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Ovine *Mannheimia haemolytica* isolates from lungs with and without pneumonic lesions belong to similar genotypes

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ABSTRACT

This study investigated the genetic characteristics of 121 ovine *Mannheimia haemolytica* isolates from lungs with (n = 75) and without pneumonic lesions (n = 46) using multilocus sequence typing (MLST), virulence-associated gene typing and pulsed-field gel electrophoresis (PFGE). Twelve STs were identified with most isolates (81%) belonged to ST16, ST28 and ST8. Analysis of the *M. haemolytica* MLST Database indicate a wide distribution of these genotypes in small ruminants, never reported in bovine isolates. This could suggest the adaptation of certain genetic lineages of *M. haemolytica* to small ruminants. e-BURST analysis grouped most STs into three clonal complexes (CC2, CC8 and CC28), consistent with a clonal population structure of *M. haemolytica.* Virulence-associated gene typing identified five virulence profiles in 64% and 65.1% of the *M. haemolytica* isolates from lungs with and without pneumonic lesions, respectively. These data suggest that *M. haemolytica* isolates from the lungs with and without pneumonic lesions are genetically homogeneous. By PGFE analysis a high level of genetic diversity was observed not only within isolates from lungs without pneumonic lesions but also among isolates from pneumonic lesions (GD 0.69 and GD 0.66, respectively; $P > 0.05$). These results indicate that multiple strains of *M. haemolytica* may be associated with individual cases of pneumonia in sheep.

1. Introduction

Mannheimia haemolytica is a normal inhabitant of the upper respiratory tract of ruminants that maintains a commensal relationship with its host (Singh et al., 2011; Klima et al., 2011). *M. haemolytica* is also a ruminant-specific pathogen mainly associated with respiratory diseases (Singh et al., 2011). It is considered the most important bacterial pathogen in bovine respiratory disease (BRD) (Klima et al., 2014; Rainbolt et al., 2016). In sheep, *M. haemolytica* is associated to pneumonic pasteurellosis, a complex disease involving host factors and other infectious agents, which originate important losses due to mortality, reduced weight gain, treatment costs and condemnations in abattoirs (Goodwin-Ray et al., 2008; Marru et al., 2013; Hussein et al., 2017). Different predisposing factors such as transportation, malnutrition, adverse physical, environmental or climatic conditions as well as previous or co-infections with other pathogens can allow *M. haemolytica* that inhabits the upper respiratory tract to overcome the host's immune system, establish infection in the lungs, and cause pneumonia (Singh et al., 2011).

For epidemiological investigations, it is essential to be able to identify specific strains of pathogens. Serotyping has been traditionally used for the characterization of *M. haemolytica* isolates. Serotype A1 is most frequently isolated from BRD lungs, with other serotypes, such as A5, A6, A7, A9, A11, and A12 less frequently reported (Katsuda et al., 2008; Klima et al., 2014; Singh et al., 2011). Serotypes A1, A2, A5, A6, A7, A8, A9, A11 and A12 of *M. haemolytica* are the most commonly isolated strains from sheep with pneumonia (Angen et al., 2002; Fernández et al., 2016; Zheng et al., 2015; Berhe et al., 2017). While serotyping yields useful epidemiologic data, it is not discriminatory enough and it needs to be complemented with more discriminative molecular typing techniques such as random amplified polymorphic DNA (RAPD) (Katsuda et al., 2003; Kodjo et al., 1999), rep-PCR (Klima et al., 2010; Taylor et al., 2014), ribotyping (Kodjo et al., 1999; DeRosa et al., 2000), pulsed-field gel electrophoresis (PFGE) (Rainbolt et al., 2016; Katsuda et al., 2003; Timsit et al., 2013; Klima et al., 2010; Kodjo et al., 1999), and multi-locus sequence typing (MLST) (Petersen et al.,

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2009). Strain typing studies have predominantly been done in cattle to investigate molecular epidemiology of *M. haemolytica* associated to BRD outbreaks in feedlots (DeRosa et al., 2000; Katsuda et al., 2003; Timsit et al., 2013; Klima et al., 2014; Rainbolt et al., 2016). Studies concerning genetic characteristics and population structure of *M. haemolytica* isolates from sheep are scarce (Kodjo et al., 1999; Petersen et al., 2009). Therefore, the aim of this study was to investigate and compare the genetic characteristics of ovine *M. haemolytica* isolates within pneumonic and healthy lung tissue collected from sheep.

2. Material and methods

2.1. Sample collection and M. haemolytica isolates

A total of 263 lungs, with (n = 151) and without (n = 112) pneumonic lesions were collected from slaughtered lambs at three different abattoirs over 22 months. The abattoirs received animals from seven different farms located in three provinces of Spain (Supplementary Table S1). Lungs were observed for the presence or absence of gross pneumonic lesions and selected for sampling. In each visit 30–40 lungs were selected, 50% of them with pneumonic lesions and the rest without pneumonic lesions. Lungs were placed into individual impermeable plastic bags and transported to the laboratory to be processed for bacteriology within four hours from collection. Pneumonic lesions were defined as the presence of clearly demarcated areas of consolidation in craneoventral lung lobules.

Lung samples were inoculated onto Columbia sheep blood agar 5% (BioMèriux) and incubated at 37 °C for 24 h. A total of 121 ovine *M. haemolytica* isolates, 75 from pneumonic lesions and 46 from lungs without pneumonic lesions were analysed in this study. All the isolates selected were haemolytic, catalase positive and Gram-negative rods. *M. haemolytica* isolates were biochemically identified using Diatabs Diagnostic Tablets (Rosco Diagnostica, Taastrup, Denmark) as described by Angen et al. (2002) and further confirmed by a species-specific PCR assay (Dassanayake et al., 2010). Detailed information on the isolates is given in Supplementary Table S1.

2.2. Multi-locus sequence typing

All isolates were characterised by MLST following the scheme described by Petersen et al. (2009) and seven housekeeping genes were amplified and sequenced for each isolate (*adk*, *aroE*, *deoD*, *gapDH*, *gnd*, *mdh*, *zwf*). PCR products were purified using a commercial kit (QIAquickR PCR, Purification kit, Qiagen, Spain) and sequenced on both strands at Macrogen Europe (Amsterdam, The Netherlands). Sequence data were imported into the *M. haemolytica* MLST database website (https://pubmlst.org/mhaemolytica/) to determine the allele designation and the MLST type. Sequence types (STs) identified in this study and those available in the *M. haemolytica* MLST database website (https://pubmlst.org/mhaemolytica/) were analysed using eBURST v3 (http://eburst.mlst.net/) to determine the population diversity and analyse clonal complexes (CC). The default eBURST setting identified groups of related STs using the most stringent (conservative) definition, where all members assigned to the same group share identical alleles at six of the seven loci with at least one other member of the group. Phylogenetic analyses of isolates were done from concatenated sequences of the seven MLST loci using the MEGA software program (MEGA6 v6.0.6). This software was used to build a neighbour-joining tree with the Kimura 2-parameter distance measure using 1000 bootstrap replicates.

2.3. Pulsed field gel electrophoresis

M. haemolytica isolates were analysed by pulsed field gel electrophoresis (PFGE) with protocols described previously (Klima et al., 2010) with some modifications. Briefly, a single colony from an overnight blood agar culture was inoculated into 5 ml of LB medium (DIFCO) and incubated at 37 °C at 200 rpm for 18 h. Bacterial cells were washed and resuspended in 0.1 M Tris-1M NaCl, pH7.6, and low-melting agarose (BioRad, Madrid, Spain) was added to prepare plugs at a final concentration of 1% agarose. Bacterial cells within plugs were lysed by EC lysis solution and extensively washed. The DNA samples were digested overnight at 37 °C in 300 ml of buffer containing 15 units of *Sal*I endonuclease (New England Biolabs, USA). The digested DNAs were loaded in 1% molecular biology-grade agarose (BioRad, Spain) dissolved in 0.5 TBE gels and were separated by PFGE in a contour-clamped homogeneous electric field with the CHEF DRIII apparatus (Bio-Rad, Madrid, Spain). The gels were run in 0.5 TBE buffer at 14 °C and voltage was maintained at 6 V/cm with pulse times of 4–44 s for 22 h. Bacteriophage Lambda PFG Ladder (New England Biolabs, USA) was used as a size marker. The gels were stained in SyBR Gold (Invitrogen, Spain) and photographed on a UV transilluminator. The PFGE profiles were analysed using the BioNumerics Software Package (Applied Maths, Belgium).

2.4. Virulence-associated gene typing

A total of 16 genes (*lktA*, *adhes*, *fhaC*, *gcp*, *hf*, *irp*, *lpsA*, *nanH*, *pilA*, *plpD*, *pomA*, *sodA*, *sodC*, *tbpA*, *tbpB* and *tonB)* associated with virulence (VAG) in *M. haemolytica* were investigated (Fisher et al., 1999; Highlander, 2001; Ewers, 2006). Primers were synthesised by Sigma-Aldrich Co. The primer pairs used for amplification of the different genes are indicated in Table 1. Three different pairs of primers (TBP1, TBP2 and TBP3) were designed for the suitable amplification of the *tbp* gene based on the three genetic variants (*tbp1*, *tbp2* and *tbp3*) of the gene detected among the available sequences of the gene at GenBank (Pinto, 2016). PCRs were carried out using AmpliTaq® Gold DNA Polymerase with GeneAmp® 10X PCR buffer (Applied Biosystems). Amplifications were performed using Multiplex PCRs (Pinto, 2016). PCR products were visualised by electrophoresis in 1% agarose gels supplemented with 1X Syber safe gel stain (Invitrogen SA). The strain *M. haemolytica* NCTC 9712 was used as the reference positive control for PCRs of all the VAGs. Strain PH344, kindly provide by Dr Robert Davies of the University of Glasgow (UK), and the reference strain NCTC10636 were also used as positive controls for variants of the *tbp* gene.

2.5. Data analysis

Genetic diversity (GD) was calculated as the ratio between total MLST or PFGE patterns and total isolates (Martínez et al., 2002). The frequency and association between variables were analysed using the Epi InfoTM 7 program of the Centers for Disease Control and Prevention (CDC) (http://wwwn.cdc.gov/). Association analysis between categorical variables was done using the Fisher exact test. The significance level was set at $P < 0.05$.

3. Results

All STs identified in the 121 *M. haemolytica* isolates from sheep are indicated in Table 2. The isolates belong to 12 STs, with ST16 ($n = 50$, 41.3%), ST 28 ($n = 29$, 24.0%) and ST8 ($n = 19$, 15.7%) the most common identified among isolates from lungs without and with pneumonic lesions. The remaining STs were represented by fewer than ten isolates each (Table 2). The differences in the ST frequencies between isolates from lungs with and without pneumonic lesions were not statistically significant (Table 2, $P > 0.05$). STs detected in this study were compared with those available on the *M. haemolytica* MLST Database (https://pubmlst.org/mhaemolytica/) by e-BURST analysis. Sixty-six out of the 121 *M. haemolytica* isolates (54.5%) grouped into three clonal complexes: CC28 comprised 31 isolates (25.6%), CC8 comprised 29 isolates (24.0%), and CC4 comprised six isolates (5.0%)