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

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**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 pneumatic 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...
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 pneumonia 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 (tpbA, 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 tpbA 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 pneumatic 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 pneumatic 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 H₂O. 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 + MgCl₂, 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).
Table 1. Primers used for identification of *P. multocida* and detection of capsular types and virulence-associated genes in *Pasteurella* strains

<table>
<thead>
<tr>
<th>Description tested features</th>
<th>Gene</th>
<th>Primers</th>
<th>Sequences (5'→3')</th>
<th>Primer length</th>
<th>Location within the gene</th>
<th>Amplicon length (bp)</th>
<th>Access number</th>
<th>GenBank</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subspecies kmt1</td>
<td>KMT1T7</td>
<td>ATCCGCTATTTACCAGTGG</td>
<td>GCTGTAAACGAACACTGCAC</td>
<td>20</td>
<td>213–232</td>
<td>457</td>
<td>AF016259</td>
<td>Townsend et al. 1998</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KMT1SP6</td>
<td>GCTGTAAACGAACACTGCAC</td>
<td>GCTGTAAACGAACACTGCAC</td>
<td>20</td>
<td>669–649</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serogroup A hyaD-hyaC</td>
<td>CAPA-FWD</td>
<td>TGGCAAAATCGCAGTCA</td>
<td>TTGCCACATCTGTCAGTG</td>
<td>18</td>
<td>8846–8863</td>
<td>1046</td>
<td>AF067175</td>
<td>Townsend et al. 2001</td>
<td></td>
</tr>
<tr>
<td>Serogroup B bcbD</td>
<td>CAPB-FWD</td>
<td>GCCCGAGAGTTCCAATCC</td>
<td>CATTATCCAAGCTCCACC</td>
<td>19</td>
<td>12258–12275</td>
<td>759</td>
<td>AF169324</td>
<td>Townsend et al. 2001</td>
<td></td>
</tr>
<tr>
<td>Serogroup D dcbF</td>
<td>CAPD-FWD</td>
<td>TTACAAAGAGAGACTGGAGGCC</td>
<td>CATCTACCCACTCAACCATCCAG</td>
<td>24</td>
<td>3142–3165</td>
<td>648</td>
<td>AF302465</td>
<td>Townsend et al. 2001</td>
<td></td>
</tr>
<tr>
<td>Serogroup E ecbD</td>
<td>CAPE-FWD</td>
<td>TCCGAGAAAATTATTGACTCT</td>
<td>GCTTGCTGTTGGATTTC</td>
<td>21</td>
<td>4388–4408</td>
<td>514</td>
<td>AF302466</td>
<td>Townsend et al. 2001</td>
<td></td>
</tr>
<tr>
<td>Serogroup F fcbD</td>
<td>CAPF-FWD</td>
<td>AATCGGAGAACGCAGAAATCAG</td>
<td>TCCGCCGCTCAATTACCTAG</td>
<td>22</td>
<td>2882–2903</td>
<td>852</td>
<td>AF302467</td>
<td>Townsend et al. 2001</td>
<td></td>
</tr>
<tr>
<td>Dermonecrotxin toxA</td>
<td>TOXA-FWD</td>
<td>TCTTAGATGAGGAGCAAG</td>
<td>GAATGCACCAACCATCTATAG</td>
<td>19</td>
<td>1877–1895</td>
<td>865</td>
<td>AF240778</td>
<td>Lichtensteiger et al. 1996</td>
<td></td>
</tr>
<tr>
<td>Outer membrane protein</td>
<td>TBPA-FWD</td>
<td>TGGTGGAACGGGTAAAGGC</td>
<td>TAAACGTTGACGGAAGAAGCC</td>
<td>19</td>
<td>970–988</td>
<td>728</td>
<td>AY007725</td>
<td>Ewers et al. 2006</td>
<td></td>
</tr>
<tr>
<td>involved in iron acquisition</td>
<td>TBPA-REV</td>
<td>TGGTGGAACGGGTAAAGGC</td>
<td>TAAACGTTGACGGAAGAAGCC</td>
<td>19</td>
<td>1697–1679</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>involved in iron acquisition</td>
<td>HGBB-REV</td>
<td>TCAATTGAGTACGGCCTTGAC</td>
<td>CTTCACGTCAGTACACTCG</td>
<td>19</td>
<td>396152–396134</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Filamentous haemagglutinin</td>
<td>PFHA-FWD</td>
<td>AGCTGATCACTGGTGGAAC</td>
<td>TGGTACATTTGGTAATGCTG</td>
<td>19</td>
<td>2408–2427</td>
<td>276</td>
<td>AY035342</td>
<td>Ewers et al. 2006</td>
<td></td>
</tr>
<tr>
<td>adhesin pfhA</td>
<td>PFHA-REV</td>
<td>AGCTGATCACTGGTGGAAC</td>
<td>TGGTACATTTGGTAATGCTG</td>
<td>20</td>
<td>2684–2665</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The PCR reaction mixture contained DNA template, 1 x Qiagen PCR Master Mix (providing a final concentration of 3 mM MgCl₂, 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 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 Pasteurella mutocida, i.e., NCTC10322 (pfhA), NCTC10323 (tbpA) and CECT 962 (toxA, tbpA, hgbB and pfhA), were obtained from NCTC and CECT.

**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 health status. These results were considered as not significant at a P-value of 0.505.

The PCR reaction mixture contained DNA template, 1 x Qiagen PCR Master Mix (providing a final concentration of 3 mM MgCl₂, 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™ 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*: NCTC10322 (capA), NCTC10323 (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 follow-
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 sero-groups 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

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

<table>
<thead>
<tr>
<th><em>P. multocida</em> isolation (n)</th>
<th>Capsular type</th>
<th>Number of isolates (%)</th>
<th>Virulence factors (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>toxA</em></td>
</tr>
<tr>
<td>Healthy lambs (410)</td>
<td>D</td>
<td>2 (5.4)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Diseased lambs (188)</td>
<td>D</td>
<td>20 (54)</td>
<td>1 (0.1)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>15 (40.5)</td>
<td>10 (90.9)</td>
</tr>
<tr>
<td>Total (598)</td>
<td></td>
<td>37 (6.2)</td>
<td>11 (29.7)</td>
</tr>
</tbody>
</table>

**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.
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.
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 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* tpbA (+) (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 *tpbA* 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 animals. 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 *tpbA* 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.

**REFERENCES**


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