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Comparison of bacteriological culture and PCR for detection of bacteria in ovine milk—Sheep are not small cows

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ABSTRACT

Mastitis, inflammation of the mammary gland, is an important cause of disease, mortality, and production losses in dairy and meat sheep. Mastitis is commonly caused by intramammary infection with bacteria, which can be detected by bacterial culture or PCR. PathoProof (Thermo Fisher Scientific Ltd., Vantaa, Finland) is a commercially available real-time PCR system for the detection of bovine mastitis pathogens. Sheep differ from cattle in the bacterial species or bacterial strains that cause mastitis, as well as in the composition of their milk. The aim of this study was to evaluate whether the PathoProof system was suitable for detection of mastitis pathogens in sheep milk. Milk samples were collected aseptically from 219 udder halves of 113 clinically healthy ewes in a single flock. Aliquots were used for bacteriological culture and real-time PCR-based detection of bacteria. For species identified by culture, the diagnosis was confirmed by species-specific conventional PCR or by sequencing of a housekeeping gene. The majority of samples were negative by culture (74.4% of 219 samples) and real-time PCR (82.3% of 192 samples). Agreement was observed for 138 of 192 samples. Thirty-four samples were positive by culture only, mostly due to presence of species that are not covered by primers in the PCR system (e.g., *Mannheimia* spp.). Two samples were positive for *Streptococcus uberis* by culture but not by PCR directly from the milk samples. This was not due to inability of the PCR primers to amplify ovine *Streptococcus uberis*, as diluted DNA extracts from the same samples and DNA extracts from the bacterial isolates were positive by real-time PCR. For samples containing *Staphylococcus* spp., 11 samples were positive by culture and PCR, 9 by culture only, and 20 by PCR only. Samples that were negative by either method had lower bacterial load than samples that were positive for both methods, whereas no clear relation with species identity was observed. This study provides proof of principle that real-time PCR can be used for detection of mastitis pathogens in ovine milk. Routine use in sheep may require inclusion of primer sets for sheep-specific mastitis pathogens.
Key words: mastitis, sheep, culture, PCR

INTRODUCTION

Mastitis, inflammation of the mammary gland, is an important cause of disease and production losses in sheep and is mostly caused by IMI by viruses or bacteria. Clinical mastitis is a painful condition and can lead to the death of the animal (Arsenault et al., 2008; Omaleki et al., 2011). In dairy sheep, mastitis leads to direct losses due to reduced milk production (Cuccuru et al., 2011). In meat sheep, reduced milk production affects the welfare, growth, and survival of the suckling lamb (Moroni et al., 2007). Clinical and subclinical mastitis are also causes of premature culling of otherwise healthy sheep (Bergonier et al., 2003).

Current mastitis control strategies strongly depend on use of antimicrobial compounds for treatment of infections and, in dairy sheep, on milking management (Bergonier et al., 2003). Risks inherent in the use of antimicrobials in livestock production include the selection and dissemination of pathogens or commensals carrying antimicrobial resistance genes, which may subsequently affect human as well as animal health (Aarestrup et al., 2008). Alternative methods to reduce the incidence of mastitis in dairy as well as meat sheep include breeding for resistance and, experimentally, vaccination (Bergonier et al., 2003; Conington et al., 2008; Pérez et al., 2009). To select or develop targeted

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mastitis control measures, the causative agents of mastitis need to be known.

Some of the major mastitis pathogens in sheep are also found in cattle, whereas other mastitis pathogens are predominantly limited to one of those host species. For example, the most common etiological agents of mastitis in sheep include *Staphylococcus aureus*, which is also commonly found in cattle, and *Mannheimia haemolytica*, which is rarely found in cattle (Mørk et al., 2007; Arsenault et al., 2008; Omaleki et al., 2011). The distribution of CNS differs between cattle and sheep (Zadoks and Watts, 2009; Cuccuru et al. 2011). In addition, for pathogen species that affect both host species, strain differences between isolates from the 2 hosts may exist. For example, cattle and sheep have different subpopulations of *Staph. aureus* and *Streptococcus uberis* (Smyth et al., 2009; Gilchrist et al., 2013).

Detection of bacterial species in ovine milk is commonly based on microbiological culture, whereas routine detection of bacterial species in bovine milk can be based on microbiological culture or PCR (Koskinen et al., 2010; Spittel and Hoedemaker, 2012). The commercially available PathoProof Mastitis PCR Assay (Thermo Fisher Scientific Ltd., Vantaa, Finland; Koskinen et al., 2009) is a real-time PCR assay that is increasingly used for diagnostic testing of bovine milk. Its performance on ovine milk has not been evaluated. Considering that many bacterial species can cause mastitis in cattle as well as sheep, it is reasonable to expect that a large proportion of mastitis pathogens in ovine milk will be detected with the current commercial assay. However, differences in bacterial species and strains affecting cattle and sheep may also result in missed diagnoses in ovine milk. Furthermore, the composition of ovine milk differs from the composition of bovine milk (e.g., in fat and protein content; Jenness, 1985), which could potentially affect diagnostic test performance. The aim of the current study was to conduct a preliminary investigation into the performance of bacteriological culture and commercially available PCR to detect pathogens in ovine milk and to provide a critical discussion of differences in results obtained with the 2 methods.

MATERIALS AND METHODS

Milk Sample Collection

Ovine milk samples were collected from a flock of Scottish Blackface-Leicester cross sheep at the Moredun Research Institute Firth Mains farm (Penicuik, UK) on a single occasion in June 2013. Ewes were in late lactation and had at least 1 lamb at foot. Udders were inspected and palpated to detect gross lesions and teats were disinfected using gauze swabs soaked in 70% ethanol. The first streak or drops of milk were discarded and samples were collected in sterile vials. Samples were kept on ice packs and transported to the laboratory within hours, where they were split aseptically into aliquots for bacterial culture and PCR, respectively. Apart from local lesions to the udders or teats of some animals, all ewes were clinically healthy.

Bacteriological Culture

Milk samples were vortexed for 5 s and 10 μL of each sample was plated onto 5% sheep blood agar (**SBA**) and MacConkey agar plates (Oxoid Ltd., Basingstoke, UK). After streaking for individual colonies, plates were incubated at 37°C and examined for growth after 24 and 48 h. Purity of the bacterial growth was assessed and samples showing growth of 3 or more species were considered contaminated. Profusion of bacterial growth was assessed semiquantitatively based on growth in each of 4 plate quadrants using the quadrant streak technique. The limit of detection was 100 cfu/mL of sample or 1 cfu/plate.

Subculturing of individual colonies was carried out on 5% SBA. Gram stains, oxidase tests (Oxoid Ltd.), and catalase tests were carried out on all cultured bacterial isolates. Further identification was carried out based on the results of these tests. Differentiation of *Staph. aureus* from other staphylococcal species was based on production of pigment, hemolysin, hyaluronidase, DNase, clumping factor, and coagulase. Streptococci were differentiated from enterococci based on the absence of growth on MacConkey agar. Identification of streptococci to Lancefield group level was carried out using the Streptococcal Grouping Kit (Oxoid Ltd.). Streptococci reacting with antisera to group B were assumed to be *Streptococcus agalactiae* and those reacting with group C were assumed to be *Streptococcus dysgalactiae*. Streptococci that failed to react with antisera to groups A, B, C, or D were tested for esculin hydrolysis and, if positive, assumed to be *Strep. uberis*. Identification of enteric gram-negative organisms was carried out using the API20E system (bioMérieux UK Ltd., Basingstoke, UK) and identification of nonenteric gram-negative organisms was completed using the API 20NE system (bioMérieux UK Ltd.). This system does not distinguish between *M. haemolytica* and *Bibersteinia trehalosi*. Therefore, growth on MacConkey agar and hydrolysis of trehalose was also assessed. Presumptive identification of gram-positive bacilli was carried out to genus level based on colonial characteristics and catalase testing (Barrow and Feltham, 2004).

Table 1. Polymerase chain reaction primers used for confirmation of species identity of bacteria cultured from ovine milk with or without sequencing of the PCR amplicon

Target species	Target gene	Primer (5′ to 3′)[1]	PCR only or amplicon sequencing	Reference
Staphylococcus aureus	*nuc*	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAAAGC	PCR only	Brakstad et al. (1992)
Streptococcus dysgalactiae	16S	GAACACGTTAGGGTCGTC AGTATATCTTAACTAGAAAAACTATTG	PCR only	Phuektes et al. (2001)
Streptococcus uberis	16S	TAAGGAACACGTTGGTTAAG TTCCAGTCCTTAGACCTTCT	PCR only	Phuektes et al. (2001)
Non-*aureus* staphylococci	*rpoB*	AACCAATTCCGTATNGGTTT GCNACNTGNTCCATACCTGT	Sequencing	Drancourt and Raoult (2002)
Non-*aureus* staphylococci	*tuf*	GCCAGTTGAGGACGTATTCT CCATTTCAGTACCTTCTGGTAA	Sequencing	Heikens et al. (2005)
Other species	16S	TGAAGAGTTTGATCATGGCTCAG AGGAGGTGATCCAACCGCA	Sequencing	Wiedmann et al. (2000)

[1]Forward primers are listed first and reverse primers are listed second.

Confirmation of Species Identity of Bacterial Isolates

Genomic DNA of bacteria was prepared by lysis of bacterial cells with lysozyme and proteinase K. Bacteria were cultured at 37°C overnight in brain-heart infusion broth (Sigma-Aldrich, Gillingham, UK). Five hundred microliters of bacterial suspension was centrifuged at 11,000 × *g* for 2 min at ambient temperature. Supernatant was discarded and pellets were resuspended in 200 μL of Tris-EDTA buffer (10 m*M* Tris-Cl and 1 m*M* EDTA, pH 8.0) containing 2 mg/mL of recombinant human lysozyme (Sigma-Aldrich) and incubated at room temperature for 15 min. Three microliters of proteinase K (Sigma-Aldrich), for a final concentration of 0.3 mg/mL, was added to the suspension and incubated at 58°C for 1 h. Enzymes were inactivated by incubation at 95°C for 8 min. Lysates were briefly centrifuged at 11,000 × *g* for 15 s at ambient temperature and stored at −20°C. Species identity of *Staph. aureus*, *Strep. dysgalactiae*, and *Strep. uberis* isolates was confirmed by species-specific conventional PCR. Species identity of non-*aureus* staphylococci and other bacterial species was determined by conventional PCR and sequencing of the *rpoB* and *tuf* or 16S ribosomal DNA genes. A list of primers is provided in Table 1. Primers were ordered from Eurofins Genomics (Ebersberg, Germany) and bidirectional sequencing of purified PCR amplicons was also performed by Eurofins Genomics, using the same primers as in the PCR reactions. Quality of sequence data was inspected using Lasergene (DNAStar Inc., Madison, WI), and blastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to determine species identity, using >98% sequence similarity for 16S-based species identification and >94% similarity or >5% difference between best and next-best match for *rpoB*-based species identification (Mellmann et al., 2006; Zadoks and Watts, 2009).

PCR-Based Diagnostics Using Milk Samples

The PathoProof Mastitis Complete-12 kit (Thermo Fisher Scientific, Vantaa, Finland) and plate format for DNA extraction (PathoProof kit F-870L) were used as per the manufacturer's instructions, with 1:5 dilution of DNA extracts if undiluted extracts showed inhibition of the internal amplification control (**IAC**). Slight modifications were made to the DNA extraction protocol, as per the diagnostic laboratory's routine procedure, whereby plates were centrifuged at 2,862 × *g* in steps 13 through 16 of the protocol. Also, at step 15 of the protocol, plates were incubated with elution buffer for 5 min at room temperature before the 4-min centrifugation step, and this initial centrifugation to wash the ethanol through the spin columns was followed by a 15-min centrifugation step to ensure that the ethanol completely evaporated. The PCR-positive samples were classified as +, ++, or +++ as per the supplier's guidelines. For samples that were culture positive but PCR negative for a target that should be detectable by real-time PCR, additional analyses were conducted on the original DNA extract from milk, on a new aliquot of milk, if available, or on DNA extracts from bacterial isolates. Where appropriate, based on the number of observations, the association between PCR results and culture results was analyzed by chi-squared analysis using Statistix 10 software (Analytical Software, Tallahassee, FL).

RESULTS

Clinical Observations

Milk samples were collected from 113 ewes. Samples were obtained from both halves of the mammary gland for 106 ewes and for a single udder half for 7 ewes,

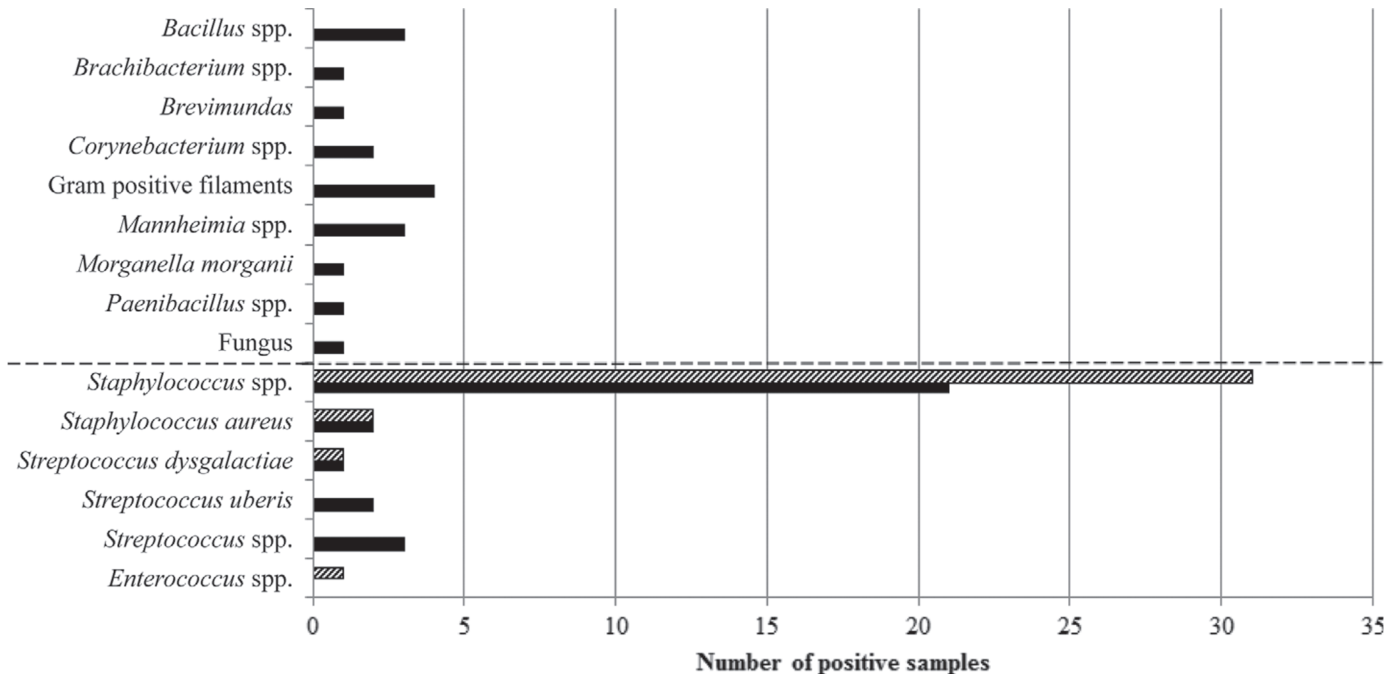

Figure 1. Distribution of diagnoses based on culture and molecular confirmation of species identity of isolates (black bars) or PCR on milk samples (hashed bars) from sheep (n = 186 samples; 6 contaminated samples, not shown). Diagnoses shown under the dashed line can be made by PCR based on primers included in the test, whereas diagnoses above the dashed line are not covered by the real-time PCR method.

resulting in a total of 219 samples for testing. Most udders appeared symmetrical and normal in size, with 5 udder halves described as shrunken and 3 described as swollen compared with the adjacent half. Milk from those glands appeared normal, with the exception of 1 gland that produced milk with clots, flakes, and discoloration. Three healthy looking glands also produced abnormal milk (i.e., watery with or without clots and blood). Several animals had teats that were chapped (n = 2 teats) or showed areas of raised scar tissue (n = 6), warts (n = 3), or wounds (n = 2).

Culture Results

Of 219 samples submitted for bacteriology, 163 (74.4%) were culture negative on SBA and MacConkey agar, 48 (21.9%) showed growth of 1 or 2 species, and 8 (3.7%) were considered contaminated due to growth of more than 2 species. Coagulase-negative staphylococci were the most common group of organisms (n = 23 samples or 10.5% of all samples), followed by unidentified gram-positive filamentous organisms (n = 5 samples, 2.3%) and *Mannheimia haemolytica* (n = 3, 1.4%). Species that would be considered major pathogens in bovine mastitis (i.e., *Staph. aureus*, *Strep. dysgalactiae*, and *Strep. uberis*) were isolated from 6 samples (2.7%; Figure 1). Seven additional bacterial species or genera were detected, as well as 1 fungus (Figure 1). Both samples that yielded *Staph. aureus* were abnormal in appearance (watery and pink or bloody) and 2 samples that yielded *Staphylococcus* spp. were watery but not pink or bloody.

Molecular Confirmation of Isolate Identity

Species-specific conventional PCR confirmed the culture-based identification of both *Staph. aureus* isolates, the *Strep. dysgalactiae* isolate, and 2 of 3 *Strep. uberis* isolates. The third isolate that was identified as *Strep. uberis* based on culture was identified as *Streptococcus gallolyticus* ssp. *macedonicus* based on 16S sequencing (99% homology with reference sequence). Of 23 isolates identified as *Staphylococcus* spp. based on culture, 21 were confirmed by *rpoB* or *tuf* sequencing, whereas the remaining 2 were identified as *Corynebacterium* spp. based on 16S sequencing. Nine *Staphylococcus* species were identified, with *Staphylococcus chromogenes*, *Staphylococcus simulans*, and *Staphylococcus warneri* as the most common species and *Staphylococcus devriesii* reported for the first time from sheep (Table 2). Using 16S sequencing, genus identity was confirmed for *Mannheimia* spp. isolates, although without distinction between *M. haemolytica* and *Mannheimia glucosida*, and for *Morganella morganii, Bacillus*, and *Corynebacterium* spp. (Appendix Table A1).

Table 2. Identification of *Staphylococcus* spp. detected in ovine milk based on culture and sequencing of housekeeping genes

Species	No. of ewes	No. of samples	Basis of identification[1]	Detected by PCR[2]
Staphylococcus auricularis	2	2	*rpoB*, >98% homology	No
Staphylococcus chromogenes	5	5	*rpoB*, 100% homology (n = 4) *tuf*, 100% homology (n = 1)	Yes (n = 3) No (n = 2)
Staphylococcus cohnii ssp. *urealyticus*	1	1	*rpoB*, 100% homology	No
Staphylococcus devriesii	1	1	*rpoB*, >97% homology	Yes
Staphylococcus equorum	1	1	*rpoB*, 100% homology	Yes
Staphylococcus haemolyticus	1	1	*rpoB*, >98% homology	Yes
Staphylococcus simulans	4	5	*rpoB*, >99% homology	Yes (n = 3) No (n = 2)
Staphylococcus vitulinus	1	1	*tuf*, 100% homology	No
Staphylococcus warneri	4	4	*rpoB*, >99% homology	Yes (n = 1) No (n = 3)

[1]Sequencing of *tuf* was performed if *rpoB* PCR failed to yield an amplicon.

[2]*Staphylococcus* spp. detected in milk sample using the PathoProof system (Thermo Fisher Scientific, Vantaa, Finland).

PCR-Based Diagnoses from Milk Samples

Due to small sample volumes, DNA extraction and PCR could only be conducted on 192 milk samples. Fifty-three DNA extracts were diluted 1:5 because use of neat DNA extracts yielded unsatisfactory results for the IAC. Among 192 samples with correct IAC results, 158 (82.3%) were negative by real-time PCR. The organisms that were detected in the remaining 34 samples included *Staph. aureus* (n = 2), *Staphylococcus* spp. (n = 31), *Strep. dysgalactiae* (n = 1), and *Enterococcus* spp. (n = 1, in combination with *Staphylococcus* spp.; Figure 1). None of the samples tested positive for *Strep. agalactiae*, *Strep. uberis*¸ *Escherichia coli, Klebsiella* spp., *Serratia marcescens*, *T. pyogenes* or *P. indolicus* (or both), *Corynebacterium bovis*, or the β-lactamase gene (*blaZ*).

Comparison of Bacteriological and PCR-Based Diagnoses

Of 192 samples processed by culture as well as real-time PCR, 138 (72%) yielded concordant results, with negative results by both methods for 124 samples and positive results for 14 samples, including 11, 2, and 1 samples positive for *Staphylococcus* spp., *Staph. aureus*, and *Strep. dysgalactiae*, respectively. Twenty samples were positive for *Staphylococcus* spp. by PCR but not by culture. When PCR results were categorized as weak versus strong positive results, a significant association existed between PCR results and culture results (n = 31; df = 1; $\chi^2 = 15.8$; $P < 0.001$), whereby culture-positive samples mostly yielded strong real-time PCR results [++ or +++, equivalent to cycle-threshold ($\mathbf{C_T}$) values of <31.0] and culture negative samples mostly yielded weak real-time PCR results (+, equivalent to a C_T value of 31.3 to 37.0). Culture-positive samples that were real-time PCR negative included 7 contaminated samples, 15 samples that yielded species that are not covered by the current real-time PCR method, 3 samples with bacteria that were phenotypically identified as *Strep. uberis*, and 9 *Staphylococcus* spp.-positive samples. No obvious relationship existed between the species identity of *Staphylococcus* isolates and real-time PCR results (Table 2). For 7 of the 9 *Staphylococcus* spp. culture-positive, real-time-PCR-negative samples, a second aliquot of milk was available and negative PCR results were confirmed, again in the presence of a positive IAC. Dilution of DNA extracts (1:2 or 1:5) resulted in positive results for 4 of 6 samples. Similarly, when real-time PCR was performed on diluted DNA extracts from *Strep. uberis*-positive milk samples, positive PCR results were obtained for 2 samples that were negative in the initial analysis, despite satisfactory IAC results in the initial analysis. The third sample continued to be negative, in agreement with 16S-based species identification of the isolate as *Strep. gallolyticus* ssp. *macedonicus* rather than *Strep. uberis*.

DISCUSSION

This study provides proof of principle that a real-time PCR-based method for detection of mastitis pathogens can be used for ovine milk. It also suggests that further optimization of the method may be needed in terms of primer selection. Notably, *Mannheimia* spp. were not detected by real-time PCR, although several samples contained the organism, as determined by culture and confirmed by 16S ribosomal DNA sequencing of bacterial isolates. *Mannheimia* spp. are an important cause of mastitis in sheep, often resulting in high morbidity and mortality (Arsenault et al., 2008; Omaleki et al.,

2011). A diagnostic test for sheep mastitis should be able to detect this pathogen genus. In our flock, all *Mannheimia*-positive animals were clinically healthy, showing that subclinical infections may also occur. Another major pathogen that went undetected by PCR was *Strep. uberis*. Primers targeting this species are included in the PCR and we postulated that strain differences between ovine and bovine *Strep. uberis* might contribute to false-negative results. Some PCR-based methods for detection of *Strep. uberis* in milk target *pauA*, the gene encoding plasminogen activator (Gillespie and Oliver, 2005). This gene is absent from a considerable proportion of ovine *Strep. uberis* isolates, which carry an alternative plasminogen activator gene, *pauB*, instead (Gilchrist et al., 2013). However, after dilution of DNA extracts from milk, positive results were obtained by real-time PCR, implying that the sample processing method rather than strain divergence explained the negative results observed in our study. The modifications that were made to the recommended DNA-extraction protocol, specifically the lower centrifugation speed, may have contributed to false-negative results.

Discordant results from culture and PCR-based diagnostics have previously been reported for bovine milk. Nonviable organisms are detectable by PCR but not by culture (Taponen et al., 2009). This phenomenon did not seem to play an important role in the current study of ovine milk, possibly due to the fact that samples were from clinically healthy animals and to the short delay between sampling and culturing. Another explanation for real-time-PCR-positive, culture-negative results is higher sensitivity of real-time PCR compared with culture (Cederlöf et al., 2012; Keane et al., 2013). The number of positive samples in our study was too small for meaningful analysis of the comparative sensitivity and specificity of real-time PCR and culture. Considering that both PCR-positive, culture-negative (n = 18) and PCR-negative, culture-positive (n = 9) samples were found for *Staphylococcus* spp. and that *Strep. uberis* was detected by culture but not by real-time PCR, the difference in results is not simply a matter of one test being more sensitive than the other. Culture-negative samples tended to have higher C_T values, indicative of lower bacterial load, than culture-positive samples for *Staphylococcus* spp. Similarly, PCR-negative samples tended to show fewer colonies on culture than PCR-positive samples for *Staphylococcus* spp. (results not shown). Because the predominant staphylococcal species may differ between cattle and sheep, with *Staphylococcus caprae* common in sheep but not in cattle (Zadoks and Watts, 2009), we explored the hypothesis that negative real-time PCR results could be due to a particular species. In our study, *Staph. caprae* was not detected and no evidence existed for species-specific diagnostic test bias. As for *Strep. uberis*, dilution of DNA extracts seemed to affect PCR results. A final explanation for discrepancies between culture and real-time PCR-based results is inaccuracy of culture-based identification. In the current study, one isolate was classified as *Strep. uberis* based on culture but subsequently shown to be *Strep. gallolyticus* ssp. *macedonicus* based on conventional PCR and 16S-sequencing, proving that the culture result was a false positive and that the real-time PCR result was a true negative. Similarly, 2 isolates that were phenotypically identified as staphylococci were genotypically identified as corynebacteria. Both staphylococci and corynebacteria are gram-positive, catalase-positive, oxidase-negative organisms that may have similar colony morphology and pigment production. Appearance on a Gram stain is a key differentiator, but in this case, the organisms had a coccoid appearance and were identified as staphylococci. These results illustrate that culture-based diagnoses should not be used as a gold standard for evaluation of real-time PCR results without confirmation of species identity of isolates.

Numerous organisms were detected by culture but not by real-time PCR because many species can grow on SBA or MacConkey agar, whereas PCR targets 11 selected species or genera. Most species were isolated in pure culture, indicating that their presence was unlikely to be due to sample contamination. In addition to the major mastitis pathogen *Mannheimia*, several other species were detected (Figure 1; Appendix Table A1), including species previously associated with ovine milk, such as *Bacillus* spp. (Fotou et al., 2011) and *Corynebacterium* spp. (Quigley et al., 2013). Other species had previously been detected in cow or goat milk but not in sheep milk, including *Brachybacterium* (Callon et al., 2007), *Empedobacter brevis* (Quigley et al., 2013), *Morganella morganii* (Tornadijo et al., 1993), and *Staph. devriesei* (Supré et al., 2010). Some of these species affect cheese quality rather than udder health and detection of such organism by means of diagnostic PCR is not a priority.

CONCLUSIONS

Real-time PCR methods can be used to detect pathogens in ovine milk, including *Staph. aureus* and other staphylococcal species. Ovine mastitis pathogens that were detected only by culture in this flock included *M. haemolytica*, which is not included in the PCR panel, and *Strep. uberis*. Routine use of PCR for ovine milk may require development of primer sets that target sheep-specific mastitis pathogens.

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APPENDIX

Table A1. Comparison of species identification of bacterial isolates from ovine milk using phenotypic methods and 16S ribosomal DNA sequencing based on >99% homology with reference sequence[1]

Phenotypic identification	16S-based identification	No. of samples
Bacillus sp.	*Bacillus* sp.	1
Brevundimonas vesicularis	*Empedobacter brevis*	1
Burkholderia cepacia	*Bacillus* sp.	1
Corynebacterium sp.	*Corynebacterium* sp.	1
Lactobacillus sp.	*Streptococcus* sp.	1
Mannheimia haemolytica	*Mannheimia glucosida* or *Mannheimia haemolytica*[2]	3
Morganella morganii	*Morganella morganii*	1
Sphingomonas paucimobilis	*Brachybacterium* sp.	1
Sphingomonas paucimobilis	*Paenibacillus xylanexedens*	1

[1]Identification based on > 99% homology with reference sequence, with the exception of the *Brachybacterium* sp. (98% homology). For 7 isolates (1 *Bacillus* sp., 1 *Streptococcus* sp., and 5 unidentified gram-positive filamentous organisms), no results were obtained.

[2]Homology with 16S sequence of *M. glucosida* 1,358/1,366, 1,358/1,361, and 1,346/1,368 nucleotides (nt), respectively, and homology with *M. haemolytica* 1,357/1,366, 1,357/1,361, and 1,334/1,368 nt, respectively.