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Bacterial species and their associations with acute and chronic mastitis in suckler ewes

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ABSTRACT

Acute mastitis in suckler ewes is often detected because of systemic signs such as anorexia or lameness, whereas chronic mastitis, characterized by intramammary abscesses with no systemic disease, is typically detected when ewes are inspected before mating. The aims of the current study were to identify the species and strains of culturable bacteria associated with acutely diseased, chronically diseased, and unaffected mammary glands to investigate whether species and strains vary by state. To investigate acute mastitis, 28 milk samples were obtained from both glands of 14 ewes with acute mastitis in one gland only. To investigate chronic mastitis, 16 ovine udders were obtained from 2 abattoirs; milk was aspirated from the 32 glands where possible, and the udders were sectioned to expose intramammary abscesses, which were swab sampled. All milk and swab samples were cultured aerobically. In total, 37 bacterial species were identified, 4 from acute mastitis, 26 from chronic mastitis, and 8 from apparently healthy glands. In chronic mastitis, the overall coincidence index of overlap of species detected in intramammary abscesses and milk was 0.60, reducing to 0.36 within individual glands, indicating a high degree of species overlap in milk and abscesses overall, but less overlap within specific glands. *Staphylococcus aureus* was detected frequently in all sample types; it was isolated from 10/14 glands with acute mastitis. In 5 ewes, closely related strains were present in both affected and unaffected glands. In chronic mastitis, closely related *Staphylococcus aureus* strains were detected in milk and abscesses from the same gland.

Key words: suckler ewe, acute mastitis, chronic mastitis, *Staphylococcus aureus*, matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF)

INTRODUCTION

Intramammary infections in suckler ewes are usually caused by bacterial infection of the mammary gland. The IMI can have a wide range of presentations from no detectable clinical abnormality (subclinical disease) to a hot swollen gland and, occasionally, sudden death (acute mastitis). The incidence of acute clinical mastitis ranges from 0 to 6.6% of sheep per flock (Onnasch et al., 2002; Arsenault et al., 2008).

Intramammary abscesses are a presentation of chronic mastitis and thought to form following an IMI, although not all IMI lead to abscess formation. These abscesses are often only detected when a gland is palpated, typically when farmers inspect ewes at the end of lactation, or when selecting ewes for mating. Few reports are available on the prevalence of ewes with intramammary abscesses. Onnasch et al. (2002) reported a prevalence of chronic mastitis of 2.8%, and Saratsis et al. (1998) reported abnormalities (including nodules, lumps, diffuse hardness, abscesses, and cysts) in the udders of 162/3,529 (4.6%) ewes. Anecdotally, British farmers indicate that 20 to 30% of ewes culled from the flock (i.e., 4–6% of the total flock if 20% are culled) at weaning have intramammary abscesses.

More than 130 bacterial species have been associated with IMI in dairy cows (Watts, 1988) and 20 to 30 with IMI in suckler sheep to date (Mørk et al., 2007; Arsenault et al., 2008; Marogna et al., 2010). The main organisms associated with acute mastitis in sheep are *Staphylococcus aureus*, *Mannheimia haemolytica*, *Streptococcus uberis*, and *Escherichia coli*. In dairy ewes, signs of chronic mastitis defined as nodules, abscesses, sclerosis, and atrophy have been significantly associated with isolation of *Staph. aureus* from milk (Marogna et al., 2010), and Onnasch et al. (2002) stated that the most frequently isolated pathogens from cases of

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chronic mastitis were *Staph. aureus*, *Streptococcus* species, and *Arcanobacter pyogenes*. Abscesses that result from infection with the endogenous flora of a body site are reported to be polymicrobial (Brook, 2002), and it is hypothesized that polymicrobial abscesses persist, in part, because the species act synergistically.

The aims of the current study were to characterize the culturable bacterial species associated with acute mastitis and associated unaffected glands, and intramammary abscesses (chronic mastitis) and associated milk, of suckler ewes to investigate whether species and strains were common or varied between these presentations.

MATERIALS AND METHODS

Sample Collection and Bacterial Isolation

Study 1: Investigation of Acute Mastitis. Milk samples were aseptically collected from both glands of 14 sheep with acute clinical mastitis in one gland only. Glycerol (final concentration ~10% vol/vol) was included in each sample as a cryopreservant (Smith et al., 2011), and all samples were stored at −20°C until culture. One hundred microliters of each sample was spread across the surface of a brain heart infusion (**BHI**) agar plate containing 5% sheep blood and incubated at 37°C for 48 h. Growth was observed at 24 and 48 h and the number of colony-forming units estimated for each morphologically distinct colony type; within a sample, unique morphological types were considered to be a single species. Where colony-forming units were too numerous to count, a figure of 1,000 cfu was used for calculations. One example of each morphologically distinct colony type per sample was purified by streaking across a second BHI agar plate containing 5% sheep blood to produce isolated colonies, and stored at −80°C.

Study 2: Investigation of Chronic Mastitis. Sixteen ovine udders (32 glands, 2 glands per udder) were obtained from 2 abattoirs in England. On arrival at the laboratory, each udder's surface was thoroughly cleaned with 70% ethanol, and one sample of milk was aspirated from each gland where possible, using a sterile 18-gauge needle and syringe. One hundred microliters of each milk sample was cultured as described above. Each gland was then sliced into parasagittal sections using a sterile blade, sterilized between cuts with 70% ethanol. The number of abscesses was recorded, and each abscess was swabbed using a sterile cotton-tipped swab that was immediately plated on a BHI agar plate containing 5% sheep blood and incubated at 37°C for 48 h. Morphologically distinct colony types were purified and stored at −80°C as described above.

Identification of Bacterial Isolates

Matrix-assisted laser desorption/ionization time-of-flight (**MALDI-ToF**) mass spectrometry was used to identify isolates (Alatoom et al., 2011). Pure isolates were cultured on BHI agar and a loop of each isolate was suspended in 75% ethanol and pelleted at >20,000 × *g* for 5 min at room temperature; the supernatant was discarded and the pellet resuspended in 1 volume (equal to pellet size) of 70% formic acid (Sigma-Aldrich Company Ltd., Dorset, UK) and 1 volume of acetonitrile (Sigma-Aldrich Company Ltd.). Samples were then pelleted at >20,000 × *g* for 2 min at room temperature, 1 μL of supernatant was placed in a well on a steel target plate (Bruker UK Ltd., Coventry, UK) and air-dried. Once dry, 1 μL of α-cyano-4-hydroxycinnamic acid matrix (Bruker UK Ltd.) was overlaid on each sample and air dried.

Target plates were loaded into a Microflex LT instrument (Bruker UK Ltd.) with protein mass profiles obtained using the MALDI Biotyper wizard classification and FlexControl software (Bruker UK Ltd.) with default settings. The MALDI Biotyper compares the spectra obtained with a database of known species [February 2014 update: 5,627 isolates, 1,951 species in 388 genera, with 20 to 24 replicates per (predominantly human) isolate] and produces a top-10 list of matches for each sample plus a confidence score (range: 0–3). Using the recommended cut-off values, a score ≥2.30 indicates highly probable species identification, a score of 2.00 to 2.29 indicates probable species identification, and scores of 1.70 to 1.99 indicate probable genus identification (Bruker Daltonik GmbH, 2011). Scores <1.70 are not considered a reliable identification. To overcome the lack of veterinary isolates for some species in the database, the criteria used in the current study were modified for isolates with a score of 1.70 to 1.99. If ≤3 of the identified species were in the database, the species designation was accepted. If >3 species were in the database and the top ≥3 identifications were the same species, then the species designation was also accepted. For all cases outside of these criteria, only the genus designation was accepted.

Where species were not identifiable using MALDI-ToF, DNA was extracted from overnight cultures using the NucleoSpin Tissue Kit (Machery-Nagel GmbH & Co. KG, Düren, Germany) as recommended. The 16S gene was amplified using primers 27F and 1525R and sequenced using the internal primers PSL and PSR (Moore et al., 2008). Sequences were assembled in SeqMan Pro (DNASTAR Inc., Madison, WI), manually trimmed, and compared with the National Center for Biotechnology Information 16S ribosomal RNA se-