DETECTION AND GENETIC CHARACTERIZATION OF MAJOR PATHOGENS ISOLATED FROM BOVINE MASTITIS IN RAJASTHAN

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ABSTRACT

The present study was contemplated with an aim to detect and evaluate the major pathogens associated with clinical mastitis in bovines. The milk samples were obtained from the cows suffering from mastitis from different areas of Bikaner and veterinary clinical complex RAJUVAS, Rajasthan. After subjecting the samples to microbiological detection protocols, their 16S–23S rRNA gene internal transcribed spacer (ITS) regions were characterized and the feasibility of using ITS sequences to discriminate between five bacterial species was validated. The conventional bacteriological and PCR based examinations showed incidence of 20 (40%) isolates of *E. coli*, 16 (32%) isolates of *S. aureus*, 8 (16%) isolates of *P. aeruginosa*, and 1 (2%) isolate of *Streptococcus* spp. The sensitivity and specificity of the test was evaluated using standard cultures.PCR was found to be more sensitive than conventional culture methods for detection of common mastitic pathogens. The results suggest that the PCR assay could be used as an alternative method in routine diagnosis for rapid, sensitive and specific simultaneous detection of *S. aureus, S. agalactiae, S. dysgalactiae, S. uberis, E. coli* and *P. aeruginosa* in milk samples.

KEY WORDS: Bovine, Culture, *E. Coli*, Milk, PCR, Subclinical Mastitis.

INTRODUCTION

Mastitis, also known as “intramammary infection” is a highly frequent and economic disease that is aptly called as a “Dairyman’s grief” owing to the heavy financial losses caused by it. It has also been identified as the most common cause of death of an adult dairy cow (Esslemont and Kossaibati,1997).

The bacterial pathogens responsible for bovine mastitis can be environmental (*E. coli*, *S. dysgalactiae*, *S. parauberis* and *S. uberis*) or contagious (*S. aureus* and *S. agalactiae*). Watts (1988) identified 137 different organisms as causal agents of mastitis. Although 80% of the bacteria detected in the samples from mastitic animals include *S. aureus, S. agalactiae, S. dysgalactiae, S. uberis* and *E. coli* (Anon, 2001); the *E. coli* and *S. uberis* remain the two most important and common cause of mastitis.

To overcome the limitations of cultural and biochemical identification of organisms, the molecular methods of identification of bacteria based on amplification of the specific segments of the genome of bacteria by polymerase chain reaction have been developed and found useful as these are more reliable and less laborious (Ghadersohi *et al*., 1997; Anand Kumar, 2009; Kozytska *et al*., 2010). The main advantage of PCR detection assay lies in its direct, rapid and cost effective application as compared to conventional microbiological culture methods which include cumbersome and lengthy steps. Therefore, the present study was designed to isolate and detect the common pathogens from the clinical mastitic milk samples by a PCR based method using species specific primers derived from 16S rRNA genes. The aim was to achieve and develop a sensitive, specific and low cost, fast turnaround time procedure for the detection of mastitic pathogens.

MATERIALS AND METHODS

50 milk samples were collected aseptically from the milking cows by cleaning the teats with cotton swabs dipped in 70% ethanol. The teats were dried completely before collecting the milk samples. The samples were collected from different areas of Bikaner city and Veterinary clinical complex from cows suffering from clinical mastitis. The samples were immediately taken thereafter to the laboratory on ice for further bacterial culturing and PCR assays.

Bacteriological culture- The isolates were characterized with established biochemical procedures described by Buchanan and Gibbons (1974), Cowan and Steel (1974), Balows *et al*. (1992) and Quinn *et al*. (1994). The bacteria were identified tentatively according to the colonial morphology, hemolytic characteristics, catalase test and Gram staining characteristics.

DNA Extraction- DNA isolation of *Staphylococcus* spp. and *Streptococcus* spp. was carried out as per the method of Nachimuttu *et al*. (2001) and DNA isolation of *E. coli* and *Pseudomonas* spp. was carried out by the method of Chen and Kuo (1993) with some modifications.

Quantification of genomic DNA- Before subjecting the isolated DNA to PCR assay, the optical density (OD) was recorded both at 260 nm and 280 nm and ratio $A_{260}/A_{280}$by UV-1800 spectrophotometer (Shimadzu). A ratio of around 1.9 (1.85-1.95) indicated best quality of DNA.

Oligonucleotide primers - The primers for *S. aureus*, *S. agalactiae*, *S. dysgalactiae* and *S. uberis were* derived from the published sequences of Phuektes *et al*. (2001) and the primers for *E. coli* were derived from Khaled *et al*. (2010). The primers for *P. aeruginosa* were obtained from Spilker *et al*. (2004). The primers were synthesized by Sigma-Aldrich Co. (Bangalore, India) and obtained in lyophilized form. The primer specificity, the sequences, the annealing temperatures and the lengths of the amplified products are summarized in Table 1.

Table 1-Bacterial species examined, corresponding primer sequence, annealing temperature and size of the amplified products (in base pairs) of various primers used for PCR assay.

Bacterial species examined	Primer sequences	Annealing temperature (°C)	Product size (bp)
S. aureus	TCT TCA GAA GAT GCG GAA TA	56	420
	TAA GTC AAA CGT TAA CAT ACG		
S. agalactiae	AAG GAA ACC TGC CAT TTG	55	270
	TTA ACC TAG TTT CTT TAA AAC TAG AA		
S.dysgalactiae	GAA CAC GTT AGG GTC GTCAGT ATA	55	264
	TCT TAA CTA GAA AAA CTA TTG		
S. uberis	TAA GGA ACA CGT TGG TTA AG	55	330
	TTC CAG TCC TTA GAC CTT CT		
E.coli	GCT TGA CAC TGA ACA TTG AG	57	662
	GCA CTT ATC TCT TCC GCA TT		
P. aeruginosa	GGGGGATCTTCGGACCTCA	58	956
	TCCTTAGAGTGCCCACCCG		

Standard strain of *S. aureus* (MTCC No. 3103), *E. coli* (MTCC No.443*)* and *P. aeruginosa (*MTCC No. 7453*)* were procured from IMTECH Chandigarh, India. Standard strains of *S. agalactiae (*ATCC NO. 13813*)* and *S. uberis (*ATCC No. 700407*)* were procured from HiMedia (Mumbai, India).

The simplex PCR assay was performed in an Applied Biosystems PCR in 0.2 ml tubes in a total reaction volume of 50 µl. All PCR reaction master mixtures were prepared using 3 µl of extracted DNA template, 10 µl of Flexi Buffer, 3 µl of 25 mM $MgCl_2$, 1 µl of dNTP mix, 1 µl of each primer, followed by addition of 0.25 µl of *Taq* polymerase. The remaining master mix was substituted by distilled Milli Q water. Except the primers, all the reagents were procured from Promega (USA).The annealing temperature was separately optimized for each individual assay. A total of 40 PCR cycles were run under the following conditions: denaturation at 96°C for 4 min, annealing temperatures 55 to 58 °C (as mentioned in Table No. 1) for 30 Sec. and extension at 72°C for 1 min. After the final cycle, the preparation was kept at 72°C for 7 min to complete the reaction. To validate the specificity of the primers, the PCR products were analyzed by electrophoresis on 1.8% (w/v) agarose gel in 0.5X Tris-borate-EDTA (TBE) buffer at 100 volts for 1 hour along with 100 bp DNA ladder (Promega, USA). After staining with (0.5 µg/ml) ethidium bromide (Sigma-Aldrich Co.), the amplicons were visualized under UVP Gel Doc Bioimaging System and documented.

RESULTS AND DISCUSSION

The milk samples were collected from clinical bovine mastitis and were subjected to PCR detection assay. Out of total 50 samples processed, 26 (52%) samples were positive for at least one species (*S. aureus, Streptococcus* spp*., P. aeruginosa* and *E. coli*) from clinical mastitic samples. The rest of the 24 (48%) samples of mastitis were negative for all four bacterial species. *Streptococcus* spp*.* was identified at presumptive level in the present study. The bacterial examinations showed incidence of 40%, 32%, 16%, and 2% for *E. coli, S. aureus, P. aeruginosa,* and *Streptococcus* spp*.* respectively. Of these, *E. coli* showed maximum recovery followed by *S. aureus, P. aeruginosa,* and *Streptococcus* spp*.*

The results are in close agreement to the findings of Bradley (2002), who reported an incidence of 34.7% for *E. coli* infection followed by *S. uberis* (12.7%), *S. aureus* (5.02%) and *S. dysgalactiae* (4.76%). Ranjan *et al.* (2011), observed out of 190 milk samples, *S. aureus* to be the most prevalent followed by coagulase negative *Staphylococcus* spp., *E. coli*, *Pseudomonas* spp*.* and *Streptococcus* spp. Sumathi *et al*. (2008) reported highest prevalence of *S. aureus* (24%) followed by *E. coli* (20%) and *S. epidermidis* and *Streptococcus* spp. (16%) in bovine mastitis. Benhamed *et al.* (2011) also recorded similar prevalence of *S. aureus* (30.76%)*, Streptococcus* spp.(30.76%) *and E. coli* (23.07%) in mastitic cattle. Khaled *et al.* (2010) reported an incidence of 23.75% for *E. coli* infection followed by *S. aureus* (15.62%), *S. agalactiae* (3.12%) and *S. dysgalactiae* (0.62%) in sub-mastitic buffaloes in Egypt.

23S rRNA based genotyping of *E. coli*- In the present investigation, the standard strain (MTCC No. 443) as well as all the *E. coli* isolates were subjected to PCR amplification targeting the 23S rRNA gene using species specific primers. With the PCR, the standard strain and all isolates yielded species specific amplicon of 662 bp (Fig.1).

16S-23S rRNA spacer region (ITS) based genotyping of *S. aureus*-The standard strain (MTCC No. 3103) and all the isolates of *S. aureus* were subjected to PCR amplification targeting 16S-23S rRNA spacer region using species specific primers. With the PCR, a species specific amplicon of 420 bp was produced by standard strain and all the isolates (Fig. 2).

16S rRNA based genotyping of *P. aeruginosa*- All the eight isolates and the standard strain (MTCC No. 7453) were subjected to PCR using species specific primers which produced the product of 956 bp. (Fig.3). In the present study, 100 per cent of the isolates could be amplified by species specific primers, that showed concordance with the earlier reports that had recorded 100 per cent specificity (Spilker *et al.,* 2004).

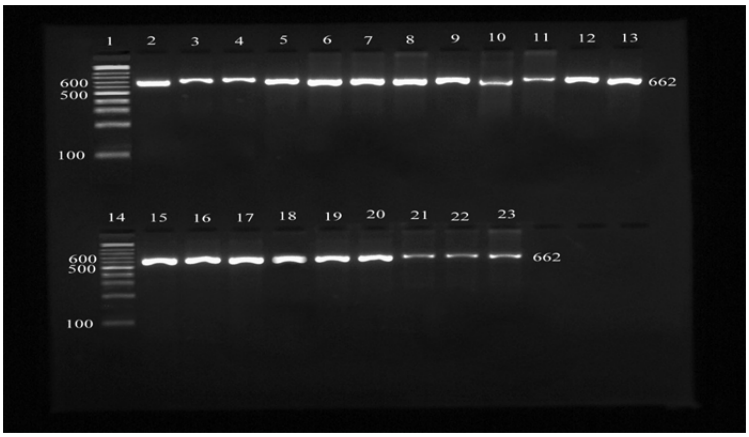

Fig. 1- Agarose gel showing Amplification products (662 bp) of 20 E. coli isolates. Lane 1, 14- 100 bp DNA ladder, Lane 2- standard strain of E. coli, Lane 3 to 23- E. coli isolates.

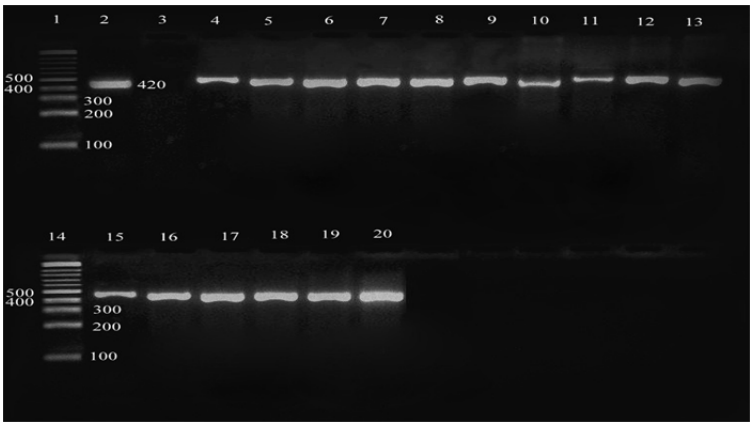

Fig. 2- Agarose gel showing Amplification products (420 bp) of 16 S. aureus isolates. Lane 1, 14-100 bp DNA ladder, Lane 2-standard strain of S. aureus, Lane 4 to 20 - S. aureus isolates.

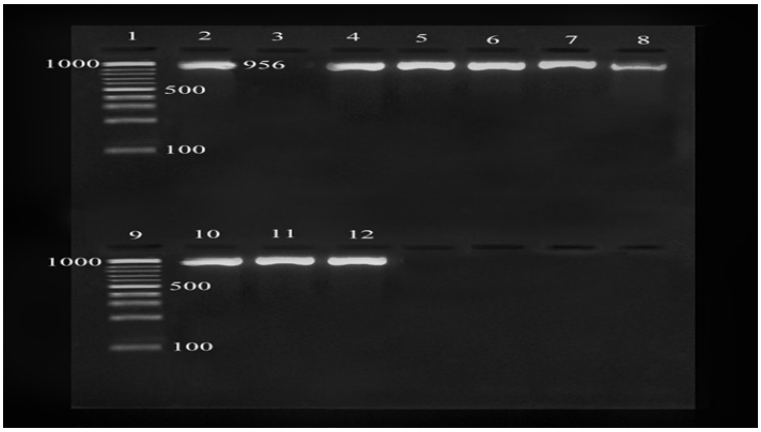

Fig. 3- Agarose gel showing Amplification products (956 bp) of 8 P. aeruginosa isolates. Lane 1, 9 - 100 bp DNA ladder, Lane 2-standard strain of P. aeruginosa, Lane 4 to 12 -P. aeruginosa isolates.

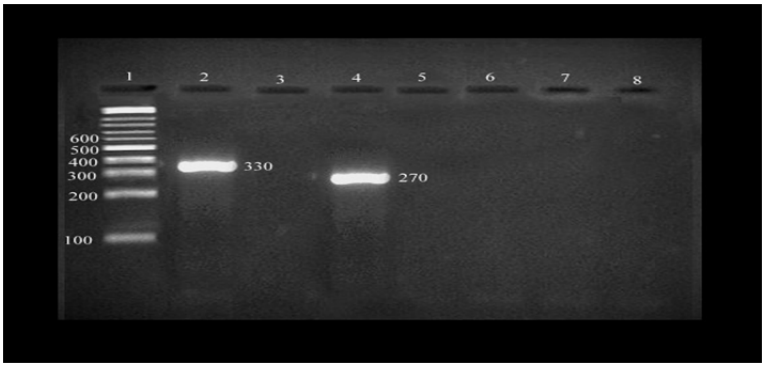

Fig. 4- Agarose gel showing Amplification product (330 bp) of the standard strain of S. uberis (Lane 2) and amplification product (270 bp) of standard strain of S. agalactiae, Lane 5 to7 - Streptococcus spp. isolate which did not show amplification with S. uberis, S. galactiae and S. agalactiae, Lane 1- 100 bp DNA ladder.

16S-23S rRNA spacer region (ITS) based genotyping of *Streptococcus* spp.- In the present investigation, standard strains of *S. agalactiae* (ATCC No.13813) and *S. uberis* (ATCC No.700407) and *Streptococcus* spp. isolate were subjected to PCR amplification targeting 16S-23S rRNA spacer region using *S. agalactiae, S. dysgalactiae* and *S. uberis* species specific primers. With all three species specific primers, *Streptococcus* spp. isolate did not show amplification (Fig.4). So, it can be concluded that it can be other species of *Streptococcus* rather than above three species. While standard strains of *S. agalactiae* and *S. uberis* produced 270 bp and 330 bp amplicon, respectively with their specific primers (Fig.4).

Specificity of PCR-The PCR primers were also examined for their specificity with bacterial species commonly found in milk samples *viz. S. aureus,S. agalactiae, S. dysgalactiae, S. uberis, E. coli* and *P. aeruginosa.* A total of six primers were examined in the present study. No PCR products were amplified from DNA of other bacterial species at optimized conditions. Specificity of all the primers at optimized conditions was found to be 100%.

It can be concluded that PCR method besides being rapid and highly specific, is equally sensitive to the inadequate DNA sample size as the amplification does not depend upon the presence of viable microorganisms. The 16S rRNA gene can be used in identification of most common mastitic pathogens as these genes are highly conserved and are present in multiple copies. Over the last few years, simultaneous detection of more than one type of bacteria in cases of mixed infections has yielded good results using multiplex PCR which can be applied in mastitis also.

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