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Occurrence and aetiology of subclinical mastitis in water buffalo in Bangladesh

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Research Article

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Abstract

Subclinical mastitis (SCM) in water buffalo is a production disease associated with decreased milk yield and impaired milk quality and safety. Water buffalo is an important livestock species in Bangladesh, but information about the occurrence and aetiology of SCM in this species is scarce. A cross-sectional study was conducted as part of the Udder Health Bangladesh Programme to (i) determine the occurrence of SCM and bulk milk somatic cell count (SCC) in water buffalo in Bangladesh, (ii) identify pathogens causing SCM and (iii) evaluate penicillin resistance in isolated staphylococci strains. Sixteen buffalo farms in the Bagerhat and Noakhali regions of Bangladesh were selected for study and a bulk milk sample was collected from each farm. In addition, 299 udder quarter milk samples were collected from 76 animals. The bulk milk samples were assessed by direct SCC and the quarter milk samples by California mastitis test (CMT). The occurrence of SCM calculated at quarter and animal level was 42.5 and 81.6%, respectively. Milk samples from 108 CMT-positive quarters in 48 animals and 38 randomly selected CMT-negative quarters in 24 animals were investigated using bacteriological culture. Estimated mean bulk milk SCC was 195 000 cells/ml milk (range 47 000– 587 000 cells/ml milk). On culture, estimated quarter-level intramammary infection (IMI) was 40.4%. The identity of isolated bacteria was confirmed by MALDI-TOF mass spectrometry. Non-aureus staphylococci (NAS) were the most common pathogens (24.7%) and, among 36 NAS tested, 36.1% were resistant to penicillin. Thus there was high occurrence of SCM on the study farms, with relatively high penicillin resistance in NAS. Further studies are needed to identify underlying risk factors and develop an udder health control strategy for water buffalo in Bangladesh.

Water buffalo (*Bubalus bubalis*) farming in Asian countries has grown exponentially over the past half century, and contributes around 13% of global milk production (Siddiky and Faruque, 2018). Water buffalo farming is also becoming popular in Bangladesh due to increasing demand for buffalo milk and milk products, greater resistance to many diseases compared with cows and lower management efforts and feeding costs (Hamid *et al*., 2016). A significant impediment to milk production by water buffalo herds is mastitis, which affects the quantity, quality, and safety of milk, causes heavy economic loss, leads to increased use of antibiotics, and impairs animal welfare (Salvador *et al*., 2012). Clinical mastitis (CM) can be diagnosed by visible changes in the milk, udder, and systemic condition of animals, but subclinical mastitis (SCM) remains undetectable in most cases due to lack of clinical signs (Patel *et al*., 2019). In water buffalo, SCM is around three-fold more common than CM (Ali *et al*., 2014) and is responsible for declining milk production, deteriorating milk quality and reduced milk processability. Subclinical mastitis is also a milk safety concern because of the presence of pathogenic microorganisms (Sharma *et al*., 2011). As the clinical signs remain unnoticed, affected animals may act as reservoirs that shed microorganisms continuously to the environment and affect their herd mates (Ali *et al*., 2014). Persistent infection also limits the efficacy of antimicrobial treatment by creating a fibrous barrier between the organism and the antibiotic (Putz *et al*., 2020).

Somatic cells, a combination of leucocytes and epithelial cells released during regeneration of udder secretory tissue, provide a second line of defense of the mammary gland, so their numbers increase in response to intramammary infection (IMI). These cells are present in high amounts in normal milk, but IMI or stress results in a significant increase in the quantity of somatic cells present in milk (Dang *et al*., 2007; Alhussien and Dang, 2018). Therefore, milk

somatic cell count (SCC) is regarded as a reliable parameter to determine milk quality and identify SCM (Sahin *et al*., 2017). However, it has been suggested that a combination of direct assessment of SCC and indirect assessment by the California mastitis test (CMT) may be the best option to diagnose SCM (Preethirani *et al*., 2015). As there is no fixed standard for SCC in water buffalo bulk milk, the threshold level of 200 000 cells/ml for cows (Adkins *et al*., 2017) has been used as the cut-off value for SCM, for example in a study on water buffalo in Nepal (Dhakal, 2006) and on Murrah buffalo in India (Dang *et al*., 2010).

Several studies have investigated the prevalence of SCM in buffalo populations around the world, with most reporting a prevalence of between 36 and 48% (Ali *et al*., 2011; Salvador *et al*., 2013; Preethirani *et al*., 2015). The most important aetiological agents of SCM are reported to be non-aureus staphylococci (NAS), *Staphylococcus aureus*, *Streptococcus* spp. and *Escherichia coli* (Dhakal, 2006; Patel *et al*., 2019). Among the staphylococci, NAS have been found to be dominant pathogens in IMI (Moroni *et al*., 2006; Ali *et al*., 2011; Guha and Guha, 2012; Locatelli *et al*., 2013) as well as in SCM and mild forms of clinical mastitis (Frey *et al*., 2013; Gindonis *et al*., 2013; Xu *et al*., 2015). In contrast, *S. aureus* is considered a significant pathogen of buffalo IMI and a concern due to its greater resistance to β-lactam antibiotics (Turutoglu *et al*., 2009).

A few previous studies have examined SCM prevalence in water buffalo in Bangladesh (Talukder *et al*., 2013; Islam *et al*., 2016; Biswas *et al*., 2020). However, there are no data available on buffalo herd-level SCC and studies investigating the susceptibility to β-lactam antibiotics of staphylococci in water buffalo in Bangladesh are scarce. Identifying SCM-causing pathogens and investigating antimicrobial susceptibility in these pathogens are critical prerequisites for implementing effective control of buffalo mastitis. The present study was undertaken to (i) determine the occurrence of SCM and bulk milk SCC in water buffalo in Bangladesh, (ii) investigate the pathogens causing SCM, and (iii) evaluate penicillin resistance in isolated staphylococci strains.

Material and methods

Study area and population

Around 40% of the existing water buffalo population in Bangladesh is concentrated in coastal areas, including the Noakhali, Bagerhat, and Bhola regions (Faruque *et al*., 1990). Two of these regions, Noakhali and Bagerhat, were selected for this study, based on ease of communication and time limitations. Bagerhat (22.8456°N, 89.5403°E) and Noakhali (22.8246°N, 91.1017°E) are located in the southern coastal part of Bangladesh and have buffalo populations of 21 570 and 13 168 head, respectively (Census, 2010; Huque and Khan, 2017). In Bagerhat, buffalo are reared mainly on household farms, where 1–3 milking buffalo are tethered inside the residence boundaries with minimum roughage and concentrate supplements, or in a semi-intensive system with ≥7 buffalo in enclosed sheds, with or without wallowing facilities in nearby ponds, swamp/marshy land, or rivers. In Noakhali, buffalo are mainly reared in a free-range system (*bathan*), with 50–500 buffalo in each *bathan*. Several closely located *bathans* combine to form a '*killa*', and there are several *killas* on each island in the Noakhali region. There are also scattered household farms (Habib *et al*., 2017).

Study design and sampling strategy

A cross-sectional pilot study was performed during September −October 2019 to make a preliminary mapping of the SCM status of buffalo in the study regions. A total of 16 buffalo farms were included. The sampling population was 76 lactating buffalo with 299 functional udder quarters (five blocked quarters). In selection of participating farms, a list of buffalo farmers was obtained from Upazila Veterinary Hospital and 14 household/*bathan* farms (12 in Noakhali, 2 in Bagerhat) with at least one lactating buffalo were selected, based on accessibility and farmer willingness. Two semi-intensive buffalo farms were also selected, one in the Bagerhat region and one in Noakhali (online Supplementary Figure S1). A convenient sampling technique (1–3 lactating buffalo from each *bathan*/household and all lactating buffalo from the semi-intensive farms) was applied, due to time limitations and inadequate data on the location of the buffalo farms. In both study regions, the sampling period was seven days.

Data collection and processing

A structured questionnaire in English was used to collect data on farm location (Noakhali/Bagerhat region), education level of the farmer (no formal education/primary/secondary/veterinarian), herd size (total number of buffalo), rearing system (household/*bathan*/semi-intensive), and number of lactating buffalo on each farm. Participation was voluntary and participants were informed that withdrawal was possible at any time. Oral and written consent were obtained from each participant before applying the questionnaire. Data collection was performed in face-to-face interviews with the farmers (farm owner or acting manager) and through on-farm observations following a checklist. One of the authors (SS), administered the questionnaire and made observations on-farm. All interviews were conducted in Bengali, but the questionnaire was completed in English. The completed observation checklists were re-checked for potential inconsistencies at the end of each day.

Analysis and case definition of subclinical mastitis

The SCM status of each buffalo quarter was determined and categorized on a scale from 1 to 5 using the CMT, following the process described by Baloch *et al*. (2016). A buffalo was considered positive for SCM if at least one of its four quarters had CMT ≥2. The same threshold was used to categorize SCM in individual udder quarters.

Sample collection and preservation

Samples (each 3–5 ml) of quarter milk for bacteriological culture were collected aseptically using 70% ethyl alcohol, following the NMC protocol (Adkins *et al*., 2017), from quarters with CMT score ≥2. CMT-negative samples were also randomly collected from 24 lactating buffaloes. Collected samples were immediately transferred to an insulated icebox, and transferred to a freezer (−10 to −15 °C) within two hours. Within seven days of field visits, the frozen milk samples were transported to the Udder Health Bangladesh laboratory at Chattogram Veterinary and Animal Sciences University. At the laboratory, the milk samples were stored at −20 °C and bacteriological culture was performed within 24 h.

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Table 1. Status of subclinical mastitis at animal and quarter level, based on 299 active udder quarters and 76 lactating water buffalo, on 16 buffalo farms in the Bagerhat and Noakhali regions of Bangladesh

	SCM (CMT ≥2)		IMI[†] (Culture positive)		CMT[††] score 1 (Healthy quarters) + IMI		CMT score ≥2 (SCM quarters) + IMI	
Trait	% (*N*)	95% CI	% (*N*)	95% CI	% (*N*)	95% CI	% (*N*)	95% CI
Animal	81.6% (76)	71.0 to 89.5	–	–	–	–	–	–
Quarter	42.5% (299)	36.8 to 48.3	40.4% (146)	32.4 to 48.8	31.6% (38)	17.5 to 48.7	43.5% (108)	34.0 to 53.4

SCM, Subclinical mastitis; CMT, California mastitis test (score range 1–5); IMI, Intramammary infection; CI, Confidence interval.

Table 2. Status of subclinical mastitis according to the California mastitis test at quarter level, based on 299 active water buffalo udder quarters and cultures of 146 quarter milk samples from 16 buffalo farms in the Bagerhat and Noakhali regions of Bangladesh

	Quarter		IMI	
Trait	% (No.)	95% CI	% (No.)	95% CI
CMT-1	57.5 (172)	51.7 to 63.2	31.6 (38)	17.5 to 48.7
CMT-2	32.4 (97)	27.2 to 38.1	40.0 (80)	29.2 to 51.6
CMT-3	7.7 (23)	4.9 to 11.3	45.5 (22)	24.4 to 67.8
CMT-4	2.0 (6)	0.7 to 4.3	80.0 (4)	28.4 to 99.5
CMT-5	0.3 (1)	0.008 to 1.8	100.0 (1)	2.5 to 100

CMT, California mastitis test (score range 1 to 5); IMI, Intramammary infection; CI, Confidence interval.

Bulk milk somatic cell count

After aseptically collecting the composite morning milk sample on each farm, an on-farm bulk milk SCC was performed using a DeLaval cell counter (DeLaval Group, Stockholm, Sweden; sensitivity 73%, specificity 86%) (Adkins *et al.*, 2017). This automated device determines SCC optically on a cassette loaded with 60 µl of milk stained with a DNA-specific fluorescent reagent. A digital camera takes pictures of the cell nuclei one-by-one and immediately displays the SCC results, expressed per µl milk.

Bacterial isolation and identification

A total of 146 quarter milk samples, comprising all CMT-positive samples ($n = 108$) and all randomly selected negative samples ($n = 38$) from 72 lactating buffalo (no negative samples were taken from the other four buffalo) were cultured in 5% bovine blood agar following the NMC protocol (Adkins *et al.*, 2017). After 24 h of incubation at 37 °C, at least two morphologically similar bacterial colonies were re-cultured on bovine blood agar. The pure cultures were enriched in brain heart infusion broth and incubated at 37 °C, after which 300 µl 50% buffered glycerol were added and the samples were stored at −80 °C until further use. The pure cultures were also cultured on selective mannitol salt agar, to identify *S. aureus* and NAS. Bacterial colonies were identified by colony morphology, colony colour, and haemolytic pattern. All culture media and reagents were manufactured by Oxoid®, Oxoid Ltd, Basingstoke, United Kingdom.

Species confirmation

The isolates from pure cultures were transported to the National Veterinary Institute, Uppsala, Sweden, in Copan Transsystem® (Capon, Brescia, Italy) transport media tubes, for species confirmation using a MALDI-TOF Biotyper 3.0 (Bruker Daltonics GmbH, Bremen, Germany) for validation (score >1.8) of phenotypic identification accuracy.

Penicillinase test of Staphylococcus spp.

All staphylococci were tested for β-lactamase production, using the cloverleaf method described by Mee'aad *et al.* (2018). For quality control, the strains *S. aureus* ATCC 29213 and *S. aureus* ATCC 25923 were used.

Statistical analysis

Collected data were imported into Microsoft Excel 2019 (MicrosoftCorp., Redmond, Washington, USA). The data were then cleaned, sorted, coded, and checked for inconsistency before export into STATA-IC-13 (StataCorp, 4905, Lakeway Drive, College Station, Texas 77845, USA) for descriptive statistical analysis. Results are presented as frequency and percentage, with 95% confidence intervals (CI). Demographic characteristics of farmers (location, education, farming system) are presented as frequencies and percentages.

Results

Descriptive statistics on farms and farmers

Of the 16 buffalo farms studied, three were in the Bagerhat region and 13 were in the Noakhali region. Farm type was mainly household farms ($n = 11$), followed by *bathan* ($n = 3$) and semi-intensive farms ($n = 2$). Among the farmers, seven had primary-level education, four had no formal education, four had achieved secondary-level education, and one farm was managed by a registered veterinarian. Twelve of the farms had 1–5 lactating buffalo, two had 6–10 lactating buffalo and the remaining two farms had ≥7 lactating buffalo. The numbers of animals sampled on each farm are listed in online Supplementary Table S1.

Occurrence of SCM and intramammary infection

Estimated SCM occurrence in the buffalo at animal and quarter level was 81.6 and 42.5%, respectively. At quarter level, the estimated occurrence of IMI was 40.4% and the combined occurrence of SCM (CMT ≥2) and IMI was 43.5% (Table 1). Among the SCM-affected quarters (CMT score 2–5), a CMT score of 2 was the most frequent (Table 2).

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Table 3. Pathogen distribution and penicillin resistance identified in quarter milk samples collected from water buffalo in the Bagerhat and Noakhali regions of Bangladesh (confirmed by MALDI-TOF mass spectrometry)

Trait	% (No.)	95% CI	Penicillin-sensitive NAS	Penicillin-resistant NAS
Non-aureus staphylococci	24.7 (36)	17.9 to 32.5		
Staphylococcus hyicus	(8)		8	0
Staphylococcus hominis	(6)		5	1
Staphylococcus chromogenes	(4)		4	0
Staphylococcus epidermidis	(4)		0	4
Staphylococcus sciuri	(3)		3	0
Staphylococcus arlettae	(3)		0	3
Staphylococcus kloosii	(3)		0	3
Staphylococcus warneri	(1)		1	0
Staphylococcus hemolyticus	(2)		2	0
Staphylococcus capitis	(2)		0	2
Total NAS	(36)		23	13
Streptococcus spp.	0.7 (1)	0.02 to 3.8		
Micrococcus spp.	5.5 (8)	2.4 to 10.5		
Bacillus spp.	2.7 (4)	0.8 to 6.9		
Corynebacterium spp.	2.1 (3)	0.4 to 5.9		
Arcanobacterium spp.	1.4 (2)	0.2 to 4.9		
Klebsiella spp.	0.7 (1)	0.02 to 3.8		
Moraxella spp.	1.4 (2)	0.2 to 4.9		
Others (*Rothia* spp., *Deinococcus* spp.)	2.1 (3)	0.4 to 5.9		
Non-specific pathogens	6.2 (9)	2.9 to 11.4		
No growth in sub-culture	6.8 (10)	3.3 to 12.2		

NAS, Non-aureus Staphylococci. 108 subclinical mastitis-positive quarters and 38 negative quarter samples were tested. Frequency of 36 NAS species was below the overall NAS estimates in the table.

Bulk milk somatic cell count

Mean bulk milk SCC on the 16 farms was 195 000 cells/ml milk (range 47 000– 587 000 cells/ml milk). In quartile terms, the lowest 25, 50 and 75% of farms had a bulk milk SCC value of 129 000, 184 000, and 233 000 cells/ml milk, respectively. The individual bulk milk SCC count for each of the 16 farms is shown in online Supplementary Figure S2.

Pathogen distribution and penicillinase production by Staphylococcus *spp.*

Among the 146 quarter milk samples, 79 (54.1%) bacterial isolates were obtained and 60 were confirmed by the MALDI-TOF results. Ten isolates yielded no bacterial growth on re-culture after shipment and nine isolates were non-identifiable on a MALDI-TOF spectral scale of >1.8. The dominant bacteria were NAS, specifically *S. hyicus* (Table 3). All 36 NAS isolates were subjected to a penicillinase test, in which 13 isolates (36.1%) tested positive for β-lactamase production (Table 3).

Discussion

The SCM status in two of Bangladesh's main buffalo farming regions was successfully estimated at animal- and quarter-level, and pathogen distribution and penicillin resistance by NAS were determined. The quarter- and animal-level occurrence of SCM was found to be rather high, which is consistent with previous reports from neighbouring countries and around the world (Sharma *et al.*, 2007; Preethirani *et al.*, 2015; Ahmed *et al.*, 2018). The quarter-level occurrence of 42.5% in the present study was in line with the 44–46% reported for other sub-tropical countries such as India and Egypt, where water buffalo production systems resemble those in Bangladesh. Other studies in India have reported higher occurrence (59–78%) (Bhanot *et al.*, 2012; Charaya *et al.*, 2015), while lower prevalence (28%) has been reported in a small-scale study in Bangladesh (Islam *et al.*, 2019). Compared with the quarter-level prevalence, the animal-level SCM prevalence was much higher in this study (81.6%), which is in line with previously reported values ranging from 70 to 78% (Bachaya *et al.*, 2005; Sentitula *et al.*, 2012). These differences in estimated SCM could be due to differences between study sites, study years, geographical areas, the criteria applied for SCM detection, climate variations, and underlying animal- or quarter-level risk factors (Hussain *et al.*, 2018). Investigating such differences was beyond the scope of this study, but further studies are needed to assess and quantify the farm- and animal-level factors that might influence SCM occurrence. Such knowledge could be valuable in controlling mastitis in water buffalo.

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Mean bulk milk SCC estimated in the present study was 195 000 cells/ml milk. This is in line with Mujeeb *et al.* (2009) and within the acceptable threshold of 200 000– 500 000 cells/ml buffalo milk established in India (Panchal *et al.*, 2016; Jhambh *et al.*, 2017). However, it exceeds the cut-off value of 100 000 cells/ml milk recently proposed for Murrah buffalo by NDRI-India and barely meets the cut-off of 200 000 cells/ml milk suggested for neighbouring countries such as Nepal and India (Dhakal, 2006; Alhussien and Dang, 2018). The median level of bulk milk SCC was 184 000 cells/ml milk, representing slightly better status. Increased SCC is positively correlated with IMI in water buffalo (Moroni *et al.*, 2006) but the increase in SCC level recorded here was still much lower than that in cows and goats (Moroni *et al.*, 2005). However, there was large variation in SCC level between the individual farms (online Supplementary Figure S2), an issue which was not explored further in this study. The level of SCC increases with entry of IMI-associated pathogens into the udder through the teat canal and with stress conditions due to seasonal diurnal variations in climate conditions and parity of the lactating buffalo (Alhussien and Dang, 2018; Bombade *et al.*, 2018). To date, relevant data on SCM in water buffalo in Bangladesh have been insufficient. The present study therefore makes a timely contribution, especially considering the increasing consumer demand for buffalo milk.

The occurrence of IMI in buffalo was shown to be high in this study (40.4%). Relative IMI occurrence in healthy and SCM-affected buffalo quarters was similar (40 and 44%, respectively). In water buffalo in India, IMI occurrence in SCM quarters of 38–45% has been reported (Singh *et al.*, 2014; Jhambh *et al.*, 2017), which is consistent with our findings. The most common pathogens in this study were NAS, which is also consistent with findings in other studies on water buffalo (Bansal *et al.*, 2007; Sharma *et al.*, 2007; Kumar, 2009; Singh *et al.*, 2014). *Staphylococcus hyicus* was the most dominant species among the NAS and has been identified previously in IMI in water buffalo in India (Singh *et al.*, 2014) and Brazil (Coimbra-e-Souza *et al.*, 2017). Non-aureus staphylococci, which are commonly found in the teat canal, are an important group of SCM pathogens and are often resistant to one or more antimicrobials (Condas *et al.*, 2017; Stevens *et al.*, 2018; Traversari *et al.*, 2019). In the present study, 36% of NAS exhibited penicillin resistance, which is in line with results reported in other studies in India and Brazil (Pizauro *et al.*, 2014; Singh *et al.*, 2014). This level of resistance is high in terms of public health safety and needs to be confirmed in future studies, which should also explore the underlying cause of dissemination of antibiotic resistance in buffalo herds in Bangladesh.

The discrepancies between the low bulk milk SCC in the buffalo herds studied (195 000 cells/ml milk) and the higher IMI at quarter level were not further assessed in the present study. However, a relatively low level of SCC and higher IMI in buffalo has been found in a previous study by Moroni *et al.* (2006). Presence of contagious *S. aureus* or *Streptococcus agalactiae* plays a major role in increases in SCC level above 500 000 cells/ml milk (Moroni *et al.*, 2018), so the absence of these pathogens in the present study could partly explain the lower SCC level. Moreover, the increase in SCC in IMI-associated quarters in buffalo may be lower than in dairy cows due to limited phagocytic activity. According to Sahoo *et al.* (1998), there is a core difference in lysosome containing hydrolases between cattle and buffalo, with lower concentrations of these enzymes in buffalo, resulting in lower phagocytic activity than in other ruminant species. The requirements of convenient sampling and small sample size imposed limitations in this pilot-scale study. However, the data obtained reflect the regional SCM situation in water buffalo in Bangladesh and can act as the basis for further national-level research.

In conclusion, high occurrence of SCM in water buffalo at quarter and animal level (42.5 and 81.6%, respectively) was found on buffalo farms in the Noakhali and Bagerhat regions of Bangladesh. A moderate rate of SCC was detected in buffalo bulk milk on these farms (7 out of 16 farms exceeded the SCC threshold), which is reasonably good for pre-harvesting buffalo milk in Bangladesh. These findings suggest that appropriate treatment and control strategies are needed to control mastitis in water buffalo in Bangladesh. NAS was the most frequently identified pathogen and the NAS strains detected frequently tested positive for penicillinase production, which emphasizes the importance of prudent use of antibiotics to maintain public health when consuming buffalo milk. A systematic assessment of the direct and indirect transmission pathways of these pathogens between water buffalo is urgently needed.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029921000698.

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