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## Subclinical mastitis in dairy cows: somatic cell counts and associated bacteria in Mymensingh, Bangladesh

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### Abstract

Subclinical mastitis is an economically important disease of dairy cows and has a prominent place amongst the factors that limit milk production. This study was undertaken to determine the association of somatic cell counts (SCC) and occurrence of bacteria with SCM in smallholder dairy cows in Mymensingh, Bangladesh. A total number of 240 quarters milk samples from apparently healthy lactating cows were subjected to SCC using NucleoCounter<sup>®</sup> SCC-100™ (Chemo Metec). A quarter was considered SCM positive if the quarter had SCC > 100 × 10<sup>3</sup> cells/ml. All subclinical mastitis positive quarter milk samples were subjected to bacteriological examination and isolates were classified into major, minor, uncommon and mixed pathogens. The overall quarter-level prevalence of subclinical mastitis of dairy cows in Mymensingh district was 25% (95% CI, 19.52% to 30.48%). The most frequently isolated bacterial species were *Staphylococcus aureus* (18.33%) followed by coagulase-negative staphylococci (10%), *Enterobacter* spp. (6.67%), *Escherichia coli* (5%), *Bacillus* spp. (5%) and *Pseudomonas aeruginosa* (5%). Different bacterial isolates were associated with 90% cases of subclinical mastitis as mono infections or mixed infections. Mono and mixed infections significantly influenced SCC and were the most prominent factors responsible for increasing SCC. Mean SCC was the highest for *Bacillus* spp. (713.67 × 10<sup>3</sup> cells/ml) followed by *Enterobacter* spp. (395.75 × 10<sup>3</sup> cells/ml), *Escherichia coli* (386.00 × 10<sup>3</sup> cells/ml), *Staphylococcus aureus* (373.82 × 10<sup>3</sup> cells/ml), coagulase-negative staphylococci (182.67 × 10<sup>3</sup> cells/ml) and *Pseudomonas aeruginosa* (138.67 × 10<sup>3</sup> cells/ml). Major pathogens induced higher SCC (380.72 × 10<sup>3</sup> cells/ml) than minor and other pathogen groups.

### Introduction

Mastitis is an inflammation of the mammary gland which, together with physical, chemical and microbiological changes, is characterized by an increase in the number of somatic cells in the milk and by pathological changes in the mammary tissue (Giannecchini *et al.*, 2002). About 140 microbial species, subspecies and serovars have been isolated from the bovine mammary gland (Radostits *et al.*, 2007). Among infectious agents, bacterial pathogens are considered to be the major threat to mammary gland. In Asia, major mastitis causing organisms are *Staphylococcus aureus*, *Streptococci*, *E. coli*, *Corynebacterium* spp. and *Klebsella* spp. (Sharma *et al.*, 2012). Different pathogens can cause chronic, subacute, acute, peracute and subclinical forms of the disease (Radostits *et al.*, 2007). In clinical mastitis all the five cardinal signs (redness, swelling, heat, pain and loss of milk production) of udder inflammation are present (Bachaya *et al.*, 2011). There are no visible abnormalities of the milk or udder instead there is a high somatic cell count in subclinical mastitis (Radostits *et al.*, 2007). Although a large number of bacteria have been isolated as causal agents of SCM in dairy cows throughout the world *Staphylococcus* spp., *Streptococcus* spp., *E. coli*, *Bacillus* spp., and *Corynebacterium* spp. have mostly been isolated from

SCM cases of cows from Bangladesh (Kayesh *et al.*, 2014; Rahman *et al.*, 2010).

SCM is 3–40 times more common than clinical mastitis and causes the greatest overall losses in most dairy herds (Bachaya *et al.*, 2011). It is responsible for 70% of economic losses and has a prominent place amongst the factors that limit milk production (Heleili *et al.*, 2012). In Bangladesh, the annual economic losses due to reduced milk production by SCM have been estimated to be Taka 122.6 (US \$ 2.11) million (Kader *et al.*, 2003). Besides causing huge losses to milk production, the sub-clinically affected animals remain a continuous source of infection to other herd mates.

SCC can be used to monitor the status of SCM in herds or individual cows which is an important component of assessing milk quality, hygiene and mastitis control (Sharma *et al.*, 2011). The SCC of healthy quarters is usually below 100,000 cells/ml or even lower (Hamann, 2005; Leitner *et al.*, 2003). The high SCC is mostly related to the presence of microorganism in the udder but also the type of microbes could affect the SCC in milk (Ariznabarreta *et al.*, 2002). The major pathogens generally cause the greatest SCC increase and infection by minor pathogens usually causes considerably less SCC elevation and rarely result in

clinical mastitis (Supr *et al.*, 2011; Sargeant *et al.*, 2001). In some cases, high SCC is detected in milk samples without presence of microorganisms and it does not indicate that the gland is healthy (McDougall *et al.*, 2001). However, cows with low SCC can also harbor the mastitis pathogen (Katsande *et al.*, 2013). Therefore, bacteriologic culture is necessary to accurately diagnose the source of the mastitis even though SCC profiles suggest lower probability of SCM. This paper describes the association of SCC and occurrence of bacteria with SCM in smallholder dairy cows in Mymensingh, Bangladesh.

## Materials and Methods

### Collection of samples

A cross sectional study was conducted on 13 dairy farms, one is Bangladesh Agricultural University (BAU) Dairy Farm and other 12 from surrounding villages (Boira, Charkalibari, Dhigarkanda and Salakanda) of Mymensingh sadar upazila around BAU. A total of 240 quarter milk samples were collected from each quarter of 60 randomly selected apparently healthy lactating cows. Breed of the study cows included indigenous (zebu) and cross-bred cows, i.e. crosses of zebu cows with Holstein-Friesian, Jersey or Shahiwal. Cows with the history of ongoing antibiotic treatment or antibiotic treatment within 96 hours were not included for sampling. Grossly dirty teats and udder were thoroughly washed and dried using a single, dry paper towel per cow with particular emphasis on the teat end. The teat end and orifice was carefully and vigorously scrubbed with a cotton pad moistened with 70% ethyl alcohol. Separate swab was used for each teat being sampled, even within the same cow. The teat end was cleaned until the swab was completely clean and white. Three or four streams of milk were discarded from the quarter being sampled to minimize chances of sample contamination from bacteria in the teat end. After that, collection vial was held at a 45° angle to avoid debris (hair, manure, dirt) contamination and about 10 ml of milk from each quarter was collected aseptically in sterile screw-capped collection vials. They were marked as right front (RF), left front (LF), right rear (RR) and left rear (LR) and transported immediately to the laboratory in ice-box at 4°C.

### Detection of subclinical mastitis

Subclinical mastitis was determined by SCC of milk using NucleoCounter® SCC-100™ (ChemoMetec). Somatic cell count was performed for all collected quarter milk samples as per instruction of manufacturer. About 50 µl of milk sample and 50 µl of reagent C (1:1 dilution) were taken in an eppendrop tube using micropipette and mixed properly. Then SCC-Cassette was loaded with the diluted milk sample by immersing the tip of the cassette into the solution and pressing the

piston. The SCC cassette was placed in the instrument and pressed the “Run” key. After 30 seconds the somatic cell count was presented on the instrument display. Individual quarter having SCC > 100 x 10<sup>3</sup> cells/ml were considered as SCM positive to reduce the occurrence of false-negative results (Malek dos Reis *et al.*, 2011).

### Bacteriological examination

All SCM positive quarter milk samples were analyzed bacteriologically. Isolation and identification of the bacteria were performed on the basis of culture characteristics, morphology and staining reactions and on the basis of biochemical tests (catalase, coagulase, oxidase, indole, methyl red, Voges-Proskauer, and sugar fermentation tests) as described by Quinn *et al.*, (2002). About 100 µl of milk samples from each SCM positive quarter was inoculated into nutrient broth (NB) within 24 hours after collection and incubated under aerobic condition at 37°C overnight for growth of organisms. Inoculums from nutrient broth were then spread into blood agar (BA) and MacConkey agar. After that, culture and subculture was performed into differential and selective media like Blood agar (BA), Mannitol salt agar (MSA), Eosin-methylene blue (EMB) agar and Triple sugar iron (TSI) agar. Isolates were classified into (1) major pathogens: *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter* spp.; (2) minor pathogens: coagulase-negative staphylococci (CNS); (3) uncommon pathogens: *Bacillus* spp., *Pseudomonas aeruginosa* and unknown and (4) mixed pathogens (Rahman *et al.*, 2010; Radostis *et al.*, 2007).

### Statistical analysis

SCC values of subclinical mastitic quarters and bacteriological culture results were entered into Statistical Package for the Social Sciences (SPSS 20.0) for statistical analysis. Independent samples t-test as well as One Way ANOVA followed by Duncan's Multiple Range Test were done to find out the significant variation in SCC corresponding to mammary quarters and different bacteria.

## Results

### Prevalence of subclinical mastitis and associated bacteria

Results of SCC of 240 quarter milk samples revealed that the overall quarter-level prevalence of SCM was 25% (Table 1). Among SCM positive quarters, 90% were culture positive. The most prevalent bacteria were *Staphylococcus aureus* (18.33%) followed by CNS (10%), *Enterobacter* spp. (6.67%), *Escherichia coli* (5%), *Bacillus* spp. (5%) and *Pseudomonas aeruginosa* (5%) (Table 2). However, overall isolation of major pathogens (30%) were higher than minor pathogens (10%) (Table 3).

**Table 1. Prevalence of SCM and status of quarters analyzed bacteriologically**

Parameter	No. of Quarters	Percentage
Somatic cell count (SCC) performed	240	
SCM positive (SCC $\geq 100 \times 10^3$ cells/ml)	60	25.00
Quarter analyzed bacteriologically	60	
Culture positive	54	90.00
Culture negative	6	10.00

**Effect of bacterial isolates on mean SCC**

The mean SCC of subclinical mastitic mammary quarters according to the types of individual bacteria and bacterial group are presented in Table 2 and 3, respectively. Significant variation in SCC was observed with mono infections by different bacterial spp. and was the highest for *Bacillus* spp. ( $713.67 \times 10^3$  cells/ml) followed by *Enterobacter* spp. ( $395.75 \times 10^3$  cells/ml), *Escherichia coli* ( $386.00 \times 10^3$  cells/ml), *Staphylococcus*

*aureus* ( $373.82 \times 10^3$  cells/ml), CNS ( $182.67 \times 10^3$  cells/ml) and *Pseudomonas aeruginosa* ( $138.67 \times 10^3$  cells/ml). Among mixed infections, SCC was significantly higher caused by *Bacillus* spp. and *Pseudomonas aeruginosa* ( $384.33 \times 10^3$  cells/ml) than other mixed infections. Considering pathogen groups, major pathogens induced significantly higher SCC ( $380.72 \times 10^3$  cells/ml) than minor pathogen groups ( $182.67 \pm 29.79$ ) as shown in Table 3

**Table 2. Bacterial isolates and mean SCC of subclinical mastitic mammary quarters according to the types of bacteria causing SCM in dairy cows**

Bacterial isolates	No. of quarter	% of SCM positive quarter	SCC ( $\times 10^3$ cells/ml) (Mean $\pm$ SEM)
<b>Mono infection</b>			
<i>Staphylococcus aureus</i>	11	18.33	$373.82^b \pm 97.33$
<i>Escherichia coli</i>	3	5.00	$386.00^b \pm 109.99$
<i>Enterobacter</i> spp.	4	6.67	$395.75^b \pm 126.02$
CNS	6	10.00	$182.67^c \pm 38.47$
<i>Bacillus</i> spp.	3	5.00	$713.67^a \pm 173.63$
<i>Pseudomonas aeruginosa</i>	3	5.00	$138.67^c \pm 6.96$
Unknown	6	10.00	$161.33^c \pm 21.83$
<b>Mixed Infections</b>			
<i>Staphylococcus aureus</i> and <i>E. coli</i>	3	5.00	$146.33^b \pm 11.61$
<i>Staphylococcus aureus</i> and <i>Enterobacter</i> spp.	2	3.33	$315.00^a \pm 14.50$
<i>Staphylococcus aureus</i> and <i>Pseudomonas aeruginosa</i>	2	3.33	$236.00^a \pm 34.00$
<i>E. coli</i> and CNS	3	3.33	$237.00^a \pm 36.00$
<i>E. coli</i> and <i>Bacillus</i> spp.	2	3.33	$189.00^b \pm 51.00$
<i>E. coli</i> and unknown	2	3.33	$263.00^a \pm 57.00$
<i>Enterobacter</i> spp. and unknown	2	3.33	$341.00^a \pm 39.00$
<i>Bacillus</i> spp. and <i>Pseudomonas aeruginosa</i>	3	5.00	$384.33^a \pm 48.67$

Values with different superscripts within a column in different types of pathogens vary significantly ( $p \leq 0.05$ ).

**Table 3. Mean SCC of subclinical mastitic mammary quarters according to the pathogen groups**

Pathogen groups	No. of quarter	% of SCM positive quarters	SCC ( $\times 10^3$ cells/ml) (Mean $\pm$ SEM)
Major pathogens <sup>1</sup>	18	30.00	$380.72^a \pm 65.35$
Minor pathogen <sup>2</sup>	6	10.00	$182.67^b \pm 38.47$
Uncommon pathogens <sup>3</sup>	12	20.00	$293.75^{ab} \pm 82.66$
Mixed pathogens	18	30.00	$264.17^{ab} \pm 22.25$

1= *Staphylococcus aureus*, *Escherichia coli* and *Enterobacter* spp.; 2= CNS; 3= *Bacillus* spp., *Pseudomonas aeruginosa* and unknown. Values with different superscripts within a column vary significantly ( $p \leq 0.05$ ).

**Effects of mammary quarter location on mean SCC**

The mean SCC distribution of SCM positive samples according to the mammary quarter's location are presented in Table 4. The mean SCC was significantly higher for rear quarters ( $341.25 \times 10^3$  cells/ml) than

front quarters ( $226.54 \times 10^3$  cells/ml). Among four quarters, right rear quarter had the higher mean SCC ( $365.63 \times 10^3$  cells/ml) than other quarters though it was not statistically significant.

**Table 4. Quarter-wise variation in mean SCC in SCM positive cows**

Quarter location	SCC (x 10 <sup>3</sup> cells/ml) (Mean ± SEM)	p-Value
Right front	239.29 ± 32.93	0.237
Left front	208.70 ± 33.38	
Right rare	365.63 ± 65.50	
Left rare	314.00 ± 51.96	
Front quarters	226.54 ± 23.43	0.050
Rear quarters	341.25 ± 42.03	

## Discussion

In this study, the quarter level prevalence of SCM based on SCC was 25% which is almost similar to the prevalence (26.2%) in Uruguay (Giannechini *et al.*, 2002). Another study conducted by Katsande *et al.*, (2013) showed comparatively lower prevalence (16.3%) of SCM in Zimbabwe. Previous studies in different regions of Bangladesh reported that the prevalence of SCM based on California Mastitis Test (CMT) varies from 29% to 37.6% (Islam *et al.*, 2011; Islam *et al.*, 2010) and based on SCC was 55% (Hoque *et al.*, 2015). However, comparative higher prevalence of SCM indicates lack of awareness about SCM and farmers are not adapted to use different screening tools to detect SCM in timely fashion and maintain the udder and teat hygiene.

Among SCM positive quarters, 90% were culture positive and 10% were negative. Abrahamsen (2012) also reported that the 75.1% of SCM positive quarter milk samples were culture positive. Another study shown that all (100%) SCM positive composite milk samples were culture positive (Kastande *et al.*, 2013). However, SCM positive quarter milk samples with no bacterial growth (22% and 55.1% respectively) were also obtained by Persson *et al.*, (2011) and Giannechini *et al.*, (2002). The reason behind the elevated SCC in the absence of bacterial isolates may be due to the numbers of bacteria being below detectable levels (10–100 organisms/ml) at collection time or the organisms may have been killed by the cow's immune system. Most frequently isolated bacteria was *Staphylococcus aureus* (18.33%) which suggests that the prevention of spread of contagious bacteria during milking was not effective. Similar to this, *Staphylococcus aureus* was the most frequently isolated pathogen in Sweden (19%), Jordan (22.2%), and Brazil (30.8%) (Persson *et al.*, 2011; Malek dos Reis *et al.*, 2011; Alekish, 2015). Besides other minor pathogens, *Staphylococcus aureus* was still the most prevalent pathogen in clinically healthy animals and both environmental and contagious forms of mastitis may be caused by *Staphylococcus aureus* (Rall *et al.*, 2013; Barkema *et al.*, 2009). Isolation of CNS (10%) is compatible with the results reported in Netherland (10.8%), Sweden (16%) and Germany (17.17%) (Persson *et al.*, 2011; Schwarz *et al.*, 2010; Sampimon *et al.*, 2009). Although Katsande *et al.*,

(2013) and Abrahamsen *et al.*, (2012) reported higher prevalence of CNS (28.7% and 27.6% respectively); these discrepancies may be related to changes in herd management and bacteriological ecology in the herd environment (Pitkala *et al.*, 2004). In this study, the result of isolation of *Escherichia coli* was almost similar to another study (6.67%) in Bangladesh (Kayesh *et al.*, 2015). Although, the frequency of isolation of *Enterobacter* spp., *Escherichia coli*, *Bacillus* spp. and *Pseudomonas aeruginosa* was almost higher than other studies reported in different parts of the world (Umar *et al.*, 2013; Shrestha and Bindari, 2012; Schwarz *et al.*, 2010; Iqbal and Siddique, 1999). However, this variation of isolation may be attributed by poor cleanliness, drainage and manure disposal in farms. Besides, milking practices in Bangladesh are also poor, only few farmers practice hygienic procedure before and after milking and post dipping is usually not adapted in most of the farms because calves stay with the cows after milking to suck residual milk. Therefore cow's teats are most prone to contaminated by environmental pathogens.

The main indicator of mastitis is the high SCC but it may vary according to the type of microbes infecting quarter (Sharma *et al.*, 2011). Variation in SCC to infection by different bacteria in this study may be due to the individual immune response and mechanism of development of infection by pathogens. In this study, we have found higher SCC for quarter infected by major pathogens ( $380.72 \times 10^3$  cells/ml) compared to minor ( $182.67 \times 10^3$  cells/ml) and other pathogen groups. Sargeant *et al.*, (2001) reported that quarters infected with minor pathogens tend to have lower SCC values than quarters infected with major pathogens. Malek dos Reis *et al.*, (2011) and Djabri *et al.*, (2002) had also reported that log SCC was highest (5.51–5.79) in sample with major pathogen isolations (5.51–5.79) compared with the minor pathogen (5.04–5.31). The probability of isolating a major pathogen increases as SCC exceeds  $200.00 \times 10^3$  cells/ml. The magnitude of SCC response to major pathogens varies among cows, however, differentiation of types of pathogens seem impossible based on SCC alone (Sharma *et al.*, 2011).

In this study, the mean SCC ( $341.25 \times 10^3$  cells/ml) was higher for rear quarters than front quarters. Almost similar to this result, Malek dos Reis *et al.*, (2011) reported that rear quarter had higher log SCC (5.10) compared with front quarters (5.03). Schepers *et al.*, (1997) also reported a higher SCC in rear quarters. Rear quarters might be more susceptible to infections than front quarters because of larger capacity and mass and greater exposure to environmental effects. In addition, teats of the rear quarters are frequently nearer to the floor, especially in older cows, and would thus be contaminated or subjected to injury more readily. Here we included the SCM positive samples only for the bacteriological investigation. It would be worthy if we

could investigate the SCM negative samples for identification of bacteria.

## Conclusion

The study indicates a high prevalence (25%) of subclinical mastitis in selected area. *Staphylococcus aureus*, *Enterobacter* spp., *Escherichia coli*, CNS, *Bacillus* spp. and *Pseudomonas aeruginosa* were associated with SCM as single infections or mixed infections. Of them *Staphylococcus aureus* were the most frequent bacteria causing single infection. It is interesting to note that 10% SCM positive quarters based on SCC had no infection. As in most of the cases of SCM there was higher SCC due to bacterial infection therefore special care should be taken in regards to quarter and teat hygiene to prevent ascending infection through mammary quarters. In addition, regular screening test should be adapted for early detection of SCM otherwise it may develop clinical disease.

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