Development of polymerase chain reaction for detection of predominant streptococcal isolates causing subclinical bovine mastitis

K Nithin Prabhu1, S Isloor1*, Raveendra Hegde1, D Rathnamma1, B M Veeregowda1, H N Narasimha Murthy2, Rajeswari Shome3 and V V S Suryanarayana4

1Department of Veterinary Microbiology and 2Department of Poultry Science, Veterinary College, KVAFSU, Hebbal, Bangalore 560 024, India
3Project Directorate on Animal Disease Monitoring And Surveillance (PD_ADMAS) Hebbal, Bangalore 560 024
4Molecular Virology Laboratory, Indian Veterinary Research Institute, Hebbal, Bangalore 560 024, India

Received 29 March 2012; revised 23 June 2012; accepted 29 August 2012

Bovine mastitis is the most important source of loss for the growing dairy industry. Streptococci, with special reference to Streptococcus agalactiae, S. dysgalactiae and S. uberis, are the predominant pathogens causing bovine mastitis. A rapid, sensitive and specific test for the detection of these pathogens needs to be developed. To accomplish this, initially 163 milk samples were collected from various organized and unorganized sectors in and around Bangalore, India. These milk samples were screened for subclinical mastitis by somatic cell counting (SSC) and electro conduction (EC). Of these, 131 samples selected based on SCC and EC values were subjected for isolation of the organisms. Two sets of specific primers, targeting streptococcal 16S rRNA gene were designed for detection of S. agalactiae, S. dysgalactiae and S. uberis. The results of the study showed S. agalactiae as the predominant streptococci among the generally identified streptococcal species associated with subclinical bovine mastitis in dairy cattle in and around Bangalore.

Keywords: Mastitis, PCR, 16S rRNA, Streptococcus

Introduction

India continues to be the largest producer of milk in the world with 185 millions cattle and 98 million buffaloes as Livestock Census, 20071. Animal Husbandry and Dairy development plays a prominent role in the rural economy in supplementing the income of rural households, particularly, the landless, small and marginal farmers. Bovine mastitis (BM) is an inflammation of the mammary gland, usually due to a microbial infection2 and causes considerable economic loss to Indian dairy industry. These losses are primarily due to lower milk yields, reduced milk quality and higher production costs3-5. 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 bovine mastitis can be classified as environmental (Streptococcus dysgalactiae, S. parauberis, S. uberis & Escherichia coli) or contagious (S. aureus & S. agalactiae) depending on their primary origin, viz., environment or infected mammary gland quarter6,7.

Identification of bacterial pathogens in milk from cows with mastitis is regarded as the ‘golden standard’ in diagnosis of mastitis. It also provides important information for prevention and control of this disease. In most clinical laboratories, identification methods are based on microbiological culturing of milk and biochemical tests. However, there are several disadvantages associated with microbiological culture. Subclinically infected cows are intermittent shedders of organisms and may cycle through low and high shedding patterns during lactation. Culture of milk may not yield any bacteria from subclinically infected glands due to the presence of very low numbers of pathogens when samples are collected. Failure to isolate bacteria from mastitic milk may also be due to the presence of residual therapeutic antibiotics in the submitted samples that may inhibit bacterial growth in vitro. The presence of leukocytes in milk samples from cases of clinical mastitis and in milk samples with high somatic cell counting may also potentially inhibit growth of bacteria. Moreover, microbiological culture...
of milk samples is time consuming and species level identification by standard biochemical methods is time honoured approach.

For the limitations of cultural methods, polymerase chain reaction (PCR) has been developed to identify various mastitis pathogens. The development of PCR-based methods provides a promising option for the rapid identification of bacteria. With this method, identification of bacterial pathogens can be made in hours, rather than the days required for conventional cultural methods. PCR can also improve the level of detection due to its high sensitivity. Different PCR-based methods have been developed for specific and sensitive detection of mastitis pathogens in milk.

Majority of the bovine mastitis cases caused by streptococcus species are mainly subclinical and, therefore, can be diagnosed only in the laboratory. Streptococci are a highly infectious bovine mastitis pathogen that can rapidly spread throughout the herd from an infected animal. In view of this, early detection of involvement of streptococci in subclinical bovine mastitis in a herd is important for effective control. The objective of this study was to develop a sensitive, rapid PCR as a molecular diagnostic method for specific identification of S. agalactiae, S. dysgalactiae and S. uberis from cases of bovine mastitis and compare it with the conventional method of bacterial culture.

Material and Methods

Milk Samples

In all, 163 milk samples were collected from 3 organized and 1 unorganized sectors in and around Bangalore and subjected for somatic cell counting (SCC) and electrical conductivity (EC) tests. As per the International Dairy Federation criteria of SCC value >500,000 cells/mL and EC of >6.5 mS (milli Siemens)/cm as the indicators of subclinical mastitis, >500,000 cells/mL and EC of >6.5 mS (milli Siemens)/cm as the indicators of subclinical mastitis, 131 milk samples were subjected for bacterial isolation.

Bacterial Cultures

After initial enrichment in Streptococcus selection broth for 6 h, 10 µL of enriched milk samples were streaked onto blood agar plates, incubated at 37° C for 48 h. The colony morphology and haemolytic patterns were recorded, and colonies were further streaked onto Brain heart infusion agar plates to obtain pure cultures. Conventional biochemical assays like catalase test, Voges-Proskauer test (VP), hydrolysis of esculin and hippurate, and fermentation of sugars like glucose, lactose, mannitol, ribose, sorbitol, raffinose, sucrose and pyrrolidonyl reduction tests were employed to differentiate various streptococcal isolates.

Bacterial Strains

The reference streptococci, viz., S. agalactiae (HM 355961), S. dysgalactiae (HC 359248) and S. uberis (HC 355971 & HC 355972), were procured from the Project Directorate on Animal Disease Monitoring And Surveillance (PD_ADMAS), Bangalore. E. coli (JF926686) and S. aureus (JN247783) were obtained from the cultures maintained at the Department of Microbiology, Veterinary College, Bangalore.

DNA Extraction

The bacterial DNA was extracted using QIAamp DNA Mini and Blood mini kit (Qiagen, Inc) as per the manufacturer’s instructions.

Designing of PCR Primers

Genus specific and species specific primers were designed by targeting tuf and 16S rRNA genes, respectively for identification of streptococci. The primers were designed from highly divergent and species specific regions of the DNA coding for 16S rRNA based on previously published sequence entries available in the NCBI-GenBank database (S. agalactiae, S. dysgalactiae & S. uberis). The primer sequences and the sizes of the amplified products are shown in Table 1.

Polymerase Chain Reaction (PCR)

The PCR reaction mixture contained, 2.5 µL of 10× PCR Taq buffer A, 0.5 µL (10 pmol) of each SagaF/SdysF/SubF and SagaR/SdysR/SubR primers and 1 µL (100 µM) of each dNTPs, 3 µL (150 ng) of DNA samples. The PCR reaction mixture contained, 2.5 µL of 10× PCR Taq buffer A, 0.5 µL (10 pmol) of each SagaF/SdysF/SubF and SagaR/SdysR/SubR primers and 1 µL (100 µM) of each dNTPs, 3 µL (150 ng) of DNA samples.

Table 1—Nucleotide sequences of Streptococcus genus-specific (tuf) and species-specific (16S rRNA) primers

<table>
<thead>
<tr>
<th>Genus-specific primer (tuf)</th>
<th>Nucleotide sequence</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S tuf-F</td>
<td>5'-CAA CTT GAC GA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AGGT CCT GCA-3'</td>
<td>110</td>
</tr>
<tr>
<td>S tuf-R</td>
<td>5'-TGG GTT GAT TG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AACC TGG TTT A-3'</td>
<td></td>
</tr>
<tr>
<td>Species-specific primers (16S rRNA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saga F</td>
<td>5'-GCC TCA TAG CG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GGG ATA AC-3'</td>
<td>329</td>
</tr>
<tr>
<td>Saga R</td>
<td>5'-ACG TTC TTC TCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AAC AAC AGA-3'</td>
<td></td>
</tr>
<tr>
<td>Sdys F</td>
<td>5'-GGA GTG GAA AAT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCA CCA T-3'</td>
<td>549</td>
</tr>
<tr>
<td>Sdys R</td>
<td>5'-CGG TCA GGA GGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TGT CAA GAC-3'</td>
<td></td>
</tr>
<tr>
<td>Sub F</td>
<td>5'-GTA CCC TAT TTA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AAA GGG GCA AAT-3'</td>
<td>854</td>
</tr>
<tr>
<td>Sub R</td>
<td>5'-C TCC GAT GTA CCG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AAG TAA AGC TCT-3'</td>
<td></td>
</tr>
</tbody>
</table>
streptococcal DNA and filtered quartz water was added to make final volume to 25 µL. The amplification reactions were carried out using a programmable thermal cycler (Master Cycler pro, M/s Ependorff, Germany). The amplification was programmed for 30 cycles with temperature cycles of denaturation at 94°C for 30 sec, annealing at 49.5°C for 30 sec and extension at 72°C for 30 sec. A final extension step of 10 min was included. The amplified products were electrophoresed on a 1.8% agarose gel containing 0.5 µg/mL of ethidium bromide and the images were captured (Gel Doc XR, M/s, BioRad., USA).

Analysis of Nucleotide Sequences
The PCR products were sequenced (M/s, Chromous Biotech, Bangalore) and the sequence results were analyzed using Basic Local Alignment Search Tool (BLASTn).

Results and Discussion
Of 131 milk samples subjected for isolation, a total of 40 streptococci were isolated from 32 milk samples. Of these 32 milk samples, 6 revealed mixed streptococcal isolates, remaining 26 milk samples yielded pure streptococcal isolates. These isolates were confirmed to the genus level by plethora of biochemical tests. However, observations made in this study, despite thorough biochemical characterization of streptococcal isolates, could not lead to their precise identification up to the species level due to variability in their biochemical profiles. Three isolates (isolate no. 17, 31 & 32) were confirmed as S. agalactiae by conventional biochemical tests, which were further confirmed by genotypic methods. Furthermore, conventional biochemical tests identify not only typical but also atypical streptococcal isolates. Hence, they are considered dubious for identification of streptococcal species. These observations are also supported by the studies of Odierno et al.18

The findings in the present study indicate that the conventional biochemical tests like esculin hydrolysis, hydrolysis of sodium hippurate, VP and sugar fermentation are ambiguous and lack repeatability. Further, these findings also contradict the fact that the conventional biochemical assays are the gold standard in the isolation and identification of the bacterial pathogens and support the debate about the inconsistency of these test profiles, which are labour intensive and time consuming.11,14,19,21 These findings emphasize the need for development of molecular methods based on 16S rRNA gene for precise identification of streptococci since this is one of the most useful tools applied to the revision of the bacterial classification system22.

Elongation factor-Tu (EFTu), a GTP-binding protein, is encoded by tuf gene, which plays a central role in the protein synthesis. In the bacterial genome, there are up to three tuf genes present in various copy numbers, whereas only one is present in the majority of Gram-positive bacteria with the low GC content23. In the present study, the tuf gene based genus-specific PCR precisely identified all the 40 isolates as Streptococcus with an amplicon of 110 bp (Fig. 1), which very well correlated with the conventional biochemical identification methodologies. Many authors have reported the usefulness of tuf gene in the development of PCR assays21,23,24.

Further, a house keeping 16S rRNA gene was targeted for designing species-specific primers for the identification of the streptococci. The primers designed yielded amplicons of 329 bp for S. agalactiae, 549 bp for S. dysgalactiae and 854 bp for S. uberis reference strains (Figs 2-4). The species-specific PCR amplification of 110 bp tuf gene of Streptococcus sp. isolated from bovine mastitis cases. [Lane M: 100 bp DNA ladder; lanes 1, 2 & 3: S. agalactiae (HM 355961), S. dysgalactiae (HC 359248) and S. uberis (HC 355971), respectively; lanes 4 to 16: streptococcal isolates; lane SAU: Negative control, S. aureus (JN247783); lane E. coli: Negative control, E. coli (JF926686)]

Fig. 1—PCR amplification of 110 bp tuf gene of Streptococcus sp. isolated from bovine mastitis cases. [Lane M: 100 bp DNA ladder; lanes 1, 2 & 3: S. agalactiae (HM 355961), S. dysgalactiae (HC 359248) and S. uberis (HC 355971), respectively; lanes 4 to 16: streptococcal isolates; lane SAU: Negative control, S. aureus (JN247783); lane E. coli: Negative control, E. coli (JF926686)]

Fig. 2—PCR amplification of 329 bp 16S rRNA gene of S. agalactiae isolated from bovine mastitis cases. [Lane 1: Positive control S. agalactiae (HM 355961); lanes 2, 3 & 4: S. agalactiae isolates (17, 31 & 32); lane 5: 100 bp DNA ladder; lane 6: Negative control, S. aureus (JN247783); lane 7: Negative control, E. coli (JF926686); lane 8: No template control (NTC)]
specific PCR identified only three isolates as *S. agalactiae*, which were further confirmed by sequence BLAST analysis of amplified products and sequences submitted to GenBank database (Acc. Nos JN247788, JN247789 & JN247790). Interestingly, none of the isolates were identified as *S. dysgalactiae* or *S. uberis*. The results showed high specificity of 16S rRNA based PCR in identification of streptococci. 16S rRNA has been a reliable site for the development of rapid and accurate detection methodologies. Compared to the time-consuming and costly procedures used to diagnose mastitis due to streptococci, the PCR-based methodology presented here is highly accurate and requires only a single reaction, followed by the product analysis. The present results were completely specific and consistent and did not result in any false-positive or false-negative reactions.

**Conclusion**

The results of the present study indicated the higher prevalence of *S. agalactiae* in subclinical cases of bovine mastitis among the reported predominant species of streptococci. Furthermore, the PCR method can be successfully used for the identification of the major streptococcal isolates both at genus and species levels, especially the predominant streptococcal species, such as, *S. agalactiae*, *S. dysgalactiae* and *S. uberis*. Use of such rapid, sensitive and specific method may be helpful in the field studies of diagnosis and effective treatment, antibiotic selection and ultimately the control of mastitis.

**Acknowledgement**

The authors acknowledge the financial support provided under the grant of National Agricultural Innovation Project (NAIP) by Indian Council of Agricultural Research (ICAR), Government of India, New Delhi to the Sub-project entitled, ‘Bovine mastitis: Unraveling molecular details of host-pathogen interaction and development of molecular diagnostic methods’.

**References**

12. Riffon R, Sayasith H, Khalil H, Dubreuil P, Drolet M *et al*, Development of a rapid and sensitive test for identification of...


