RESEARCH ARTICLE

Isolation and characterization of *Staphylococcus aureus* from bovine mastitis in Andhra Pradesh

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Abstract: *Staphylococcus aureus* is the key causative agent for contagious mastitis and responsible for subclinical infections leading to economic loss to dairy industry worldwide. Hence, the present work was planned to isolate and characterize the *Staphylococcus aureus* from bovine mastitis cases of *S. aureus* origin. From non therapeutic areas, a total of 381 staphylococcal isolates were recovered out of 438 milk samples with percentage positivity of 86.98, whereas, out of 60 milk samples in selected therapeutic areas 40 staphylococcal isolates with positivity of 66.67% were recovered based on cultural and biochemical tests. A total of 290 out of 381 and 30 out of 40 culturally positive isolates respectively from non therapeutic and therapeutic areas were confirmed as *S. aureus* by PCR method targeting the 16S rRNA with an expected amplicon size of 229bp. Sequencing and nucleotide analysis of 16S rRNA amplicons of non therapeutic area isolates showed 99.3% identity whereas therapeutic isolates showed 95-100% identity with gene bank reference strains. On phylogenetic analysis, isolates from non therapeutic area were not closely related to reference gene bank strains whereas isolates of therapeutic area were shown close evolutionary relationship with gene bank reference strains. Further, Peruru isolates of therapeutic area have shown close evolutionary relationship with Spain isolate KX348312.1 and formed separate group in cladogram.

Keywords: Contagious mastitis, *Staphylococcus aureus*, Subclinical mastitis, 16srRNA, Phylogenetic analysis

Introduction: Mastitis is the predominant intra mammary infection in dairy cattle in India causing huge economic loss to the dairy industry. Both clinical and subclinical bovine mastitis are responsible for reduction in milk production, deterioration of quality of milk and milk products, increased amount of health care expenditure, financial loss due to culling of sick animals and even mortality of cows. *S. aureus* is the major contagious agent of bovine mastitis. *S. aureus* is the most important and lethal agent (Jaradat et al. 2014) causing chronic and deep infections in mammary tissue and becomes difficult to treat successfully and is responsible for dairy scourge in the livestock industry (Husain et al. 2012a; Raza et al. 2013). It originates from the cows environment and infect the udder via the teat canal (Padhy et al. 2014). The staphylococci have been adapted to survive in the udder, usually establish chronic, subclinical infection and are shed in the milk becoming the source of infection for other healthy cows during milking process. The main source of infections for *S. aureus* mastitis is the udder of infected cows which is transferred via milkers hands, utensils, towels and the environment (floor) where the cows are kept (Radostitis et al. 2007).

The bacterial culturing of the raw milk is the standard procedure for diagnosis of the bacterial pathogens, but the method is time consuming. Several approaches regarding phenotyping and genotyping are being used to subtype the *S. aureus* isolates recovered from animals and human (Kalorey et al. 2007; Saei et al. 2009). The molecular diagnosis could be the most suitable technique for the identification of various circulating strains of pathogens which are difficult to identify by conventional methods. The molecular based techniques are much effective in pursuing the spread of bacterial infections and developing diagnostic methods. The 16S rRNA genes are highly conserved throughout bacterial evolution; hence it was targeted for molecular identification of *S. aureus* using PCR method.

Extensive and indiscriminate use of antibiotics in the treatment and control of mastitis lead to accumulation undesirable of drug residues in milk, emergence of antibiotic resistant bacteria and

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necessary withholding period. To avoid indiscriminate use of antibiotics and to reduce the cost of treatment, practice of antibiotic sensitivity testing should be adopted before initiation of treatment. Antimicrobial therapy is a primary tool for controlling Staphylococcal mastitis. Wide spread and indiscriminate use of antimicrobial agents has resulted in the emergence and transfer of resistant organisms to humans through milk. The emergence of resistance to antibiotics in gram-positive pathogens has become a major global issue (WHO, 2015). Hence, the present study was undertaken to isolate and identify *S. aureus* from bovine mastitis cases by both cultural and molecular methods. Further, antibiogram of the recovered *S. aureus* isolates was conducted to know the antibiotic of choice, sensitivity and resistance pattern for effective treatment and control of mastitis.

Materials and Methods

Collection of milk samples

A total of 498 milk samples were collected randomly from milch cattle in non therapeutic and therapeutic areas of Andhra Pradesh during the period from November, 2017 to July, 2018 shown in Table 1.

Isolation and Identification of *Staphylococcus aureus*

Processing of mastitis milk samples

Milk samples from bovine mastitis cases were subjected for isolation and identification of *Staphylococcus aureus* using conventional and cultural isolation methods. The collected milk samples were inoculated into BHI broth (5ml) and incubated at 37°C for 24 hrs (Cruickshank et al. 1975). After incubation a loopful of inoculated broth culture was streaked on to the selective differential media like Blood agar, MacConkey's agar and selective media like Mannitol salt agar media and incubated at 37 °C for 48 hrs in incubator and observed for morphology, cultural characteristics, Gram's staining, Biochemical confirmation by Catalase, Oxidase and Coagulase tests etc (Quinn et al. 2004).

Extraction of bacterial DNA

Table 1 Details of the milk samples screened against subclinical & clinical mastitis in non therapeutic and therapeutic areas of Andhra Pradesh

Area	No. of milk samples collected	No. of milk samples tested positive	Percent Positive
Non Therapeutic area	438	381	86.98
Therapeutic area	60	40	66.67
Total	498	421	

Table 2 Details of Oligonucleotide primers for amplification of 16SrRNA gene of *Staphylococcus aureus*

Target Gene	Primer Sequence (5'-3')	Amplicon Size (bp)	Reference
16S rRNA	16S-F-GTAGGTGGCAAGCGTTACC 16S-R CGCACATCAGCGTCAG	229	Lovesth et al. 2004

DNA was extracted according to the method of Arora et al (2006). Single pure bacterial colony was picked and dissolved in 50µl of distilled water in 1.5ml eppendorf tubes. The mixture was boiled at 96°C for 8 min in water bath. The tubes were removed and immediately chilled by placing on to the ice for 10min. All the bacterial lysates were centrifuged at 10,000 rpm for 5 min in refrigerated centrifuge at 4°C. From this snap chilled bacterial lysate, the supernatant containing genomic DNA was collected and stored at -20°C for conducting PCR.

Molecular Detection of *Staphylococcus aureus*

The culturally and biochemically positive *S. aureus* isolates were further confirmed genotypically using PCR with genus specific primers according to method of Pati and Reena Mukherjee (2016).

Primers

Oligonucleotide primers were obtained from Sigma Aldrich Chemical India Pvt.Ltd., Bangalore. Details of the primer sequences are shown in Table 2.

Polymerase chain reaction (PCR)

The PCR method is standardized according to the method of Lovesth et al 2004. The total reaction volume was set up to 25µl consisting of 2.5 µl 10x PCR buffer, 0.5 µl of 10mM dNTP mix, 0.3 µl of Taq polymerase, 100µM concentration of forward and reverse primer (0.5µl each), 1.5µl of MgCl₂ (25mM), 3µl of template DNA and remaining volume was made up with nuclease free water. The DNA template obtained by boiling and snap chilling extraction technique was subjected to PCR with genus specific primers, for the identification of *S. aureus*.

Steps and thermal cyclic conditions for 16SrRNA gene PCR

The amplification reactions were carried out in 0.2ml micro centrifuge tubes using programmable thermal cycler (Proflex PCR, applied bio-systems life technologies, Singapore) with initial denaturation at 95°C for 5min, followed by 35 cycles with

denaturation at 95°C-1min, 72°C-30sec and 72°C -90 seconds. A final extension step was also carried out at 72°C, for 90Sec.

Analysis of PCR products

Amplified PCR products were subjected to agarose gel electrophoresis as described by Sam brook and Russell (2001). PCR amplified product specific to *S. aureus* was visualized under U.V trans-illuminator and photographed with gel documentation system (BIO-RAD, Gel Doc™).

DNA sequence analysis

Amplified PCR products of 16SrRNA gene were sequenced and sequence analysis was done using NCBI BLAST for similarity and percent identity searches with the gene sequences available in the Gene bank. After confirmation of *Staphylococcus aureus*, the nucleotide sequences were submitted to gene bank through sequin submission portal for obtaining accession numbers. Nucleotide sequencing and Phylogenetic (Cluster) analysis was done by multiple sequence alignment using online software Clustal X 2.1. The phylogenetic analysis was done using the MEGA 6.06 program.

Results and Discussion

Cultural isolation

A total of 381 *Staphylococcal* isolates were recovered on cultural isolation with positivity of 86.98% from 438 milk samples collected from non therapeutic areas. Similarly, a total of 40 *Staphylococcal* isolates from therapeutic areas were obtained from 60 milk samples on cultural isolation with positivity of 66.67% (Table 1). Various workers reported the positivity rate of 80.98% from Maharashtra (Yadav 2018) and Sanotharan et al. 2016 from Srilanka (90.5%). However, Qudri et al.2017 recorded low positivity rate of 42.2 from Srinagar. Inconsistency and irrational use of antibiotics indicated the variations in positive percent of our findings and earlier studies from non therapeutic areas. Whereas, the positivity rate (66.67%) of our study in the therapeutic areas were in coincidence with the earlier reports of Jyothi et al. (2018) from Hyderabad with 62.9%; Bhat et al. (2017) from Jammu with 60.80% and Mubarak et al.(2012) from Tamilnadu with 60.80%. However, high prevalence was reported by Mpatswenumugab et al. (2017) from Rwanda with 72.1% and Pati and Reena Mukherjee (2016) from Uttar Pradesh with 71.87%. Whereas low prevalence rate was recorded by Harini and Sumati 2011 from Karnataka (58%) and 46.3% by Jena et al. 2015 from Rajasthan.

The variability in occurrence of *S. aureus* in mastitis cows among different reports might be that *S.aureus* is a contagious pathogen transmitted from one cow to another in a herd by contact via unhygienic milking procedures and poor farm management practices, environmental inconsistency and lack of awareness among dairy farmers.

Lane M: 100bp

DNA ladder

Lane 1-5:

positive *S. aureus* isolates

Fig. 1 PCR amplification of 16SrRNA gene of *S. aureus* isolates-Non therapeutic areas

All the 421 isolates from non therapeutic and therapeutic areas were shown hemolytic colonies on blood agar media; on mannitol salt agar media, yellow color round glistening colonies indicative of Coagulase Positive; pink color colonies indicative of Coagulase Negative and no growth on Macconkey's agar suggesting positive for *S. aureus*. On Gram's staining reaction all positive isolates revealed gram positive cocci with grape like clusters and biochemically positive to catalase test and negative to oxidase test.

Similarly, 271 isolates of *S. aureus* (71.0%) out of 381 isolates from non therapeutic areas and 27 isolates out of 40 isolates from therapeutic areas were positive for Coagulase test

Molecular Characterization of *S.aureus* isolates

All the *S. aureus* isolates which positive phenotypically were subjected to genotypic confirmation using 16SrRNA PCR. Out of 381, 291 (73.5%) *S. aureus* isolates from non therapeutic areas (Fig 1) and 30 isolates (75%) out of 40 *S.aureus* isolates from therapeutic areas yielded expected amplicon size of 229 bp (Fig 2). It has been used by several workers (Pati and Reena Mukerjee, 2016; WHO 2015; Lange et al.2015; Ciftci et al. 2009; Zhang et al. 2004; Krimmer et al. 1999). During the present study the identification rate of 73.5 to 66.67% was reported and it was less than those reported by earlier workers Lange et al. 2015 (95%); Pati and Reena Mukerjee 2016 (83.8%) and Mellmann et al. 2006 (91%) respectively.

The results of multiple nucleotide alignment of 16SrRNA gene of all the isolates (291) from non therapeutic areas showed nucleotide identity in the range of 99.3% with reference gene bank strain of Assam (Accession no.KP337596.1), Bihar strain (Accession no .MH 255793.1),UttarPradesh (Accession no. KX 181851.1), German (GU 459255.1), Spain (Accession no.KX 348312.1) and USA strain (KJ 83377.1). Similarly multiple nucleotide alignment of 16Sr RNA Gene of all the isolates from therapeutic

Lane M: 100 bp
DNA ladder
Lane 2-7: positive
S. aureus isolates
Lane 1: Negative
control

Fig. 2 PCR amplification of 16S rRNA gene of *S. aureus* isolates -Therapeutic areas

areas (LFC,SKP&Peruru) showed a nucleotide identity in the range of 95-100% on comparison with reference strains in the gene bank database. The partial 16Sr RNA Sequencing differentiated 95% of the isolates tested for species identification in the present study. The identification rate of 95% obtained in this study was higher than those reported by Mellmann et al. (2006) with 91%.

On phylogenetic analysis, isolates from non therapeutic areas were not closely related to reference gene bank strains (Fig 3). Whereas isolates of therapeutic areas viz., Livestock Farm Complex (LFC) and Sorakayalapalem (SKP) shown close evolutionary relationship with gene bank reference strains. However, Peruru isolates have shown close evolutionary relationship with Spain isolate KX348312.1 and formed separate clade.

During the study, antibiotic sensitivity test was conducted on *S. aureus* isolates recovered from non therapeutic areas and selected therapeutic areas. The antibiotic sensitivity test results revealed that the isolates recovered from non therapeutic areas showed higher sensitivity to Amoxicilli 60.38% (64/106) followed by Ampicillin 56.60% (60/106), Enrofloxacin 53.77% (57/106), Ciprofloxacin 46.23% (49/106), Gentamycin 26.42% (28/106), Tetracycline 20.75% (22/106), Streptomycin 12.26% (13/106), Kanamycin 9.43% (10/106) and Amikacin 7.55% (8/106%) but were resistant to Amikacin 92.45% (98/106) followed by Kanamycin 90.57% (96/106), Streptomycin 87.74% (93/106), Tetracycline 79.25% (84/106), Gentamycin 73.58% (78/106), Penicillin-G 56.60% (60/106), Ciprofloxacin 53.77% (57/106), Enrofloxacin 46.23% (49/106) Ampicillin 43.40% (46/106) and Amoxicillin 39.62% (42/106) respectively. This was in accordance with reports of Qayyum et al. (2016); Hussain et al. (2012a) and Idriss et al (2014). But in therapeutic areas higher sensitivity to Enrofloxacin 66.67%

Fig.3 Phylogenetic analysis of 16S rRNA gene of *S. aureus* isolates

followed by Ciprofloxacin 59.25%, Amoxicillin 44.44% and Ampicillin 40.74%. Similar sensitivity pattern of *S. aureus* isolates to Enrofloxacin was reported by Baghel et al.2018; Dar et al. 2014; Bhanot et al. 2012 ; Kumar and Sharma 2002 ; Sahoo et al. 2009; Joshi et al. 2006 and Ranjan et al. 2010. It could be due to lesser usage of these antibiotics in majority of districts of Andhra Pradesh in treating the mastitis cases.

In the present study among Quinolones, Enrofloxacin 66.67% Ciprofloxacin 59.25% and beta lactamase antibiotics like Amoxicillin 63.7% and Ampicillin 56.6% were found to be effective in therapeutic study areas. The extensive use of antibiotics in treatment and control of mastitis has possible implications on human health through emergence of antibiotic resistant bacteria. The antibiotic sensitivity profile of isolated pathogens can serve as a guide for field veterinary practitioners to provide effective and timely treatment of mastitis cattle.

Conclusions

The present work was undertaken with an objective of isolation and characterization of *S. aureus* from clinical and subclinical cases of bovine mastitis and to conduct antibiogram of all *S. aureus* isolates to know the antibiotic of choice for the treatment of bovine mastitis caused by *S. aureus*. Hence, the present study revealed the isolation, identification and phenotypic characterization of *S. aureus* strains by cultural and biochemical tests; genotypically using 16SrRNA PCR. Further, it was reported that 16SrRNA PCR was found to be powerful and simple tool for the identification of *S. aureus* from bovine mastitis. Thus the findings of this study in non therapeutic and therapeutic areas would be helpful in detecting the existence and emergence of *S. aureus* isolates, antibiotic resistant pathogens and useful in selection of isolate for preparation of vaccine for effective control of clinical and sub clinical mastitis caused by *S. aureus*.

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