Development of a Rapid and Sensitive Test for Identification of Major Pathogens in Bovine Mastitis by PCR

RENÉE RIFFON,¹ KHAMPOUNE SAYASITH,¹ HAYSSAM KHALIL,¹ PASCAL DUBREUIL,²
MARC DROLET,¹ AND JACQUELINE LAGACE^{1*}

Department of Microbiology and Immunology, Faculty of Medicine, University of Montreal, Succursale Centre-Ville, Montreal, Quebec H3C 3J7,¹ and Department of Clinical Science, Faculty of Veterinary Medicine, University of Montreal, 3200, Saint-Hyacinthe, Montreal, Quebec J2S 2M2,² Canada

Received 4 December 2000/Returned for modification 8 April 2001/Accepted 29 April 2001

Bovine mastitis is the most important source of loss for the dairy industry. A rapid and specific test for the detection of the main pathogens of bovine mastitis is not actually available. Molecular probes reacting in PCR with bacterial DNA from bovine milk, providing direct and rapid detection of *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus parauberis*, and *Streptococcus uberis*, have been developed. Two sets of specific primers were designed for each of these microorganisms and appeared to discriminate close phylogenetic bacterial species (e.g., *S. agalactiae* and *S. dysgalactiae*). In addition, two sets of universal primers were designed to react as positive controls with all major pathogens of bovine mastitis. The sensitivities of the test using *S. aureus* DNA extracted from milk with and without a pre-PCR enzymatic lysis step of bacterial cells were compared. The detection limit of the assay was 3.125×10^2 CFU/ml of milk when *S. aureus* DNA was extracted with the pre-PCR enzymatic step compared to 5×10^3 CFU/ml of milk in the absence of the pre-PCR enzymatic step. This latter threshold of sensitivity is still compatible with its use as an efficient tool of diagnosis in bovine mastitis, allowing the elimination of expensive reagents. The two PCR tests avoid cumbersome and lengthy cultivation steps, can be performed within hours, and are sensitive, specific, and reliable for the direct detection in milk of the six most prevalent bacteria causing bovine mastitis.

Bovine mastitis (BM) is an inflammation of the mammary gland, usually due to a microbial infection (28), which causes North American dairy producers to lose billions of dollars every year. These losses are primarily due to lower milk yields, reduced milk quality, and higher production costs. BM often becomes chronic, and it is important to identify quickly the new clinical cases in order to control infection in the herd. The bacteria responsible for BM can be classified as environmental (*Escherichia coli*, *Streptococcus dysgalactiae*, *Streptococcus parauberis*, and *Streptococcus uberis*) or contagious (*Staphylococcus aureus* and *Streptococcus agalactiae*) depending of their primary reservoir (environment versus infected mammary gland quarter) (11, 25).

The suitability of a detection method for routine diagnosis depends on several factors, such as specificity, sensitivity, expense, amount of time, and applicability to large numbers of milk samples. The most common but unspecific method (2) to identify potential chronic infections is a somatic cell count: the California Mastitis Test in field conditions and the automated method in the diagnosis laboratory. Currently, the method of identification of the mammary gland pathogens is by in vitro culture, which provides the “gold standard”; however, this technique is labor-intensive and time-consuming. Two other problems can be encountered when these methods of identification are used: first, 2 to 3 days are required to grow, isolate, and identify the pathogen; second, some bacteria, like *S. uberis*

and *S. parauberis* (*S. uberis* type II), cannot be distinguished by biochemical assays (16). It has been demonstrated that early detection procedures have been shown to enhance cure rates and reduce the time required to return to normal milk when coupled with appropriate antimicrobial therapy (20). It is important to identify the pathogen not only for antimicrobial therapy purposes but also to monitor and control the rate of infection at the farm level.

During the last 7 years, many tests have been developed for the diagnosis of BM. However, a rapid (less than 1 day), simple, and specific test for each kind of bacterium involved has not been achieved. Many tests for the detection of human pathogens already exist. Some of these tests have been applied to pathogens of a bovine origin, such as the Minitex Gram-Positive Set for *Streptococcus* (29), but with no success because of a lack of information on veterinary pathogens in the database. Enzyme-linked immunosorbent assay methods exist for *S. aureus* detection in cases of BM (10), but the antibody titer does not correlate with the amount of infecting bacteria (10, 15). Other enzyme-linked immunosorbent assays were developed to screen milk for contamination with *Listeria* organisms (1, 9). Most PCR and API methods used for the detection of microorganisms in milk or in other organic samples need a step of multiplying the bacteria in culture media (3, 18, 21, 22, 27, 30) and are, therefore, time-consuming. Rapid identification methods, in particular nucleic acid-based tests, have the potential to be extremely specific and can also discriminate between closely related organisms, such as *S. parauberis* and *S. uberis*. It has been previously shown that milk samples could serve as substrate for the amplification of specific DNA sequences using PCR (6, 17). For all these reasons, we selected

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Faculty of Medicine, University of Montreal, Case Postal 6128, Succursale Centre-Ville, Montreal, Quebec H3C 3J7, Canada. Phone: (514) 343-2180. Fax: (514) 343-6358. E-mail: jacqueline.lagace@umontreal.ca.

TABLE 1. Oligonucleotide primer sequences used for PCR

Primer	Specificity	Sequence(5'-3')	G+C content (%)
Eco 223	<i>E. coli</i>	ATC AAC CGA GAT TCC CCC AGT	52
Eco 455	<i>E. coli</i>	TCA CTA TCG GTC AGT CAG GAG	52
Eco 2083	<i>E. coli</i>	GCT TGA CAC TGA ACA TTG AG	45
Eco 2745	<i>E. coli</i>	GCA CTT ATC TCT TCC GCA TT	45
Sau 234	<i>S. aureus</i>	CGA TTC CCT TAG TAG CGG CG	60
Sau 1501	<i>S. aureus</i>	CCA ATC GCA CGC TTC GCC TA	60
Sau 327	<i>S. aureus</i>	GGA CGA CAT TAG ACG AAT CA	45
Sau 1645	<i>S. aureus</i>	CGG GCA CCT ATT TTC TAT CT	45
Sag 40	<i>S. agalactiae</i>	CGC TGA GGT TTG GTG TTT ACA	48
Sag 445	<i>S. agalactiae</i>	CAC TCC TAC CAA CGT TCT TC	50
Sag 432	<i>S. agalactiae</i>	CGT TGG TAG GAG TGG AAA AT	45
Sag 1018	<i>S. agalactiae</i>	CTG CTC CGA AGA GAA AGC CT	55
Sdy 105	<i>S. dysgalactiae</i>	AAA GGT GCA ACT GCA TCA CTA	43
Sdy 386	<i>S. dysgalactiae</i>	GTC ACA TGG TGG ATT TTC CA	45
Sdy 519	<i>S. dysgalactiae</i>	GGC TCA ACC ACT NTA CGC TT	50
Sdy 920	<i>S. dysgalactiae</i>	ATC TCT AGA CCG GTC AGG AG	55
Spa 301	<i>S. parvauberis</i>	GCG ACG TGG GAT CAA ATA CT	50
Spa 1219	<i>S. parvauberis</i>	TAC CAT TAC CTC TAA AGG TA	35
Spa 2152	<i>S. parvauberis</i>	TTT CGT CTG AGG CAA TGT TG	45
Spa 2870	<i>S. parvauberis</i>	GCT TCA TAT ATC GCT ATA CT	35
Sub 302	<i>S. uberis</i>	CGA AGT GGG ACA TAA AGT TA	40
Sub 396	<i>S. uberis</i>	CTG CTA GGG CTA AAG TCA AT	45
Sub 1546	<i>S. uberis</i>	TGA TGG GGA GCG AAA ATA AG	45
Sub 2170	<i>S. uberis</i>	CCC AAC AAC GCC TCA AAC GA	55
Uni 678	Universal	AGT GGA ATT CCA TGT GTA GC	45
Uni 888	Universal	GAG TGC TTA ATG CGT TAG CT	45
Uni 1870	Universal	TGG AAG GTT AAG AGG AGT GG	50
Uni 2308	Universal	GCC TCC GTT ACC TTT TAG GA	50

a PCR technique as a method for identifying BM pathogens because of its precision, high limit of detection, and rapidity.

The aim of this study was to develop molecular tools to identify with rapidity, sensitivity, and specificity the major pathogens involved in intramammary infections in cows. To reach this objective, two sets of universal primers used as positive controls and two sets of specific primers for each bacterium species were developed to identify, by PCR, *E. coli*, *S. aureus*, *S. agalactiae*, *S. dysgalactiae*, *S. parvauberis*, and *S. uberis* in milk samples inoculated with these bacteria. Finally, the sensitivity of two PCR assays performed on *S. aureus* DNA samples prepared with and without a pre-enzymatic lysis step in milk and in Tryptic Soy Broth (TSB) was analyzed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The organisms used in this study include *E. coli* DH5 α , *Staphylococcus epidermidis* ATCC 35984, *S. parvauberis* ATCC 13386, *S. uberis* ATCC 9927, and three clinical isolates from cases of BM, *S. aureus* 97-6609, *S. agalactiae* 91-0121, and *S. dysgalactiae* 97-6617, which were identified biochemically by the API Staph and Strep systems accordingly. All organisms were cultured in TSB (Difco Laboratories, Detroit, Mich.) at 37°C for about 17 h before DNA extraction. Cell numbers were determined by the preparation of serial dilutions of an overnight culture in phosphate-buffered saline (PBS) and plating on blood agar (Columbia agar base supplemented with 5% defibrinated sheep blood).

Preparation of bacterial DNA for PCR using the Dneasy Tissue kit from Qiagen. Bacteria were grown overnight in 20 ml of TSB, and then 1.5 ml of this culture was centrifuged at 5,000 $\times$ g for 10 min. The Dneasy Tissue kit from Qiagen was used with some modifications to the protocol for gram-positive bacteria: the pellet was resuspended in 200 μ l of enzyme incubation buffer (20 mM Tris-HCl, pH 8.0, 1.2% Triton, 20 μ g of lysozyme/ml, and 200 μ g of lysostaphin/ml) followed by an incubation period of 30 min at 37°C. Then, 20 μ g of RNase H (GIBCO Life Technologies, Montreal, Canada)/ml was added with 200 μ l of AL buffer (supplied with the kit) and incubated at 70°C for 10 min followed by the addition of 25 μ l of proteinase K (supplied with the kit) and incubation at 70°C for 30 min. This mixture was transferred into columns and

eluted with deionized autoclaved water by following the manufacturer's recommendation.

Preparation of bacterial DNA for the comparative study. A culture of *S. aureus* (1.6×10^8 CFU/ml) in TSB was distributed in tubes and centrifuged at 5,000 $\times$ g for 15 min and the pellets were kept at -20°C until subsequent use. CFU per milliliter was determined by limit dilution and plating on blood agar (Columbia agar base supplemented with 5% defibrinated sheep blood). Thawed cells were resuspended in sterile bovine milk (ultra high temperature-treated [UHT] milk; Grand-Pré, Québec, Canada) or in TSB and submitted to twofold serial dilutions to reach concentrations of 4×10^4 to 3.125×10^2 CFU/ml. Aliquots of 1 ml of these dilutions were centrifuged for 5 min at 7,000 $\times$ g and submitted to four washes, and then the pellets were resuspended in 1 ml of 1 $\times$ PBS and centrifuged at 5,000 $\times$ g for 5 min. PBS rather than water was used for washes to precipitate calcium ions from the milk, which is known to be a PCR inhibitor (8), and to protect the bacterial membranes from lysis during these steps. After the last centrifugation, the pellet was resuspended in 1 ml of autoclaved distilled water to allow the burst of bacterial cells. This preparation was directly used for PCR or treated with the Dneasy Tissue kit. At the end of this latter step, 600 μ l of deionized autoclaved water was added to the DNA preparation to obtain a total volume of 1 ml ready for PCR use. Sample of 5 μ l of these preparations were used for PCR.

PCR primers. PCR primers were designed from highly divergent and species-specific regions of the DNA coding for 16S and 23S rRNA (16S or 23S rRNA) based on previously published sequence entries available in the GenBank database (*E. coli*, GI no. 42756; *S. aureus*, GI no. 288516; *S. agalactiae*, GI no. 2353759; *S. dysgalactiae*, GI no. 560494; *S. parvauberis*, GI no. 433515; and *S. uberis*, GI no. 433703 and 2668550). Genes encoding rRNA were used as target sequences rather than genes encoding mRNA because of the signal enhancement due to the presence of several copies of genes encoding rRNA in the genome (4). The sequences, specificities, and G+C contents are summarized in Table 1. The primer combinations, the annealing temperatures, and the lengths of the amplified products are summarized in Table 2. These primers were synthesized by AlphaDNA (Montreal, Canada). All these primers were resuspended to a final concentration of 200 μ M in deionized autoclaved water. The working concentration of the primers for PCR was 5 μ M.

PCR amplification. PCR was performed in a GeneAmp PCR System 2400 (Perkin-Elmer). All reactions were carried out in a final volume of 100 μ l. Volumes of 200 ng of extracted DNA template or 5 μ l of bacterial preparation, 5 μ M primer, 2.5 U of *Taq* DNA polymerase (GIBCO Life Technologies, Montreal, Canada), 10 μ l of 10 $\times$ PCR buffer minus Mg (GIBCO Life Technologies), 3 μ l of 50 mM MgCl₂, and all four deoxynucleotide triphosphates (supplied by GIBCO Life Technologies) were added to a 0.5-ml microcentrifuge tube. A pre-PCR step at 94°C for 2 min was applied. A total of 35 PCR cycles were run under the following conditions: denaturation at 94°C for 45 s, annealing (at the temperature in Table 2) for 1 min, and extension at 72°C for 2 min. After the final cycle, the preparation was kept at 72°C for 10 min to complete the reaction. The PCR products were stored in the thermocycler at 4°C until they were collected.

Detection of PCR products. Twenty microliters of the PCR-amplified product was analyzed by electrophoresis on a 1.7% agarose gel stained with 0.5 μ g of ethidium bromide/ml. Electrophoresis was carried out in 1 $\times$ TAE (pH 8.0; 0.04 M Tris-acetate, 0.001 M EDTA) at 80 V for 2 h. The molecular size marker, a

TABLE 2. Primer conditions during PCR

Forward primer	Reverse primer	Annealing temp (°C)	Size of product amplified (bp)
Eco 223	Eco 455	64	232
Eco 2083	Eco 2745	57	662
Sau 234	Sau 1501	70	1,267
Sau 327	Sau 1645	64	1,318
Sag 40	Sag 445	60	405
Sag 432	Sag 1018	65	586
Sdy 105	Sdy 386	57	281
Sdy 519	Sdy 920	57	401
Spa 301	Spa 1219	57	918
Spa 2152	Spa 2870	57	718
Sub 302	Sub 396	56	94
Sub 1546	Sub 2170	59	624
Uni 678	Uni 888	56	210
Uni 1870	Uni 2308	58	438

FIG. 1. Demonstration of the specificity of the molecular probes in PCR assay with purified bacterial DNA from different species. Amplification products of the different primer combinations were analyzed by electrophoresis on a 1.7% agarose gel. Lanes: A, 100-bp DNA ladder (GIBCO Life Technologies); B and C, primers Eco 223 and Eco 455 with *E. coli* (B) and *P. aeruginosa* (C); D and E, primers Eco 2083 and Eco 2745 with *E. coli* (D) and *P. aeruginosa* (E); F and G, primers Sau 234 and Sau 1501 with *S. aureus* (F) and *S. epidermidis* (G); H and I, primers Sau 327 and Sau 1645 with *S. aureus* (H) and *S. epidermidis* (I); J and K, primers Sag 40 and Sag 445 with *S. agalactiae* (J) and *S. dysgalactiae* (K); L and M, primers Sag 432 and Sag 1018 with *S. agalactiae* (L) and *S. dysgalactiae* (M); N, 100-bp DNA ladder (Life Technologies, Inc.); O and P, primers Sdy 105 and Sdy 386 with *S. dysgalactiae* (O) and *S. agalactiae* (P); Q and R, primers Sdy 519 and Sdy 920 with *S. dysgalactiae* (Q) and *S. agalactiae* (R); S and T, primers Spa 301 and Spa 1219 with *S. parauberis* (S) and *S. uberis* (T); U and V, primers Spa 2152 and Spa 2870 with *S. parauberis* (U) and *S. uberis* (V); W and X, primers Sub 302 and Sub 396 with *S. uberis* (W) and *S. parauberis* (X); and Y and Z, primers Sub 1546 and Sub 2170 with *S. uberis* (Y) and *S. parauberis* (Z).

100-bp, 1-kb DNA ladder (GIBCO Life Technologies), was run concurrently. Gels were visualized under UV illumination (Alphadoc; Alpha Innotech Corporation, San Leandro, Calif.) and photographed.

RESULTS

Specificity of primers. The specific oligonucleotide primer sequences designed to amplify and identify the main bacteria causing BM are presented in Table 1. Twelve pairs of primers were tested (2 pairs/bacterium). Prior to PCR, purified DNA was run on 0.8% agarose gel and quantified by UV absorbance at 260 and 280 nm to confirm its quantity and presence in PCR. The specificity of the primer pairs was confirmed by the positive amplification of the DNA from bacteria found in BM, whereas no DNA amplification was observed with the closest phylogenetic bacteria tested, as demonstrated in Fig. 1. The assay

was controlled as follows: *Pseudomonas aeruginosa* was tested as a negative control for *E. coli*, *S. epidermidis* was a negative control for *S. aureus*, *S. dysgalactiae* was a negative control for *S. agalactiae*, *S. agalactiae* was a negative control for *S. dysgalactiae*, *S. uberis* was a negative control for *S. parauberis*, and finally, *S. parauberis* was a negative control for *S. uberis*. Amplification of the DNA templates using proper primers produced the expected fragments (Fig. 1) between 94 and 1,318 bp as shown in Table 2.

Universal primer amplification. Universal primer pairs Uni 678 plus Uni 888 and Uni 1870 plus Uni 2308 were tested against six bacterial species: *E. coli*, *S. aureus*, *S. agalactiae*, *S. dysgalactiae*, *S. parauberis*, and *S. uberis* (Fig. 2). A product of 210 bp was observed when Uni 678 and Uni 888 were tested, and a product of 438 bp was observed when Uni 1870 and Uni

FIG. 2. PCR amplification of purified bacterial DNA by using two pairs of universal primers. Amplification products for the different primer combinations were analyzed by electrophoresis on a 1.7% agarose gel. Lanes: A, 100-bp DNA ladder (GIBCO Life Technologies); B to H, primers Uni 678 and Uni 888 with *E. coli* (B), *S. aureus* (C), *S. agalactiae* (D), *S. dysgalactiae* (E), *S. parauberis* (F), *S. uberis* (G), and a negative control without DNA (H); I to O, primers Uni 1870 and Uni 2308 with *E. coli* (I), *S. aureus* (J), *S. agalactiae* (K), *S. dysgalactiae* (L), *S. parauberis* (M), *S. uberis* (N), and a negative control without DNA (O); P, 100-bp DNA ladder (GIBCO Life Technologies).

FIG. 3. Sensitivity of the PCR assay in detecting DNA from two different media (milk and TSB) artificially inoculated with *S. aureus* 97-6609 with and without a pre-PCR enzymatic lysis step of bacterial cells. Primers Sau 327 and Sau 1645 were used. The amounts of bacteria used were 4×10^4 (lanes B, K, and T), 2×10^4 (C, L, U), 1×10^4 (D, M, V), 5×10^3 (E, N, W), 2.5×10^3 (F, O, X), 1.25×10^3 (G, P, Y), 6.25×10^2 (H, Q, Z), and 3.125×10^2 (I, R, AA) CFU. Lanes: A, J, and S, 1-kb DNA ladder (GIBCO Life Technologies); B to I, *S. aureus* samples resuspended in milk at the concentrations indicated above and extracted with the pre-PCR enzymatic step; K to R, *S. aureus* samples resuspended in milk at the concentrations indicated above and not subjected to the pre-PCR treatment; T to AA, *S. aureus* samples resuspended in TSB at the concentrations indicated above and not subjected to the pre-PCR treatment; AB, PCR mixture without bacteria in milk; AC, PCR mixture without bacteria in TBS; AD, positive control using 200 ng of *S. aureus* DNA.

2308 were used. Amplification was not observed in negative controls of PCR mix. The universal primers used in this study, Uni 678 plus Uni 888 and Uni 1870 plus Uni 2308, were able to detect all BM pathogens cited above. The intensity of each amplification was good with all the bacteria tested. The strongest signal was obtained with the primers Uni 678 and Uni 888 for *S. uberis* and Uni 1870 and Uni 2308 for *S. aureus* (Fig. 2). These primer sets were designed from DNA regions coding for 16S and 23S rRNA, respectively. Nucleotide sequence data comparison (BLASTN 2.0) showed that the primers Uni 678, Uni 888, Uni 1870, and Uni 2308 are conserved for all pathogens of BM.

Sensitivity of PCR assay depending on pretreatment used.

In the aim to make the test as simple and cheap as possible, we tried to eliminate the pre-PCR enzymatic lysis step of bacterial cells. Secondly, in comparing milk with the TSB as support medium, it was possible to measure the inhibitory effect of milk, particularly calcium ions, on the PCR test. For control purposes, uninoculated samples of UHT milk and TSB media were also subjected to the sample preparation methods and to PCR. No amplification could be observed (Fig. 3c). Figure 3a shows the PCR results obtained following the pre-PCR enzy-

matic lysis step using the Qiagen kit: a very high level of sensitivity corresponding to 1.56 CFU/5 μ l of PCR mixture or 3.125×10^2 CFU/ml of milk was obtained, as demonstrated by the twofold serial dilutions of the *S. aureus* samples. The sensitivity of the assay fell to 6.25 CFU/5 μ l of PCR mixture or 1.25×10^3 CFU/ml of TSB in the absence of the pre-PCR enzymatic lysis step of the *S. aureus* cells (Fig. 3c). In milk, the elimination of the pre-PCR enzymatic lysis step of *S. aureus* gave a level of sensitivity of 25 CFU/5 μ l of milk, or 5.0×10^3 CFU/ml of milk (Fig. 3b).

DISCUSSION

An efficient vaccine against BM is not yet available, and prevention as a measure of control needs sensitive, rapid, and specific tests to identify the main bacteria that cause heavy losses in the dairy industry. Conventional procedures for the identification of BM pathogens are labor-intensive, and most of the commercial identification systems are not designed to identify important veterinary pathogens (16, 29). We aimed to develop a detection and identification test for BM pathogens that produced results in 1 day, did not need a culture step, and

was as specific, sensitive, and cheap as possible. Molecular methods are very efficient tools for the development of newly improved diagnosis tests. When methods such as ribotyping are laborious, new methods using PCR based on the 16S or the 23S rDNA region sequences have been successfully applied for the identification of many bacteria (5, 7, 12, 19, 23, 24, 26, 31). The major advantages of PCR lay in the possibility of using only nanograms of nucleic acid samples, allowing the elimination of culture, rapidity, and easy analysis. Therefore, we selected PCR amplification of DNA regions coding for rRNA because of the presence of hypervariable regions, which facilitates the design of highly specific oligonucleotide probes (13) and common regions for the design of universal probes (14). Moreover, rDNA is present in many copies, which permits signal enhancement (4).

Specific primers described here (Table 1 and Fig. 1) were proven to be specific since on agarose gel only one band was observed for each set of primers and no signal was detected with negative controls. The primers for *E. coli*, *S. aureus*, *S. parauberis*, and *S. uberis* were designed based on a DNA sequence coding for 23S rRNA, and primers for *S. agalactiae* and *S. dysgalactiae* were based on DNA coding for 16S rRNA. All the signals were very obvious even though the bands produced by the primers Eco 2083 and Eco 2745 with *E. coli* and Spa 301 and Spa 1219 with *S. parauberis* were of lower intensity (Fig. 1). The difference of signal cannot be explained by the amount of DNA used for PCR because the same amount (200 ng) was used for all bacteria. Therefore, it is possible that the difference in the copy numbers of the coding regions for these particular probes could explain the phenomenon (4). Negative controls made with the closest phylogenetic bacteria found in BM confirmed the specificity of each primer set. While the bacteria *S. parauberis* and *S. uberis* are genotypically and phenotypically close, PCR performed with the described primers made it possible to distinguish between them. The universal primer sets used as positive controls, Uni 678 plus Uni 888 and Uni 1870 plus Uni 2308, were designed from DNA regions coding for 16S and 23S rRNA, respectively. Even though the nucleotide sequence data comparison (BLASTN 2.0) showed that the four universal primers are conserved in all pathogens of BM, the sequences can vary by 1 or 2 nucleotides, therefore varying the signal intensity.

In the aim to evaluate the sensitivity level of the PCR test and to reduce the cost and the duration of the test, a study to compare the sensitivities of the test with and without a pre-PCR enzymatic lysis step of the bacterial cells was performed. It appeared that the limits of detection of the PCR test performed on infected milk samples treated with a pre-PCR enzymatic lysis step were 1.56 CFU/5 μ l of PCR mixture and 3.12×10^2 CFU/ml of milk. Although these very high levels of sensitivity were diminished to 1.25×10^3 CFU per ml of TSB and 5×10^3 CFU per ml of milk (Fig. 3) in the absence of the pre-PCR enzymatic lysis step, these levels of the detection limit are sensitive enough to be used as a diagnosis tool for BM. The level of this detection limit was obtained by significantly reducing the quantity of calcium ions from milk by performing four washes with PBS. We think that it would be possible to increase the sensitivity level of CFU detection in milk in the absence of the pre-PCR enzymatic lysis step to the same level observed in TSB by improving the method to eliminate calcium

ions. Even though PCR is less labor-intensive than bacterial culture and conventional methods of bacterial identification, the elimination of the pre-PCR enzymatic lysis step leads to a more important economy of expensive reagents and time.

In conclusion, a PCR-based assay for the detection of the major pathogens involved in BM is described here. The test, directly performed from milk samples without a culture step, is specific for *E. coli*, *S. aureus*, *S. agalactiae*, *S. dysgalactiae*, *S. parauberis*, and *S. uberis*. The primers were designed from the 16S and 23S rRNA sequences available in GenBank. The test can be performed with and without a pre-PCR enzymatic lysis step of the bacterial cells. The greatest advantage of the latter version is the elimination of expensive reagents. When used with the pre-PCR enzymatic lysis step, the test can be performed in 6 h and reaches a limit of detection of 3.12×10^2 CFU/ml of milk. The test without the pre-PCR enzymatic lysis step can be performed in 4.5 h and reaches a limit of detection of 5×10^3 CFU/ml of milk. Considering the level of detection obtained in TSB without the pre-PCR enzymatic lysis step, it could be possible to improve the detection limit of the latter assay in milk by using a better method to eliminate calcium ions. Secondly, it will be important to test in the near future the detection limit of the molecular probes in milk from actual mastitis cases. If the results comply with the present study, these PCR tests could be readily implemented in clinical veterinary microbiological laboratories and be of great value for promoting prevention of BM.

ACKNOWLEDGMENTS

This work was supported by the Fonds pour la formation de chercheurs et l'aide à la recherche (FCAR, Québec, Canada) and Novalait.

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