



Subclinical mastitis in crossbred dairy cows: *Staphylococcus* spp. and risk factors in small holder dairy farms

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ABSTRACT

Subclinical mastitis (SCM) is one of the major economic diseases of dairy cows. In the present cross-sectional study, dairy cows (n=205) of small holding dairy farms were screened using California mastitis test (CMT) and prevalence of 12.19% was recorded. Holstein Friesian and Jersey crossbred cows showed highest CMT score of 2 followed by 3, 1 and T; linear scores of somatic cell counts (SCC) of 3.14 ± 0.08 , 4.72 ± 0.25 , 6.17 ± 0.12 , 7.99 ± 0.11 and 9.84 ± 0.45 were observed for CMT scores N, T, 1, 2 and 3, respectively. Risk factors associated with SCM were studied in 25 randomly selected cows. *Staphylococcus* species were detected in 54% of quarters based on PCR amplification of 16s rRNA gene; thermonuclease (*nuc* gene) PCR categorized *Staphylococcus* species as *S. aureus* (SA, 37%) and non-*aureus* *Staphylococcus* (NAS, 26%). Significant association between CMT score 2 and *Staphylococcus* species was established. *Staphylococcus* species, SA and NAS, were detected in 30.7, 20.5 and 17.9% of SCM negative as well as 68.85, 47.54 and 31.14% SCM positive quarters, respectively. Significant association between SCM and *Staphylococcus* species and *Staphylococcus aureus* was observed. Study indicated strong association between SCM, *Staphylococcus aureus* and certain risk factors among cows reared under small holding dairy farms of the tropical region.

Keywords: Association, Crossbred cow, PCR, *Staphylococcus*, Subclinical mastitis

Mastitis is one of the most common health conditions of dairy cows characterized by physico-chemical changes in milk or pathological alterations in the udder parenchyma. Subclinical mastitis (SCM) occurs without obvious changes in milk or udder and occurs at higher frequencies (15–40 times) than clinical mastitis in dairy cows (Halasa *et al.* 2007). SCM affected cows show marked reduction (15–45%) in milk yield and its prevalence in dairy farms varies from 19 to 78% (Tuteja *et al.* 1993). Infected cows transmit mastitis pathogens to healthy penmates, unattended SCM cases would eventually lead to clinical mastitis (Rowlands *et al.* 1986).

Staphylococci usually colonize skin and mucous membranes of humans and animals (Weidenmaier *et al.* 2012). *S. aureus* is an opportunistic pathogen, some strains cause mild to severe life threatening infections (Archer 1998), cause foodborne disease, pneumonia and wound infections in humans; mastitis, arthritis and urinary tract infections in animals (Sutra and Poutrel 1994). Emergence of zoonotic antimicrobial resistant, methicillin-resistant *S. aureus* (MRSA), hospital or community-acquired MRSA

in humans or epidemic strains of livestock-acquired MRSA affirm need for interventions to protect the public health (Klein *et al.* 2007). Methicillin resistance encoding *mecA* and *mecC* homologue genes in staphylococci isolated from mastitis cows (Waller *et al.* 2011) require surveillance in dairy production systems.

S. aureus is one of the primary agents of mastitis in dairy cows (Karabasanavar *et al.* 2019); SCM affected cows shed organisms in milk that could eventually reach humans leading to foodborne disease. Entry of *S. aureus* into the udder mostly occurs from the environment, predominantly through newly introduced cows (Middleton *et al.* 2002); extra-mammary sources include cow, farm environment, people, feedstuff, farm air, etc (Roberson *et al.* 1998). Coagulase negative staphylococci (CoNS) have been incriminated in SCM and also harbour elements of multidrug resistance (MDR) posing public health implications (Sawant *et al.* 2009). Present cross-sectional study was undertaken with the objective to elucidate SCM and risk factors in crossbred cows of small holder dairy farms of the tropical region of India.

MATERIALS AND METHODS

Sample analysis: Milk samples were collected from crossbred (Holstein Friesian or Jersey) lactating cows (n=205) maintained under small holding dairy farms of the

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tropical region (Harave village, Chamarajanagar district, Karnataka). Individual cows were tested for mastitis using California mastitis test (CMT) and scored as T, 1, 2 and 3 (Schalm and Noorlander 1957, Schneider and Jasper 1964). Somatic cell count (SCC) was performed using Newman's stain (Sharma and Rajani 1969). Cows having CMT score of T, 1, 2 or 3 and SCC of >2 lakh cells per millilitre (linear score >4) were clinically examined for cardinal signs of mastitis, viz. udder inflammation, changes in cow health and organoleptic changes in milk. Inclusivity-exclusivity criterion of cows was based on absence of visible changes in milk, abnormalities of udder or teat, clinical signs of mastitis, malformed teat or inability to secrete milk from the quarter.

Isolation, identification and categorization of *Staphylococcus* spp.: Individual teat orifice sanitized with surgical spirit swab was dried and milk sample drawn into sterile tube, labeled and transported under chilled conditions to the laboratory. Staphylococci were isolated on Baird-Parker agar in accordance with the Bacteriological Analytical Manual (2001).

Simultaneous detection of *Staphylococcus* genus (16s rRNA gene), *S. aureus* species (thermonuclease *nuc* gene) and methicillin resistance encoding gene (*mecA* gene) was performed using multiplex polymerase chain reaction (Zhang *et al.* 2004, Murugadas *et al.* 2016). Isolates were screened for methicillin resistance *mecC* gene using specific PCR (Malyszko *et al.* 2014).

Statistical analysis: The SCC per milliliter of milk was converted into linear score (SCC-LS) for comparison

(Radostitis *et al.* 2007). Descriptive statistics and t-test were calculated using MS-Excel program (Microsoft Corp.); odds ratio (OR) and chi-square (χ^2) exact test were calculated using Epi-info epidemiological software (Centre for Disease Prevention and Control, CDC, USA).

RESULTS AND DISCUSSION

Based on the case definition (milk CMT score T/1/2/3 and absence of clinical signs of mastitis), cow-wise SCM prevalence of 12.19% was estimated in crossbred dairy cows. Cross-sectional study performed on 25 randomly selected cows (100 quarters) showed highest CMT score of 2 (27%) followed by 3 (18%), 1 (10%) and T (6%) as shown in Fig. 1. All the four quarters of cows were equally susceptible to SCM (Pearson's χ^2 0, p1) as evidenced by non-differing CMT scores between quarters (Pearson's χ^2 2.36, p 0.98). The SCC linear scores (SCC-LS) were significantly correlated with CMT scores (η 0.95, $p < 0.01$) as shown in Table 1 and Fig. 1.

Staphylococcus spp.: *Staphylococcus* spp. isolated from milk samples were confirmed using PCR for genus *Staphylococcus* (amplification of 756 bp PCR product of 16S rRNA), *Staphylococcus aureus* (SA) (amplification of 279 bp of thermonuclease *nuc* gene) and non-*aureus* Staphylococci (NAS). *Staphylococcus* spp. were isolated from 54% of quarter milk samples and categorized as SA (37%) and NAS (26%); prevalence was higher (68.85% comprising of SA 47.54% and NAS 31.14%) in SCM positive quarters than SCM negative quarters (30.76% with SA 20.51% and NAS 17.94%). Occurrence of

Table 1. Relationship between CMT score, SCC-LS and *Staphylococcus* spp.

SCC and <i>Staphylococcus</i>	CMT Score	N	T	1	2	3
SCC-LS	Mean±SE	3.14±0.08	4.72±0.25	6.17±0.12	7.99±0.11	9.84±0.45
	95% CI	2.98, 3.29	4.07, 5.37	5.89, 6.45	7.77, 8.22	8.88, 10.80
	Minimum	2.44	4.18	5.51	6.95	5.76
	Median	3.18	4.62	6.26	8.15	9.40
	Maximum	3.86	5.56	6.84	9.77	13.83
Prevalence (%)	<i>Staphylococcus</i> spp.	30.76	66.66	30.00	85.18	66.66
	SA	20.51	33.33	20.00	62.96	44.44
	NAS	17.94	50.00	10.00	37.03	27.77

SCC-LS, Somatic Cell Count as Linear Score; SA, *Staphylococcus aureus*; NAS, Non-*aureus* Staphylococci.

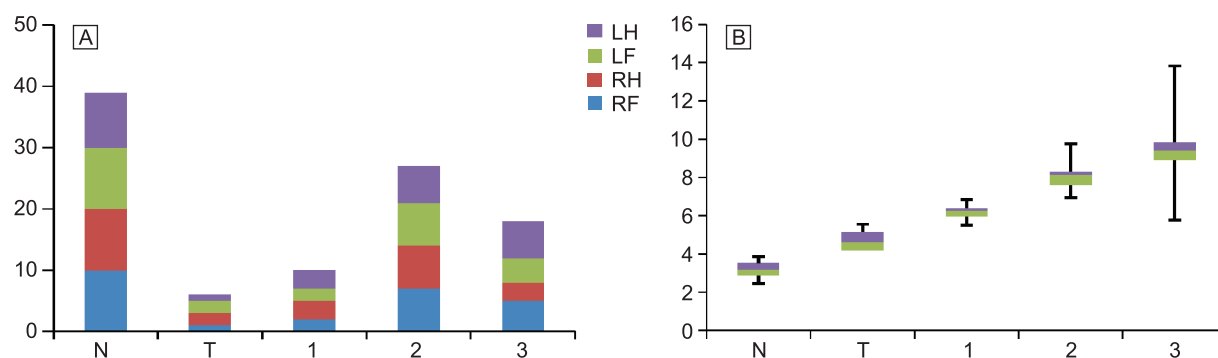


Fig. 1. Diagram showing CMT scores vis-à-vis different quarters (A) and SCC-LS in crossbred cows across different CMT scores (B). N, Negative; T, Traces; 1/2/3, CMT scores.

Staphylococcus spp. was significantly higher ($p < 0.05$) in SCM positive quarters than SCM negative quarters (OR 4.97, χ^2 13.89, p 0.0001). Further, association was highly significant ($p < 0.05$) between SA and SCM positive quarters (OR 3.51, χ^2 7.45, $p < 0.05$) but not with NAS ($p < 0.05$) (OR 2.06, χ^2 2.15, $p > 0.05$). These findings indicate *S. aureus* as the predominant cause of SCM among crossbred dairy cows maintained in the tropical small holding dairy farms. Mastitic *Staphylococcus* isolates are usually known to carry methicillin resistance; but specific PCRs showed absence of *mecA* or *C* genes in any of the isolates.

Characteristics of dairy farms: Farmers engaged in dairying were self-employed and invariably reared cattle since generations; dairying was either primary (59%) or secondary (subsidiary to agriculture) occupation (41%); with varying education, viz. none (22.7%), $<6^{\text{th}}$ (22.7%), $<10^{\text{th}}$ (25%), $<12^{\text{th}}$ standard (18.1%) and graduation (9%). Cows were milked twice daily and milk sold to the nearest cooperative milk collection center; cows by artificial insemination (85.7%) or natural service (14.3%). Source of new stock was neighboring farm (82.3%), animal fair (11.8%) or purchase from commissioned agents (5.9%). Criteria considered for purchasing new stock included animal characteristics (body condition, hair coat, teeth, gait, leg and udder conformation, 11.7%); recommendation of an experienced farmer (53%) and milk yield or udder

conformation (35%). Farmers reared Jersey (54.5%) or Holstein Friesian (45.5%) crossbreds having daily milk yield of 3–5 (40.9%), 5.1–7.5 (22.7%), 7.6–10 (31.8%) and >10 (4.6%) liters per cow. Prophylactic vaccination received by about 90% animals when young (19%) or as and when insisted by veterinarians (81%). Nevertheless, farmers used antibiotics without knowing their correct indications/usage.

Risk factors: Table 2 shows risk factors of SCM; breed-wise, Holstein Friesian or Jersey crosses with local zebu cattle were found equally susceptible to SCM. Households reared 1–8 dairy cows (predominantly 1–2) in their farms and holdings rearing >2 cows showed higher SCM than 1–2 rearings (OR 2.69); nevertheless, no such association was observed for *Staphylococcus*. Cows reared as single species showed higher SCM than mixed species in the farm/shed (OR 4.33, $p < 0.05$). Grazing system did not influence SCM in cows; morning grazing was invariably with other cattle (92.08%) or sheep/goats (79.01%) in the common grazing area.

Housing factors influenced occurrence of SCM; cows sheltered under roof made of metal sheets (18.18%), asbestos sheets (36.36%), tiles (31.81%) or plant materials such as coconut trees (13.63%) showed variation with respect the occurrence of SCM. Significant association between SCM and roofing using metal or plant material was noticed. However, shelters roofed with asbestos or plant

Table 2. Risk factors vis-à-vis SCM and *Staphylococcus* spp.

Risk factor	Parameter	Odd's ratio (OR)*	
		SCM	<i>Staphylococcus</i> spp.
No. of animals in the farm	<2 vs >2	2.69 (0.031)	0.84 (0.703)
Species in the farm	Single vs multiple (>2)	4.33 (0.018)	0.55 (0.364)
Grazing of cows	Grazing vs non-grazing	2.52 (0.233)	3.28 (0.150)
	Grazing with other species (sheep, goat, etc.)	2.15 (0.327)	4.05 (0.078)
Roof material	Metal sheet	0.15 (0.001)	0.58 (0.337)
	Asbestos sheet	1.77 (0.216)	0.33 (0.015)
	Tile	0.67 (0.383)	2.25 (0.086)
	Plant material	–1.00 (0.002)	5.00 (0.031)
Roof height	<7 ft. vs >7 ft	3.78 (0.005)	1.57 (0.303)
Roof area per animal	20–60 ft ²	1.29 (0.559)	0.40 (0.038)
	61–100 ft ²	1.16 (0.741)	3.66 (0.008)
	>100 ft ²	0.62 (0.359)	1.00 (1.000)
Floor type	Even vs. undulated	0.44 (0.181)	1.25 (0.686)
Floor height	<0.50 ft	14.05 (<0.001)	1.05 (0.911)
	0.51–1 ft	0.06 (<0.001)	1.00 (1.627)
	1.1–1.5 ft	0.55 (0.419)	2.56 (0.253)
Floor space per animal	<20 ft ²	0.91 (0.882)	1.69 (0.416)
	21–60 ft ²	0.48 (0.124)	0.23 (0.002)
	>61 ft ²	2.58 (0.076)	4.34 (0.006)
Distance from compost pit	<20 ft. vs >20 ft	7.78 (<0.001)	1.28 (0.615)
Milk yield per cow per day	<5 L vs. >5 L	1.58 (0.304)	3.54 (0.005)
Milking method	Full hand	0.67 (0.460)	0.42 (0.114)
	Stripping	1.32 (0.558)	1.97 (0.154)
	Stripping and knuckling	1.07 (0.915)	1.00 (1.000)
Cow hygiene score (mean)	1	1.13 (0.854)	1.56 (0.358)
	2–3	1.44 (0.403)	0.28 (0.005)
	3–4	0.64 (0.641)	11.59 (0.005)

*Chi-Square values in parenthesis.

materials ($p < 0.05$) were found associated with *Staphylococcus* species. Lesser roof heights (5–7 ft) were significantly associated with SCM ($p < 0.05$) compared to heighted (>9 feet) roofs; roof heights exceeding 9 feet could be recommended for sheds to ensure effective ventilations in the tropical climate. Surprisingly, none of the shed had ventilator or opening in the roof even though exhaled light stale air must escape through such openings in the tropical region. Roof area per animal (calf=0.5×adult) showed wide variation (20–360 ft²/head); no association between SCM and roof area was established. Nevertheless, 20–100 ft²/head roof area was found associated with *Staphylococcus* spp. opposed to >100 ft²/head. No association was established between roof height and *Staphylococcus* spp. Floor of cow shed having <1 feet height (relative to ground surface) was found significantly associated with SCM. Each shed occupied about 1–8 adult animals and up to 4 calves.

Floor space allocated to each cow varied widely (21–750 ft²) and it had no influence on the SCM; however, cows housed in sheds with larger floor spaces (>21 ft²) were found associated with *Staphylococcus* probably due to negligence in maintaining hygiene and sanitation by farmers; building of up of litter that might act of source of contamination. Incidentally, no bedding material was used for cows in the study area. Distance between shed and compost pit (<20 feet) significantly influenced prevalence of SCM but not *Staphylococcus* spp.

Farmers practiced full-hand (27.7%), stripping (55.5%) and stripping-cum-knuckling (16.5%) methods of milking; however, such methods did not influence SCM; farmers applied castor oil as lubricant while milking. Milk yield and milking method did not show any association with SCM or *Staphylococcus* spp. Nevertheless, cow hygiene scores of >2 showed association with *Staphylococcus* spp.

Rectal temperature of dairy cows ranged 100.4–102.8°F (mean 101.5°F); variation in temperature across SCM positive (101.33±1.38°F) and negative (100.79±1.03°F) cows was insignificant. Likewise, mean teat temperature of SCM positive cases (97.8±0.4°F) was significantly higher than SCM negative (95.8±1.8°F) teats. Further, udder temperature in SCM positive cases (100.3±2.4°F) was significantly higher than SCM negative (96.9±3.0°F) udders. However, association between SCM and teat score was insignificant.

Farmers did not undergo any kind of milking hygiene training (95%); nevertheless practiced washing of udder and teats prior to milking. Cleaning of cows prior to milking with water was not practiced. Milking was by wet milking method; drying of teats and udder was not practiced; pre- or post-milk teat dipping was also not practiced.

Pathogenic strains of *S. aureus* arise from various sources in the dairy farm, viz. animal body, human handlers, fomites and other environmental sources. Virulent *S. aureus* strains get transmitted from animals to animals or humans through animal, human or environmental sources (Lim *et al.* 2013). Infection with *S. aureus* most frequently leads to mastitis and directly affects animal health, milk quality and safety

of human consumers (Jorgensen *et al.* 2005). Virulent *S. aureus* strains or serotypes could be acquired through raw milk leading to food poisonings, *S. aureus* is known to produce over 20 enterotoxins (Hennekinne *et al.* 2012). Transmission of *S. aureus* occurs from infected udders through milk to humans; that could subsequently result in foodborne outbreaks. Decreased milk production, treatment of animals, animal culling and other expenses incurred in managing SCM induce huge economic loss to dairy farmers (McMillan *et al.* 2016).

Emergence of antimicrobial resistant *S. aureus* in subclinical infections and their low cure rates is an area of concern (Zhang *et al.* 2018). Of the several antibiotic resistance genetic elements, *mecA* gene that encodes penicillin binding protein (PBP2a) and livestock origin *mecC* genes have drawn considerable attention in the recent past (Stegger *et al.* 2012). Dominant strains of livestock-associated methicillin resistant *S. aureus* (LA-MRSA) could even get transmitted from livestock to humans (He *et al.* 2018). Interestingly, none of the *Staphylococcus* isolates showed methicillin resistance (*mecA* or *C* gene) as also observed by Schmidt *et al.* (2017).

Cows in the study area were driven to grazing area typically 1–2 kilometer away from sheds. Nevertheless, tie-housed cows had significantly higher (OR 3.23, $p < 0.001$) mastitis (Fadlelmoula *et al.* 2007). Cows that suffered mastitis in the previous parity were found at greater risk of acquiring mastitis in the subsequent lactations (Berry and Meaney 2005) and reason for such clinical infections could be attributed to the increased susceptibility or prolonging of subclinical infections through dry period (Rowlands *et al.* 1986). Higher *S. aureus* infection rate was reported in those quarters that were previously infected (Zadoks *et al.* 2001) and significant influence of inadequate sanitation of dairy environment with mastitis has been established (χ^2 8.7, $p < 0.001$) (Biffa *et al.* 2005).

In conclusion, observations made in the present study indicated *S. aureus* as the major cause of SCM among crossbred dairy cows maintained under the tropical climate in small holding dairy farms. Farmers need to be educated and trained about milk hygiene practices. Risk factors related housing and hygiene need to be promptly addressed to curtail the SCM. Significant association between *S. aureus* and SCM was observed among crossbred cows of small holder farms raising concerns about health, productivity and welfare of cows coupled with food safety and public health requiring prompt attention.

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