Diagnostic Microbiology and Infectious Disease 75 (2013) 227–234

Contents lists available at SciVerse ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Bacteriology

High-resolution melt analysis for species identification of coagulase-negative staphylococci derived from bovine milk☆

Praseeda Ajitkumar [a], Herman W. Barkema [a], Ruth N. Zadoks [b], Douglas W. Morck [c], Frank J.U.M. van der Meer [d], Jeroen De Buck [a,*]

[a] Department of Production Animal Health, Faculty of Veterinary Medicine, University of Calgary, Calgary, Alberta, Canada AB T2N 4N1
[b] Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik EH26 0PZ, UK
[c] Department of Comparative Biology and Experimental Medicine, Faculty of Veterinary Medicine, University of Calgary, Calgary, Alberta, Canada
[d] Department of Ecosystem and Public Health, Faculty of Veterinary Medicine, University of Calgary, Calgary, Alberta, Canada

ARTICLE INFO

Article history:
Received 21 September 2012
Received in revised form 21 November 2012
Accepted 22 November 2012
Available online 27 December 2012

Keywords:
Bovine
Mastitis
High-resolution-melt analysis
Coagulase-negative staphylococci

ABSTRACT

Coagulase-negative staphylococci (CNS) are the most frequently isolated pathogens isolated from bovine milk. In this study, we report a rapid assay for species identification of CNS using high-resolution melt analysis (HRMA) of 16S rDNA sequences. Real-time polymerase chain reaction amplification of 16S rRNA gene fragment, spanning the variable region V1 and V2, was performed with a resulting amplicon of 215 bp. A library of distinct melt curves of reference strains of 13 common CNS species was created using HRMA. Sequencing of 16S rRNA and *rpoB* genes, and, when needed, *tuf* gene, of 100 CNS isolates obtained from Canadian Bovine Mastitis Research Network was done to determine their species identity, allowing for subsequent evaluation of the performance of HRMA for field isolates of bovine CNS. A combination of HRMA and sequencing revealed that *Staphylococcus chromogenes*, *S. xylosus*, *S. simulans*, and *S. sciuri* had multiple genotypes, complicating their resolution by HRMA. As the 3 genotypes of *S. chromogenes* had distinct melt curves, the 3 distinct genotypes were employed as reference strains in a blinded trial of 156 CNS isolates to identify *S. chromogenes*. HRMA correctly identified all *S. chromogenes* isolates which were later confirmed by sequencing. *Staphylococcus chromogenes* (68%) was most frequently found among the CNS isolates, followed by *S. haemolyticus* (10%) and *S. xylosus* (6%). The present study revealed that HRMA of 16S rRNA gene (V1–V2) could be used as a rapid, efficient, low-cost, and minimally cumbersome technique for *S. chromogenes* identification, the most common CNS derived from bovine milk.

1. Introduction

Coagulase-negative staphylococci (CNS) are the most frequently isolated pathogens from udder quarters (Reyher et al., 2011; Sampimon et al., 2009a). CNS are considered pathogens of minor importance in dairy production especially when compared to *Staphylococcus aureus*, streptococci, and coliforms (Taponen et al., 2007); however, their role in udder health is debated (Piepers et al., 2010; Sampimon et al., 2010; Taponen et al., 2007). Since CNS are a large group of distinct staphylococci, it is likely that the individual species interact variably with the host and, consequently, will have specific effects leading to different courses of the intramammary infection (IMI). In order to determine the specific effects of different CNS species on udder health and production in heifers and cows, more reliable species identification of these organisms needs to be undertaken.

Phenotypic identification of CNS (DeVriese et al., 1994) requires numerous media, is labor intensive, and, as a result, is relatively expensive. Commercial test kits like API Staph ID 32 (API Test, BioMérieux, France) and the Staph-Zym™ test (Rosco, Taastrup, Denmark) for phenotypic identification of CNS lack accuracy (Sampimon et al., 2009c; Zadoks and Watts, 2009). For identification of CNS species, sequence data of housekeeping genes such as *rpoB*, *cpn60*, *dnaJ*, or *tuf* can be used (Drancourt and Raoult, 2002; Shah et al., 2007; Zadoks and Watts, 2009).

Genomic DNA fingerprinting methods, such as amplified fragment length polymorphism (AFLP), tRNA-intergenic spacer polymerase chain reaction (PCR), and $(GTG)_5$-PCR fingerprinting, have been proposed as valid alternatives for genomic identification with a high typeability and accuracy for CNS isolates from bovine milk (De Braem

☆ This research was supported by the Natural Sciences and Engineering Research Council of Canada, Alberta Milk, the Dairy Farmers of New Brunswick, the Dairy Farmers of Nova Scotia, the Dairy Farmers of Ontario, the Dairy Farmers of Prince Edward Island, Novalait Inc., Dairy Farmers of Canada, Canadian Dairy Network, Agriculture and Agri-Food Canada, Public Health Agency of Canada, Technology PEI Inc., Université de Montréal, and University of Prince Edward Island, through the Canadian Bovine Mastitis Research Network.

* Corresponding author. Tel.: +1-403-220-5393; fax: +1-403-210-6693.
E-mail address: jdebuck@ucalgary.ca (J. De Buck).

0732-8893/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.diagmicrobio.2012.11.008

Table 1
Panel of reference strains used in the study.

Number	Bacterial species	Source
1	*Staphylococcus chromogenes*	CBMRN22705099
2	*Staphylococcus hyicus*	CBMRN32902167
3	*Staphylococcus epidermidis*	CBMRN11211860
4	*Staphylococcus simulans*	CBMRN11110774
5	*Staphylococcus capitis*	CBMRN20309206
6	*Staphylococcus warneri*	CBMRN32107630
7	*Staphylococcus xylosus*	CBMRN10613450
8	*Staphylococcus haemolyticus*	CBMRN11501077
9	*Staphylococcus sciuri*	CBMRN11501046
10	*Staphylococcus auricularis*	CBMRN41808610
11	*Staphylococcus cohnii*	CBMRN41813355
12	*Staphylococcus hominis*	CBMRN32411508
13	*Staphylococcus saprophyticus*	CBMRN31915182

CBMRN = Isolate obtained from the Canadian Bovine Mastitis Research Network Mastitis. Pathogen Collection, St-Hyacinthe, QC, Canada.

et al., 2011; Piessens et al., 2010; Supré et al., 2009). We recently demonstrated that a high-resolution melt analysis (HRMA) using a DNA-incorporating fluorescent dye can discriminate reliably all major mastitis pathogens (Ajitkumar et al., 2012). Slany et al. (2010) tested the potential of using HRMA in distinguishing staphylococcal species targeting the V8–V9 region of the 16S rRNA gene, but limited the analyses to reference strains alone and the most common CNS in bovine, *S. chromogenes*, was not included. We hypothesized that HRMA targeting the V1–V2 region of 16S rRNA gene could be a relatively rapid, inexpensive alternative for genomic identification of CNS species from bovine milk.

2. Materials and methods

2.1. Bacterial strains and isolates

To generate a set of reference strains, the 13 most common species in bovine milk were selected based on the work of Sampimon et al. (2009b). Isolates representing those 13 species were obtained from the Canadian Bovine Mastitis Research Network (CBMRN) mastitis pathogen collection (Reyher et al., 2011) and were used to create a library of melt curves for these species (Table 1). To evaluate the usefulness of the reference library for identification of field isolates, a collection of 100 CNS isolates was used. These isolates were obtained through the CBMRN mastitis pathogen collection and were originally derived from clinically normal milk samples. Genotypic confirmation of these isolates was carried out by sequencing of *rpoB* and 16S rRNA genes and comparing them with the sequences in GenBank. For *rpoB*, homology values >94% were considered reliable (Mellmann et al., 2006). For 16S sequence, an isolate was declared identified if it had >98.7% similarity with one of the online sequences (Jousson et al., 2005). Subsequently, the isolates were subjected to HRMA of 16S rRNA gene along with reference species to evaluate the clustering pattern.

Based on the results of the above experiment, and because *S. chromogenes* appeared to exhibit 3 distinct melt curves, a set of 3 reference strains of *S. chromogenes* was further used to evaluate the efficacy of HRMA in identifying these strains in a blinded trial using a third set of 156 CNS isolates from clinically normal milk samples from the CBMRN mastitis pathogen collection. All isolates that did not cluster with the 3 reference melt curves of *S. chromogenes* in HRMA were genotyped by sequencing of *rpoB* and 16S rRNA genes. Sequencing of the *tuf* gene was performed on the isolates in which *rpoB* and 16S rRNA gene sequencing gave conflicting results. In the case of *tuf*, homology values >97% were considered reliable (Heikens et al., 2005). The isolates that were identified as *S. chromogenes* by HRMA were also further genotypically confirmed by sequencing of the *rpoB* and 16S rRNA genes.

2.2. Bacterial culture, DNA extraction protocol, amplification, and sequencing of 16S rDNA, rpoB, and tuf genes

Isolates were grown on Colombia agar with 5% sheep blood (BD Biosciences, USA) for 24 h at 37 °C. DNA extracts were prepared according to the procedure described by Sampimon et al. (2009c). Briefly, 1 calibrated loop (1 μL) of cells was suspended in 20 μL lysis buffer (0.25% SDS, 0.05N NaOH), heated for 5 min at 95 °C, and diluted with 180 μL distilled water. Cell debris was isolated by centrifugation at 16,000 × *g* for 5 min, and supernatants were used as PCR template. PCR of a 751-bp fragment of the *rpoB* gene was performed using primers and run parameters of either Drancourt and Raoult (2002) or Mellmann et al. (2006). For the 16S rRNA gene sequencing, universal primers 27F and 1392R were used for amplification and species confirmation. In the case of *tuf*, amplification and sequencing were performed according to Heikens et al. (2005). PCR products were purified with the Qiagen PCR purification kit (Qiagen, Mississauga, ON, Canada). DNA sequencing was carried out at Eurofins (Eurofins MWG Operon, Huntsville, AL, USA). Assembled sequence data were compared with sequence data in GenBank using the nucleotide-nucleotide BLAST algorithm of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov).

2.3. Amplification of 16S rRNA gene using real-time PCR and HRMA

Reference sequences of the 16S rRNA gene for the 13 most common CNS species were obtained from the Ribosomal Database Project (http://rdp.cme.msu.edu/), imported in ClustalW2 for multiple sequence alignment (Larkin et al., 2007) and compared with the partial 16S rDNA sequences obtained from the reference isolates. TheV1–V2 region of the 16S rRNA gene was identified based on sequencing as having the genetic differences which can discriminate the common CNS species. The 16S sequences were aligned, compared, and a cladogram was created using the ClustalW program (http://www.ebi.ac.uk/clustalw) (Fig. 1). A sequence alignment of the HRMA amplicon was created with *S. epidermidis* as the reference strain (Table 2).

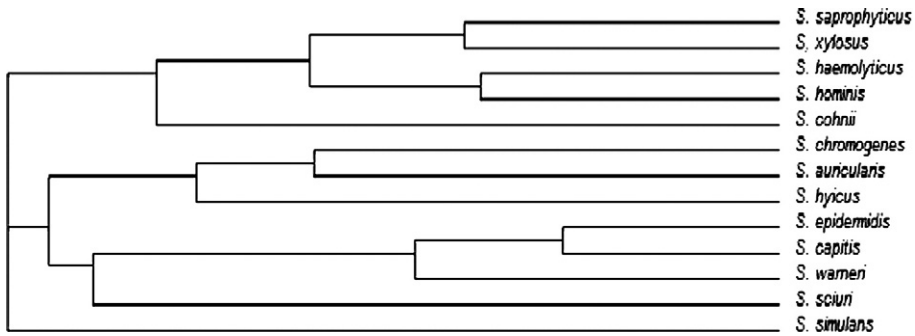

Fig. 1. Cladogram of the 16S rDNA sequences (V1–V2 region) of the bovine CNS isolates included in the study (reference species).