Molecular Characterization and Toxicity Confirmation of LukM/F'-PV Producing *Staphylococcus aureus* Isolated from Bovine Mastitis Samples in Mysore, India

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Abstract The widespread status of subclinical condition of bovine mastitis is often associated with the production of leukotoxin M/F'-PV producing *Staphylococcus aureus*. The present study aims for the profiling of such leukotoxin producers through conventional and molecular methods in parallel to their leukotoxicity. The incidence of this particular pathogen was assessed in mastitis infected Holstein–Friesian cattle, where eight isolates of staphylococci were found to be present in 20 % of collected samples. Being intermediately resistant to vancomycin, they showed characteristic double zone hemolysis on 7 % sheep blood agar and typical type II reaction for coagulase test indicating the pathogenic attributes. Further with RAPD-PCR and 16S rDNA-RFLP, epidemiological specificity and genotypic relatedness of isolates to *S. aureus* was confirmed. Subsequently, the presence of leukotoxin (*lukM*) gene in native isolates was detected by leukotoxin gene specific PCR. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay evaluated for secreted leukotoxin in cell free supernatant was estimated to be 223 toxic units which had an LD₅₀ cytotoxic activity on bovine neutrophil. Thus, the data acquired during study can be of prime diagnostic method for timely and accurate analysis of subclinical mastitis samples which goes undetected at consumer level.

Keywords Vancomycin · RAPD · MTT · Leukotoxicity

Introduction

Staphylococcus aureus being ubiquitous among the bacterial genera ranks the highest order for causing infectious disease

outbreaks around the world. At the site of infection, *S. aureus* secretes several exoproteins [1] among which heterodimeric beta-pore forming toxins (PFT) constitutes one of the most important groups besides the superantigens [2]. Apart from human source, the reported pathogenic *S. aureus* strains isolated from animal counterparts of bovine, ewe and goat secretes Luk M/F'-PV, a highly active bicomponent leukotoxin pair which is considered as the primary contributor for virulent attributes in mastitis [3–5]. The toxin once secreted, causes intensive damage of bovine mammary tissues which generally result in discharge of pathogen to other areas of mammary glands. Therefore, the mastitis would be often categorized to sub-clinical or clinical conditions [6, 7]. Hence, the mastitis inducing leukotoxin producers are considered to be a threat to the bovine especially of high milk yielding varieties where the quantity and quality of the milk produced will be greatly reduced and at some extremes can even cause the death of cattle. In such instances, the identification of the specific organism during intramammary infection is subjected to error because of unavailability of appropriate diagnostic procedures. Therefore, conventional and reliable molecular means were aimed in this study for the easier diagnosis and characterization of leukotoxin producing *S. aureus* for controlling the further development of disease from mastitis to non mastitis herds.

Materials and Methods

Sample Collection

A total of 21 mastitis cases of bovine were investigated for a period of 6 months at individual quarter level and information on age, parity with lactation period was gathered. The milk samples collected under study were from

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subclinical cases of Holstein–Friesian cows. Sampling of milk was carried out with the aid of veterinary experts where the infected quarter was washed, dried and few milk streams were forcibly stripped. Thereafter a 10 ml of milk stream was collected into sterile screw capped bottle after the teat was scrubbed with sterile cotton swabs soaked in 70 % alcohol. The collected samples were immediately transferred to the laboratory for bacterial analysis under sterile conditions.

Screening and Isolation of Bacteria

Bacterial Analysis

Approximately 10 µl of fresh milk sample was inoculated to nutrient agar supplemented with 7 % defibrinated sheep blood, Baird parker agar with 1 % potassium tellurite and egg yolk emulsion as additives and MacConkey agar plates for selective screening of *S. aureus*. The plates were incubated at 37 °C for 24 h and examined for characteristic bacterial growth on the respective inoculated plates.

Morphological and Biochemical Characterization

The isolated cultures were preliminarily characterized by routine laboratory methods following Gram's staining, scanning electron microscopy, catalase and tube coagulase tests. Deoxyribonuclease and thermonuclease activity on nucleic acids were performed with the help of HIMEDIA instruction manual. Further for all the experiments carried out, *S. aureus* (FB278 and MTCC96), *Staphylococci sp* (Ssp)—a native strain of bovine obtained from Veterinary college, Bangalore and *Lactobacillus casei* (MTCC 1423) were taken as positive and negative controls respectively.

Scanning Electron Microscopy

For preparation of the sample, protocol described by [8] was followed. The culture isolate grown in BHI broth was fixed with 2 % Glutaraldehyde and subjected to alcoholic dehydration and analyzed under scanning electron Microscope (Leo-435, Zeiss Ltd, UK).

Deoxyribonuclease and Thermonuclease Tests

The DNase agar was inoculated with 0.2 ml of inoculum to the wells made on the agar plate. The remaining inoculum was boiled for 15 min, centrifuged and 0.2 ml of the supernatant was inoculated to one more plate with DNase medium. Both the inoculated plates were incubated at 37 °C for 24 h and the plates were flooded with 1 % HCl before observation. Any zone observed around the well

indicated positive result for the degradation of DNA incorporated in the medium.

Disk Diffusion Susceptibility Testing

Antibiotic susceptibility profiling was carried out for one of the representative isolate using the HiMedia Octodiscs G-III plus. The following eight antibiotic agents that target the pathogenic bacteria was included in the test: Amikacin (AK; 10 mcg), Amoxicillin (AM; 10 mcg), Bacitracin (B; 10 units) Cephalothin (CH; 30 mcg) Erythromycin (E; 15 mcg), Novobiocin (NV; 30 mcg), Oxytetracycline (O; 30 mcg) and Vancomycin (VA; 30 mcg). The octodisc with the antibiotic was impregnated at the center of the inoculated plate with the putative native isolate of *S. aureus* and was incubated at 37 °C for 24 h. After the incubation period, based on inhibition zone sizes formed around the discs the results were recorded as sensitive, intermediate and resistant using the interpretation chart supplied by the antibiotic disc manufacturers.

Molecular Characterization

RAPD-PCR, 16S rDNA PCR and 16S rDNA RFLP was employed for the identification and differentiation of the isolates at species and strain level. The Genomic DNA used as template was extracted from *S. aureus* as described by Ravinder Kumar et al. (Unpublished data) with slight modifications where sonication was used as the strategy to lyse the cell wall.

RAPD-PCR Analysis of Genomic DNA

The arbitrary universal oligonucleotide M13 primer (5'-GAGGGTGGCGGTTCT-3') was used to study the genetic diversity and polymorphism of the selected isolates [9]. The reaction was prepared in a final volume of 25 µl with 20 pmol primer of M13. The PCR condition was programmed in a thermal cycler (MWG-Primus), comprising one cycle of denaturation at 95 °C/3 min, followed by 35 cycles of 95 °C/1 min, 43 °C/1 min 45 s, 72 °C/2 min and a final extension of 72 °C/5 min. Amplified PCR products were separated by gel electrophoresis on 1.8 % (w/v) agarose gel along with 10 kb DNA ladder (Fermentas, Canada) and visualized under UV trans-illuminator (Genei, Bangalore) after staining with ethidium bromide.

PCR-Ribotyping

The 16S rDNA gene was amplified using primers BSF-5'-GAGTTTGATCCTGG CTCAGG-3' and BSR-5'-TCAT CTGTCGTCCCACCTTCGGC-3' [10]. Amplification reactions were performed in a total volume of 25 µl with

10 pmol primers and were carried out in thermal cycler for 1 cycle of 94 °C/3 min, followed by 30 cycles of 94 °C/1 min, 55 °C/1 min and 72 °C/1 min and a final extension of 4 min for 72 °C. The amplicon size was visualized corresponding to the 10 kb DNA ladder. The fragment was eluted, cloned and sequenced at VIMTA labs, Hyderabad-India and BLAST search [11] was employed for the analysis of ribotyped sequences.

PCR–RFLP

The obtained 16S rDNA PCR-amplified product (5 µl) was subjected to digestion at 37 °C for 6 h with *Alu1* in a 20 µl reaction mixture containing 2 U of enzyme, 2 µl 10× RE buffer and distilled water. The digested products were separated on 1.5 % agarose gel and were documented using Biorad gel doc system.

Leukotoxin Gene Detection by PCR

The detection primers for the specific amplification of *S. aureus lukM* component of LukM/F'-PV were designed based on the available sequences of NCBI database. The thermal cycling conditions were standardized for the amplification of *lukM* gene with 25 µl reaction mix containing 10 pmol of primers LukMF1 (5'-CAACTTTGTCGCTAG GTCT-3') and LukMR1 (5'-AAGATTCAGGCGATACG AG-3'). The amplification was programmed at one denaturation step of 94 °C/3 min followed by 35 cycles of 94 °C/1 min, 54 °C/45 s, 72 °C/1 min and a final extension step of 72 °C/10 min. Further, a 4 µl of PCR product diluted 100 times was taken for nested PCR with LukMF2 (5'-AAGATATTGGCGACGATG-3') and LukMR1 as primers of 10 pmol each. The PCR programme was maintained as per the amplification of *LukM* gene. The expected gene specific amplicon was cloned and sequenced.

MTT Assay for Leukotoxicity

To determine the functionality of leukotoxin M/F'-PV secreted by the native isolate of *S. aureus*, the colorimetric MTT dye reduction assay was performed as described by [12] with slight modifications. Briefly, the target neutrophil cells were isolated from the EDTA treated bovine blood was dissolved in 1 ml of RPMI1640 medium for further assays. Subsequently cell viability was assayed with varying number of neutrophil counts for 4 h to evaluate the reduction of yellow tetrazolium to purple formazan salt indicated by the optical density (OD) of 0.4–0.6 units measured at 570 nm using a spectrophotometer (Biorad).

The cell free supernatant of the late exponentially grown staphylococcal culture was used as toxin source for cell cytotoxicity assay. In a 96 well flat bottomed plate, the cell

free supernatant was serially diluted to two fold in 1× PBS buffer and the cell count which showed an OD of 0.5 units was added and incubated at 37 °C for 1 h. Untreated cells were used as control and each of the experiments were carried out in three trials. Further to the treated and untreated cells, 20 µl of 5 mg/ml of MTT was added and the plates were reincubated in a humidified chamber with 5 % CO₂ at 37 °C for 4 h. Later the formed crystals of purple formazan were solubilized in 200 µl of DMSO and after 15 min of incubation at room temperature the reaction was read at 570 nm. The % cytotoxicity is inversely proportional to the log₂ dilution of the leukotoxin preparation and is calculated by the formula $1 - (\text{OD of cells treated with leukotoxin} / \text{OD of untreated cells}) \times 100$. Therefore one toxin unit is defined as the amount of leukotoxin preparation that can cause death of 50 % (LD₅₀) bovine neutrophil cells.

Results

Screening, Isolation and Susceptibility of Bacteria

Among the 21 mastitis milk samples screened, about 142 putative staphylococci isolates were subjected to further studies. Based on colony morphology, presumptive staphylococcal colonies from Sample#01(01 dh, 01, 05), Sample#09 (9C), Sample#12 (12, 06) and Sample#17 (17a, 17b, 17c) showed characteristic double zone hemolysis on 7 % sheep blood agar (Fig. 1a, b) from the 4 h of incubation in 6–10 cfu/10 µl of sample tested. Distinguished grayish black colonies upon potassium tellurite reduction with a halo zone surrounding them on Baird parker agar (Fig. 1c) along with no growth on MacConkey agar were selected for further morphological and biochemical characterization. The selected isolates were Gram-positive and showed clustered grape like morphology under scanning electron microscope (Fig. 1d). The isolates were positive for catalase and showed typical type II reaction by coagulating rabbit plasma which is due to the free coagulase produced by them. A complete clear zone was observed around the wells on DNase media indicating that the organism produces both DNase and thermonuclease (Fig. 1e, f) for degrading nucleic acid surrounding them. Additionally, the in vitro antibiogram studies analyzed for isolates revealed an intermediate resistance to vancomycin and high sensitivity pattern in the range of 16–40 mm (in dia) zone of inhibition for the rest of antibiotics tested (Table 1).

Molecular Characterization and PCR Detection of *lukM*

The isolates characterized with M13 RAPD showed the prominent fingerprints of size 600, 800 bp, 1.2, 1.5 and

3 kb with 10 kb marker (Fig. 2a), suggesting that the same species of staphylococci prevailed in different localities of Mysore responsible for bovine mastitis. BLAST searches for the sequenced 1.2 kb ribotyped product of the representative isolate indicated high sequence similarity towards *S. aureus* (GenBank: FJ899095.1). Thereafter, the RFLP for *AluI* digested 16S rDNA product with band size of 100, 200 and 700 bp (Fig. 2b) revealed similar intraspecific restriction pattern among the screened strains of *S. aureus*. Finally, PCR assay developed for the detection of *lukM* from native isolates, yielded the expected amplicon of 725 bp and with the nested PCR, a band size of 625 bp (Fig. 3) was obtained. Later, the sequences of 725 bp product analyzed with BLAST, revealed a score of high identity towards the *lukM* gene of RF122 which indicated the presence of *lukM* gene coding sequences in all the examined cultures of *S. aureus*.

Leukotoxicity Assay

The reduction of MTT to purple formazan was linearly correlated to the number of neutrophil cells seeded to each well. In order to accomplish 0.5 units at OD₅₇₀ an optimum number of 3×10^5 cells were found to be necessary (Fig. 4) for the assay. The 50 µl sample tested containing log₂

dilution of *S. aureus* leukotoxin showed $2^{7.8} = 223$ toxic unit which represents 50 % cytotoxic activity (Fig. 5).

Discussion

Bovine mastitis has become a major threat to the dairy world ever since the cattle were exploited for high yield in milk production. As reported, mastitis is found to be more predominant in Friesian breeds when compared to Jersey and Ayrshire heifers [13]. In general, the cows more than 10 years were prone to subclinical phase than the younger ones and which have calved more than seven had a greater chance for developing udder infections with more possibility for the mounting of clinical mastitis during the first 3 months of lactation [14, 15]. These reports supported the present finding where LukM/F'-PV coding *S. aureus* was found to be more prominent in the sub clinical samples collected from the Friesian breeds between the age group of 6–10 years and have calved 4–6 times with 3–7 weeks of lactation time period.

India being one of the largest producers of milk is at risk with respect to bovine mastitis and in particular, subclinical mastitis is found to be more important varying from 10 to 50 % in cows and 5 to 20 % in buffaloes than clinical

Fig. 1 a Double zone hemolysis on 7 % blood agar, b Comparison with positive and negative isolates for hemolysis, c Colonies on Baird Parker Agar, d SEM Photograph of *S. aureus* (Magnification: $\times 25k$), e, f DNase and TNase activity on Deoxyribonuclease Agar

Table 1 Antibigram profile for the isolated culture of *Staphylococcus aureus* (17c)

S. no.	Antibiotic tested	Disc conc	Zone of inhibition (in mm)	Range of sensitivity (in mm)	Interpretation
1.	Amikacin	10 µg	25	20–26	Sensitive
2.	Amoxycillin	10 µg	40	28–36	Sensitive
3.	Bacitracin	10 units	25	12–22	Sensitive
4.	Cephalothin	30 µg	40	29–37	Sensitive
5.	Erythromycin	15 µg	28	22–30	Sensitive
6.	Novobiocin	30 µg	30	22–31	Sensitive
7.	Oxytetracycline	30 µg	30	24–30	Sensitive
8.	Vancomycin	30 µg	16	17–21	Intermediate

Fig. 2 Molecular characterization of the native isolates. **a** M13 RAPD-PCR. Lanes 1 Marker (10 kb), 2–9 Native isolates of *S. aureus*, 10 *S. aureus* MTCC96, **b** PCR-RFLP: Lanes 1 Marker (10 kbp), 2–9: *AluI* digested products of 16S rDNA amplicon of the native isolates **Fig. 3** PCR detection of *lukM*. Lane M Marker 10 kb, 1–5 725 bp *lukM* amplicon with LukMF1/LukMR1, 6 Nested PCR of 625 bp amplicon with LukMF2/LukMR1 **Fig. 4** Cell viability assay. Increase in MTT formazan in well with relation to number of neutrophil cells

stages [16]. During such subclinical forms, the latent inflammatory reaction within the mammary gland is detectable only through the culturing of milk for the identification of the residing pathogen [17]. Hence, an attempt made in the study with the direct culturing of milk

to identify as well as to characterize the major mastitis pathogen LukM/F'-PV producer *S. aureus* with reliable methods was successful at quarter level. The data acquired from the conventional procedure for the double zone hemolysis observed on 7 % blood agar after 4–24 h incubation at 37 °C with lukM/F'-PV *S. aureus* can become an

Fig. 5 Leukotoxicity assay. Percent leukotoxicity of bovine neutrophil (30×10^4) in relation to the *S. aureus* leukotoxin added for the MTT assay

important criteria for screening the bacteria at preliminary stages. Further, failure in treatment for mastitis is due to indiscriminate use of antibiotics without prior testing for in vitro sensitivity and not considering the documented antibiotic resistance strains [18, 19]. Thus, the antibiogram profile of the present study provides a suitable antibiotic for effective control of *S. aureus* at the onset of subclinical mastitis.

In recent years, molecular approaches have become a major concern for bacterial identification and to type pathogens at generic and species level for epidemiological studies; RAPD-PCR and ribotyping have been extensively used in the detection of various pathogens [20, 21]. Henceforth for sensitive and rapid diagnosis, M13 RAPD-PCR along with 16S rDNA-RFLP applied for genetical identification of the isolates clearly indicated the phylogenetic homogeneity among the *S. aureus* associated with the spread of bovine mastitis in different localities of Mysore. Gene specific PCR carried out for the leukotoxin trait, confirmed the presence of *lukM* in all the strains screened from subclinically affected milk samples owing to the first report for identification of LukM/F'-PV producing *S. aureus* from the region studied. The cell free supernatant of one of the representative *lukM* positive isolate analyzed by MTT assay showed that the leukotoxin was produced and they were toxic to neutrophil cells with LD₅₀ of 223 toxic units. This data was supported by the results of Rainard et al. [5] that the presence of *lukM* gene is responsible for strong leukotoxicity in LukM producing isolates.

In conclusion, the early diagnosis of the toxigenic LukM/F'-PV producing *S. aureus* along with antimicrobial therapy can be of great importance in the prevention of mammary gland tissue damage and thereby subsequently to protect against secondary infections caused by other invading pathogens. Apart from conventional methods, the potential molecular techniques can become an ideal

characterization tool for the rapid identification of the organism at an accurate level for epidemiological specificity. Thus, these reliable methods can be utilized for population monitoring studies to control the mastitis spread from quarter to herd level.

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References

- Dinges MM, Orwin PM, Schlieveret PM (2000) Exotoxins of *Staphylococcus aureus*. Clin Microbiol Rev 13:16–34
- Prevost G (2005) Toxins in *Staphylococcus aureus* pathogenesis. In: Proft T (ed) Molecular and cellular biology. Horizon Bioscience Publishers, Norfolk, pp 243–284
- Barrio B, Rainard P, Prevost G (2006) LukM/LukF'-PV is the most active *Staphylococcus aureus* leukotoxin on bovine neutrophils. Microbes Infect 8:2068–2074
- Kaneko J, Muramoto K, Kamio Y (1997) Gene of LukF-PV-like component of Panton–Valentine leukocidin in *Staphylococcus aureus* P83 is linked with LukM. Biosci Biotechnol Biochem 61:541–544
- Rainard P, Corrales JC, Barrio B, Cochard T, Poutrel B (2003) Leukotoxic activities of *Staphylococcus aureus* strains isolated from cows, ewes, and goats with mastitis: importance of LukM/LukF'-PV leukotoxin. Clin Diagn Lab Immunol 10:272–277
- Bachaya HA, Raza MA, Murtaza S, Akbar FUR (2011) Sub-clinical bovine mastitis in Muzaffargarh district of Punjab (Pakistan). J Anim Plant Sci 21:16–19
- Lam TJGM, Olde Riekerink RGM, Sanpimon OC, Smith H (2009) Mastitis diagnostics and performance monitoring: a practical approach. Irish Vet J 62:34–39
- McDougall LA, Holzapfel WH, Schillinger U, Feely DE, Rupnow JH (1994) Scanning electron microscopy of target cells and molecular weight determination of bacteriocin produced by *Lactococcus lactis* D53. Int J Food Microbiol 24:294–308
- Schillinger U, Yousif NMK, Sesar L, Franz CMAP (2003) Use of group specific and RAPD-PCR analysis for rapid differentiation of *Lactobacillus* strains from probiotic yoghurts. Curr Microbiol 47:453–456
- Halami PM (2008) Production of polyhydroxyalkanoate from starch by the native isolate *Bacillus cereus* CFR06. World J Microbiol Biotechnol 24:805–812
- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped blast and PSI-blast: a new generation of protein database search programs. Nucleic Acid Res 25:3389–3402
- Chung W, Backstrom LR, McDonald J, Collins MT (1993) The (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) colorimetric assay for the quantification of *Actinobacillus pleuropneumoniae* cytotoxin. Can J Vet Res 57:159–165
- Compton CWR, McDougall S, Parker K, Heuer C (2007) Risk factors for peripartum mastitis in pasture-grazed dairy heifers. J Dairy Sci 90:4171–4180
- Biffa D, Debelo E, Beyene F (2005) Prevalence and risk factors of mastitis in lactating dairy cows in southern Ethiopia. Int J Appl Res Vet Med 3:189–198

15. Corbett R (2009) Minimizing the effects of immunosuppression through management and nutrition. In: Proceedings NMC Annual Meeting, pp 113–119
16. Joshi S, Gokhale S (2006) Status of mastitis as an emerging disease in improved and periurban dairy farms in India. *Ann N Y Acad Sci* 1081:74–83
17. Sudhan NA, Neelesh S (2010) Mastitis—an important production disease of dairy animals. SMVS' Dairy Year Book, Ghaziabad, pp 72–88
18. Hoekstra KA, Paulton RJL (2002) Clinical prevalence and antimicrobial susceptibility of *Staphylococcus aureus* and *Staphylococcus intermedius* in dogs. *J Appl Microbiol* 93:406–413
19. Sudhakar PA, Narendra VK, Vikas MS, Mangesh SM (2009) Prevalence and current antibiogram trend of mastitis agents in Udgir and its vicinity, Maharashtra state, India. *Int J Dairy Sci* 3:117–122
20. Sabat G, Rose P, Hickey WJ, Harkin JM (2000) Selective and sensitive method for PCR amplification of *Escherichia coli* 16S rDNA genes in soil. *Appl Environ Microbiol* 66:844–849
21. Biswajit M, Malathi S, Karunasagar I, Indrani K (2009) Evaluation of RAPD-PCR and protein profile analysis to differentiate *Vibrio harveyi* strains prevalent along the south west coast of India. *J Genet* 8:273–279