl primer KMT1T7 5 pM, 0.5 μl Primer KMT1SP6 5 pM, 0.5 μl Taq polymerase 0.5 U and 2 μl DNA.

PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide and photographed. Distilled water without any DNA was used as a negative control. Electrophoresis conditions were 90 V for 60 minutes. Strain 10322 from the National Collection of Type Cultures (NCTC) was used as a positive control for *P. multocida.*

Capsular typing by multiplex PCR. The capsular type of *P. multocida* isolates was determined according to the technique described by Townsend et al. (2001) using a specific pair of primers for each capsular type (*capA*, *capB*, *capD*, *capE* and *capF*).

https://doi.org/10.17221/142/2016-VETMED

Table 1. Primers used for identification of *P. multocida* and detection of capsular types and virulence-associated genes in *Pasteurella* strains

Description tested features	Gene	Primers	Sequences (5'→3')	Primer length	Location within the gene	Amplicon length (bp)	Access number GenBank	References
Subspecies	*kmt1*	KMT1T7	ATCCGCTATTTACCCAGTGG	20	213–232	457	AF016259	Townsend et al. 1998
		KMT1SP6	GCTGTAAACGAACTCGCCAC	20	669–649			
Serogroup A	*hyaD-hyaC*	CAPA-FWD	TGCCAAAATCGCAGTCAG	18	8846–8863	1046	AF067175	Townsend et al. 2001
		CAPA-REV	TTGCCATCATTGTCAGTG	18	9891–9874			
Serogroup B	*bcbD*	CAPB-FWD	GCCCGAGAGTTTCAATCC	19	12258–12275	759	AF169324	
		CAPB-REV	CATTTATCCAAGCTCCACC	18	13016–12998			
Serogroup D	*dcbF*	CAPD-FWD	TTACAAAAGAAAGACTAGGAGCCC	24	3142–3165	648	AF302465	
		CAPD-REV	CATCTACCCACTCAACCATATCAG	24	3789–3766			
Serogroup E	*ecbJ*	CAPE-FWD	TCCGCAGAAAATTATTGACTC	21	4388–4408	514	AF302466	
		CAPE-REV	GCTTGCTGCTTGATTTTGTC	20	4901–4882			
Serogroup F	*fcbD*	CAPF-FWD	AATCGGAGAACGCAGAAATCAG	22	2882–2903	852	AF302467	
		CAPF-REV	TTCCGCCGTCAATTACTCTG	20	3733–3714			
Dermonecrotoxin	*toxA*	TOXA-FWD	TCTTAGATGAGCGACAAGG	19	1877–1895	865	AF240778	Lichtensteiger et al. 1996
		TOXA-REV	GAATGCCACACCTCTATAG	19	2742–2724			
Outer membrane protein involved in iron acquisition	*tbpA*	TBPA-FWD	TGGTTGGAAACGGTAAAGC	19	970–988	728	AY007725	Ewers et al. 2006
		TBPA-REV	TAACGTGTACGGAAAAGCC	19	1697–1679			
Outer membrane protein involved in iron acquisition	*hgbB*	HGBB-FWD	TCATTGAGTACGGCTTGAC	19	395653–395671	500	AE004439	Atashpaz et al. 2009
		HGBB-REV	CTTACGTCAGTAACACTCG	19	396152–396134			
Filamentous haemagglutinin adhesin	*pfhA*	PFHA-FWD	AGCTGATCAAGTGGTGAAC	19	2408–2427	276	AY035342	Ewers et al. 2006
		PFHA-REV	TGGTACATTGGTGAATGCTG	20	2684–2665			

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Table 2. PCR conditions used for the identification of *P. multocida* and detection of capsular types and virulence-associated genes in *Pasteurella* strains

Primers	N° cycles	Conditions of PCR (°C/s)					References
		Initial denaturation	Denaturation	Annealing	Extension	Final extension	
Identification of *P. multocida*	30	95/300	94/60	55/60	72/60	72/540	Townsend et al. 1998
Capsular typing by multiplex PCR	30	95/180	95/30	54/60	72/90	72/300	Townsend et al. 2001
Virulence genes detection using multiplex PCR	30	95/300	94/45	54/50	72/50	72/600	Lichtensteiger et al. 1996; Ewers et al. 2006; Atashpaz et al. 2009

The PCR reaction mixture contained DNA template, 1 × Qiagen PCR Master Mix (providing a final concentration of 3 mM $MgCl_2$, 0.2 mM of each dNTP and Hot Star Taq DNA polymerase), RNA-free water and the set of five primer pairs at a final concentration of 0.2 μM. PCRs were carried out in an MJ Mini Thermal Cycler (Bio-Rad). The primer sequences used in the multiplex PCR for capsular typing are shown in Table 1. Reactions were run according to Townsend et al. (2001) with minor modifications (Table 2). PCR products were separated by electrophoresis using a 1.5% agarose gel (Invitrogen, USA) at 90 V stained with SYBR Safe DNA gel stain (10 000 ×) (Thermo Fisher Scientific, USA), and visualised using Imager^{TM} Safe UV equipment (Invitrogen, USA). The images were obtained using the GENEFLASH Syngene Bio Imaging application (TopoGEN, USA).

As positive controls, the following reference strains were used for each capsular type of *P. multocida*: NCTC 10322 (*capA*), NCTC 10323 (*capB*), NCTC 10326 (*capE*), Spanish Type Culture Collection (CECT) 962 (*capD*) and C2040103 (*capF*).

Virulence gene detection. The detection of virulence genes was carried out using multiplex PCR techniques. PCR-amplified DNA fragments with sizes of about 275, 499, 728 and 846 bp indicated the presence of *pfha1*, *hgbB*, *tbpA* and *toxA* genes in the isolates, respectively (Atashpaz et al. 2009). PCR conditions were as described by Atashpaz et al. (2009) (Table 2) and primers used for multiplex PCR are shown in Table 1.

AmpliTaq Gold DNA Polymerase 5 U/μl with Gold buffer (Applied Biosystems) and the GeneAmp dNTP blend (Applied Biosystems) were. Reagents volumes in the 25-μl reactions were the following: 13 μl of sterile ultrapure miliQ water, 2.5 μl of PCR + $MgCl_2$ 1 ×/2 mM, 1 μl dNTPs 10 mM, 0.5 μl 5 primers (F) c/u 5 mM, 0.5 μl 5 primers (F) c/u 5 mM, 0.5 μl Taq-Polymerase 1 U and 3 μl DNA.

The amplified products were analysed using electrophoresis on a 1% agarose gel run at 90 V for 45 minutes, and the results were recorded using the Geneflash Syngene Bio Picture documentation system (TopoGEN, USA). All tests were repeated twice in parallel with the corresponding positive and negative controls.

The reference strains for each capsular type of *P. multocida*, i.e., NCTC10322 (*pfhA*), NCTC10323 (*tbpA*) and CECT 962 (*toxA*, *tbpA*, *hgbB* and *pfhA*), were obtained from NCTC and CECT.

Data analysis. The statistical program SPSS 19.0 was used. Fisher's exact test was applied to analyse the relationship between the presence of *P. multocida* and health status. The results were considered as significant at a *P*-value of less than 0.001. The same test was applied to analyse the relationship between the capsular serotype of *P. multocida* and health status. These results were considered as not significant at a *P*-value of 0.505.

RESULTS

Frequency of *P. multocida* isolation

Of the 598 specimens obtained from healthy (410) and diseased (188) lambs, *P. multocida* was isolated and identified in 37 samples (6.2%): two (0.49%) from healthy lambs and 35 (18.62%) from diseased animals. A statistically significant association was found between the presence of serotypes of *P. multocida* and the development of disease (Table 3).

The presence of a DNA band of about 460 bp in size (amplification of a specific fragment of the

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Table 3. Capsular type and virulence factors of the 37 *P. multocida* ovine isolates

P. multocida isolation (*n*)	Capsular type	Number of isolates (%)	Virulence factors (%)			
			toxA	*tbpA*	*hgbB*	*pfhA*
Healthy lambs (410)	D	2 (5.4)	0 (0.0)	2 (5.4)	0 (0.0)	0 (0.0)
	A	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Diseased lambs (188)	D	20 (54)	1 (0.1)	20 (54)	0 (0.0)	0 (0.0)
	A	15 (40.5)	10 (90.9)	15 (40.5)	0 (0.0)	0 (0.0)
Total (598)		37 (6.2)	11 (29.7)	37 (100)	0 (0.0)	0 (0.0)

kmt1 gene) further confirmed the identification of isolates as *P. multocida* (Figure 1).

Capsular typing by multiplex PCR

Amplified DNA products of ~1044 and ~657 bp corresponding to *P. multocida* capsular groups A and D, respectively, were observed (Figure 2). Expected amplicons of ~760, ~511 and ~854 bp corresponding to *P. multocida* capsular serogroups B, E and F, respectively, were not detected.

Only two isolates were obtained from healthy lambs and both (2/2) showed the capsular type D. On the other hand, of the isolates obtained from diseased lambs, 42.9% (15/35) showed the capsular type D and 57.1% (20/35) the capsular type A. Capsular types B, E and F were not detected in the population sample. These results show that isolates of both serogroups A and D are generally isolated from diseased animals with similar frequencies. The results of the PCR analysis for the presence of different capsular types in the present study are summarised in Table 3.

Virulence gene detection using PCR analyses

The *tbpA* gene was detected in all isolates investigated (Figure 3), both from healthy (2/2) and diseased animals (35/35). On the other hand, the *toxA* gene was exclusively detected in isolates obtained from diseased animals (11/35), and was also present in most isolates of capsular type A (10/11), but in only one D-type isolate (1/11) ($P < 0.001$). The remaining genes (*hgbB* and *pfhA*) were not detected in any of the investigated isolates.

DISCUSSION

This report describes the prevalence, capsular typing and virulence factor profile of ovine *P. multocida* in Extremadura (Southwestern Spain). *P. multocida* is a pathogen of the respiratory tract of mammals and birds with a worldwide prevalence. *P. multocida* has been isolated from numerous species, including cats, cattle, pigs, sheep and humans (Davies et al. 2003; Ewers et al. 2006; Ekundayo et al. 2008). In our study, out of 37 isolates, 35 were

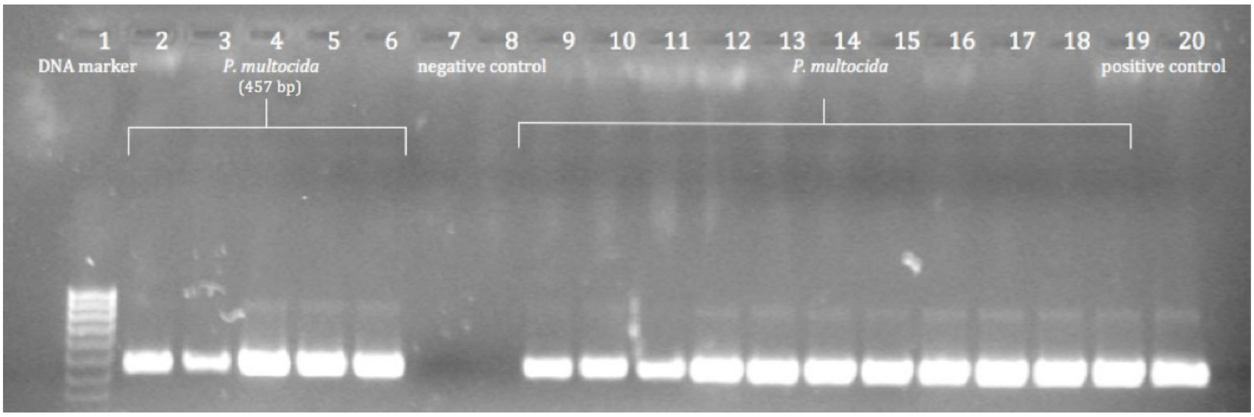

Figure 1. *P. multocida*-specific PCR assay. This figure depicts fragments specifically amplified by PCR in all *P. multocida* subspecies and serotypes by means of the primers KMT1SP6 and KMT1T7. The upper panel shows the following: Lane 1: 100-bp DNA marker; lane 20: positive control; lanes 7–8: negative control; lanes 2–6, 9–19: *P. multocida.* Samples were electrophoresed at 90 V/cm for 45 min on a 1% agarose gel (1 × TAE) stained with ethidium bromide, visualised by UV illumination and photographed

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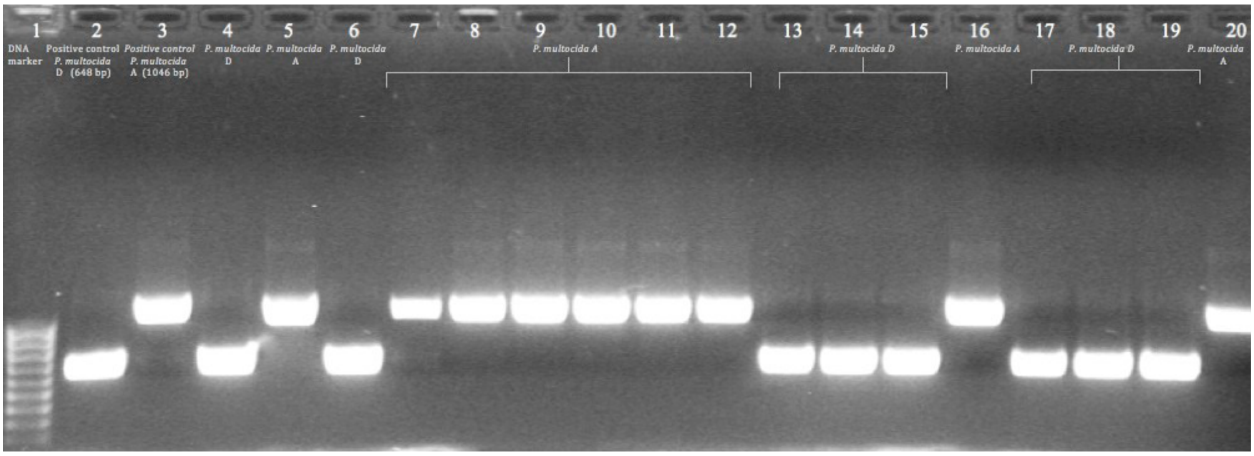

Figure 2. Capsular groups A and D identified in a *P. multocida*-specific PCR assay. Lane 1: 100-bp DNA marker; lane 2: positive control *P. multocida* capsular type D; lane 3: positive control *P. multocida* capsular type A; lanes 4–6, 13–15, 17–19: *P. multocida* capsular type D; lanes 5, 7–12, 16, 20: *P. multocida* capsular type A. Samples were electrophoresed at 90 V/cm for 45 min on a 1% agarose gel (1 × TAE) stained with ethidium bromide, visualised by UV illumination and photographed

obtained from diseased lambs and two isolates from healthy lambs, confirming a close relationship ($P < 0.001$) between the presence of pathogens and development of disease. This fact has been noted by other authors (Chanter et al. 1989; Frank 1989) in studies in which this pathogen was demonstrated to be responsible for pneumonia in cattle and sheep.

Only two *P. multocida* isolates were from healthy animals and both were capsular type D (2/2), whereas isolates obtained from diseased animals were in similar proportions type D (42.9%) and type A (57.1%). The relationship between capsular type and disease status, however, could not be measured statistically due to the small number of isolates obtained from healthy animals. Capsular types have been studied in several species. The results of previous studies have shown that capsular types A and D are common among isolates recovered from sheep and goats (Chandrasekaran et al. 1991; Zamrisaad et al. 1996; Shayegh et al. 2008), in agreement with the results of our study. However, in most previous studies no strong relationship could be determined between capsular type and the presence or absence of disease, both in ruminants as well as in other species. Thus, Shayegh et al. (2008) reported that type D was found only in diseased cases in Iran, while type A was found in diseased and healthy animals (Shayegh et al. 2008). These results are exactly the opposite of those re-

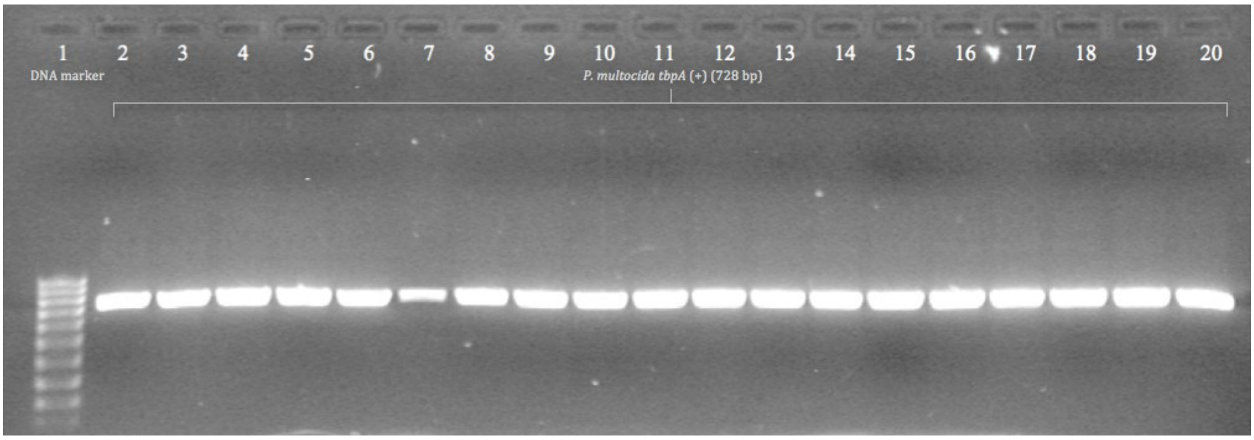

Figure 3. Virulence gene detection using PCR. Lane 1: 100-bp DNA marker; lanes 2–20: *P. multocida* tbpA (+). Samples were electrophoresed at 90 V/cm for 45 min on a 1% agarose gel (1 × TAE) stained with ethidium bromide, visualised by UV illumination and photographed

https://doi.org/10.17221/142/2016-VETMED

ported here, in which we have found both types in diseased animals, whereas in healthy lambs we only detected type D. Our results indicate that the A and D capsular types are the most common in the studied feedlots.

Differing frequencies of *P. multocida* serotypes have been observed in other studies. Values for *P. multocida* serotype D vary from 0% in rabbits in Brazil (Porfida et al. 2012) to 82.6% in cattle in India and South Asia (Verma et al. 2013). Similar results were obtained with *P. multocida* serotype A, which exhibit a prevalence ranging from 17.4% in cattle (Verma et al. 2013) to 95.24% in geese in Hungary (Varga et al. 2013). These differences between serotypes of *P. multocida* may be due to several factors, including the prevalence of the microorganism in the breed and processing techniques.

Each of the virulence genes considered in this study has a distinct role in the pathogenesis of *P. multocida*. The results of our study with respect to virulence factors are in accordance with those of some previous studies described in the literature (Shayegh et al. 2008).

The *toxA* gene is encoded within a lysogenic bacteriophage (Pullinger et al. 2004). Apparently, the phage that encodes *toxA* can engage in horizontal gene transfer resulting in the transfer of the gene between strains of different capsular serotypes and different host animals (Ewers et al. 2006). In previous research, a high prevalence of *toxA* among all of the studied *P. multocida* isolates was found (Sahragard et al. 2012). This virulence gene profile was similar to the previous reports in diseased ovine (Shayegh et al. 2008). In our study, we found that the *toxA* gene was detected exclusively in isolates from diseased animals (29.7%), similar to Stepniewska and Markowska-Daniel (2013); further, we found that this gene was frequently associated with capsular type A (10/11), which may be used as a molecular marker of virulence.

We observed a remarkably high prevalence of *P. multocida tbpA* (+) (100%) in the lungs of healthy and diseased lambs. This gene is related with iron acquisition and was studied with respect to the pathogenesis of *P. multocida* (Bosch et al. 2002; Cox et al. 2003). The mechanism involves iron-binding proteins expressed on the outer membrane of the bacterial cell that interact directly with host iron-binding glycoproteins. Previous studies reported the presence of *tbpA* in bovine isolates of *P. multocida* associated with pneumonia and haemorrhagic septicaemia (Ogunnariwo et al. 2001). Ewers et al. (2006) found the *tpbA* gene exclusively in *P. multocida* strains isolated from ruminants, including cattle, buffalo, sheep and goats. The present results confirm the strong prevalence of the *tpbA* gene in *P. multocida* isolated from small ruminants.

The second gene related with iron acquisition, *hgbB*, encodes a bacterial protein that binds haemoglobin and the haemoglobin complex to the host glycoprotein (Cox et al. 2003). However, in this study, the *hgbB* gene was not observed in diseased sheep. The absence of the *hgbB* gene in these isolates could be due to the fact that its prevalence in diseased animals is lower than in healthy animals and in contrast to the bovine strain, this gene may be not important in ovine disease and might not be valuable as an epidemiological marker.

The last virulence factor gene studied was *pfhA*; the prevalence of this gene among *P. multocida* isolates from sheep was reported to be very low in previous studies (Ewers et al. 2006; Shayegh et al. 2008). Its absence in our study could indicate that *pfhA* is not important for the virulence of ovine isolates, and it is not likely to be a suitable candidate gene for epidemiological studies in sheep.

According to the results of this project *P. multocida* is associated with respiratory disease in fattening lambs. Capsular types A and D are common in ovine isolates. Of the virulence genes investigated, the *tbpA* gene was identified in all isolates, confirming its specificity for ruminant isolates, and the *toxA* gene was detected relatively frequently in isolates obtained from diseased animals.

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