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Mannheimia Species Associated with Ovine Mastitis[▽]

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***Mannheimia glucosida*, *M. haemolytica*, and *M. ruminalis* were isolated from cases of acute mastitis in ewes. *M. glucosida* was found to be a common cause of clinical mastitis in sheep. Selected phenotypic tests in addition to genotyping were needed to definitively identify *Mannheimia* species causing ovine mastitis.**

Mastitis is an important disease of sheep in dairy, wool, and meat production systems. Several studies have found that the prevalence of *Mannheimia haemolytica* as a cause of ovine mastitis is similar to or greater than that of *Staphylococcus aureus* (6, 7, 13, 14). In a preliminary study, our laboratory also demonstrated the significance of this species in mastitis in Poll Dorset ewes in southeastern Australia (7).

The genus *Mannheimia* contains several species from the family *Pasteurellaceae* that have been recently reclassified (4). The five named species within this genus are *M. haemolytica*, *M. glucosida*, *M. ruminalis*, *M. varigena*, and *M. granulomatis* (4). There are also several unnamed taxa that are distinct from these named species (2).

M. haemolytica is regarded as the most important species in this genus, as it is the major organism involved in pneumonia in feedlot cattle (10) and can cause pneumonia and mastitis in sheep (10, 14). The former species *Pasteurella haemolytica* was separated into two biotypes, A and T, based on the capacity to ferment arabinose or trehalose and the results of some other phenotypic tests (19). Sixteen serotypes within these two biotypes were originally defined using indirect hemagglutination assays (10), with a new serotype, A17, added later (22). Biotype A was divided into 9 biogroups (1). Biogroup 1 of the former *P. haemolytica* was later renamed *M. haemolytica* and includes serotypes 1, 2, 5 to 9, 12 to 14, and 16 (4, 5). Serotypes 3, 4, 10, and 15 of biotype T of the former *P. haemolytica* were classified as *P. trehalosi* (20) and later as *Bibersteinia trehalosi* (9).

Serotype A11, as well as biogroups 3A to H and 9 of the former *P. haemolytica*, were reclassified as *M. glucosida* (1), which is a heterogeneous species that has been isolated from in ruminants case of pneumonia (2) and from the nasal cavities of healthy sheep (2, 18).

M. ruminalis has not been associated with disease and can be isolated from the rumen of sheep (2), and *M. ruminalis*-like organisms have been isolated from the nasal cavities of healthy sheep (18).

This study investigated the phenotypic and genetic characteristics of *Mannheimia* species isolated from cases of clinical mastitis in sheep, with the aim of definitively identifying the most prevalent species responsible for this disease.

Twenty-four bacterial isolates from cases of mastitis, mainly in Poll Dorset ewes, were used in this study. The isolates were all Gram-negative rods with no significant growth on MacConkey agar and produced mucoid, gray colonies on sheep blood agar (SBA). Seventeen were from a previous survey of mastitis in Poll Dorset ewes (7), and the other seven isolates were collected from ewes with mastitis in 2008. The isolates were from 8 different farms, which were designated A to F, H, and I.

Each isolate was grown on SBA at 37°C overnight. A set of discriminative biochemical tests were chosen based on data from previous studies (1, 2, 8). The type strains of *M. haemolytica* and *M. ruminalis* (kindly provided by Pat Blackall) were used as positive controls, and uninoculated broths were used as negative controls. All tests were performed in duplicate and repeated twice. Peptone-sugar media were made using standard methods. India ink wet-film staining was used to detect the presence of a capsule (11).

The presence of the leukotoxin gene (*lktA*) was investigated using a pair of primers to amplify the full coding region and part of the flanking region of *lktA* (Table 1). Amplification was performed with 50-μl reaction mixtures containing 1 μM (each) primers, 200 μM (each) deoxynucleoside triphosphates (dNTPs), 1.5 μM $MgSO_4$, 5 μl of 10× High-Fidelity PCR buffer, and 1.5 U of Platinum *Taq* high-fidelity DNA polymerase (Promega). The DNA template was obtained by extracting DNA from an overnight growth of each strain in brain heart infusion (BHI) broth using High Pure PCR template preparation kits (Roche). Reaction mixtures were incubated at 94°C for 1 min and then subjected to 35 cycles of 94°C for 45 s, 60°C for 45 s, and 68°C for 3.5 min, followed by a final extension step at 72°C for 7 min. PCR products were visualized by electrophoresis in a 1% agarose gel.

All strains were subjected to phylogenetic analysis by amplification and sequencing of two housekeeping genes, *rpoB* and *rrnA* (the 16S rRNA gene) (15, 16). The primers used are listed in Table 1. Amplification was performed in 25-μl volumes containing 1 μM (each) primers for *rpoB* or 0.25 μM (each) primers for *rrnA*, 200 μM (each) dNTPs, 2 μM $MgCl_2$, 5 μl of 5× GoTaq Flexi buffer, and 1 U of *Taq* polymerase (Promega). A single colony was used as the template for the reaction. Amplification was performed in a Bio-Rad thermal cycler. Reaction mixtures were incubated at 94°C for 1 min and then subjected to 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 45 s for amplification from *rpoB* and 60 s for amplification from *rrnA*, with a final extension at 72°C for 7 min. Amplified

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TABLE 1. Primers used for amplification of two housekeeping genes, *rpoB* and *rrnA*, and the *lktA* (leukotoxin) gene

Gene	Primer	Sequence	Reference
rpoB	Pasrpob-L	5′-GCAGTGAAAGARTTCTTTGGTTC-3′	15
	Rpob-R	5′-GTTGCATGTTNGNACCCAT-3′	
rrnA	16SUNI-L	5′ AGAGTTTGATCATGGCTCAG 3′	16
	16SUNI-R	5′ GTGTGACGGGCGGTGTGTAC 3′	
lktA	lktA F1	5′-TCAAGAAGAGCTGGCAACAGCACT-3′	This study
	lktA R1	5′-AAACGGCAACTCCATAATGTGCGA-3′	This study

DNA was purified using Qiaex II gel purification kits (Qiagen), and sequencing reactions were performed using BigDye Terminator version 3.1 kits.

Sequences were compared and aligned with published sequences for type strains (Table 2). Maximum-likelihood analysis was performed using the DNAml program in the Phylip package, and the best transition-transversion ratio was determined based on the value that yielded the best log likelihood. Bootstrap analysis of each set of sequences (from 100 resamplings) was performed using Phylip. Nucleotide distances were calculated using the Jukes-Cantor gamma distance model in MEGA version 4 (21), assuming that the rate of nucleotide substitution was the same for all pairs of the four nucleotides.

A summary of the phenotypic and genotypic characteristics of the isolates is shown in Table 3. Isolates were divided into those that produced and those that did not produce β-glucosidase. Nine (A2, A3, A4, A5, B1, D2, F1, H1, and H2) of these 24 isolates were β-glucosidase positive and also hydrolyzed selected glycosides.

Twelve L-arabinose-negative isolates had similar capacities to ferment other selected sugars and were identified as *M. haemolytica*. All isolates were encapsulated. A 3-kbp product was amplified using the *lktA* PCR from all isolates except A6 and A7.

The distances between the DNA sequences of the *rpoB* genes from all the isolates obtained from cases of ovine mastitis and the *rpoB* genes from *M. varigena* and *M. granulomatis* were 9 to 12%, so these two species were omitted from phenotypic comparisons and phylogenetic analyses. Twelve isolates (C1, C2, C3, E1, G1, G2, I1, I2, F2, D1, A8, and A9) had *rpoB* sequences identical to that of *M. haemolytica* but could be divided into three groups on the basis of one or two nucleotide differences in their *rrnA* genes.

The distance between the *rpoB* sequences from isolates A6 and A7, which showed no hemolysis on SBA and were phenotypically similar to *M. ruminalis*, and that from *M. ruminalis* was 1.9%, but the distances between the *rrnA* sequences from these isolates, as well as that from isolate A1, which showed hemolysis on SBA, and that from *M. ruminalis* were less than 1%.

Of the β-glucosidase-producing isolates, A2, A3, A4, and B1 were the most similar to *M. glucosida* (with 0.2, 0.2, 0.2, and 0% nucleotide distances between *rpoB* sequences, respectively). The distance between the *rpoB* gene sequence from isolate A5 and that from *M. glucosida* was 5%, but the *rrnA* sequence from A5 was identical to that from *M. glucosida* strain P733. Phylogenetic trees for *rrnA* and *rpoB* sequences are shown in Fig. 1.

The results of this study have established that *M. glucosida* is a significant cause of ovine mastitis. Previous studies have demonstrated the importance of *Mannheimia* (*Pasteurella*) species in ovine mastitis (6, 7, 13, 14). In 1998, Jones and Watkins (13) isolated different serotypes of *M.* (*P.*) *haemolytica*, including serotype A11, from cases of ovine mastitis in the United Kingdom. Nine (37.5%) of 24 *Mannheimia* isolates in our study were *M. glucosida*, with 6 isolated in pure culture, suggesting that this species can be as significant a cause of ovine mastitis as *M. haemolytica*.

TABLE 2. *Mannheimia* type strain sequences used for phylogenetic analysis

Gene	Species	Strain	GenBank accession no.	Reference
rpoB	*M. haemolytica*	ATCC 33396	AY170217.1	3
	M. glucosida	CCUG38457	AY362959	15
	M. ruminalis	CCUG38470	AY362961	15
	M. varigena	CCUG38462	AY362962	15
	M. granulomatis	ATCC 49244	AY362960	15
rrnA	*M. haemolytica*	PH213[a]	DQ301920.1	17
	M. haemolytica	NCTC9380[b]	M75080	12
	M. glucosida	CCUG38457	AY362912	15
	M. glucosida	P733	AF053892	4
	M. ruminalis	CCUG38470	AF053900	4
	M. varigena	CCUG38462	AF053893	4
	M. granulomatis	ATCC 49244	AY362913	15

[a] Serotype A1.
[b] Serotype A2.

TABLE 3. Phenotypic and genotypic characteristics of isolates of *Mannheimia* species from animals with ovine mastitis

Characteristic	Result[b] for:			
	M. haemolytica (12 isolates)	*M. glucosida* (9 isolates)	*M. ruminalis* (2 isolates)	*M. ruminalis*-like strain (1 isolate)
Beta-hemolysis[a]	w	w	−	w
Oxidase	+	+	−	+
Ornithine decarboxylase	−	V (6)	−	−
β-Glucosidase	−	+	−	−
Acid from:				
L-Arabinose	−	V (6)	+	+
Trehalose	−	−	−	−
Maltose	+/w	V (6)	V (1)	+
L-(+)-Rhamnose	−	−	V (1)	−
D-Sorbitol	+/w	+	−	+
D-Xylose	+/w	+	−	+
Hydrolysis of:				
Esculin	−	+	−	−
Salicin	−	+	−	−
Presence of capsule	+	+	+	+
lktA gene	+	+	−	+

[a] Hemolysis on SBA.
[b] V, variable (numbers in parentheses show the number of isolates yielding a positive reaction); +, positive results within 2 days; w, weakly positive.

In the study from which these isolates were derived, 74 of 166 milk samples from ewes with clinical mastitis yielded cultivable bacteria, 48 of which were *Mannheimia* species, 12 of these being *M. glucosida*. Only 1 of 1,900 milk samples from clinically healthy ewes yielded *M. glucosida*, and this sample had a somatic cell count of 6.7 million cells/ml. Thus, *Mannheimia* species and *M. glucosida* are significantly more prevalent in milk from ewes with acute mastitis than in milk from clinically normal ewes ($P < 0.000001$; Fisher's exact test).

The classification *M. glucosida* includes other members of the former *P. haemolytica* classification, in addition to members of serotype A11, and some strains within this species are not typeable by indirect hemagglutination. Thus, our study has established the association between this species and ovine mastitis for the first time since the definition of the species was published. *M. glucosida* is a heterogeneous species but is consistently β-glucosidase and *meso*-inositol positive. The *M. glucosida* isolates in our study differed in their capacities to ferment L-arabinose and produce ornithine decarboxylase.

There are no previous reports of any association between *M. ruminalis* and disease. Although the milk samples yielded light growths of the *M. ruminalis* and *M. ruminalis*-like organisms,

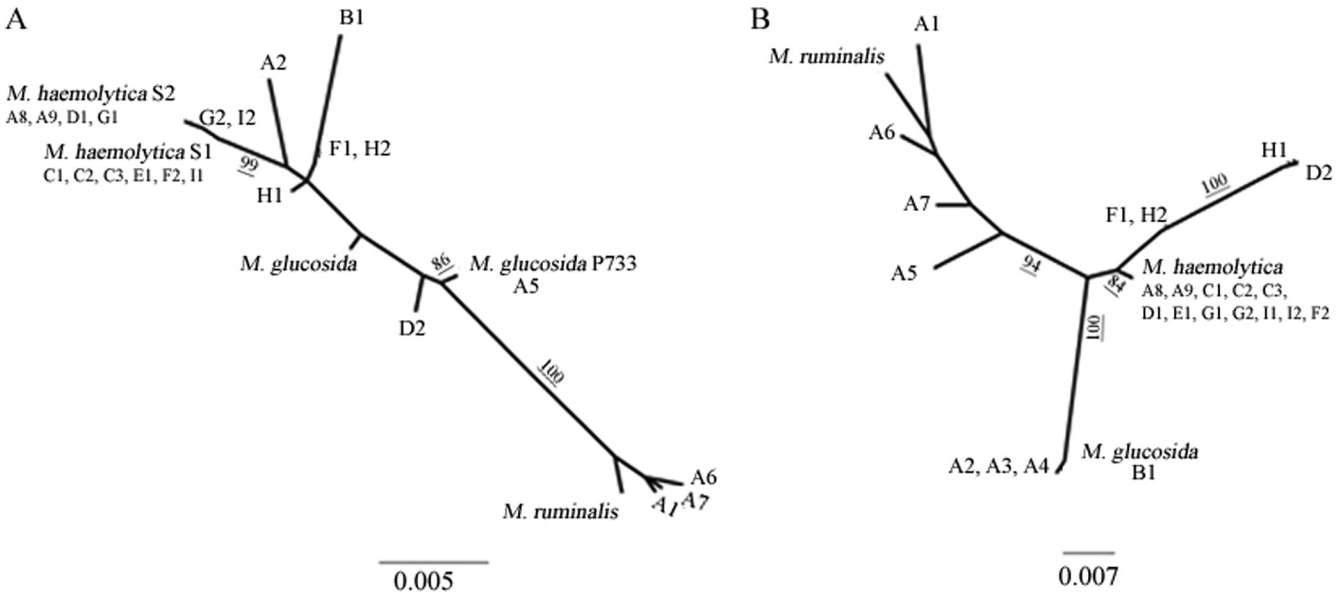

FIG. 1. Maximum-likelihood trees for *rrnA* (A) and *rpoB* (B) gene sequences, assuming transition-transversion ratios of 1.2 and 5, respectively. Bootstrap values for each branch are shown when they are greater than 75%.

the isolates were obtained in pure culture from animals with mastitis on the same farm.

To assess whether the isolates that were not *M. haemolytica* had any of the known virulence factors of *M. haemolytica*, we examined them for the presence of a capsule as well as the structural gene for the leukotoxin. The hemolytic *M. ruminalis* isolate (A1) and all the *M. glucosida* and *M. haemolytica* isolates contained the *lktA* gene. Although the two nonhemolytic *M. ruminalis* isolates did not contain the *lktA* gene, they, like all the other isolates, were encapsulated.

Korczak et al. suggested that *rpoB* sequences were more reliable for discrimination between genera within the family *Pasteurellaceae* than *rrnA* sequences (with 10 to 12% difference between *rpoB* gene sequences from different genera, compared to 6 to 7% between *rrnA* gene sequences) (15). *M. haemolytica* isolates were easily identified using *rpoB* sequencing, but identification of *M. glucosida* and *M. ruminalis* remained difficult, with some isolates, such as A5, unable to be identified to the species level. The phenotypic and genotypic heterogeneity of strains of *M. glucosida* suggests that this classification may consist of more than one taxon.

Definitive identification of isolates in this genus remains difficult (2), and we found that multiple phenotypic tests, including tests for production of β-glucosidase and ornithine decarboxylase, fermentation of L-arabinose, D-sorbitol, and D-xylose, and hydrolysis of salicin and esculin, were needed for differentiation of *M. glucosida* from *M. haemolytica* and *M. ruminalis* whenever there was any doubt about identification of isolates based on *rpoB* and *rrnA* sequences.

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