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Isolation and Identification of Common Mastitis Causing Pathogens from Clinical Bovine Mastitic Milk

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Bovine mastitis is an important disease of cattle which affects economy of the country. The purpose of study was isolation and identification of the common bacterial causes of clinical bovine mastitis. To this end, 50 bovine milk samples from CMT confirmed-clinical mastitis cases were collected and cultured on Nutrient agar, Blood agar and Mac-Conkey agar plates for bacterial isolation. The major pathogens isolated were *Escherichia coli* (28%), *Staphylococcus aureus* (24%), *Pseudomonas aeruginosa* (18%), *Klebsiella pneumoniae* (10%) and *Streptococcus sp*. (2%) as per the biochemical tests and PCR. In conclusion, high percentage of positive samples and relatively high occurrence of environmental microorganisms indicates urgent need to emphisize udder hygiene for clean milk production.

Keywords: Bovine Mastitis, PCR, *Staphylococcus, E. coli, Pseudomonas.*

Mastitis is one of the most important diseases in dairy animals resulting into huge economic losses to the country. The losses due to mastitis are temporary or permanent loss of milk production, poor milk quality, discarding of milk from affected animals prior to or after antibiotic treatment and pre-mature culling of the cow or reduced productive life of animals. Mastitis is defined as an inflammation of the parenchyma of mammary gland, which not only reduces milk yield but alters milk composition (Souto *et al*., 2010) also. The microbial species that commonly cause bovine mastitis, such as *Escherichia coli, Klebsiella pneumoniae, Streptococcus agalactiae* and *Staphylococcus aureus* also occur as commensals or pathogens of humans. Whereas other causative species, such as *Streptococcus uberis, Streptococcus agalactiae* are almost exclusively found in animals (Zadoks *et al*., 2011).

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Compared to culture, the PCR assays are less time consuming takes less than 24 hours to complete, while identification of bacteria to the species levels by conventional microbiological and biochemical methods requires more than 72 hours. Thus a PCR based method act as a useful tool for confirmatory diagnosis of pathogens of mastitis.

MATERIALS AND METHODS

Clinical samples

A total of 50 mastitic milk samples were collected from different locations in and around the Bikaner city, Rajasthan. The bacteria were isolated in pure culture on Nutrient Agar. Further identification of bacteria was done on the basis of colony characters; Gram's staining reaction, growth patterns on Blood Agar and Mac Conkey Agar (i.e., hemolysis and lactose fermentation, respectively) followed by motility, morphology, and biochemical tests (Indol , MR, VP, Citrate and Catalase).

Genotypic characterization

Although, phenotypic identification of bacterial cultures using biochemical tests is one of the standard methods for identification of any bacteria, variability in biochemical tests at times lead to misidentification of these organisma, therefore genotypic tests such as PCR was attempted for the confirmation.

23S rRNA based genotyping of *E. coli*

In the present investigation all *E. coli* isolates were subjected to PCR amplification targeting 23S rRNA gene using species specific primers. All isolates yielded species specific amplicon of 232bp by using primers reported by Riffon *et al.* (2001).

23S rRNA based genotyping of *S. aureus*

In the present investigation all isolates of *S. aureus* were subjected to PCR amplification targeting 23S rRNA using species-specific primers reported by Riffon *et al.* (2001).

16S rRNA based genotyping of *P. aeruginosa*

In the present study, all 9 isolates, which had been biochemically identified as *P. aeruginosa,* were subjected for genotyping by using species-specific primers as reported in earlier studies (Spilker *et al.,* 2004).

16-23S rDNA inter transcribed spacer (ITS) region based genotyping of *K. pneumoniae*

In the present investigation, all 5 *K. pneumoniae* isolates were subjected to PCR amplification targeting 16S–23S rDNA internal transcribed spacer (ITS) region based specific primers (Liu *et al*., 2008) .

23S rRNA based genotyping of *Streptococcus agalactiae*

Streptococcus spp. isolates were subjected to PCR amplification targeting 23S rRNA spacer region by using *S. agalactiae, S. dysgalactiae* and *S. uberis* species-specific primers with 587 bp, 403 bp and 95 bp 23S rRNA spacer region based studies were conducted by Riffon *et al.* (2001) for genotypic confirmation of *Streptococcus agalactiae* isolated from bovine mastitic milk and they observed 587 bp PCR product.

RESULTS

In the present study, mastitic causing pathogens were isolated from 66% cases, while no growth was evident in 34%. The failure of pathogens to grow in-vitro in high percentage of samples may be because of premedication of the animals with antibiotics, non-bacterial causes and the type of media that did not support the growth of whole range of bacteria associated with mastitis.

All 14 isolates of *E. coli* on preliminary biochemical characterization revealed characteristic IMViC pattern, growth on TSI (Y/Y/-) and fermented different sugars. Preliminary biochemical characterization of all 12 isolates of *S. aureus* revealed characteristic growth pattern on mannitol salt agar, showed haemolysis on blood agar, DNAse test and fermented different sugars. All 9 *P. aeruginosa* isolates revealed standard biochemical characterization as growth at 42^{o}C, pigment production, growth on triple sugar iron (TSI) agar (R/R/-), citrate utilization, arginine hydrolysis, gelatin liquefaction, haemolysis and nitrate reduction. Negative reaction was observed for aesculin hydrolysis and urease tests. All 5 isolates of *K. pneumoniae* on preliminary biochemical characterization revealed characteristic IMViC pattern, growth on TSI (Y/Y/-) and fermented different sugars. One *Streptococcus agalactiae* isolate showed dew drop like colonies on Edward's medium, 6.5% sodium chloride medium, hydrolysed aesculin, and fermented different sugars.

A total of 41 bacterial isolates could be cultured and identified by biochemical and genotypic test, which consisted of 14 (28%) isolates of *E. coli*, 12 (24%) isolates of *S. aureus*, 9 (18%) isolates of *P. aeruginosa*, 5 (10%) isolates of *K. pneumoniae* and 1(*2%)* isolate of *Streptococcus agalactiae*.

DISCUSSION

Bovine mastitis is an important disease that afflicts dairy industry through decrease in milk quality and quantity. Various species of bacteria such as *E. coli ,S. aureus, S. agalacticae, P. aeruginosa and K. pneumoniae* are involved in causing mastitis and affecting milk production consequently.In the present investigation, all *E. coli* isolates were subjected to PCR amplification targeting 23S rRNA gene using species-specific primers and amplicon size matched with those reported by Riffon *et al. (*2001). Prevalence of *E. coli* in mastitis cases generally suggests poor udder

hygiene and the results obtained in this study are in agreement with those obtained by ,Bradely *et al*., 2002, Khaled *et al.,* 2010., Sayed, 2014, who have also reported higher incidence of *E.coli*. The biochemical characterization of isolates and fermentation of sugars are comparable with studies of Kruthi (2006) and Pachaury (2011).

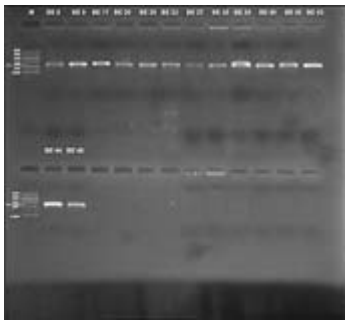
All 12 isolates of *S. aureus* complied with standard biochemical and sugar fermentation tests, and results obtained were in concurrence with that of Upadhyay (2009), who carried out fermentation studies with *S. aureus* obtained from milk of mastitic cattle and goat. There have been reports of amplification of other genomic fragments *viz.*,23S rRNA for genotypic confirmation of *S. aureus* strains isolated from bovine mastitis (Straub *et al.*,1999; Stephan *et al.,* 2001; Salasia *et al.,* 2004; Sanjiv *et al.,* 2008; Bhanderi *et al.,* 2009; Momtaz *et al.,* 2010 and Upadhyay *et al.,* 2010), that generated species specific amplicon of 1250 bp. Khaled *et al.* (2010) have also used 23S rRNA gene based system, albeit generated species-specific amplicon of 1318 bp using a different set of primers.

Streptococcus spp. isolates were biochemically confirmed and subjected to PCR amplification, targeting 23S rRNA spacer region, by using *S. agalactiae, S. dysgalactiae* and *S. uberis* species-specific primers that generated 587 bp , 403 bp and 95 bp amplicons. 23S rRNA spacer region based studies were conducted by Riffon *et al*. (2001) for genotypic confirmation of *Streptococcus agalactiae* organisms isolated from bovine mastitic milk and they observed 587 bp PCR product. 16S-23S rRNA spacer region based studies were conducted by Forsman *et al.* (1997) for genotypic confirmation of *Streptococcus* spp. isolated from bovine mastitic milk and they observed 280 bp, 270 bp, and 330 bp PCR product for *S. agalactiae, S. dysgalactiae* and *S. uberis* respectively. Phuektes *et al.* (2001) also used 16S-23S rRNA spacer region for differentiating *S. agalactiae, S. dysgalactiae* and *S. uberis* based on amplicon size There have been reports (Khaled *et al.,* 2010), where amplification of other genomic sites *viz.,* 16S rRNA gene for differentiating *S. agalactiae (405 bp)* and *S. dysgalactiae (281 bp) by amplicon size.* . 16S rRNA , 23S rRNA and 16S-23S rRNA spacer region based PCR have been developed by Hassan *et al.* (2001) for *S. uberis* and they observed 445 bp, 451 bp, and 330 bp respectively for 16S rRNA , 23S rRNA and 16S-23S rRNA spacer region.

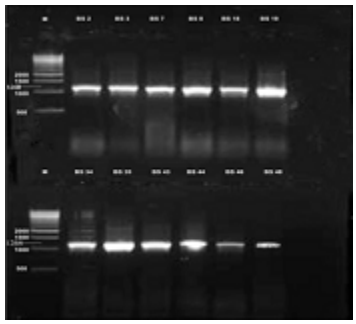
In the present study, all 8 isolates which had been biochemically identified as *P. aeruginosa* were subjected to genotyping using species-specific primers reported earlier (Spilker *et al.,* 2004). All 8 isolates subjected to PCR using species specific primers produced the product of 956 bp as reported by Spilker *et al.* (2004). All the isolates could be amplified using the said PCR indicates higher sensitivity and specificity of this test.

In the present investigation, all 5 isolates, which had been biochemically identified as *K. pneumoniae* were subjected to PCR amplification targeting 16S–23S rDNA internal transcribed spacer (ITS) region based specific primers (Liu *et al*., 2008) and our results are completely in agreement with that of Liu *et al*., 2008.

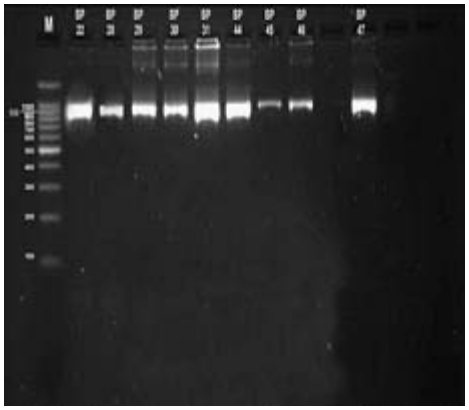
In conclusion, high percentage of positive samples and relatively high occurrence of environmental microorganisms indicates urgent need to emphisize udder hygiene.



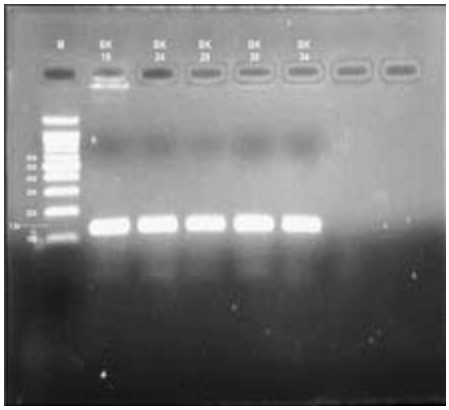
Agarose Gel electrophoresis of PCR amplified 23SrRNA gene (232 bp) of *E.coli* isolates. M: 100 bp DNA ladder



Agarose Gel electrophoresis of PCR amplified 23SrRNA gene (1268 bp) of S.aureus isolates. M: 1000 bp DNA ladder



Agarose Gel electrophoresis of PCR amplified 16SrRNA gene (956 bp) of *P. aeruginosa* isolates. M: 100 bp DNA ladder



Agarose Gel electrophoresis of PCR amplified 16S-23S rDNA inter transcribed spacer (ITS) region (110 bp) of *K. pneumoniae* isolates. M: 100 bp DNA ladder

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