A PCR-Based Method for the Detection of *Streptococcus agalactiae* in Milk

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ABSTRACT

Bovine mastitis caused by *Streptococcus agalactiae* is mainly subclinical and therefore can be diagnosed only in the laboratory. We developed a polymerase chain reaction (PCR)-based method for specific and sensitive detection of *S. agalactiae* in raw milk. The specificity of the PCR reaction is based on unique *S. agalactiae* DNA sequences within the 16S subunit of the rRNA genes. Two pairs of sequences were used as positive controls; general streptococci primers, which anneal to conserved areas within the 16S rRNA subunit gene, and primers, which anneal to sequences within bovine mitochondrial DNA. The method of detection includes selective enrichment of *S. agalactiae* in the milk sample, followed by DNA extraction using a rapid and simple procedure developed for this purpose, and specific PCR reaction with appropriate controls. The method enables the detection of one bacterium in 1 ml of raw milk. The method developed can be easily incorporated as part of routine screening of bulk milk collection tanks for early detection of infected cows in a herd.

(**Key words:** *Streptococcus agalactiae*, bovine mastitis, polymerase chain reaction diagnosis)

Abbreviation key: CAMP = Christie, Atkins, and Munch-Peterson.

INTRODUCTION

Bovine mastitis is considered the major cause of economic loss to the dairy industry (Keefe, 1997; Allore and Erb, 1998; Robert and Yancey, 1999) through reduced milk yield and quality, cost of drugs and veterinary treatment, discarded milk, and forced culling. Among the various pathogens causing mastitis, *Streptococcus agalactiae* is of particular importance because

it is highly infectious (spreading from cow to cow by machine milking, unless care is taken to disinfect the teat cups), and causes mainly subclinical infections, which are not identified by the herdsman (National Mastitis Council, 1998). As a result, *S. agalactiae* can spread widely within a herd, causing immediate loss due to reduced milk yield, and eventual large losses as listed above, when it is finally recognized. For this reason, it is important to identify the presence of *S. agalactiae* in a herd with the appearance of the first infected animal. Because of its subclinical nature, such identification must rely upon laboratory diagnosis. Current methods for identifying *S. agalactiae* are based on bacteriological examination of blood agar plates including the hemolysis caused by an exocellular product [the Christie, Atkins, and Munch-Peterson (CAMP) test], or the lack of ability to hydrolyze esculin, and on the production of colored colonies when grown anaerobically on starch (Keefe, 1997). Serological methods based on surface polysaccharide antigens (Skinner and Quesnel, 1978) are often used to confirm the biochemical identification.

Because of its subclinical manifestation and high infectivity, control of *S. agalactiae* requires early diagnostic identification of infected cows. At present, a bacteriological screen of each milking cow is performed usually every 12 to 18 mo, using standard methods. Clearly, this is not sufficient for early detection. As an alternative, we attempted to diagnose *S. agalactiae* in samples taken from the bulk milk collection tank, with the intent of identifying an infection with its first appearance in the herd. However, trials showed that using standard bacteriological procedures as described by the National Mastitis Council (Hogan et al., 1999), we were only able to identify a single infected animal in bulk milk of 10 to a maximum of 50 cows (A. Saran, unpublished data). This is far below the level of sensitivity required to identify a single infected animal or quarter in the more usual Israeli herd of 300 or more milking cows. In addition, the current methods are labor intensive and take at least 2 to 3 d to yield a positive result. Thus, a less

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Table 1. Bacterial strains used in the study, their abbreviations, and sources.

Line	Abbreviation	Source
<i>S. agalactiae</i> lines		
AZ.AR.KN	AgKN	1
AH.SH.225	Ag225	1
AH.SH.109	Ag109	1
AZ.YO.174	Ag174	1
AZ.YO.132	Ag132	1
Agalactiae 245 no. 150	Ag245	1
Agalactiae 239 no. 148	Ag239	1
Streptococcus B ATCC 13813	Ag138	2
Other bacterial lines		
<i>Strep. uberis</i>	Ub166	1
<i>Strep. dysgalactiae</i>	Dys164	1
<i>Enterococcus</i> (unknown species)	Ent96	1
<i>Escherichia coli</i> K12:DH5 α	<i>E. coli</i>	3

¹National Mastitis Research Center (Kimron Veterinary Institute, Bet Dagan, Israel).

²American Type Culture Collections.

³Collection of the Faculty of Food Engineering and Biotechnology (Technion, Haifa).

costly and more rapid diagnostic test for *S. agalactiae* is needed, one that is highly sensitive and can be applied to bulk milk samples.

Recently, a number of PCR-based methods for diagnosis of group B streptococci have been presented (Hall et al., 1995; Ahmet et al., 1999; Bäckman et al., 1999; Ke et al., 2000). Of particular interest in our context are studies based on rRNA sequences (Jayarao et al., 1992; Jensen et al. 1993; Gürtler and Stanisich, 1996; Forsman et al., 1997). In this study, we describe an efficient PCR method for specific and sensitive detection of *S. agalactiae* in raw milk based on the DNA sequence of the 16S rRNA subunit.

MATERIALS AND METHODS

Bacterial Strains

Table 1 shows the bacterial strains used in this study and their abbreviations. *Escherichia coli* was obtained from the collection of the Faculty of Food Engineering and Biotechnology (Technion, Haifa). The remaining cultures were provided in lyophilized form by the National Mastitis Reference Center (Kimron Veterinary Institute, Bet Dagan, Israel). The *S. agalactiae* strains were all of bovine origin and were isolated from four independent outbreaks in Israel, during 1990 to 2000, except for one strain (ATCC 13813) from the ATCC (American Type Culture Collection, MD).

Bacterial Growth and Selective Enrichment

All streptococcal strains were grown at 37°C on blood agar plates (tryptic soy agar + defibrinated sheep blood)

obtained from Chai Laboratories (Rehovot, Israel). For plate counting, colonies were spread on the plates and incubated for 24 to 48 h. In some cases, in order to increase sensitivity, milk samples containing *S. agalactiae* were selectively enriched by adding 1 ml of the sample to 1 ml of Strep Select Broth (BP 212/100, Chai Laboratory), mixing gently, and incubating overnight at 37°C without shaking. The enrichment step was followed by plate counting or DNA extraction for PCR.

DNA Extraction

DNA was extracted from bacterial cultures by incubating a loopful of bacterial colony with lysozyme and proteinase K, followed by extraction with phenol then chloroform:isoamyl alcohol, and ethanol precipitation according to Jersek et al. (1996).

DNA extraction from milk was according to protocol adapted specifically for this purpose: Put 1 ml of raw milk to an Eppendorf tube. Spin down at 14,000 rpm (20,000 $\times g$) for 2 min. Discard the supernatant. Resuspend and wash with Tris-EDTA until a clear solution is obtained. Wash once with PCR buffer. Resuspend in PCR buffer (100 μ l). Add lysozyme to final concentration of 1 mg/ml. Let stand for 15 to 20 min at room temperature. Add proteinase K to final concentration of 200 μ g/ml. Incubate at 56°C for 30 to 60 min. Boil for 15 min. Centrifuge at 14,000 rpm (20,000 $\times g$) for 45 s, to pellet aggregates and transfer the upper liquid phase to a new test tube.

Primer Design

To facilitate the design of primers that are highly specific to *S. agalactiae* for clear and repeatable diagnostic amplification, the examined 16S rRNA subunit sequence was aligned [using GCG Pileup and Pretty programs (GCG Wisconsin package)] across 11 streptococcal strains (Genbank accession numbers: AB023574, AF135453, AF088900, AF015928, AB002517, AF076028, AB023576, AF003932, AF009494, AF104675, AJ243965).

The examined streptococcal 16S rRNA sequences are the ones that are likely to be in cows' milk and for which rRNA sequences are known. The consensus sequence was determined and the alignment was examined to identify regions for which homology was absent or only partial (data not shown). These regions were then examined to identify sequences unique to *S. agalactiae*. Emphasis was placed on identifying unique regions in the 16S rRNA subunit sequence characterized by multiple nucleotide differences with respect to the consensus and other streptococcal strains. In this way, two main polymorphic sites in the 16S rRNA were identified. At

these two sites, primers V1 and V2 were designed to be specific for the *S. agalactiae* sequences. One of these sites was previously identified and used for specific detection of *Streptococcus* B (Wang et al., 1999; Bentley and Leigh, 1995).

In addition, two sets of positive control primer pairs were also designed. Their function was to identify false negatives obtained with primers V1 and V2. When negatives result from some fault in the amplification reaction, the positive controls would also not give an amplification product. The first set of positive controls, primer pair C1 and C2, consisted of two fully conserved streptococcal primer sequences. These are intended to react with the *Streptococcus* spp. that are normally present in raw milk. The second set, primers BMC1 and BMC2, were prepared from a sequence within the bovine mitochondrial Cytochrome-B gene. These are intended to react with the bovine somatic cells that are normally present in milk. The three sets of primer pairs are shown below.

V1: 5'-TTTGGTGTGTTACTAGACTG-3'
 V2: 5'-TGTGTTAATTACTCTTATGCG-3'
 C1: 5'-GCGTGCCTAATACATGCAA-3'
 C2: 5'-TACAACGCAGGTCCATCT-3'
 BMC1: 5'-CGATACATACACGCAAACG-3'
 BMC2: 5'-TGTTGGGTTGTTGGAGCC-3'

The PCR

The PCR reaction mixture contained 2.5 μ l of 10 $\times$ Taq polymerase buffer (1.5 mM MgCl); 1.0 μ l of forward primer (10 μ M); 1.0 μ l of reverse primer (10 μ M); 0.2 μ l of dNTP (25 mM), 0.1 μ l of Taq polymerase (0.25 u); 5 μ l of DNA (50 to 100 ng/ μ l); add ddH₂O (sterile) to total volume 25 μ l. The reaction was carried out in a PCR thermocycler (HYBAID Omn-E), as follows: 94°C for 4 min; five cycles of 94°C, Tm°C and 72°C for 45 s each step; 20 cycles of 94°C, (Tm - 4)°C, 72°C for 45 s each step; and a step of 72°C for 5 min, at the end of the reaction. PCR products were run on agarose gel (1.8 to 2.0%) and visualized by EtBr 0.005%.

DNA Sequencing

DNA sequencing was by the BigDyeTM Terminator Cycle Sequencing Kit (Perkin Elmer) with AmliTaq Polymerase according to manufacturer's instructions using ABI Prizm 310 automatic sequencer (Perkin Elmer).

DNA Sequence Analysis

We compared Genbank sequences or sequences obtained in the course of this study to identify conserved

and variable regions with the GCG Pileup and Pretty programs (GCG Wisconsin package). Sequences were screened against the universal gene bank using the BLAST procedure of the NCBI website (www.ncbi.nlm.nih.gov).

Sensitivity Tests

Sensitivity of the PCR assay for presence of *S. agalactiae* was determined as follows. A series of concentrations of *S. agalactiae* in saline was prepared by 10-fold dilution of a culture of *S. agalactiae*. Of each concentration, three samples were taken: one for plate counting, one for immediate PCR diagnostics, and one for PCR diagnostics following overnight selective enrichment. Both series of test tubes for PCR diagnostics were subjected to DNA extraction followed by the specific *S. agalactiae* PCR reaction using V1 and V2 primers. Sensitivity was determined by the calculated number of colony-forming units present in the test tube of highest dilution for which a recognizable amount of PCR product was obtained.

A second sensitivity test was implemented to determine the ability to detect a single infected quarter or cow (four quarters) diluted with milk from uninfected cows in a bulk tank. For this purpose, milk from a known infected cow was progressively diluted with milk from a known uninfected cow. The diluted samples were subjected to selective enrichment, DNA extraction, and diagnostic PCR as above. Sensitivity was determined by the dilution factor in the test tube of highest dilution for which a recognizable amount of PCR product was obtained. The dilution factor was taken to represent the herd size in which a single infected cow (four quarters) could be detected in a sample from a bulk milk tank; one fourth of this herd size represented the herd size in which a single infected quarter could be detected.

RESULTS

The 16S rRNA subunit sequence across 11 streptococci species were aligned and compared. Examination of the consensus sequence revealed two main regions for which consensus was absent or only partial; the first extending from position 66 to 101, and the second from position 183 to 210 in the 16S rRNA subunit sequence. Examination of the *S. agalactiae* sequence for these regions showed that the *S. agalactiae* sequence was unique and differed from all other streptococcal strains at a number of nucleotide sites (data not shown).

The two *S. agalactiae* variable region sequences were then screened against the universal gene bank as described in Methods. There was complete homology to six strains of *S. agalactiae* (of bovine and human origin)

Figure 1. Agarose gel showing amplification products with *Streptococcus agalactiae* and with a series of other Streptococci strains and *Escherichia coli*, using the V1 and V2 primer pairs (lanes 1 to 7) and the C1 and C2 primer pairs (lanes 9 to 15) in separate reactions. Lanes 1 and 9, Ag225; lanes 2 and 10, Ag138; lanes 3 and 11, Ent96; lanes 4 and 12, Ub166; lanes 5 and 13, Dys164; lanes 6 and 14, *E. coli*; lanes 7 and 15, negative controls; lane 8, size marker. Strain abbreviations as in Table 1. Size of PCR products: V1-V2, 120 bp; C1-C2, 207 bp, shown by arrows.

whose 16S rRNA sequence was found in Genbank. In addition, there was complete homology to *S. difficile*. The two variable regions did not show homology to any known bovine sequences, including the 18S rRNA bovine sequence.

Within the two variable regions, primers V1 and V2 were designed that were specific to the *S. agalactiae* sequence. In addition, two fully conserved primer sequences, C1 and C2, flanking the pair of variable regions were also designed.

To confirm the conservation of the unique sequences V1 and V2 in all bovine strains of *S. agalactiae*, a 200-bp fragment containing both variable regions was amplified (using the flanking C1 and C2 primers) in seven isolates of *S. agalactiae* obtained in the course of four independent outbreaks in Israel in the years 1990 to 2000 and one strain from the ATCC collection (Table 1). The 200-bp fragment was sequenced and found to be identical in all tested strains (data not shown).

Figure 1 shows amplification products with *S. agalactiae* and with a series of other streptococcal strains and *E. coli*, using the V1 and V2 primer pairs and the C1 and C2 primer pairs in separate reactions. The single V1-V2 product was obtained only with *S. agalactiae*, while the C1-C2 product was obtained with all streptococci strains, but not with *E. coli*. Primer pairs C1-V2 and V1-C2 also gave products with *S. agalactiae* alone, and not with any of the other streptococci strains (data not shown).

Sensitivity

With the protocol described in Methods, sensitivity tests were carried out using a series of milk samples to which were added different concentrations of *S. agalactiae* colony-forming units. When amplification followed directly on addition of the *S. agalactiae* dilution

Figure 2. Sensitivity tests using V1 and V2 primer pairs. A. Sensitivity with direct PCR amplification without prior enrichment. Concentration of *Streptococcus agalactiae* per milliliter: Lane 1, 10^8 ; lane 2, 10^7 ; lane 3, 10^6 ; lane 4, 10^5 ; lane 5, 10^4 ; lane 6, 10^3 ; lane 7, 10^2 ; lane 8, 10^1 ; lane 9, 10^0 ; lane 10, milk without *S. agalactiae*; lane 11, positive control, *S. agalactiae* DNA; lane 12, negative control (no DNA). B. Amplification after selective enrichment. Lane 1, negative control (no DNA); lane 2, milk without *S. agalactiae*; lane 3, 10^8 ; lane 4, 10^7 ; lane 5, 10^6 ; lane 6, 10^5 ; lane 7, 10^4 ; lane 8, 10^3 ; lane 9, 10^2 ; lane 10, 10^1 ; lane 11, 10^0 ; lane 12, positive control, *S. agalactiae* DNA.

to the milk samples, products were obtained at a concentration of 10^4 to 10^5 cfu/ml or greater (Figure 2A). When amplification followed a selective enrichment period, products were obtained at an initial concentration of 1 cfu/ml (Figure 2B).

Positive Control

When C1, C2, V1, and V2 primers were combined in the same reaction, useful products were not obtained. Therefore, the C1-C2 positive control had to be implemented in a separate reaction mixture. When BMC1, BMC2, V1, and V2 primers were combined in the same reaction, the separate products were successfully obtained (Figure 3). Thus, when these primers are used in combination, the amplification product of BMC1-BMC2 should always appear in milk samples, while appearance of the additional PCR product of *S. agalactiae* is a positive diagnosis of *S. agalactiae* (Figure 3, lane 2). Complete absence of both products indicates a technical problem with the amplification procedure.

Figure 3. PCR amplification with primers V1, V2, BMC1, and BMC2. Lane 1, *Streptococcus agalactiae* DNA only; lane 2, DNA extracted from *S. agalactiae* inoculated milk; lane 3, DNA extracted from uninfected milk; lane 4, negative control (no DNA). Arrows indicate V1-V2 product (120 bp) and BMC1-BMC2 product (389 bp).

Field Test

For purposes of field testing, the Israel National Mastitis Reference Center (NMRC) referred us to a mastitis outbreak in a commercial herd. Samples were obtained from nine cows diagnosed as infected, three cows of unknown status, and five that were infection free. Samples were subjected to the *S. agalactiae* overnight selective enrichment procedure followed by DNA extraction, and PCR amplification with the V1-V2, C1-C2, and BMC1-BMC2 primer pairs used separately. In all cases but one, results with the V1-V2 pair conformed to the prior diagnosis when known. In addition, using the V1-V2 pair, two of the unknown cows were diagnosed as infected, and one as uninfected; these diagnoses were subsequently confirmed by blood agar plating. In the exceptional case noted above, the infected sample did not give a product with any of the three primer pairs, indicating a technical problem with the amplification procedure for this sample. Reexamination of this sample gave a positive product with V1-V2 and the two positive controls. Except for this exceptional case, the C1-C2 primer pair produced product in all samples, and the BMC1-BMC2 primer pairs produced product in all but one of the samples. We attribute lack of amplification of the BMC1-BMC2 product in the latter instance to degradation of somatic cell mitochondrial DNA by the long enrichment incubation to the point where it could no longer serve as a substrate for the PCR.

Using the above infected and uninfected samples, we implemented a study simulating a sample from a milk tank with one infected cow in the herd by appropriate dilution of the milk sample of an infected cow with milk of an uninfected cow. The results show that the infected cow could readily be identified at the equivalent of one infected cow (four quarters) in a herd of 500, or one infected quarter in a herd of 125 (data not shown). Because 1:500 was the limit dilution used in this trial, it is possible that higher dilutions would have been detected as well.

DISCUSSION

Streptococcus agalactiae is a highly infectious bovine mastitis pathogen that can rapidly spread throughout a herd from a single infected animal. Consequently, early diagnosis of the presence of the infection in a herd is important for effective control. Good farming management, with high level of veterinary monitoring and treatment, may allow eradication of this udder pathogen from the herd. Diagnosis is difficult, however, because of the normally subclinical expression of the pathogen. Current methods for diagnosis of *S. agalactiae* are based on the biochemical characteristics of the organism, such as the CAMP or esculin tests, followed by serological procedures for definitive diagnosis (Keefe, 1997).

The aim of this study was to develop a PCR-based system for a highly sensitive, low cost, rapid, and specific identification of *S. agalactiae* in milk. Our results, indeed, show high sensitivity and specificity of *S. agalactiae* identification using primers V1 and V2, specific to 16S rRNA. All *S. agalactiae* isolates and all *S. agalactiae* sequences in the Genbank had identical V1-V2 primer sequences. Furthermore, with the exception of *S. difficile*, found to be identical to *S. agalactiae* in these regions, Genbank screening did not uncover these primer sequences in any other organism. *Streptococcus difficile* belongs to Group B streptococci, and is considered serologically to be a strain of *S. agalactiae*. However *S. difficile* is a pathogen of cold-blooded species such as fish and frogs, and is not likely to be a source of infection in cows (Vandamme et al., 1997). All *S. agalactiae* isolates tested produced an amplification product with the V1-V2 specific primers; none of the other streptococcus strains included in the present study did so.

In a field test of the procedure on an outbreak of *S. agalactiae* occurring in a herd in Israel during the course of this study, the V1-V2 primer pair was tested on a set of milk samples taken directly from known infected and noninfected cows. In none of the cases did the V1-V2 primer pair give a positive reaction for the

uninfected cows. Within the infected cows, V1-V2 primers gave a positive reaction for all cases but one. The exceptional case was shown by the positive controls to be due to general failure of the PCR reaction in this sample and was found positive on reexamination. Thus, the results of the PCR method were completely specific and consistent with those of the classical bacteriological methods. The PCR procedure did not give any false-positive or false-negative reactions.

Two series of sensitivity tests were implemented. In the first, *S. agalactiae* were added directly to milk samples in decreasing concentrations. The results showed that immediate PCR amplification from milk could identify initial dilutions of 10^4 to 10^5 cfu/ml, while—after overnight selective enrichment—PCR amplification could identify initial dilutions of as few as 1 cfu/ml. In the second series, a milk sample from an infected cow was diluted with milk from an uninfected cow. The results showed that after overnight incubation, PCR amplification could, at the least, identify the equivalent of one infected quarter in bulk milk of 125 cows or one infected cow (four quarters) in bulk milk of 500 cows. These results confirm the high sensitivity of the procedure.

A variety of technical causes can lead to failure of a PCR amplification reaction. These include, for example, the DNA extraction step, faulty composition of the PCR mix, or the use of inactive polymerase enzyme. Because, in a field situation, the majority of reactions are expected to be negative, it is essential to include positive controls to avoid false negatives that are due to failure of the amplification procedure for any of the above or other reasons. Absence of the control product will indicate a technical problem in the process and will avoid a false-negative conclusion. To facilitate diagnostic field application, therefore, two sets of positive control primers were developed. One pair, C1-C2, was chosen to react with all streptococci strains, some of which are normally present in all milk samples. A second pair, BMC1-BMC2, was chosen to react with bovine mitochondrial DNA, which is present in all milk cells by virtue of their SCC. In the field test situation mentioned above, the positive controls were tested on a set of milk samples taken directly from 11 infected and six uninfected cows, after selective enrichment. In one case as noted, failure to get a reading with positive controls prevented a false negative reading by the V1-V2 pair. In a second exceptional case, the BMC1-BMC2 pair did not give a product even on retesting. In this exceptional case, we attributed the lack of BMC1-BMC2 product to degradation of mitochondrial DNA by the overnight enrichment incubation. We believe that the enrichment period can be minimized to a few hours, without reducing the method's sensitivity, which should solve this

problem as well as reduce the time needed for the whole method of detection. In retrospect, the incubation step may not even have been necessary in this case, because samples were taken directly from infected cows rather than from a highly diluted bulk milk tank.

Compared with the time-consuming and costly procedures currently used to diagnose *S. agalactiae*, the PCR-based methodology presented here is highly sensitive and requires only a single reaction followed by product analysis. Cost of materials and basic equipment to implement PCR diagnosis is low. Automated equipment is available for part and possibly the entire procedure, but such equipment is rather expensive. With automation, the marginal costs of a single test are very low, allowing widespread use of preventive screening.

The method we developed here worked well in the laboratory and performed as expected in the single field test situation examined. It is well suited for central, regional, or national milk test laboratories. Large scale implementation of the procedure will clearly require further validation of its ability to identify an infected herd on the basis of bulked milk, and of its ability to identify individual infected cows within the herd. The veterinary and the food industries are traditionally conservative in their methodology, and properly so. Thus, even if this procedure is adopted by a commercial organization, it is likely that it would be backed up by the classical methods during the transition period, allowing in situ validation and possible modification for the particular adopting organization. Alternatively, the results of this study may provide the impetus for the development of a fully validated commercial kit. The method presented here could easily enable monthly or even weekly testing of bulk milk from the collection tanks. In this way, outbreaks of *S. agalactiae* in a herd could be detected before the infection spreads. Because *S. agalactiae* is not a normal constituent of udder flora, aggressive monitoring and treatment may be able to completely eradicate this pathogen from national herds.

Although the research presented here was focused on diagnosis of *S. agalactiae* in milk, the general approach exemplified here can readily be applied to diagnosis of other pathogens in milk, and the specific V1-V2 primers can be used for diagnosis of *S. agalactiae* in human disease as well (Bentley and Leigh, 1995; Wang et al., 1999). The specific organisms for which procedures are developed will depend on the testing organization. For our purposes in Israel, we intend to extend the methodology to emerging pathogens of public health significance such as *Listeria* and *Mycobacterium paratuberculosis* and additional contagious mastitis organisms, such as *Staphylococcus aureus* and *Arcanobacterium pyogenes*.

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