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Field level interventions on subclinical mastitis and detection of *Staphylococcus* in crossbred dairy cows

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ABSTRACT

Mastitis has emerged as one of the major managemental diseases of economic importance of high yielding dairy cows. A cross sectional study was carried out on 116 cross-bred dairy cows of small scale dairy farms; subclinical mastitis (SCM) was detected in 22 animals (cow-wise prevalence, 18.96%). Of the 88 individual quarter's milk tested using California mastitis test (CMT) and somatic cell count (SCC) tests, 51 quarters showed SCM (quarter-wise prevalence, 57.9%). Quarter-wise prevalence of SCM was 29.4, 31.4, 23.5 and 15.7% in right-fore (RF), right-hind (RH), left-fore (LF) and left-hind (LH) quarters, respectively. Fore-(52.9%) and right-(60.8%) quarters showed higher prevalence of SCM than hind-(47.1%) and left-(39.2%) quarters. All 4 quarters were found affected with SCM in 47% cows followed by 3-(23.5%), 1-(17.6%) and 2-(11.8%) quarters. Staphylococci were isolated and confirmed using 16S rRNA gene based genus-specific PCR in 39.2% of SCM affected quarters. Virulence associated *nuc* gene was detected in 75% of *Staphylococcus* isolates indicating their potential pathogenicity. Antibiogram showed multiple drug resistance (≥3 antimicrobial category) in 63.6% of Staphylococci. Multiple antimicrobial resistance (MAR) was recorded in 31.8% isolates. However, none of the isolate carried *mec*A gene. Interventions, viz. clean milk production practices, antimicrobial therapy and non-specific supportive treatments resulted in 77.7, 50 and 38.8% reductions in SCM compared to the untreated control (37.5%). This study accentuated higher prevalence of SCM among dairy cattle and predominance of Staphylococcus as the major mastitogen. Early detection and management of SCM among dairy cattle is recommended so as to prevent its progression to clinical illness and curtail potential economic loss to farmers.

Key words: 16S RNA gene, Antibiogram, AMR, CMT, MAR, *mec A gene, nuc* gene, Quarter, Subclinal mastitis

Mastitis, the inflammation of the udder, is a multifactorial management related disease mostly of high yielding dairy cattle. Indian dairy sector incurs an estimated economic loss of over ₹ 7,165.51 crore annually because of bovine mastitis (Bansal and Gupta 2009); and mastitis has been identified as one of the major economically important diseases of dairy animals in India (Sasidhar *et al.* 2002). A dairy cow affected with SCM is expected to produce ~2.58 litre/day less milk causing a loss of ₹ 2,322 to 7,824/cow a month (Bardhan 2013, Das *et al*. 2018). Several microorganisms are responsible for bovine mastitis but members of the genus *Staphylococcus* account for about 50% of mastitis cases (Radostitis *et al.* 2007). *S. aureus* one of the predominant mastitogens has emerged as a major cause of bovine mastitis (Mello *et al.* 2016). Clinical mastitis is characterized by overt changes in the udder or milk; however, in subclinical mastitis (SCM) such changes are not obvious. SCM accounts for severe economic losses to the dairy industry (Mdegela *et al*. 2009).

Mastitis is diagnosed by several methods (Emanuelson *et al.* 1987, IDF 1987); however, CMT or white side test (WST) is the preferred test at the field level (Sharma *et al.* 2011). Early diagnosis of mastitis helps in the reduction of economical losses (Sharma *et al.* 2010). Milk drawn from mastitic quarters shows elevated somatic cell counts (SCC) comprising macrophages, lymphocytes, neutrophils and epithelial cells (Sordillo *et al.* 1997, Pillai *et al.* 2001). Milk drawn from healthy quarter contains SCC less than 150,000/ml; however, SCC rises to several millions during mastitis (Harmon 1994). Milk SCC helps in the detection and monitoring of mastitis including anti-mastitis interventions (Green *et al*. 2004, De Haas *et al*. 2005). SCC based California Mastitis Test (CMT) continues to be the approved method at the field level (cow side test) for the detection of mastitis. Present study was undertaken to establish prevalence of SCM in crossbred dairy cows, isolate and characterize Staphylococci and evaluate field level SCM interventions.

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MATERIALS AND METHODS

Sample analysis for mastitis: Milk samples were collected from individual quarters from crossbred (Holstein Frisian or Jersey) lactating dairy cows of Menasigi and Gadgoli villages of Gadag district (Karnataka, India). Samples were screened for mastitis using California mastitis test (CMT; Schalm and Noorlander 1957) and CMT was scored using the grading procedure (Schneider and Jasper 1964). Somatic cell count (SCC) was performed per the method outlined by Sharma and Rajani (1969).

Cows testing positive for CMT (scores 1 to 3) and SCC (count >2 lakh cells/ml, linear score >4) were clinically examined for mastitis such as cardinal signs of udder inflammation, deviations in cow's health and gross changes in milk. Cows affected with only subclinical mastitis (SCM) i.e. without any obvious signs of mastitis were identified as SCM positive cases and 22 such cows with sub-clinical mastitis in at least one of their quarters were selected for the isolation of staphylococci. Staphylococcus were isolated as per the method outlined by BAM (2001).

Characterization of Staphylococcus: Multiplex PCR (Zhang *et al.* 2004) was used for the confirmation of staphylococci. Genus *Staphylococcus* was confirmed by the specific amplification 16S rRNA gene (Murugadas *et al.* 2016). Pathogenicity of staphylococci was determined by the amplification of virulence associated thermonuclease (*nuc*) gene. The *mec*A gene was checked for the determination of methicillin resistance.

Multiplex PCR: The DNA was isolated from Staphylococci using GenElute bacterial genomic DNA kit (Sigma-Aldrich) according to manufacturer's instructions. Multiplex PCR was set in 20 µl reaction mixture comprising 5 µl of template DNA, 2.5 mM $MgCl_2$, 0.2 mM dNTPs mix, primers (Table 1) and *Taq* DNA polymerase. The PCR program comprised initial denaturation temperature at 94°C for 5 min; 10 cycles of 94°C for 40 sec, 68°C for 40 sec, and 72°C for 1 min; 25 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min; and the final extension at 72°C for 10 min. Agarose gel electrophoresis was performed for separation of amplicons in 2% agarose gel containing ethidium bromide (0.5 µg/ml) followed by capturing of images using gel documentation system.

Antimicrobial susceptibility testing: Staphylococci isolated from SCM affected milk was tested against 12 different antimicrobials using disc diffusion method (Bauer *et al.* 1966). Mueller-Hinton agar plates containing NaCl (4%) were inoculated with 0.5 McFarland density adjusted culture and incubated at 37°C for 18–24 h and zones of inhibition were measured and scored as sensitive, intermediate and resistant in accordance with CLSI (2012). Multiple antimicrobial resistance (MAR) index was calculated for each isolate as per Krumperman (1983), as the ratio of number of antimicrobials to which isolate was resistant (or intermediate) to the total number tested. Standard *Staphylococcus aureus* ATCC 25923 and ATCC 43300 were used as reference strains and staphylococci showing resistance to at least 3 classes of antimicrobials were considered as multi-drug resistant (Shittu and Lin 2006).

Interventions for SCM: In order to elucidate the effect of different field level interventions, 22 cows diagnosed as SCM positive were divided into 3 experimental groups of six each (Gr 1–3) and no treatment control (4 cows) group. Cows of Gr 1 were subjected for clean milk production (CMP) practices comprising pre-milking udder wash, discarding of foremilk, post-milking antimicrobial dipping and other standard hygienic practices. Cows of group 2 received non-specific prophylactic treatment comprising oral multi-vitamin/ mineral powder and polyherbal topical gel recommended for sub-clinical mastitis and claimed to restore milk pH, enhance udder immunity and having anti-inflammatory actions. Gr 3 cows received specific antimicrobial therapy based on the antimicrobial susceptibility testing (ciprofloxacin, enrofloxacin or gentamicin). The cows of Gr 4 were kept as no treatment controls without any intervention. Pre- and post-intervention milk samples (30 days apart) were analysed for recovery from the SCM using CMT and SCC.

Statistical analysis: The SCC/ml of milk was converted to linear score (Radostitis *et al.* 2007). Descriptive statistics were calculated using MS-Excel program. Dendrogram of hierarchical clustering of MAR index was plotted by using agglomerative hierarchical clustering algorithm (Wessa 2018).

RESULTS AND DISCUSSION

Prevalence of subclinical mastitis in crossbred dairy cows: Out of 116 cows screened for mastitis using CMT and SCC, 22 showed SCM in at least one of their quarters;

Table 1. PCR target and primers used for the multiplex PCR

Marker	Target	Primer	Oligo sequence (5'→3')	Primer concentration
Gene specific to *Staphylococcus* genus	16S rRNA gene	Staph756F	AAC TCT GTT ATT AGG GAA GAA CA	0.12 µM
		Staph750R	CCA CCT TCC TCC GGT TTG TCA CC	0.12 µM
Virulence gene encoding thermonuclease	*nuc* gene	*nuc*F	GCG ATT GAT GGT GAT ACG GTT	0.04 µM
		*nuc*R	AGC CAA GCC TTG ACG AAC TAA AGC	0.04 µM
Gene responsible for methicillin resistance	*mec*A gene	*mec*F	GTA GAA ATG ACT GAA CGT CCG ATA A	0.12 µM
		*mec*R	CCA ATT CCA CAT TGT TTC GGT CTA A	0.12 µM

cow-wise prevalence of SCM in crossbred cows was 18.96%. Of the 88 quarters tested, 51 quarters showed SCM (quarter-wise affection 57.9%). Involvement of right-hind (RH, 31.4%) quarter was the highest followed by right-fore (RF, 29.4%), left-fore (LF, 23.5%) and left-hind (LH, 15.7%) quarters. Fore-quarters (52.9%) were comparatively more affected than hind-quarters (47.1%), and right-sided quarters (60.8%) showed higher SCM than animal's left-sided (39.2%) quarters. About 47% of cows showed SCM in their all 4 quarters followed by 3 (23.5%), 1 (17.6%) and 2 (11.8%) quarters.

Subclinical mastitis is characterized by lack of cardinal signs of udder inflammation, gross changes in milk and other local or systemic health changes in affected cows. Hence, detection of SCM is aimed at detection of etiological agents or products of inflammation in milk (IDF1987). Gold standard for the detection of SCM is isolation and identification of causative agent; the procedure is laborious, time consuming and expensive requiring rapid detection tools (Radostitis *et al.* 2007). Of the reliable rapid tests CMT, WST and SCC have been the most commonly used tests for the detection of SCM. Milk leucocytes are lysed to release deoxyribonucleic acid in CMT and WST (white side test), detergent or sodium salt (sodium hydroxide) produce coagulum and aid detection (Busato *et al.* 2000). The SCC on the other hand involves staining of thin smear and quantitative expression of microscopically observed somatic cells. Of these, CMT has emerged as the most widely used cow-side test while SCC as the most reliable laboratory test. Both CMT and SCC have been considered as reliable, rapid, easy to perform and inexpensive tests for the detection of SCM (Schalm *et al.* 1971). The CMT has positive association with SCC for the detection of mastitis (Contreras *et al.* 1996); further, SCC has been accepted as standard for the determination of milk quality. SCC predominantly measures leucocytes (phagocytes, lymphocytes and neutrophils) that massively influx (mostly neutrophils) during infections in the udder (Askr 2013). CMT aid screening udders for SCM at the farm level and is based on estimation of milk SCC (Tiwari and Sisodia 2001, Leach *et al.* 2008).

Recovery of staphylococci from SCM affected quarters: Staphylococci were isolated from 22 quarters out of 51 SCM affected quarters (prevalence 43.2%) and confirmed based on amplification of 756 bp 16S rRNA gene specific to genus *Staphylococcus*. Of these, 68.2% isolates were identified as *Staphylococcus aureus* based on *Staphylococcus aureus* species-specific amplification of 279 bp virulence associated thermonuclease (*nuc*) gene that has also been identified as virulence marker marking potential pathogenicity.

Subclinical mastitis is characterized by lack of inflammation and eventually such cases lead to clinical manifestations with changes in milk, udder or cow's health. Higher percentage (43.2%) of isolation of staphylococci from SCM affected quarters in the study endorses its role as a predominant mastitogen in the study area as also concluded by others (Sori *et al.* 2005, Mekonnen *et al.* 2005). Such subclinical infections are associated with decreased milk production and lowered milk quality (Bradley and Green 2001).

Staphylococci have been identified as the principal mastitis causing bacterial pathogens followed by *Streptococcus, Proteus, Corynebacterium, Escherichia coli, Klebsiella*, *Citrobacter, Bacillus, Micrococcus,* and other species (Buragohain and Dutta1999, Lalrintluanga *et al.* 2003). Staphylococci have been isolated not only from clinically or subclinically infected udders but also from apparently healthy udders. Variation in the isolation of Staphylococci from quarters could be attributed to varied factors linked to locations, farming systems, management practices and animal's immunity. In our study, 43.2% of quarters affected with SCM showed *Staphylococcus* species. Buragohain and Dutta (1999), Abrahmsén (2014) and Thorberg (2008) showed staphylococci in 22.1, 50.65, 54.7 and 87% quarters. Moroni *et al*. (2006) reported *Staphylococcus* as the predominant bacterial pathogen among 78% clinically infected cows, however, Tadesse and Chanie (2012) isolated *Staphylococcus* species from only 11.93% of clinically infected cows.

Of the several microbial mastitogens, *Staphylococcus aureus* appears to be the most prevalent organism of public health significance worldwide (Kubota *et al.* 2007). It is associated with contagious mastitis in bovines and is shed in milk thereby acting as source of infection to other mates in the shed (Radostits *et al.* 2007, Ararsa 2018). Several studies showed prevalence of *Staphylococcus aureus* among dairy cows with SCM at variable levels, viz. 42.85% (Ararsa *et al.* 2018), 36.9% (Wubish *et al.* 2013), 31.19% (Tadesse and Chanie 2012) and 21% (Barrett *et al.* 2005).

Our study showed prevalence of *Staphylococcus aureus* in SCM affected quarters at 68.2%. *Staphylococcus aureus* was isolated from as low as 0.9% (Abrahmsén *et al.* 2014) to as high as 90% (Shem *et al.* 2001). *Staphylococcus aureus* at 19, 28.1, 34.3, 45 and 56.89%, respectively, was reported by Persson *et al.* (2011), Abera *et al.* (2012), Ahlner (2003), Khan and Muhammad (2005) and Singh *et al.* (2005). Barrett *et al.* (2005), Ali *et al.* (2011), Harini and Sumathi (2011) and Chen *et al.* (2012) showed *Staphylococcus aureus* in SCM affected quarters at 21, 28.32, 58 and 62.9%, respectively.

Antimicrobial susceptibility of Staphylococci isolated from SCM cases: Highest resistance among Staphylococci was observed against ciprofloxacin followed by ampicillin, cotrimaxazole, penicillin G, gentamicin/ ceftizoxime, cefepime and ceftrioxone/ chlromphenicol (Table 2). Agglomerative nesting (hierarchical clustering) dendrogram of MAR index is shown in Fig. 1; wherein, 22 isolates formed 2 major and 6 sub-clusters of 3 each. First major cluster comprised 2, 3 and 2 isolates having the MAR index of 0.5, 0.33 and 0.25; 7 isolates (31.8%) of this cluster were multi-resistant (against ≥3 antimicrobials) and MAR index above 0.2. While, the second major cluster had 15 isolates having MAR index of <0.2 that also included 3 pan-sensitive

Table 2. Antimicrobial susceptibility of Staphylococci isolated from SCM quarters

Isolate no.	Zone of inhibition in mm for the antimicrobial												MAR index	MDR status
	MET	CX	TE	C	CTR	CPM	CZX	GEN	P	COT	AMP	CIP		
1	S	S	S	S	S	S	S	S	S	1	S	S	0.08	–
2	S	S	S	S	S	S	S	R	S	S	S	S	0.08	
3	S	S	S	S	S	S	S	S	S	S	S	S	0.00	–
4	S	S	S	S	S	S	S	S	S	S	S	S	0.00	–
5	S	S	S	S	S	S	S	S	S	1	S	S	0.08	–
6	S	S	S	S	1	S	S	S	R	S	R	R	0.33	+
7	S	S	S	S	S	S	S	S	R	S	R	S	0.17	–
8	S	S	S	S	S	1	1	S	R	1	R	R	0.50	+
9	S	S	S	S	S	1	R	S	R	R	R	R	0.50	+
10	S	S	S	S	S	S	S	S	S	R	R	R	0.25	+
11	S	S	S	S	S	S	S	S	S	S	S	R	0.08	–
12	S	S	S	S	S	S	S	S	S	1	R	R	0.25	+
13	S	S	S	S	S	S	1	S	R	S	R	R	0.33	+
14	S	S	S	S	S	S	S	1	S	S	S	R	0.17	–
15	S	S	S	S	S	S	S	S	S	S	S	R	0.08	–
16	S	S	S	S	S	S	S	S	S	R	S	R	0.17	–
17	S	S	S	S	S	S	S	S	S	S	S	R	0.08	–
18	S	S	S	S	S	S	S	1	S	1	R	R	0.33	+
19	S	S	S	1	S	S	S	S	S	S	S	R	0.17	–
20	S	S	S	S	S	S	S	S	S	S	S	R	0.08	–
21	S	S	S	S	S	S	S	S	S	S	S	S	0.00	–
22	S	S	S	S	S	S	S	S	S	S	R	R	0.17	–
Total (%)	0	0	0	1 (4.5)	1 (4.5)	2 (9.0)	3 (13.6)	3 (13.6)	5 (22.7)	8 (36.3)	9 (40.9)	15 (68.1)	–	7 (31.8)

S, sensitive;1, intermediate; R, resistant (For calculation of MAR index, intermediate isolate was considered as resistant). MET, methicillin (5 µg); CX, cefoxitin (30 µg); TE, tetracycline (10 µg); C, chloramphenicol (30 µg); CTR, ceftriaxone (30 µg); CPM, cefepime (30 µg); CZX, ceftizoxime (30 µg); GEN, gentamicin (10 µg); P, penicillin G (1 U); COT, co-trimoxazole (25 µg); AMP, ampicillin (10 µg); CIP, ciprofloxacin (10 µg).

against 12 antimicrobials.

The MAR index values >0.2 indicates higher risk due to the frequent usage of the antimicrobials (Osundiya *et al.* 2013). To tackle infections in dairy cows, antimicrobials are used indiscriminately leading to acquisition of resistance among pathogens and widespread usage has lead to serious problem of antimicrobial resistance in the low and middle income countries (Okeke *et al.* 2005). Microorganisms use multifaceted mechanisms to develop resistance against antimicrobials and relationship between antimicrobial usage and resistance is very complex (Marshall and Levy 2011). Shamila-Syuhada *et al.* (2016) also reported MAR index in range of 0.08–0.67 with the predominance of resistance towards penicillin, ampicillin, cefoxitin and tetracycline. Widespread use of antimicrobials in the food producing animals to tackle infection has been identified as the far most serious cause for the emergence of resistance in organism including staphylococci. Although methicillin resistant *Staphylococcus aureus* (MRSA) strains have been isolated from infected and healthy cows worldwide (Ünal *et al.* 2012); interestingly, MRSA was not detected in this study as evidenced by the phenotypic and genotypic methods, i.e. detection of methicillin resistance *mec*A gene.

Therapeutic management and recovery of SCM: Three different field level interventional strategies, viz. clean milk production practices, prophylactic multi-vitamin/mineral plus non-specific polyherbal therapy and specific antimicrobial therapy were compared with untreated cows diagnosed as SCM (Table 3). The SCM affected cows (Gr 1) that were managed with clean milk production practices in the post-intervention period (one month) showed 77.7% reduction in the SCM. This was followed by 50% reduction in SCM in Gr 3 that received specific intra-mammary administration of selected antimicrobials. Non-specific multi-vitamin/mineral and polyherbal prophylactic treatment (Gr 2) resulted in 38.8% reduction in the SCM affected quarters. Nevertheless, cows that did not receive any intervention showed 37.5% reduction in

Table 3. Effect of different field level interventions on the occurrence of SCM

	No. of quarters positive for SCM		Recovery* (%)
	Before intervention	After intervention	
Clean milk production practices	9	2	7 (77.7%)
Herbal treatment	18	11	7 (38.8%)
Antibiotic therapy	16	8	8 (50%)
No treatment control	8	5	3 (37.5%)

*SCM negativity evidenced by reduction in SCC <2 Lakhs/mL (Linear score <4).

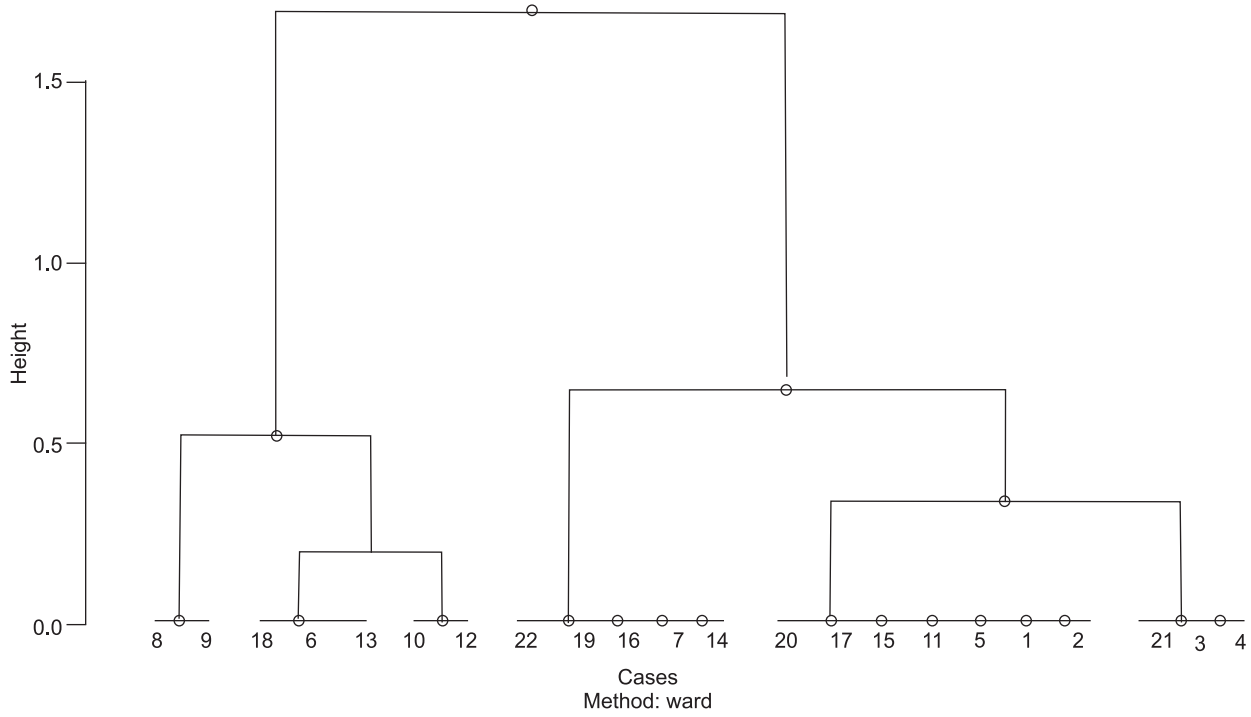

Fig. 1. Dendrogram showing agglomerative nesting (hierarchical clustering) of MAR index of staphylococci isolated from SCM positive quarters.

SCM. The study indicated implementation of prompt clean milk production practices for the effective management of the SCM in high producing dairy crossbred cows.

Dasohari *et al.* (2017) observed 66.6–100% cure from SCM by using specific antimicrobials in cows. However, Rupakala (2016) reported only 66.6% recovery from SCM in buffaloes. Das *et al.* (2018) also recorded 64% recovery of mastitis cases using antibiotics and supportive therapy. In our study, antimicrobial intervention resulted in comparatively lower recovery rate (50%) of SCM in crossbred cows than the clean milk production practices (77.7%). Antimicrobial therapy is the most recommended approach for clinical mastitis, but for SCM, antimicrobials may not always help in getting rid of the SCM. Hoeben *et al.* (2000) found marked reduction in severity of clinical mastitis using antimicrobials. Nevertheless, for the treatment of SCM induced by *Staphylococcus* species in addition to the antimicrobials other determinants like host, pathotype, efficacy of the therapeutic, pharmacokinetics, etc also play equivocal role (Zecconi *et al.* 2006). *In vitro* susceptibility testing of *S. aureus* aid in the selection of suitable antimicrobial for the therapy; nonetheless, several confounding factors influence the therapeutic success as mastitis is a multi-factorial management disease of high yielding cows (De Oliveira *et al.* 2000, Bradley and Green 2009). There is no single interventional approach that could be universally adapted for the management of SCM. Hence, attempts must be made to use novel therapeutic options. For instance, Leitner *et al.* (2018) recorded about 70% recovery using acoustic pulse therapy (APT).

In conclusion, mastitis continues to be the major economic burden of dairy farms worldwide and infections affect about 30% of dairy cattle (Hillerton and Berry 2005, Halasa *et al.* 2007). Timely interventions suiting local conditions must be discovered to tackle SCM. Present study showed higher prevalence of SCM in crossbred dairy cows with the predominance of *Staphylococcus aureus* as the mastitogen. Clean milk production practices appeared effective in reducing SCM compared to antimicrobial therapy and non-specific supportive therapies.

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