Detection of Mycoplasma bovis in Preservative-Treated Field Milk Samples

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ABSTRACT

Control of mycoplasmal mastitis requires individual cow milk sampling for culture and identification of Mycoplasma bovis. This sampling is time-consuming and expensive. Currently, some herds sample cows monthly with the dairy herd improvement (DHI) program, but a preservative is added to this milk that kills M. bovis. In this paper, a nested polymerase chain reaction (PCR) procedure that allows for rapid testing of preservative-treated milk is validated. The specificity of the nested PCR assay was confirmed by testing isolated nucleic acids of other organisms phylogenetically related to M. bovis or common to milk. A comparison against blind-passage culture on 53 field milk samples determined its sensitivity. Exposure of seeded milk samples to the procedure resulted in a sensitivity of 5.1 cfu equivalents per milliliter. Analysis of these results proved that the nested PCR assay was as sensitive as traditional culture and can be used on preservative-treated milk.

(Key words: Mycoplasma bovis, mycoplasma detection, polymerase chain reaction, preservative-treated milk)

INTRODUCTION

As the causative agent of mycoplasmal mastitis, Mycoplasma bovis causes considerable economic losses to the dairy industry (Brown et al., 1990; Gonzalez et al., 1992). One hundred colony-forming units can colonize the udder and cause disease (Bennett and Jasper, 1980), and the incubation period for M. bovis-induced disease can last from 2 to 6 d, during which time shedding may occur (Jasper, 1981). Also, chronically infected cows can shed the organism for prolonged periods, long after clinical signs have ended (Pfu¨ tzner and Sachse, 1996). Because no vaccine is available, present methods for controlling M. bovis involve preventative measures to limit infection and the culling of shedders (Sachse et al., 1993). The highly virulent and pathogenic nature of M. bovis creates a demand for the rapid identification of infected animals, because identification during early stage of infection results in lower overall impact on the herd (Feenstra et al., 1991).

The most commonly used method of diagnosing M. bovis is detection by culture. This method is time consuming and often problematic because of nonmycoplasmal bacterial overgrowth (Sachse et al., 1993). Inoculated plates must be incubated for at least 2 d and should not be considered negative until 7 d have passed (Jasper, 1981). As a result, significant efforts have been made to develop new M. bovis detection techniques, including the use of DNA hybridization probes and PCR assays (Ghadersohi et al., 1997; Hotzel et al., 1996; McCully and Brock, 1992).

In addition, collecting individual cow milk samples to identify subclinical carriers is time consuming and expensive. Currently, some producers obtain individual cow samples on a monthly basis for evaluation of SCC (DHI programs). However, these samples cannot be used for culture because a preservative is added to the milk at the time of collection to kill microbes.

Although PCR tests have been developed, none seems to solve all the problems with detecting M. bovis in milk. There either have been problems with specificity (Gonzalez et al., 1995)—the issue of sensitivity was not addressed (Subramaniam et al., 1998)—or the method was not validated on field milk samples (Hotzel et al., 1999). Furthermore, in another protocol, the milk was cultured before PCR (Baird et al., 1999), eliminating the ability to use the method on preservative-treated milk.

However, the present authors (Pinnow et al., 1998) briefly described a protocol for the detection of M. bovis in preservative-treated milk using nested PCR. The procedure eliminated the need for culture before PCR.
Table 1. Results of the nested polymerase chain reaction on reference strains using a commercial nucleic acid extraction or a cationic surfactant extraction from log phase broth culture.

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Species</th>
<th>Culture media1</th>
<th>Origin</th>
<th>Nucleic acid template</th>
<th>Cationic surfactant template</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG45</td>
<td><em>Mycoplasma bovis</em></td>
<td>a</td>
<td>ATCC 255232</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M23</td>
<td><em>M. bovis</em></td>
<td>a</td>
<td>R. Rosenbusch</td>
<td>+</td>
<td>+</td>
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<td>PG51</td>
<td><em>Mycoplasma alkalescens</em></td>
<td>e</td>
<td>ATCC 29103</td>
<td>−</td>
<td>−</td>
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<td>GM139</td>
<td><em>Mycoplasma agalactiae</em></td>
<td>a</td>
<td>ATCC 35890</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>PG11</td>
<td><em>Mycoplasma bovinigenitalium</em></td>
<td>e</td>
<td>ATCC 14173</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>PG43</td>
<td><em>Mycoplasma bovirhinis</em></td>
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<td>ATCC 29104</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>ST-6</td>
<td><em>Mycoplasma californicum</em></td>
<td>g</td>
<td>ATCC 33416</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>M165/69</td>
<td><em>Mycoplasma bovoculi</em></td>
<td>a</td>
<td>ATCC 29104</td>
<td>−</td>
<td>−</td>
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<tr>
<td>462/2</td>
<td><em>Mycoplasma dispar</em></td>
<td>a</td>
<td>ATCC 27140</td>
<td>−</td>
<td>−</td>
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<td>PG31</td>
<td><em>Mycoplasma gallisepticum</em></td>
<td>d</td>
<td>ATCC 19610</td>
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<td>−</td>
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<tr>
<td>PG21</td>
<td><em>Mycoplasma hominis</em></td>
<td>f</td>
<td>ATCC 23114</td>
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<td>PG50</td>
<td><em>Mycoplasma spp., bovine</em></td>
<td>a</td>
<td>ATCC 27367</td>
<td>−</td>
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<tr>
<td>C492</td>
<td><em>Acholeplasma laidlawii</em></td>
<td>c</td>
<td>R. Rosenbusch</td>
<td>−</td>
<td>−</td>
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<tr>
<td>AR2</td>
<td><em>Arcanobacterium pyogenes</em></td>
<td>i</td>
<td>R. Griffith3</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td></td>
<td>h</td>
<td>R. Griffith</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Klebsiella pneumoniae</td>
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<td>h</td>
<td>R. Griffith</td>
<td>−</td>
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<td>h</td>
<td>R. Griffith</td>
<td>−</td>
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<tr>
<td>Serratia marcescens</td>
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<td>R. Griffith</td>
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<td>Staphylococcus aureus</td>
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<td>R. Griffith</td>
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<tr>
<td>Streptococcus agalactiae</td>
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<td>i</td>
<td>R. Griffith</td>
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<td>−</td>
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<tr>
<td>Streptococcus dysgalactiae</td>
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<td>i</td>
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<td>−</td>
<td>−</td>
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<tr>
<td>Streptococcus uberis</td>
<td></td>
<td>i</td>
<td>R. Griffith</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

1Media: a. modified Friis medium (Knudtson et al., 1986); b. modified Friis medium at pH 6.8; c. Hayflick's broth (Hayflick, 1965); d. Hayflick's broth with 0.5% Glucose; e. Medium B (Erno and Stipkovits, 1973) with 10% fetal bovine serum (FBS); f. SP4 (Tully et al., 1977) with 20% FBS; g. M96 (Frey et al., 1973) with 10% Horse Serum; h. Tryptic Soy Broth (TSB) (Gibco BRL, Rockville, MD); i. TSB with 10% FBS.
2ATCC, American Type Culture Collection (Manassas, VA).
3R. Griffith, Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames 50010.

and used a surfactant to remove milk proteins known to inhibit PCR reactions. In the present study, the sensitivity and specificity of the method were addressed. In addition, the survival of *M. bovis* exposed to DHI milk preservative was plotted. Finally, the procedure was validated on field milk samples submitted for *M. bovis* testing.

**MATERIALS AND METHODS**

**Organisms and Culture Methods**

The mycoplasmas and other bacteria used in this study are listed, with references to their culture and media, in Table 1. All organisms were grown to log phase before extraction of nucleic acids. For culture of *M. bovis* field strains, a 0.2-ml aliquot of each field milk sample was combined with 1.8 ml of modified Friis broth with bacterial inhibitors (Knudtson et al., 1986) and incubated at 37°C for 48 h. This broth culture was streaked onto Friis plates (Knudtson et al., 1986), incubated at 37°C for 48 h, and examined for mycoplasma colony growth by microscopy. The mycoplasma species was identified using fluorescent antibodies on colony impressions on glass slides (Clark et al., 1963).

**Field Milk Samples**

Fifty-three field milk samples from Iowa farms and bovine mastitis cases submitted in 1996 to the Iowa State University Veterinary Medical Research Institute were used to validate the nested PCR procedure. Upon receipt in 1996, all field milk samples were immediately cultured by blind passage in Friis broth as described above. The samples were then frozen at −20°C. In 1998, samples were thawed, recultured, and prepared for the PCR assay. To simulate submission of a milk sample to the DHIA program, DHI milk preservative, which has an active ingredient of 18% 2-bromo-2-nitropropane-1, 3-diol (Bronopol-Boots, National DHIA, Columbus, OH), was added at the mandated 1.69 µl of preservative per milliliter of milk sample. The samples were mixed until homogenous and then placed at 4°C for 20 h.
Effect of DHI Milk Preservative

To determine the toxic effect of DHI milk preservative upon *M. bovis*, log phase *M. bovis* strain PG45 (American Type Culture Collection 25523) was used to seed 20 milliliter of milk from a known negative cow. The seeded milk was vortexed until homogenous and divided into two samples. The DHI preservative was added at 1.69 µl/ml to one of the samples. The samples were placed in a 10°C water bath while aliquots were taken every hour for titration plating on Friis agar as described (Albers and Fletcher, 1982). A toxicity curve was plotted.

Milk Sample Preparation for Nested PCR

A method previously described by the present authors and modified from a procedure for fecal samples (Uwato et al., 1996) was used to process the field milk samples (Pinnow et al., 1998). Briefly, a preservative-treated milk sample was vortexed until homogeneous. One milliliter of the sample was transferred to a 2-ml microfuge tube, and 500 µl of sterile PBS was added. After a brief vortex, the sample was centrifuged at 14,000 × g for 20 min at room temperature. The supernatant was removed, and the pellet was resuspended in 1 milliliter of PBS and centrifuged at room temperature for 10 min at 14,000 × g. One milliliter of the cationic surfactant Catrimox-14 (tetradecyltrimethylammonium oxalate, Qiagen, Hilden, Germany) was immediately used to resuspend the pellet until homogenous. After an incubation of 10 min at room temperature, the sample was centrifuged at 14,000 × g for 10 min. The supernatant was removed, and the pellet was washed twice. A wash was defined as the resuspension of the pellet in 500 µl of distilled water and then centrifugation for 10 min at 14,000 × g. After the final wash, the pellet was resuspended in 30 µl of distilled water. Five microliters of this suspension was used as the template for the outer primer set of the nested PCR.

Development of Inner Primers

The 2-kbp *HindIII* insert of pUC19, previously described as pMB920 (Hotzel et al., 1993), was sequenced at the Iowa State University DNA facility using primers corresponding to the T3 and T7 promoters flanking the multiple cloning site. This was repeated so that the insert had been sequenced a total of 4 times (2 forward, 2 reverse). The contiguous sequences were combined using the software SeqEditor (Perkin Elmer, Norwalk, CT), and the resulting sequence was analyzed using MacDNASIS (Hitachi Software, San Bruno, CA). Related nucleotide and amino acid sequences were searched for in GenBank, EMBL, DDBJ, and PDB databases using BLAST and FASTA programs. The sequenced region was submitted to GenBank and given the accession #AF130119. The inner primers PpSM5-1 and PpSM5-2 (Table 2) were designed by using Oligo 4.0 (National Biosciences, Inc., Plymouth, MN) to search for target sites internal to the previously described (Hotzel et al., 1993) primer pair PpMB920-1 and PpMB920-2 (Table 2).

Nested PCR

The conditions for the nested PCR reaction were followed according to Pinnow et al. (1998). The outside PCR reaction used primers PpMB920-1 and PpMB920-2 (Table 1). Each tube had a total volume of 50 µl, which contained 1× PCR buffer (containing 15 mM MgCl₂), 100 pM each primer, 200 µM each deoxynucleotide triphosphate, 1.9 units of AmpliTaq Gold polymerase (Perkin Elmer, Norwalk, CT), and 5 µl of template. After an initial denaturation step at 94°C for 11.5 min, the samples were exposed to 35 cycles of denaturation at 94°C for 30 s, primer annealing at 48°C for 60 s, and extension at 72°C for 150 s in a thermal cycler. The final extension step was 5 min. The PCR product obtained from the outside reaction was diluted 1:100. Five microliters of this dilution was used as the template for the inside reaction. Positive and negative controls were always included. The inside reaction used primers PpSM5-1 and PpSM5-2 (Table 2). Each tube had a total reaction volume of 50 µl and contained the same concentration of reagents as the outside reaction. The cycling conditions for the inside reaction were an initial denaturing step at 94°C for 11.5 min, followed by 30 cycles of 94°C for 45 s, 54°C for 60 s, and 72°C for 120 s. The final extension cycle was for 5 min. Ten-microliter aliquots of the inside product were separated by electrophoresis in a 1.5% (wt/vol) agarose gel. The gel was then stained with ethidium bromide (0.5 µg/ml) for photography.

Specificity

Nucleic acids from log-phase culture of bacterial species listed on Table 1 were extracted with the QIAamp blood kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions for bacterial cultures, excluding the RNase step. Gram-positive bacteria were given a lysozyme pretreatment before DNA extraction (Johnson, 1994). A total of 1 µg of nucleic acid was used as the template for the nested PCR.

Sensitivity

To determine the sensitivity of the assay, milk samples taken from a known negative cow were seeded with
Table 2. Primer pairs used in nested polymerase chain reaction assay for the detection of Mycoplasma bovis.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Nucleotide position</th>
<th>Sequence</th>
<th>Annealing temperature</th>
<th>Fragment size</th>
</tr>
</thead>
<tbody>
<tr>
<td>PpMB920-2</td>
<td>11-33</td>
<td>5′-TTTAGCTCTTTTTGAACAAAt-3′</td>
<td>48°C</td>
<td>1911 bp</td>
</tr>
<tr>
<td>PpMB920-1</td>
<td>1921–1902</td>
<td>5′-GGCTCTCATTAAGAATGTC-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PpSM5-1</td>
<td>168-192</td>
<td>5′-CCAGCTACCCCTTATACATGACGC-3′</td>
<td>54°C</td>
<td>442 bp</td>
</tr>
<tr>
<td>PpSM5-2</td>
<td>609-581</td>
<td>5′-TGACTCACATTAGAACCACGACTATTTCAC-3′</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1Nucleotide position according to GenBank accession number AF130119.
2PpMB920-1 and PpMB920-2 were previously described (Hotzel et al., 1993).

tenfold serial dilutions of *M. bovis* strain PG45 and processed in the same manner as the field milk samples. The titration of *M. bovis* was determined by dilution plating as described (Albers and Fletcher, 1982). Sensitivity was determined by the number of colony-forming units present in the 1-ml milk sample before the procedure.

RESULTS

Effect of DHI Milk Preservative

The toxicity curve of *M. bovis* exposed to DHI preservative is illustrated in Figure 1. The concentrations of mycoplasma remained relatively static in milk without preservative, while there was a steady decline in the number of viable *M. bovis* cells in the preservative-treated milk.

Figure 1. Time survivor curve of *Mycoplasma bovis* exposed to the DHI milk preservative at 10°C.

Specificity

After preliminary work in optimization of the nested PCR conditions using purified DNA, the established protocol amplified the expected region of *M. bovis* type strain PG45 and reference strain M23 to produce a specific 442-bp product. Testing of the nested PCR protocol on isolated nucleic acids from several non-*M. bovis* microorganisms did not result in detectable amplification (Table 1). However, the nested PCR assay was not strain-specific, as shown in direct testing of 26 known positive field milk samples. In all instances, the expected 442-bp amplicon was generated.

Sensitivity

As shown in Figure 2, the use of tenfold serial dilutions of *M. bovis* PG45 in preservative-treated milk resulted in amplification of the specific 442-bp product.

Figure 2. Ethidium bromide-stained, 1.5% agarose gel of nested PCR-amplified *Mycoplasma bovis* PG45 DNA purified from DHI preservative-treated milk samples by the cationic surfactant. Lane A: *Phi* X174/Hae III Markers. Lanes B to I: 442-bp products of 10-fold serially diluted mycoplasma organisms. Lane B: 5.1×10^5 cfu; Lane C: 5.1×10^4 cfu; Lane D: cfu; Lane E: 510 cfu; Lane F: 51 cfu; Lane G: 5.1 cfu; Lane H: 0.51 cfu; Lane I: 0.051 cfu.
to 5.1 cfu equivalents per milliliter of milk. No detection was found at 0.51 cfu/ml.

Validation

Of the 53 milk samples used for culture and PCR comparison, 26 samples were positive and 27 were negative for \textit{M. bovis} according to initial culture records in 1996. Only seven of the 26 samples of the original culture-positive samples from 1996 recultured positive in 1998 after frozen storage. All 26 culture-positive samples were also positive by the nested PCR assay after frozen storage. Application of the nested PCR assay showed that five of the 27 initially culture-negative samples were positive by the nested PCR assay. The remaining 22 culture-negative samples were also negative by PCR. False-positive results were ruled out by use of appropriate negative controls in each batch of PCR reactions run.

DISCUSSION

The specificity of the method was confirmed by testing purified nucleic acids from several bacterial species in the nested PCR reaction (Table 1). Other mycoplasma species that have been isolated from cattle or may be phylogenetically related to \textit{M. bovis} did not cross-react in the nested PCR assay. Bacteria predominantly associated with mastitis or found in milk samples were not amplified.

Cationic surfactant purification has been shown to be an effective and rapid way of eliminating DNA polymerase inhibitors in clinical samples (Kobayashi et al., 1996). By lysing cells and precipitating nucleic acids in one step, the time for PCR template extraction is dramatically reduced, and the speed of diagnosis is improved. The total time taken to process and evaluate eight milk samples for this assay is under 10 h; the majority of this time was accounted for by the automated PCR process. The time required for detection could be critical if there is a need to isolate or remove subclinical carriers to avoid the spread of the infective agent to the rest of the herd.

Single-step PCR amplifications may be ineffective for detection of low bacterial concentrations in cow milk samples (Willems et al., 1994). Therefore, the use of a nested PCR primer improved sensitivity of the assay to help identify subclinical carriers, which may be shedding low titters of \textit{M. bovis} without exhibiting clinical signs of mastitis. The detected sensitivity of the milk sample assay was slightly higher than five viable cells per milliliter of milk sample. The improved sensitivity of this method is illustrated by Figure 2, and our finding that 18.5% of the initial, culture-negative frozen milk samples were determined to be positive for \textit{M. bovis} by the nested PCR assay. Although the culture method used here is theoretically capable of detecting 5 cfu/ml, it has been reported that the actual detection limit in milk samples is between $10^1$ to $10^2$ cfu (Sachse et al., 1993). Therefore, this nested PCR assay appears to be more sensitive than the standard culture method.

Additionally, detection of mycoplasmas by culture is often hampered by the care needed in obtaining, preserving, and transporting diagnostic samples. This often results in difficulty or a potentially missed diagnosis (Baird et al., 1999). It is generally considered good practice to store milk samples in a refrigerator or freezer until they can be cultured. However, prolonged freezing can have detrimental effects on the accuracy of culture as a diagnostic method. Only 36.8% of the \textit{M. bovis} in the field milk samples remained viable after the 2-yr storage. Although it is unrealistic to assume that clinical samples would be stored for 2 yr, the effect of freezing on mycoplasma viability may play a role in misdiagnosis of subclinical \textit{M. bovis} carriers.

In addition, as shown in Figure 1, the DHI preservative causes a steady decrease in viable \textit{M. bovis} cells making the milk unacceptable for culture. However, this nested PCR assay was shown to work directly on preservative-treated milk. Therefore, it would be possible to process DHI samples for the presence of \textit{M. bovis}. Many herd improvement programs currently collect milk samples on a routine basis, so this method could be used in association with existing herd health evaluation procedures.

CONCLUSIONS

The nested PCR procedure has been shown to be a rapid, specific, and sensitive test that can aid in the detection of subclinical \textit{M. bovis} carriers in dairy herds. Additionally, the method can be used on preservative treated milk that is not acceptable for culture. Implementation of this test could save producers money by eliminating the need for additional individual cow milk samples while identifying subclinical \textit{M. bovis} carriers that continue to infect other animals.

ACKNOWLEDGMENT

We thank R. Griffith for providing us with the nonmycoplasma species used in this study.

REFERENCES
