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Genotypic identification of methicillin resistance and virulence factors in *Staphylococcus* spp. from bovine mastitis milk

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Abstract The aim of this study was the genotypic characterization of methicillin resistance and virulence of *Staphylococcus* spp. from bovine mastitis cases. The study was designed to collect milk samples from 120 animals (90 lactating cows and 30 buffaloes) and screened using California mastitis test and somatic cell counts. *Staphylococci* were isolated on Baird-Parker, mannitol salt and identified based on their physiologically and species-specific biochemical tests. Antibiotic resistivity assay was performed using standard antibiotic disc diffusion technique for screening. On the basis of antibiograms, 26 strains were selected. Selective isolates were identified based on 16S rDNA gene sequencing. Virulence genes associated with *Staphylococcus* sp. viz. *coa*, *ccr*, *sar*, *spa*, *clf*A, *ica* complex, *fnb*A, and *mec*A were amplified on screened isolates. A total of 26 isolates, *S. aureus*, *S. pasteuri*, *S. hominis*, *Micrococcus* sp., and *Macrococcus* sp. were selected on the basis of all in vitro assay and their 16S rDNA sequences were submitted to NCBI, GenBank under the accession number KX821621 to KX821646. Out of 26 studied typed isolates, 19, 15, 14, 21, 6, 25, and 22 isolates give amplification for *mec*A, *coa*, *spa*, *clf*A, *ccr*, *ica*, *fnb*A, and *sar* genes, respectively. Four *S. aureus* possessed all virulence genes. Presence of methicillin resistance *Staphylococci* having virulence genes revealed that mastitis is a major concern nowadays affecting animal health, milk quality, and yield. Further genomic study of these isolates will provide broad new insights on virulence.

Keywords Virulence · Mastitis · Staphylococcus · Antibacterial resistance · Resistance genes · MRSA

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Introduction

Staphylococcus genus includes several pathogenic organisms; among which, *Staphylococcus aureus* is one of the most present etiological agent of mastitis (Chu et al. 2012; Lundberg et al. 2014). Emergence of coagulase-negative *Staphylococcus* sp. (CoNS) is also a concern nowadays (Aslantaş et al. 2014). These microorganisms' pathogenicity against immune system of host animal affects animal health and milk yield which directly disturbs economy (Kelly et al. 2010).

The basis of pathogenicity depends on many virulence factors, including resistant to antibiotics, production of toxins, and many of surface proteins which help in attachment and colonization of the bacteria. These proteins act within the cellular and extracellular material of the host that affects the ability of the host to actively hinder infection by a specific immune response (Dhanawade et al. 2010).

Increasing ability of bacteria to survive in hostile environment is because of accumulation of resistance factors which has rendered the bacteria immune to a haphazard use of common antibiotics (Oliveira et al. 2006; Kumar et al. 2009).

Other than this, the pathogenesis of *Staphylococcus* mastitis is attributed to a combination of extracellular factors and properties such as adherence and biofilm formation (Dhanawade et al. 2010; Chavhan et al. 2012).

Acquisition of resistance against different antibiotics mainly methicillin is due to staphylococcal cassette chromosome mec (SCC*mec*) integration, containing *mec*A gene (Paterson et al. 2013). The expression of *mec*A in MRSA allows cell wall synthesis to continue, despite inactivation of native PBPs by *B*-lactams (Shore et al. 2011). *S. aureus* also carries two site-specific recombinase genes, cassette chromosome recombinase A and B (*ccr*A and *ccr*B) or sometimes complex *ccr* (Goudarzi et al. 2016).

In *Staphylococcus* spp., presence of coagulase as a *coa* gene, clumping factor (*clf*A), intracellular adhesion protein (*ica*), and fibronectin-binding proteins (*fnb*A) mediate bacterial adherence states pathogenicity of a strain (Feßler et al. 2010; Nemeghaire et al. 2014). Products of icaABCD complex synthesize polysaccharide intercellular adhesion which leads organisms to form biofilms (Barakat and Nabil 2016). *Staphylococcal* accessory regulator (*sar*) helps to regulate biofilm formation and protein A (*spa)* gene segment encoding the immunoglobulin G binding region is one of the vital virulence factors (Hata et al. 2010).

The work was designed to study the virulence genes in *Staphylococcus* spp. associated with mastitis.

Materials and methods

Milk sample collection

A total of 120 animals were screened for mastitis based on California mastitis test (CMT), somatic cell counts (SCC), pH of milk were determined (Bhutto et al. 2012).

Milk samples were collected and proceeded as described in our previous study (Patel et al. 2017a, b); in brief, udder were wiped with 80% ethyl alcohol, wiped and few drops of milk was discarded initially. Afterwards, in a 50-ml falcon tube, the sample was collected and proceeded further (Bhatt et al. 2011; Patel et al. 2017a, b). Simultaneously, CMT was executed on site and on the basis of CMT score samples were collected and proceeded for somatic cell count, pH measurement, and bacteriological examination (Bhutto et al. 2012).

Isolation of staphylococci from milk samples

We used Baird-Parker agar base (Himedia, Catalog No. M043, India) for screening and mannitol salt agar (Himedia Catalog No. MH118, India) for differentiation and selection of *Staphylococcus* spp. as described by (Le Maréchal et al. 2011; De Oliveira et al. 2011). Confirmed isolates were cultured in Luria broth (Himedia M575, India) and cultures were preserved in 25% glycerol (Himedia, Catalog No. MB060, India) and stored at − 20 °C (Blue-star).

Determination of antibiotic susceptibility of isolates

In vitro antibiotic susceptibility of a total of 179 *Staphylococcus* spp. was evaluated against 20 different antibiotics (HiMedia, India) which include amoxicillin (10 mcg/disc), ampicillin (10 mcg/disc), cefotaxime (30 mcg/disc), chloramphenicol (30 mcg/disc), ciprofloxacin (5 mcg/disc), cloxacillin (10 mcg/disc), enrofloxacin (10 mcg/disc), erythromycin (15 mcg/disc), gentamicin (10 mcg/disc), kanamycin (30 mcg/disc), meropenem (10 mcg/disc), methicillin (5 mcg/disc), nalidixic acid (30 mcg/disc), ofloxacin (5 mcg/disc), oxacillin (1 mcg/disc), penicillin-G (10 mcg/disc), rifampicin (5 mcg/disc), streptomycin (10 mcg/disc), tetracycline (30 mcg/disc), and vancomycin (30 mcg/disc) using disc diffusion method according to the National Committee of Clinical Laboratory Standards (NCCLS) (Shitrit et al. 2015).

Identification of bacteria based on 16S rDNA gene sequencing and phylogenetic analysis

Isolates which showed resistance against most of the antibiotic tested were selected for further study. Genomic DNA was isolated by following the proteinase K - SDS method manually by little modification as described (Patel et al. 2016). Isolated DNA samples were checked for quality and quantity using agarose gel electrophoresis (Bio-Rad, USA) and NanoDrop spectrophotometer (ND-1000, Thermo-Scientific, USA), respectively. 16S rDNA gene was amplified using universal primer 8F and 518R (Carroll et al. 2013), followed by Sanger sequencing. The sequences obtained were submitted to NCBI GenBank. Phylogenetic analysis was conducted by MEGA 7 software using the UPGMA statistical method. Test of phylogeny was done using bootstraps method obtained from 1000 replicates.

Detection of virulence genes

We selected a total of nine genes which are prevalent and confer pathogenicity to *Staphylococcus* sp. The primers were designed using primer3 using MRSA252, SA RF122, and SA 8325 as a reference strain (Haran et al. 2012; Luini et al. 2015). Primers were synthesized at Eurofins Genomics India Pvt. Ltd., Bengaluru. The specific primers with sequences are shown in Table 1.

The target genes were amplified using Applied Biosystems 2720 thermal cycler. The 25 μl PCR mixture contain 1 μl of DNA template (50–70 ng), 2.5 μl PCR Taq Buffer with $MgCl_2$ (*Gene*i), 2.5 μl DNTP mix (2.5 mM each) (*Gene*i, Bengaluru), 0.5 μl genomic DNA polymerase (Sigma-Aldrich, Germany), 1 μl forward primer (10 pM/μl), and

Table 1 Primer sequences of studied virulence gene and annealing temperature

Sr.No.	Gene	Forward sequence (5′ to 3′)	Reverse sequence (5′ to 3′)	Annealing temperature
1	*coa*	CGAGACCAAGATTCAACAAG	AAAGAAAACCACTCACATCA	55 °C
2	*spa*	CACCTGCTGCAAATGCTGCG	GGCTTGTTGTTGTCTTCCTC	58 °C
3	*clf*A	GGCTTCAGTGCTTGTAGG	TTTTCAGGTCAATATAAGC	57 °C
4	*mec*A	TTTACCTGAGATTTTGGCATTG	AGTTGTAGTTGTCGGGTTTGG	60 °C
5	*ccr*	GCGTGAAACGTTGTTGAATG	AAGCAACAATCCTTAGCAGCA	55 °C
6	*fnb*A	TTGGCTGTGCCACTTCAA	ATCGTTGTTGGGATGGGA	56 °C
7	*ica*A	TGATCCAAAACTTGGTGCAG	TTTTGGAAATGCGACAAGAA	55 °C
8	*ica*D	CAAGCCCAGACAGAGGGA	ATCGCGAAAATGCCCATA	56 °C
9	*Sar*	TGACATACATCAGCGAAAACA	TCAATTTCGTTGTTTGCTTCA	56 °C

1 μl reverse primer (10 pM/μl) sterilized milli-Q water to make up the reaction volume (Chavhan et al. 2012). PCR conditions for each gene amplification was standardized by initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 30 s, annealing by varying Tm, extension at 72 °C for 45 s with 25 cycles, and final extension at 72 °C for 10 min. Finalized annealing temperature is shown in Table 1**.** A negative control with each of the reaction components except template DNA and positive control (Accession No: KR028445) with the genomic DNA from *S. aureus* and *Staphylococcus* sp. was taken for each gene (Darwish and Asfour 2013; Patel et al. 2016). The PCR products were visualized on 1.5% agarose gel along with the molecular markers of definite length. The gel was electrophoresed in 2 x Tris-borate buffer at 70 V for 1.5 h and documentation was done using the Gel Doc system (Bio-Rad, USA).

Results

Bacterial strains

A total of 193 *Staphylococcus* spp. were isolated from 180 quarters milk samples containing somatic cells higher than 500 cells/μl, pH above 6.8, and CMT score 1 or 2 (Bhutto et al. 2012). All the isolates showed black color colony on Baird-Parker medium containing rabbit plasma and out of 193 isolates; 139 yielded yellow color on mannitol salt differential media due to fermentation of mannitol and 40 with no change in color. These isolates were phenotypically confirmed to be *S. aureus* and *Staphylococcus* sp. by performing various biochemical assays as per Bergey's manual of determinative bacteriology.

Antibiotic susceptibility of isolates

Qualitative antibiotic susceptibility test (ABST) against 179 *Staphylococcus* spp. comprising 139 *S. aureus* and 40 *Staphylococcus* spp. was achieved. Resistance against methicillin in 31 (22.30%) out of 139 *S. aureus* and in 17 (42.5%) out of 40 *Staphylococcus* spp. was observed. A total of 26 (14.53%) isolates were found resistant to more than 12–14 antibiotics thus used in further study.

Molecular identification and phylogenetic analysis

On the basis of antibiograms, 26 isolates were further selected for molecular identification using 16S rDNA gene amplification. Submitted sequences were assigned under accession number KX821628 to KX821638 for *S. aureus* (11), KX821621 to KX821627 for coagulase-negative *Staphylococcus* sp. (07), and KX821639 to KX821646 (08) for *Micrococcus* sp. including two *Macrococcus* spp. The phylogenetic tree generated from the 16S rDNA gene sequence of the *Staphylococcus* strains/isolates included in this study showed, as expected, that all *Micrococcus* spp., *S. hominis* and *S. pasteuri* were clustered in individual clade, whereas *S. aureus* were clustered individual and also found closely related with *Macrococcus* sp. in some strains (Fig. 1).

Virulence gene detection

Nine eminent virulence genes were amplified using gene-specific primers. Results revealed that all the resistant genes were present in the isolates, but not all nine in each isolate. Amplification of genomic DNA of all 26 typed isolates with antibiotic resistivity gave positive amplification for *mec*A gene resulted in the amplification of a fragment of approx. 1900 *bp* in 19 isolates; no amplification was observed in the rest of the isolates. Among the 26 isolates, 15 showed amplification of 850 *bp* fragment for *coa* gene which includes all *S. aureus* and four *Micrococcus* spp., 14 isolates to carry *spa* gene having size of 920 *bp* and 21 isolates were found positive for *clf*A gene having 980 *bp* size. Amplification of *ccr* gene with 425 *bp* amplicon size was found in 04 coagulase-negative *Staphylococcus* spp. (CNS) and 2 MRSA. Biofilm

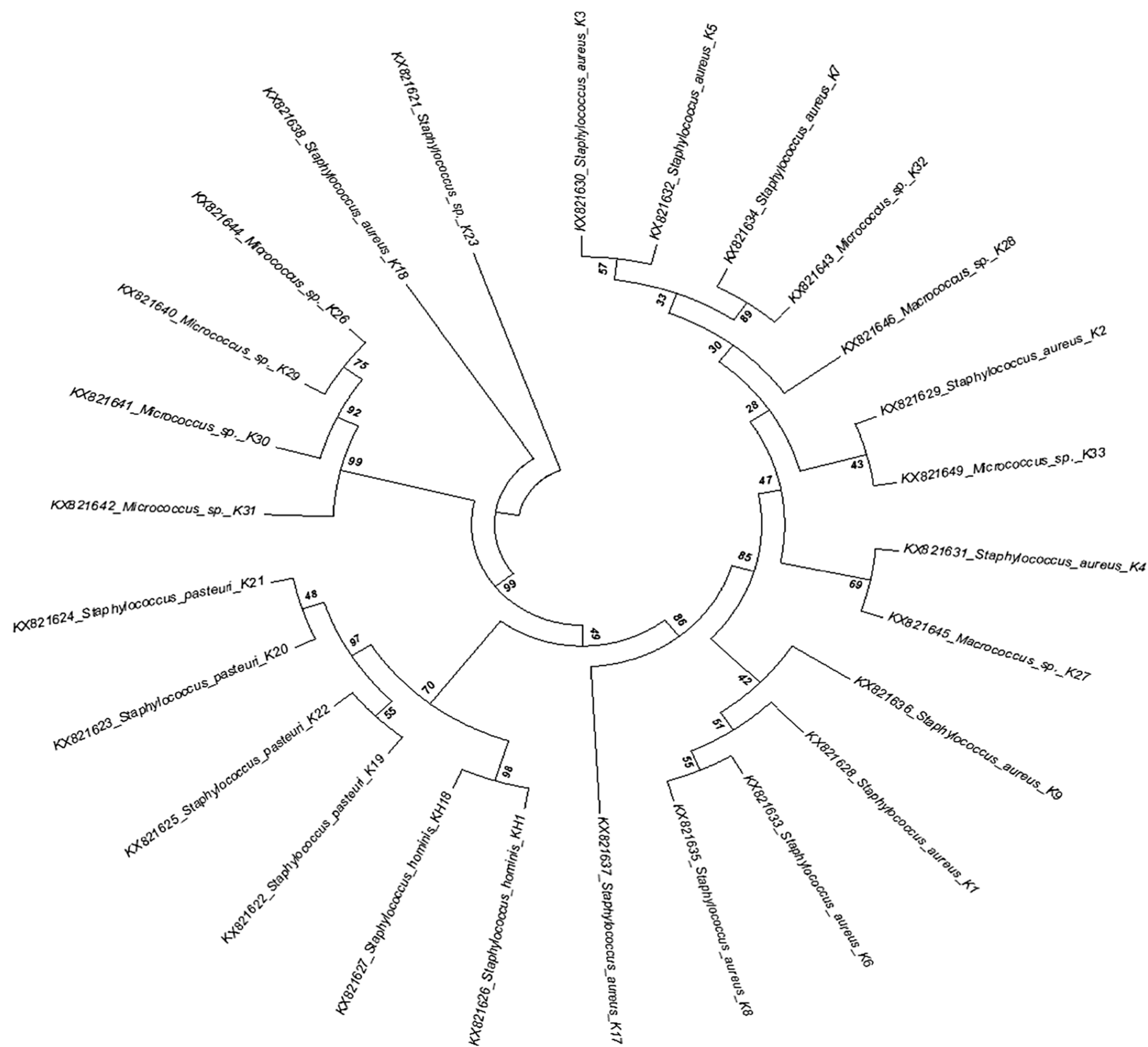

Fig. 1 Phylogenetic tree in a circular view based UPGMA; showing the relationship of Staphylococcal species isolated from mastitis cases constructed using MEGA 7

associated virulence gene amplification was observed in most of the isolates. Twenty-five isolates were found *ica* (*ica*A of approx. 550 *bp* and *ica*D of approx. 330 *bp*) and *fnb*A (520 *bp*) positive, while 22 isolates were found positive for *sar* gene of 250 *bp* amplified product (Fig. 2a–i).

Though the amplification of all nine genes was observed but not consistently in all of the isolates, different genes were existing in different isolates. However, four of the 11 *S. aureus* possessed all virulence genes.

Discussion

Virulence factors of *S. aureus* and coagulase-negative *Staphylococcus* sp. (CoNS) enable the bacteria to attach, colonize, invade, survive, and infect the host which directly intrudes immunity. In this study, we investigated virulence determinants in methicillin resistance *Staphylococcal* strains isolated from the milk having higher SCC (> 500 cells/μl) and CMT positive. Methicillin-resistant *S. aureus* are considered resistant against β-lactams antibiotics which are widely used to treat and prevent mastitis in dairy cows. Emergence of this resistant is due to irrational use of antibiotics for treatment (Chavhan et al. 2012).

Phenotypic resistance by ABST against methicillin was found in 31 (22.30%) out of 139 *S. aureus*, which is in accordance with detailed study of past 13 years on clinical *S. aureus* isolates by authors where around 22% MRSA was observed among studied isolates in UK, Slovenia, Belgium, Finland, and France (Denis et al. 2014). A study from India revealed 30% MRSA from their studied 323 strains by in vitro ABST assay, which is 8% higher than our study (Debnath and Chikkaswamy 2015).

coa gene was found to be present in all the isolates of *S. aureus* and *Micrococcus* sp. and absent in remained strains; which is confirmatory of phenotypically performed biochemical assay to distinguish coagulase-positive and negative strains. Coagulase-negative *Staphylococcus* spp. (CoNS) are

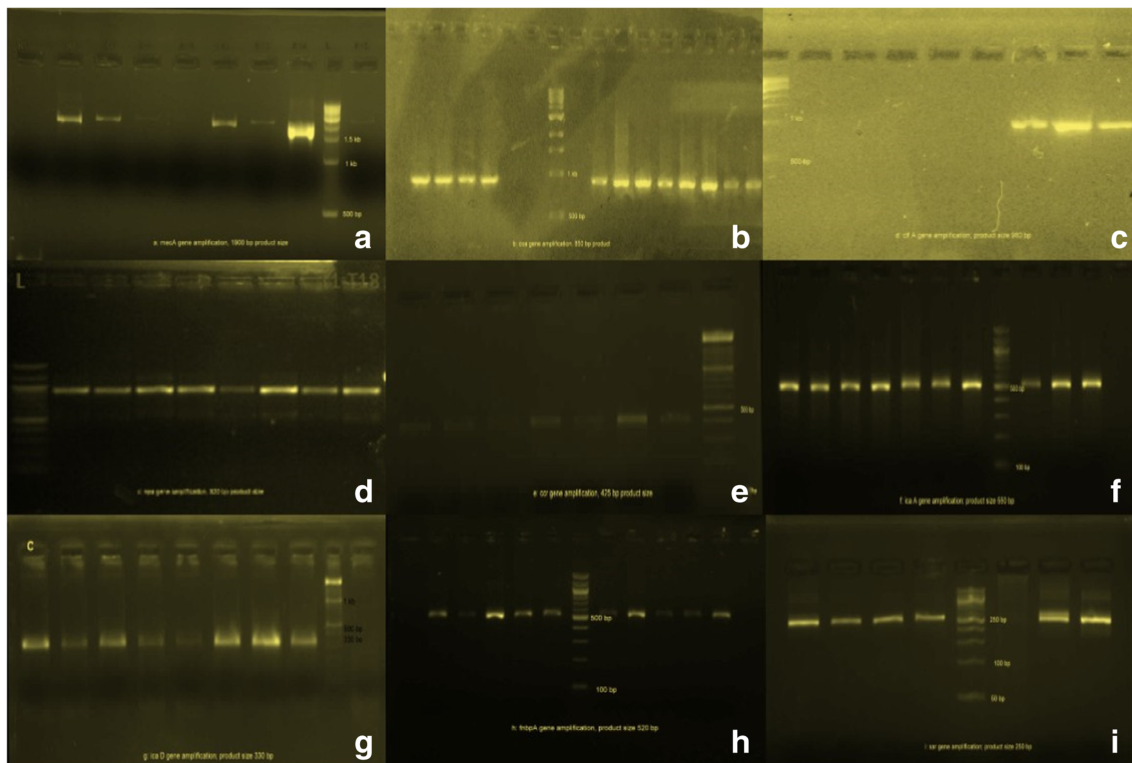

Fig. 2 Agarose gel (1.5%) of amplified products of different genes with approx. product size. **a** *mec*A; 1900 bp. **b** *coa*; 850 bp. **c** *clf*A; 980 bp. **d** *spa*; 920 bp. **e** *ccr*; 425 bp. **f** *ica*A; 550 bp. **g** *ica*D; 330 bp. **h**. *fnb*A; 520 bp. **i** *sar*; 250 bp

also becoming more prevailing with mastitis in the population of mixed animals. Two different sized *coa* amplified product is observed in few isolates, which states that *S. aureus* is having two different regions for *coa*. However, two bands of *coa* gene amplicons have also been reported by various authors (Al-Ashmawy et al. 2016; Nagaraju and Raju 2017).

Presence of *mec*A gene in *S. aureus* is the only known way to spread the gene by horizontal gene transfer mechanism. In this study, *mec*A gene encoding PBP2a (penicillin-binding protein 2a) was detected in 11 *S. aureus* can be confirmatory termed as MRSA and 07 in coagulase-negative *Staphylococcus* sp. termed as CNS. Emergence of such methicillin-resistant CNS is due to excessive use of antibiotics in treatment of *Staphylococcus* spp. A group of scientists from Brazil also observed *mec*A-positive CNS strains which were classified as methicillin-resistant by both the MIC and disc diffusion method (Fernandes Dos Santos et al. 2015). Resistance against this antibiotic is due to changes in penicillin-binding proteins (PBPs) other than PBP2 thus giving no activity against methicillin. In our study, the *mec*A gene was not detected in seven strains containing six *Micrococcus* spp., which did not show phenotypic methicillin resistance.

A genetic element that encoded methicillin resistance and carried unique site-specific recombinase designated as cassette chromosome recombinase (*ccr*) was identified in six of the 26 studied isolates containing 4 CNS and 2 MRSA having *mec*A and can be designated as the staphylococcal cassette chromosome *mec* (SCC*mec*) on the basis of studies (Chongtrakool et al. 2006; Aslantaş et al. 2014; Harrison et al. 2014; McManus et al. 2015).

Biofilm forming ability of a pathogenic strain against stress and inapt environment is considered to be one of the important virulence factors. *S. aureus* adhere to the extracellular matrix and cell components that are internalized into the mammary gland epithelial cells (Li et al. 2012). Like fibrinogen-binding proteins (*fnbp*A), clumping factor A (*clf*A) and clumping factor B (*clf*B) are important *S. aureus* bacterial adhesins: they contribute to initiate infection. Clumping factor A (*clf*A) is the major virulence factor responsible for clumping of *S. aureus* and all S. aureus clinical strains carry the *clf*A gene.

The ability of *S. aureus* to adhere to extracellular matrix proteins is essential for the colonization and establishment of infections and *S. aureus* possesses various adhesion genes. In one of the study on virulence factors, researchers found *clf*A (77.1%) and *fnbp*A (94.3%) (Wang et al. 2016). Similarly, we observed in this study too that *clf*A (80.76%) and *fnbp*A (96.15%) were found to be prevalent (Wang et al. 2016). Similarly, intracellular adhesion representing genes, *ica*A and *ica*D were also present in 96.15% of the isolates which is in accordance with a Brazilian study (Marques et al. 2017).

Staphylococcal accessory regulator (*sar*) protein is responsible for growth of strain during exponential phase that leaves it to form a layer to defend against stress condition so called as biofilm. Few studies also suggest that *sar* may also

incidentally regulate Staphylococcal protein A (*spa*) production (Bar-Gal et al. 2015). In our study, 53.8 and 84.61% isolates were found positive for *spa* and *sar* genes, respectively which states that in some cases *spa* is not regulated by *sar* gene (Morrison et al. 2012; Brackman et al. 2016).

Use of β-lactams, lincosamide, and macrolides as a therapeutic purpose is not eternally effective, as the prevalence of antibiotic resistance in various CoNS causing mastitis in cattle. Therefore, it is essential to evaluate the susceptibility of individual CoNS species against antimicrobials before applying for therapeutic application to treat mastitis. Few trials have been also applied by adding citric acid in lesser amount with beta lactum antibiotics against MRSA (Chandak et al. 2014). Moreover, the PCR technique to amplify the particular gene is not sufficient to confirm the antimicrobial resistivity of MRSA and CoNS but it is an insight into bacteria causing mastitis. Further genomic level study can help to understand the biological and functional processes of virulence gene and its mode of actions.

Conclusion

Presence of virulence factors including antibiotic resistivity in mastitis causing *Staphylococcus* spp. is an alarming spot for veterinarians, as several sources are there for spreading of microorganisms including human activities. Judicious use of antimicrobials for treatment of mastitis by eliminating the effect of pathogenic microbes is essential to control the emergence and spread of resistance against antibiotics. Antibiotic resistance and spreading of virulence have emerged in the last two decades shows alarming status in the field of medicine. Detailed genomic evaluation of particular resistant strain having virulent factors may possess a great scope for future development by targeting treatment purpose.

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Compliance with ethical standards

Ethical approval No need of ethical approval.

Conflict of interest The authors declare that they have no competing interests.

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