Hot topic: Bovine milk samples yielding negative or nonspecific results in bacterial culturing—The possible role of PCR-single strand conformation polymorphism in mastitis diagnosis

K. Schwaiger,^{*1} M. Wimmer,^{*} R. Huber-Schlenstedt,[†] K. Fehlings,[†] C. S. Hölzel,^{*} and J. Bauer^{*}

^{*}Chair of Animal Hygiene, Technische Universität München, Weißenstephaner Berg 3, 85354 Freising-Weißenstephan, Germany

[†]Bavarian Animal Health Service (TGD), Senator-Gerauer-Str. 23, 85586 Poing, Germany

ABSTRACT

A large proportion of mastitis milk samples yield negative or nonspecific results (i.e., no mastitis pathogen can be identified) in bacterial culturing. Therefore, the culture-independent PCR-single strand conformation polymorphism method was applied to the investigation of bovine mastitis milk samples. In addition to the known mastitis pathogens, the method was suitable for the detection of fastidious bacteria such as *Mycoplasma* spp., which are often missed by conventional culturing methods. The detection of *Helcococcus ovis* in 4 samples might indicate an involvement of this species in pathogenesis of bovine mastitis. In conclusion, PCR-single-strand conformation polymorphism is a promising tool for gaining new insights into the bacteriological etiology of mastitis.

Key words: PCR-single-strand conformation polymorphism, bovine mastitis, *Helcococcus ovis*, *Mycoplasma*

Hot Topic

The conventional bacteriological examination of milk samples is still the most commonly used method for the diagnosis of bovine IMI. Unfortunately, more than 30% of the milk samples are culture-negative or do not allow identification of the causative agent (Taponen et al., 2009). In this case, culture-independent molecular biological techniques like PCR assays are a useful tool to reveal bacteriological etiology (Bexiga et al., 2011). However, the PCR method is limited to the detection of specifically targeted species by using species- or genus-specific primers (Taponen et al., 2009). Therefore, the objective of this study was to apply a new molecular biological method that is geared to detect all bacteria and bacterial communities, respectively. This method, called PCR-single strand conformation polymorphism

(SSCP), was used for the analysis of microbial communities with the help of universal primers (Com1 and Com2-Ph), targeting an approximately 400-bp fragment of the bacterial 16S rRNA gene (Schwieger and Tebbe, 1998). In the present approach, we applied this technique for the investigation of milk samples for the first time.

All milk samples ($n = 11$) were initially sent by farmers to the Bavarian Animal Health Service (TGD, Grub, Germany) for routine diagnostics. In 9 cases, an IMI was suspected, either due to a positive California Mastitis Test (CMT) or due to flakes in the milk (Table 1). The 2 samples with negative CMT were from cows with abnormal milk in another quarter. All samples were from different farms. The samples were bacteriologically investigated by the TGD as described previously (Fehlings, 2011) and according to the “Guidelines for the collection of milk samples under aseptic conditions and isolation and identification of mastitis pathogens” (DVG, 2009). Briefly, 1 loop of the sample was streaked onto esculin blood agar (Oxoid, Darmstadt, Germany and Fiebig, Idstein-Niederauroff, Germany) and incubated at $36^{\circ} \pm 1^{\circ}\text{C}$ for 24 to 48 h. Identification was based on morphological appearance and approved differential tests (e.g., β -hemotoxin zone; clumping factor; coagulase of *Staphylococcus aureus*; esculin hydrolysis; hemolysis; Christie, Atkinson, Munch-Petersen (CAMP) test; and Lancefield groups of streptococci). If necessary, further differentiation was made by using the VITEK system (bioMérieux Deutschland GmbH, Nürtingen, Germany). One hundred microliters of samples 2041a and 2042a was inoculated into the selective *Mycoplasma* broth base containing *Mycoplasma* supplement (Oxoid) for 5 d at $36^{\circ} \pm 1^{\circ}\text{C}$ and 5% CO_2 . One loop of the enrichment was streaked onto *Mycoplasma pneumoniae* agar (heipha Dr. Müller GmbH, Eppelheim, Germany) and incubated for 5 d at $36^{\circ} \pm 1^{\circ}\text{C}$ and 5% CO_2 . Suspicious colonies were microscopically examined. Differentiation from *Acholeplasma* was performed by using the digitonin test.

The bacteriological examination led to a specific diagnosis in 8 cases, but 3 samples remained not diag-

Received July 7, 2011.

Accepted October 18, 2011.

¹Corresponding author: Karin.Schwaiger@wzw.tum.de

Table 1. Investigated mastitis milk samples

Sample number ¹	Milk ²	Culture diagnosis	Corresponding band in SSCP gel ³	Additional bands ⁴
2041a	F	<i>Mycoplasma</i> spp.	Yes	—
2042a	F	<i>Mycoplasma</i> spp.	Yes	—
2314d	CMT 1	<i>Streptococcus agalactiae</i>	Yes	+/- ⁵
2324a	F	<i>Arcanobacterium pyogenes</i>	Yes	+ ⁶
2377b	CMT 2	<i>Staphylococcus aureus</i>	Yes	—
2331d	F	Coliforms	Yes ⁷	—
2338a	CMT n	No mastitis pathogens identified	—	+ ⁸
2350a	F	<i>A. pyogenes</i>	Yes	+ ⁹
2350b	CMT 1	No mastitis pathogens identified	— ⁹	+ ⁹
2374a	CMT 1	<i>Staph. aureus</i>	Yes, weak	+ ⁹
2377a	CMT n	No mastitis pathogens identified	— ⁹	+ ⁹

¹a = front right; b = hind right; c = front left; d = hind left. Same sample number = 1 cow.

²F = flaky milk; CMT = California Mastitis Test; 1 = slight thickening; 2 = begins to gel; n = negative.

³SSCP = single strand conformation polymorphism.

⁴— = no (or almost invisible) additional bands; +/- = weak additional bands visible; + = clear additional bands visible.

⁵One prominent band at the position of *Strep. agalactiae*; 1 weak band at the position of *Mycoplasma bovis* (standard).

⁶Two equally strong bands: 1 at the position of *A. pyogenes*, and 1 at the position of *Staph. aureus* (standard).

⁷Corresponding band at the position of *Escherichia coli* (standard).

⁸Many different bands; 1 prominent band at the position of *Staph. aureus* (standard).

⁹See Figure 1.

nosable. After bacteriological investigation, the samples (listed in Table 1) were stored at -20°C and sent to our laboratory for SSCP-analysis. The DNA extraction of all samples was performed immediately after defrosting in 1 d.

The CTAB method (*N*-acetyl-*N,N,N*-trimethyl ammonium bromide), as described by Somerville et al. (2005), turned out to be a suitable procedure for DNA extraction from the matrix milk. A few initial modifications were necessary to eliminate matrix inhibitors and to yield a sufficient amount of bacterial DNA. Briefly, 2 mL of the milk sample was incubated with 2.9 mg of pronase (Roche Diagnostics GmbH, Mannheim, Germany) and 5.72 μL of mercaptoethanol (Merck KGaA, Darmstadt, Germany) for 90 min at 37°C on a G10 Gyrotory shaker (New Brunswick Scientific, Wesseling-Berzdorf, Germany). After 30 min of centrifugation at $5,800 \times g$ (Galaxy 16DH; VWR International AG, Darmstadt, Germany), the cream layer and supernatant were discarded; the remaining pellet was washed with 1 mL of sterile bidistilled water and centrifuged at $5,800 \times g$ for 20 min. After discarding the supernatant, the pellet was washed with 1 mL of TES buffer (Tris-HCl, EDTA, and saccharose) and again centrifuged at $5,800 \times g$ for 20 min. The pellet was dissolved in 700 μL of TES buffer and transferred to a 2-mL tube containing 0.5 g of glass beads (Sigma, Steinheim, Germany). The sample was shaken 6 times in a TissueLyser II (Qiagen GmbH, Hilden, Germany) for 30 s and cooled on ice between treatments. The remaining steps of the

CTAB extraction were performed according to Somerville et al. (2005), beginning with transferring 500 μL of the supernatant to a 2-mL tube and heating at 80°C for 20 min. Finally, the pellet was resuspended in 30 μL of TE buffer (Tris EDTA). For the development of the species standard, 1 loopful of a pure culture of 1 of the 7 reference strains (Figure 1) was added to 500 μL of TE buffer and extracted as described by Somerville et al. (2005). As a negative control, nuclease-free water (Promega GmbH, Mannheim, Germany) was extracted. The subsequent PCR-SSCP method was applied as described by Korthals et al. (2008), using mostly the same reagents, conditions, and equipment. In deviation from the protocol, the digestion was stopped using the GenElute PCR Clean-Up Kit (Sigma-Aldrich, Hamburg, Germany), and columns were eluted with 20 μL of the supplied buffer. The purified DNA single strands were denatured for 5 min at 95°C . The species standard is a mixture consisting of equal amounts of the reference strain eluates (Figure 1). Eight microliters of the extracted milk samples and of the species standard were applied to a 31.2% polyacrylamide gel. The gel running time was 17 h. Three bands of sample number 2350b were excised from the gel (Figure 1), purified, and sent for sequencing (Sequissime GmbH, Vaterstetten, Germany). Sequence similarity was compared by using the NCBI Basic Local Alignment Search Tool database (BLAST; <http://blast.ncbi.nlm.nih.gov>).

The method was suitable for the detection of the genera *Mycoplasma*, *Streptococcus*, *Staphylococcus*, and

Figure 1. Polyacrylamide gel resulting from PCR-single strand conformation polymorphism (SSCP) analysis of selected bovine mastitis milk samples. Lane S = standard. Identity or source of reference strains: *Klebsiella* spp. (WS 1340), *Corynebacterium mycetoides* (DSM 20148), *Escherichia coli* (DSM 1103), *Arcanobacterium pyogenes* (Schwaiger et al., 2009), *Staphylococcus aureus* (DSM 2569), *Streptococcus agalactiae* (DSM 2134), and *Mycoplasma bovis* (DSM 22781); Lanes 1 to 4 are samples (1 = 2350a; 2 = 2350b; 3 = 2374a; 4 = 2377a; see Table 1). Circled bands were sequenced; the results (the upper band first) were *A. pyogenes*, *Peptoniphilus*/*Peptostreptococcus* spp., and *Helcococcus ovis*, indicative of coinfection or contamination. Additional bands may also be contaminants.

Arcanobacterium, and for coliforms (*Escherichia coli* and *Klebsiella* spp.) in the mastitis milk samples (Table 1). In 4 samples, only 1 band was visible, agreeing with the culture results (2 × *Mycoplasma* spp., *Staphylococcus aureus*, coliforms). Multiple bands were visualized for samples 2314d and 2324a, but the dominant band of the lane agreed with the culture result (*Streptococcus agalactiae*, *Arcanobacterium pyogenes*; Table 1). Two samples (2350a and 2374a) had a band according to

the culture diagnosis (*A. pyogenes* and *Staph. aureus*, respectively), but more prominent bands appeared at other positions (Figure 1). Multiple bands on the SSCP gel were also visualized for the 3 clinical samples without specific culture results.

Four samples revealed 3 prominent bands; these results were repeatable in a second approach (including DNA extraction and PCR-SSCP). One band was at the level of *A. pyogenes* and of *Strep. agalactiae*, respectively, and 1 band was below the bottommost band of the standard (Figure 1). The sample with the culture diagnosis *Staph. aureus* (2374a) had a weak band at the position of *Staph. aureus* of the standard. Additional bands were seen in all 4 samples, but only the 3 above-mentioned, prominent bands of sample 2350b were sequenced. Sequence analysis of the 3 bands matched the base sequences of *A. pyogenes* (gi|300510890; position 467–876), *Peptoniphilus* spp. or *Peptostreptococcus* spp. (gi|152963853, position 475–874 or gi|2854097, position 492–891), and *Helcococcus ovis* (gi|284049430; position 464–867), respectively (Figure 1).

We demonstrated that the PCR-SSCP method is applicable for the milk matrix, with some modifications in the DNA extraction. It should be recognized that the PCR technique applied for the preparation of the species standard must be identical with that for the amplification of the field samples because, for example, the alternative use of 2'-deoxyuridine-5'-triphosphate (dUTP) and 2'-deoxythymidine-5'-triphosphate (dTTP) in real-time and endpoint PCR can lead to different migration behavior in the SSCP gel (data not shown). One prominent advantage over conventional bacteriology is the ability to detect fastidious or no-growth bacteria. This could be clearly demonstrated for *Mycoplasma* spp., which are very slow-growing, fastidious bacteria and are, therefore, not detected by routine diagnostics (Selbitz, 2010).

The fact that some samples had more than 1 specific band in the SSCP gel has to be judged with caution. The same is true for the presence of bacterial DNA in culture-negative samples. On the one hand, it is conceivable that contaminants or dead bacteria have been detected, which are irrelevant for the pathogenicity. To a certain extent, the thickness of a band provides semiquantitative information about the bacterial load (Korthals et al., 2008) and may, thus, indicate the causative agent(s). However, it has to be considered that a linkage between bacterial count and causality does not necessarily exist. On the other hand, it is also possible that some forms of mastitis are triggered by multiple, maybe still unknown, bacteria simultaneously. The PCR-SSCP method could provide valuable information, particularly about the relevance of mixed infections in bovine mastitis. However, due to the pre-

liminary character of the study, we are not yet able to explain this item satisfactorily.

A very interesting finding of this investigation is the detection of *A. pyogenes*, *Peptostreptococcus/Peptoniphilus* spp. and *H. ovis* within 1 sample. This pattern was present in 4 different samples from 3 different cows (Figure 1). Certainly it is possible that some bacteria are only contaminants of the sample, but some reports already exist on the occurrence of *A. pyogenes* and *Peptoniphilus* (formerly *Peptostreptococcus*) *indolicus* in summer mastitis. It is supposed that combined infections with *A. pyogenes* and *P. indolicus* lead to clinically more severe courses of disease with a higher frequency of systemic involvement (Hillerton and Bramley, 1989). Sequence similarity of the investigated band was 100% with *Peptoniphilus* spp., but only 99% with *P. indolicus* (gi|295126669) in the NCBI BLAST database. Therefore, it is conceivable that *Peptoniphilus* or *Peptostreptococcus*, other than the species *indolicus*, are involved in mastitis.

Helcococcus ovis is a gram-positive, catalase-negative coccus associated with endocarditis and pulmonary disease in bovines (Post et al., 2003; Kutzer et al., 2008; Zhang et al., 2009). The first described strain was recovered together with *A. pyogenes* from ovine lung, liver, and spleen (Collins et al. 1999). This is an interesting finding, as the *H. ovis* band of our study was only present in combination with a band at the level of *A. pyogenes*, but not in other samples lacking the *A. pyogenes* band. One strain of *H. ovis* was isolated from a sheep with subclinical mastitis. Because this strain was recovered in combination with other pathogenic organisms, namely with staphylococci, the clinical significance remained unknown (Collins et al., 1999). To our knowledge, no report exists on the presence of *H. ovis* in milk originating from other animal species yet. It is very likely that this organism is underreported because it grows very slowly and can easily be missed when fast-growing organisms overgrow this bacterium in a mixed infection (Zhang et al., 2009) or in a contaminated sample. The role of *H. ovis* in bovine mastitis cannot be definitely determined with the data of our study—however, the consistent combination of *A. pyogenes*, *Peptoniphilus/Peptostreptococcus*, and *H. ovis* in 4 samples, but the absence of the *H. ovis* band in all other samples strongly indicates an involvement of this species in the pathogenesis of bovine mastitis. Further investigations are necessary, but we believe that PCR-SSCP is a very useful tool for gaining new insights into the bacteriological etiology of bovine mas-

titis. Polymerase chain reaction SSCP is not competitive with conventional bacteriological methods because conventional bacteriology is faster, easier to run, and cheaper than PCR-SSCP, but only if easy-to-culture bacteria are present. In the absence of such bacteria, PCR-SSCP could help to elucidate the large proportion of unexplained mastitis cases.

REFERENCES

- Bexiga, R., M. T. Koskinen, J. Holopainen, C. Carneiro, H. Pereira, K. A. Ellis, and C. L. Vilela. 2011. Diagnosis of intramammary infection in samples yielding negative results or minor pathogens in conventional bacterial culturing. *J. Dairy Res.* 78:49–55.
- Collins, M. D., E. Falsen, G. Foster, L. R. Monasterio, L. Dominguez, and J. F. Fernandez-Garazabal. 1999. *Helcococcus ovis* sp. nov., a gram-positive organism from sheep. *Int. J. Syst. Bacteriol.* 49:1429–1432.
- DVG (Deutsche Veterinärmedizinische Gesellschaft). 2009. Leitlinien zur Entnahme von Milchproben unter antiseptischen Bedingungen und Leitlinien zur Isolierung und Identifizierung von Mastitisserregern. Fachgruppe: Milchhygiene; Sachverständigenausschuss: "Subklinische Mastitis". Vol. 2. K. Fehlings, M. Zschöck, B. Baumgärtner, M. Geringer, J. Hamann, and K. Knappstein, ed. DVG, Gießen, Germany.
- Fehlings, K. 2011. Langzeitantibiotika als Bestandteil des Trockenstellmanagements—Ist die Anwendung im Hinblick auf Resistenzen noch anzuraten? *Prakt. Tierarzt* 92:706–716.
- Hillerton, J. E., and A. J. Bramley. 1989. Infection following challenge of the lactating and dry udder of dairy cows with *Actinomyces pyogenes* and *Peptostreptococcus indolicus*. *Br. Vet. J.* 145:148–158.
- Korthals, M., M. J. Ege, C. C. Tebbe, E. von Mutius, and J. Bauer. 2008. Application of PCR-SSCP for molecular epidemiological studies on the exposure of farm children to bacteria in environmental dust. *J. Microbiol. Methods* 73:49–56.
- Kutzer, P., C. Schulze, A. Engelhardt, L. H. Wieler, and M. Nordhoff. 2008. *Helcococcus ovis*, an emerging pathogen in bovine valvular endocarditis. *J. Clin. Microbiol.* 46:3291–3295.
- Post, K. W., S. D. Rushton, and S. J. Billington. 2003. Valvular endocarditis associated with *Helcococcus ovis* infection in a bovine. *J. Vet. Diagn. Invest.* 15:473–475.
- Schwaiger, K., C. Hölzel, and J. Bauer. 2009. Resistance gene patterns of tetracycline resistant *Escherichia coli* of human and porcine origin. *Vet. Microbiol.* 142:329–336.
- Schwieger, F., and C. C. Tebbe. 1998. A new approach to utilize PCR-single-strand-conformation polymorphism for 16S rRNA gene-based microbial community analysis. *Appl. Environ. Microbiol.* 64:4870–4876.
- Selbitz, H.-J. 2010. Zellwandlose Bakterien der Klasse Mollicutes. Gattung *Mycoplasma*. Pages 320–329 in *Tiermedizinische Mikrobiologie, Infektions- und Seuchenlehre*. H.-J. Selbitz, U. Truyen, and P. Valentin-Weigand, ed. Enke, Stuttgart, Germany.
- Somerville, W., L. Thibert, K. Schwartzman, and M. A. Behr. 2005. Extraction of *Mycobacterium tuberculosis* DNA: A question of containment. *J. Clin. Microbiol.* 43:2996–2997.
- Taponen, S., L. Salmikivi, H. Simojoki, M. T. Koskinen, and S. Pyörälä. 2009. Real-time polymerase chain reaction-based identification of bacteria in milk samples from bovine clinical mastitis with no growth in conventional culturing. *J. Dairy Sci.* 92:2610–2617.
- Zhang, Y., J. Cui, A. Parkinson, J. Hayes, K. Ott, and B. Byrum. 2009. Isolation of *Helcococcus ovis* from sheep with pleuritis and bronchopneumonia. *J. Vet. Diagn. Invest.* 21:164–166.