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IDENTIFICATION AND ANTIBIOGRAM OF MICROBES ASSOCIATED WITH BOVINE MASTITIS

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An investigation of Mastitis in cattle was carried out in Anand city and in nearby villages of Gujarat state using California Mastitis Test (CMT) kit. The prevalence of clinical and subclinical mastitis was found to be 5.5% and 15.75%, respectively.* Staphylococcus aureus *was identified through strain specific polymerase chain reaction; the remaining isolates identified on the basis of molecular analysis by 16S rDNA sequencing and phylogenetic analysis were* Staphylococcus *species,* B. pumilus, Staphylococcus chromogenes, Bacillus *species, and* Pseudomonas *species. In vitro antimicrobial susceptibility pattern of all the isolates was checked against 13 different antibiotics using the agar disc diffusion method. Highest bacterial resistance was observed with penicillin G and oxacillin antibiotics. It was also observed that the patterns of bacterial resistance have not changed in India over the years. The data supports the decrease in the incidence of mastitis but the rate of decrease is minimal. More effective control strategies are required.

Keywords: Antimicrobial susceptibility; Mastitis; Prevalence; Sequencing; Strain specific polymerase chain reaction

Mastitis is derived from *masto*, referring to the mammary gland, and *itis*, meaning inflammation. Mastitis describes an inflammatory reaction in the mammary gland (1). Recognized worldwide as one of the costliest diseases affecting dairy herds, it reduces the milk yield and quality of milk and increases rate of culling and veterinary expenses. On the basis of habitat of pathogen, mastitis is classified as one of the following: (A) contagious mastitis, which is caused by contagious bacteria living on the skin of the teat and inside the udder, transmitted from one cow to another during milking (e.g., *Staphylococcus aureus* or *Streptococcus agalactiae*) and (B) environmental mastitis, which is caused by environmental pathogens normally found in

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the cow's surroundings such as bedding, manure, soil, and feed (e.g., *Escherichia coli*, *Streptococcus uberis*, *Klebsiella* sp.) (2).

A total of 137 microbial species, subspecies, and serovars have been isolated from the bovine mammary gland (3). In 1982, screening of crossbreed cows belonging to the Gujarat Agricultural University farms recorded 91.66% incidence of mastitis (4). Sources indicate 80% to 99% of all mastitis infections are caused by four types of organisms: Staphylococcal, *S. agalactiae*, other Streptococcal species, and Coliforms (5, 6).

It has been reported that the most common causes of udder disease include Staphylococci (*S.aureus* and *S. epidermidis*), Streptococci (*S. agalactiae, Streptococcus dysgalactiae*, *S. uberis*, and *Streptococcus bovis*), and Coliforms (mainly *E. coli* and *Klebsiella pneumoniae*) (7). Other etiological agents less frequently encountered include Pseudomonads, Nocardia, Mycoplasmas, and Yeast. In many countries the most common bacterial species causing mastitis are *S. aureus*, *S. dysgalactiae*, and *S. uberis* (8). Many workers have reported *S.aureus* as the most predominant mastitis agent of subclinical intramammary infection (9–11). *E. coli*, *K. pneumoniae*, and *Enterobacter aerogenes* are the most common coliforms in bovine mastitis (12).

MATERIALS AND METHODS

Sample Collection

A total of 400 quarter samples were diagnosed as subclinical mastitis using the California Mastitis Test (CMT) (13). The milk samples were obtained from the cattle farms of Anand city and in nearby villages of Gujarat state, India. Milk samples from the infected cattle were collected aseptically in sterile wide mouth glass stopper bottles. Before sample collection the udder was thoroughly washed with potassium permanganate solution (1:1000) and the teats were mopped with 70% ethyl alcohol.

Isolation and Enrichment of Microorganisms

Milk sample (0.1 mL) was spread on 5% sheep blood agar (SBA) plates and incubated at 37°C for 24–48 h. The colonies showing fine hemolytic zone were selected and purified. For enrichment of bacterial strains, 250 mL flasks containing 100 mL nutrient culture medium were inoculated with a single isolated colony of bacteria and incubated at 37°C on a rotary shaker (150 rpm) for overnight. Stable enrichment cultures were obtained after subculture.

In Vitro Antimicrobial Susceptibility Testing

Antimicrobial susceptibility patterns of all the isolates were checked using agar disc diffusion method against 13 different antibiotics: streptomycin (10 mcg/disc), lincomycin (10 mcg/disc), amoxicillin (10 mcg/disc), ampicillin (10 mcg/disc), trimethoprim (5 mcg/disc), oxacillin (30 mcg/disc), nalidixic acid (30 mcg/disc), oxytetracycline (1 mcg/disc), amikacin (10 mcg/disc), penicillin-G (10 mcg/disc), erythromycin (15 mcg/disc), tetracycline (30 mcg/disc), and gentamicin

(10 mcg/disc). The sensitivity was measured in terms of the diameter of the zone of inhibition surrounding the disc.

Molecular Characterization

DNA was extracted (14) from all the isolates and 100 ng of the genomic DNA was used to perform 16S rDNA PCR and strain specific (*S. aureus*) PCR using the specific primers (15, 16). PCR product obtained was sent to Bangalore Genei Services, Bangalore for partial length sequencing of 16S rDNA.

RESULT AND DISCUSSION

Out of 400 quarter samples, 85 (21.25%) were found infected, 22 (5.5%) showed clinical symptoms, and 63 (15.75%) were subclinically infected.

Given the fact that bacteria are responsible for the majority of the cases of mastitis, it is important to identify the genus and species of bacteria to apply effective disease control strategies and effective drug design.

For phylogenetic studies, the 16S rDNA gene was used. Universal primers were used to amplify the 16S rDNA gene providing the phylogenetic information (17). One isolate was identified as *S. aureus* by performing species specific PCR (16). The 16S rDNA products (product size 1.5 kb) (Fig. 1) of the rest of the isolates were sequenced and identified with NCBI Gene bank RDP-II database as *Staphylococcus* sp., *B. pumilus*, *S. chromogenes*, *Bacillus* sp., and *Pseudomonas* sp., Genbank accession numbers are HM234035, HM234036, HM234033, HM234034, and HM234037, respectively.

Confidence in phylogenetic analysis was assessed by Hillis and Bull (18) who stated that the bootstrap value of $\geq$70% usually corresponds to a probability of $\geq$95% that the corresponding clade is real. The species included in the present study were initially separated into clades by analyzing the entire dataset by neighbor-joining and by a simple heuristic search from maximum parsimony.

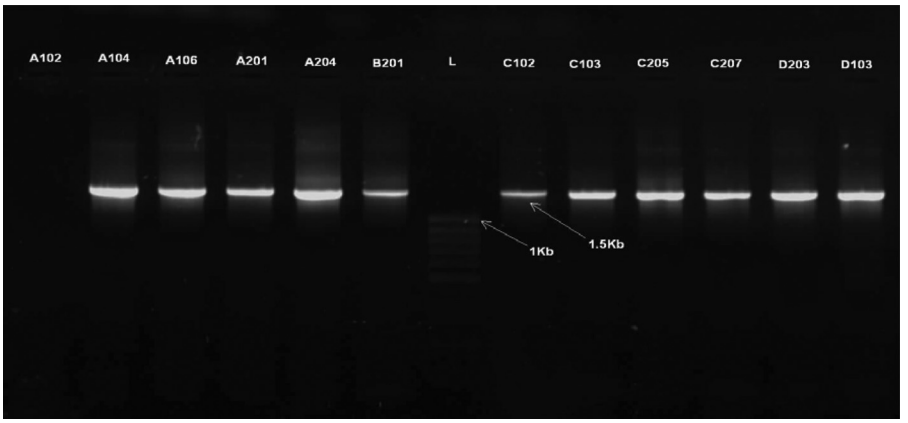

Figure 1 16S rDNA PCR product of 1.5 Kb on 1.5% agarose gel.

Sequences of the above isolates were aligned with sequences retrieved from the NCBI database and the relatedness of isolates to other organisms was determined by a phylogenetic tree based on two data sets (Fig. 2).

Out of the 12 isolates, 4 were resistant to at least three antibiotics and 2 were resistant to one antibiotic. A pattern of resistance against penicillin G, oxacillin, and trimethoprim was recorded in 2 isolates, while 1 isolate was slightly different with lincomycin resistance instead of trimethoprim. The remaining 2 isolates were resistant to nalidixic acid (Table 1). The adaptation might be due to prolonged treatment with these antibiotics in a particular area. These strains may have plasmid/s bearing the gene that imparts this resistance. Due to antibacterial usage over many decades,

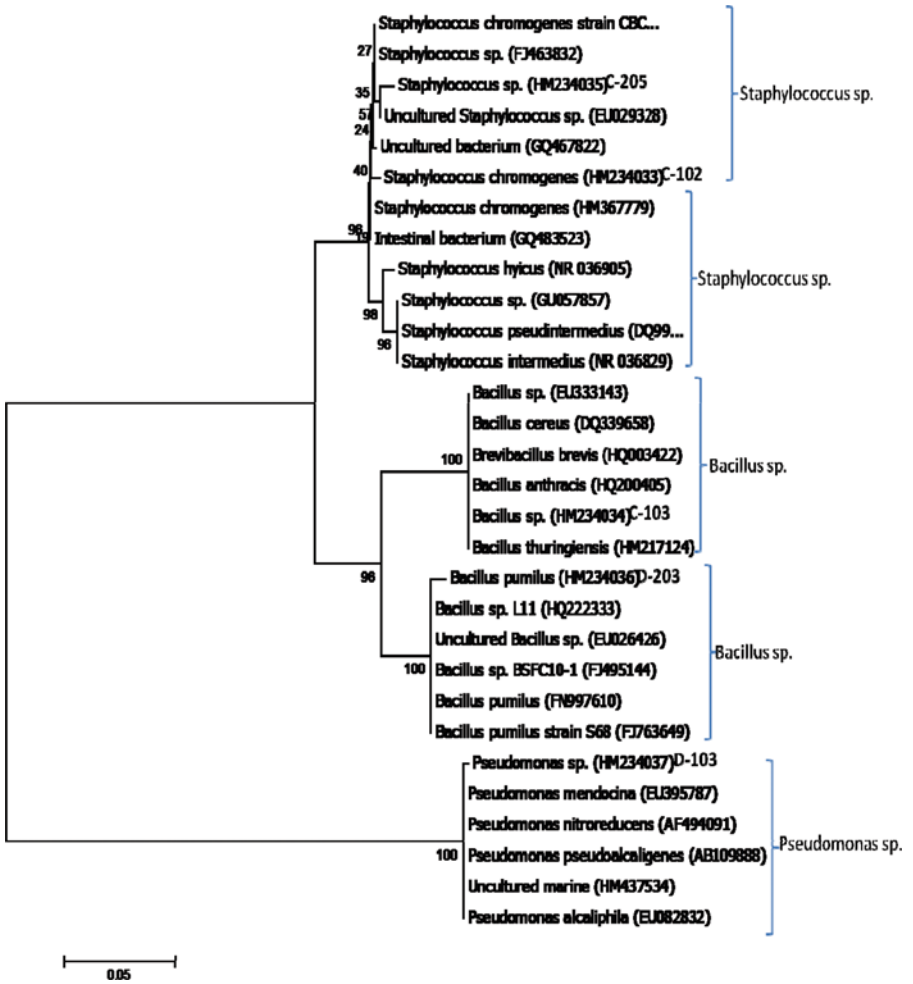

Figure 2 Phylogenetic analyses conducted in MEGA4. The numbers at the nodes indicate the levels of bootstrap support based on data for 1000 replicates to represent the evolutionary history of the taxa analyzed. Bootstrap values derived from the neighbor-joining method. The scale bar indicates 0.05 substitutions per nucleotide position.

Table 1 Antibiotic resistant pattern shows highest resistance of isolates toward Penicillin G and Oxacillin antibiotics

Isolate	Antibiotic resistance
A-102	Streptomycin (S^{10}), Lincomycin (L^{10}), Nalidixic acid (NA^{30})
A-104	—
A-106	—
A-201	—
A-204	Oxytetracycline (O^{30})
B-201	—
C-102	—
C-103	Penicillin G (P^{2}), Oxacillin (OX^{1}), Trimethoprim (TR^{5})
C-205	Nalidixic acid (NA^{30})
C-207	Penicillin G (P^{2}), Oxacillin (OX^{1}), Trimethoprim (TR^{5})
D-203	—
D-103	Penicillin G (P^{2}), Oxacillin (OX^{1}), Lincomycin (L^{10})

multiple drug resistance among the mastitis causing agents is a major problem in controlling intramammary infections. This is generally attributed to indiscriminate use of antibacterial drugs without prior drug susceptibility testing. Such antimicrobial resistant organisms can pose serious health related problems to animals as well as human beings.

The proportion of *S. aureus* isolated that were susceptible to ampicillin, erythromycin, and penicillin increased, whereas the annual proportion that were susceptible to cephalothin, ceftiofur, gentamicin, oxacillin, pirlimycin, sulfa-trimethoprim, and tetracycline did not change. The proportion of susceptible isolates to these antibacterials was consistently >95% (19). Antibiotic control programs have proved to be an effective way to reduce inappropriate use of antibiotics in hospitals. Such programs are sorely needed in the community, particularly in residence facilities for the elderly. The need for new antibiotics will continue because bacteria have a remarkable ability to overcome each new agent synthesized. Appropriate use of antibiotics will delay and in many cases prevent the emergence of the resistance.

In the present area of survey, gentamicin, enrofloxacin, ciprofloxacin, and chloramphenicol are not commonly used for treatment of mastitis, resulting in higher efficacy of these drugs. In India, agents causing mastitis were found to show the highest sensitivity to enrofloxacin (20, 21) and chloramphenicol (22) and the least sensitivity to ampicillin (20) and cloxacillin (20). Similar antibiogram patterns were also reported in Andhra Pradesh and Maharashtra states of India (23, 24), while Anakalo et al. (24) reported *Staphylococcus aureus* and Coagulase Negative *Staphylococcus* (CNS) as the major mastitis inducing pathogens, which is suggestive of a possible development of resistance from prolonged and indiscriminate use of beta-lactam antibiotics..

Ampicillin, penicillin, streptomycin, and oxytetracycline are commonly used antibiotics in bovine mastitis in the area of survey. The mastitic bacteria showed resistance to these commonly used antibiotics due to the frequent use of these antibiotics. Since Streptomycin has been extensively used along with Penicillin for treating mastitis, it may have led to the development of higher resistance in bacteria against these antibiotics.

The prevalence of clinical and subclinical mastitis were found to be 5.5% and 15.75%, respectively. Systematic records regarding the epidemiology of bovine mastitis including status of infection, antibiogram studies, and treatment patterns would provide useful management information to the producer, farmer, and veterinarian. Thus, there is a need to routinely investigate and record the epidemiology of bovine mastitis and antibiogram sensitivity of bacterial isolates in various parts of India. The data support a decrease in incidence of mastitis but the rate of decrease is minimal. More effective control strategies are required.

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