PREVALENCE OF SUB-CLINICAL MASTITIS IN LACTATING BUFFALOES DETECTED BY COMPARATIVE EVALUATION OF INDIRECT TESTS AND BACTERIOLOGICAL METHODS WITH ANTIBIOTIC SENSITIVITY PROFILES IN BANGLADESH

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ABSTRACT

Buffaloes, like cattle and goats, play a major part in the subsistance economy of rural people in Bangladesh. These livestock species are prone to the intramammary infections (IMI), which are associated with a lot of economic impact to the farmers. The prevalence and importance of clinical and sub-clinical mastitis (SCM) have been reported in cows and goats from Bangladesh and this study was undertaken to evaluate the indirect tests and bacteriological methods for the prevalence of SCM associated with host risk factors and antibiotic sensitivity profiles of bacterial isolates recovered from milk samples of apparently healthy mammary quarters of lactating buffaloes of an organized farm in Bangladesh during the period from June to November 2010. A total of 120 quarters milk samples from 30 available apparently healthy lactating cross-bred (Nili-Ravi × Murrah and Nili-Ravi × local) buffaloes were subjected to the Whiteside test (WST), the surf field mastitis test (SFMT) and the California mastitis test (CMT); those positive by the WST, SFMT and CMT were 35 (29.16%), 32 (26.66%) and 39 (32.50%) with an overall 56.66% prevalence of SCM in lactating buffaloes. The test with the highest diagnostic performance, for both animal-wise and quarter-wise prevalence of SCM was the CMT (56.66% and 32.50%), followed by the WST (53.30% and 29.16%), and the lowest was SFMT (50.00 and 26.66%), respectively. The highest prevalence of SCM was recorded at > 9 to 12 years of age (23.33%), 4th parity (16.67%) and late lactation (30.0%). The daily average milk production was insignificantly ($p > 0.05$) decreased in buffaloes (4.5 ± 0.72 liter / day) that had IMI (SCM) in comparison to buffaloes (4.8 ± 0.88 liter / day) without IMI. The CMT positive milk samples (n = 39) were subjected to bacterial culture isolation (the gold standard test for comparison of indirect mastitis test). Among the bacterial isolates of IMI, *Staphylococcus* spp. (30.77%) showed the highest frequency, followed by *Streptococcus* spp. (20.51%), *Bacillus* spp. (15.39%) and *Escherichia coli* (12.82%) as a single infection, and also recorded as mixed infection (12.82%) and 7.69% remained as unclassified bacterial growth. Moderate to high antibiotic sensitivity of *Staphylococcus* spp., *Streptococcus* spp., *Bacillus* spp. and *E. coli* was obtained with gentamicin, ciprofloxacin, enrofloxacin and chloramphenicol, but these organisms were found mostly resistant or less sensitive to ampicillin, amoxicillin and streptomycin. It may be concluded from these results that there is a high prevalence (56.66%) of SCM in buffaloes in Bangladesh and that the associated pathogens have already developed resistance due to indiscriminate use of antibiotics and accordingly, there is a need for proper attention to control of mastitis in buffaloes.

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INTRODUCTION

Buffaloes occupy a prominent place in the social, economic and cultural life of rural communities in most Asian countries. There are 170 million buffaloes in the world, 97% (164.9 million) in Asia, 2% (3.4 million) in Africa mainly in Egypt, and 0.2% (0.34 million) in Europe, mainly in Italy (FAO, 2004; Singh and Barwal, 2010). India has 56% (95.2 million), Pakistan 14% (23.8 million), China 13% (22.1 million) and Bangladesh 0.76% (1.3 million) of world buffalo population nearly 98% (166.6 million) of water buffaloes in Asia. The buffalo contributes 72 million tons of milk and three million tones of meat annually to world food, much of it in areas that are prone to nutritional imbalances. In addition, buffaloes are the source of 20 to 30% of the draught power in Southeast Asia, which is why the buffalo has been called the 'living tractor' of the east (Asia). They are also considered a 'walking fertilizer factory,' producing about 6.8 kg of dung daily (Cockrill, 1974). The 1.3 million buffaloes of Bangladesh produce about 96,000 MT of milk and 16,000 MT of meat annually (DLS, 2005). These buffaloes are mainly reared under rural conditions scattered throughout Bangladesh, although there is an organized Government Buffalo Breeding and Development Farm , which was established in 1985 and is situated in Bagerhat district of Bangladesh. Recently, the Bangladesh Government has undertaken a project to establish buffalo farms in 12 districts of Bangladesh (Anon, 2011). Research reports on buffalo health and production are very limited from Bangladesh (Samad, 2000; Islam *et al*., 2004). Among the many diseases that occur in buffaloes, mastitis is a frustrating, costly and extremely complex disease that results in a marked reduction in quality and quantity of milk (Harmon, 1994). The prevalence of mastitis has been reported to be lower in buffaloes (SCM 27% & CM 4%) as compared to cows (SCM 36% & CM 5.5%). This lower prevalence might be attributed to the tighter teat sphincter of buffaloes as compared to that of cows (Uppal *et al*., 1994). However, bubaline mastitis has been recognized as an economically serious disease as it is in cows in India (Pal *et al*., 1989; Kumar and Thakur, 2001; Sharma *et al*., 2007; Kavitha *et al*., 2009). and Pakistan (Hussain *et al*., 1984; Khan *et al*., 2004; Khan and Muhammad, 2005; Bachaya *et al*., 2005; Sharif and Ahmad, 2007; Muhammad *et al*., 2010; Sharma *et al*., 2010). but such a status has not been evaluated in buffaloes in Bangladesh (Samad, 2000). Generally mastitis occurs in two forms, clinical (overt) and sub-clinical (hidden). Clinical mastitis (CM) can be characterized by five cardinal signs of udder inflammation (redness, heat, swelling, pain and loss of milk production), while the SCM is bereft of any obvious manifestation of inflammation. SCM is 3 to 40 times more common than the CM and causes the greatest overall losses in most dairy herds (Schultz *et al*., 1978). Annual losses in the dairy industry due to mastitis was approximately 2 billion dollars in the USA and 526 million dollars in India, in which SCM is responsible for approximately 60 to 70% of these dollar losses (Merril and Galton, 1989; Varshney and Naresh, 2004). Lactating animals with SCM are those with no visible changes in the appearance of the milked /or the udder, but milk production decreases by 10 to 20% with undesirable effect on its constituents and nutritional value, rendering it of low quality and unfit for processing (Khan *et al*., 2004). Apart from causing colossal economic losses, this disease also poses the risk for the transmission of zoonotic diseases like tuberculosis, brucellosis, leptospirosis and streptococcal sore throat to human beings (Sharma *et al*., 2005; Samad, 2008). The invisible changes in SCM can be recognized indirectly by several diagnostic methods including CMT, WST, SCC, pH, chloride and catalase tests. Direct isolation and identification of intra-mammary infections (IMI) may have significant benefit as preventing CM and may lead to further understanding of the dynamics of the disease (Lam *et al*., 1996). In addition, the assessment of SCM etiological pathogens aids to classify the healthy sound milk samples from those of pH hazards. Considering these factors, the present study aimed to elucidate the prevalence of SCM in lactating buffaloes by comparative evaluation of three indirect tests, to isolate and identify the major bacterial pathogens causing IMI with their antibiotic sensitivity patterns and to investigate the association between host determinants and prevalence of SCM in lactating buffaloes.

MATERIALS AND METHODS

This study on SCM in lactating buffaloes was carried out on an organized Government Buffalo Breeding and Development Farm, situated in the district of Bagerhat, Bangladesh during the period from June to November 2010. Buffaloes were well managed under a semi-intensive husbandry system with raised floor. They were often provided with some green grass in addition to natural pasture and concentrate diet and were kept together in a common shed, but at advanced stage of pregnancy and lactation stage, they are maintained in separate sheds at a short distance from each other in a house. All the available lactating buffaloes were subjected to clinical and physical examination with special interest towards the udders and teats. A total of 30 lactating buffaloes (120 mammary quarters) which had normal udders, quarters and teats, were included for this study. The age (3 to 12 years), parities (1 to 6) and stage of lactation (early, mid and late) were recorded for each of the randomly selected 30 buffaloes. The average milk yield per day varied from 3.0 to 6.5 liters (Table 4).

Milk samples were collected after proper disinfection of teat surface with 70% ethanol. Then 20 ml of milk sample was collected aseptically from each quarter in separate sterile screw-capped vials after squiring few streams (Buswell, 1995). All indirect tests were conducted at the spot before milk sample collection for culture. The milk samples of all the 120 quarters were subjected to the White Side Test (WST), the Surf Field Mastitis Test (SFMT) and the California Mastitis Test (CMT).

White side test (WST)

The WST was performed with a prepared WST reagent (4% sodium hydrochloride) as per the procedure described by Kumar and Thakur (2001) and Sharma (2008). In brief, each milk sample was thoroughly mixed carefully to avoid violent shaking. The sample was sufficiently mixed to ensure an even distribute the sample. Then 50 µl of milk were placed on a glass slide with a dark background by micropipette. Subsequently 20 µl of WST reagent (4% sodium hydroxide) were added to the milk sample and the mixture was stirred rapidly with a toothpick for 20 to 25 seconds. A breaking up of milk in flakes, shreds and viscid mass was indicative of a positive reaction, while milky and opaque and entirely free of precipitant was indicative of a negative reaction.

Surf field mastitis test (SFMT)

The SFMT was performed and scored following the method described by Muhammad *et al.* (2010) In brief, 2.0 ml milk sample was drawn from a bottle into a cup and an estimated 2.0 ml reagent (Surf Excel®, Uniliver Bangladesh, 3.0% solution) was squirted from a polyethylene bottle. Mixing was accomplished by gentle circular motion of the paddle in a horizontal plane for a few seconds. The reaction developed almost immediately with milk containing a high concentration of somatic cells. The peak of reaction was obtained within 30 seconds and immediately scored as 1+, 2+ and 3+ and score ≥ 1+ considered positive for SCM.

California mastitis test (CMT)

The CMT was performed by using CMT kit (Leucocytest®, Synbiotics Corporation-2, France) as per the kit manufacturer's instructions as described by Rabbani and Samad (2010). In brief, 2.0 ml of milk sample was taken in the CMT paddle and equal volume (2.0 ml) of CMT reagent was added in each cup, rotated for few seconds and then the result was recorded within 30 seconds as 0 (negative) and T (trace) were considered negative or normal, while CMT scores of 1+ (weak positive), 2+ (distinct positive) and 3+ (strong positive) were taken as indicators of sub-clinical mastitis (SCM).

Bacteriological studies

The CMT positive milk samples (n = 39) were subjected to bacteriological culture as per method described by Quinn *et al*. (1994) Each bacterial colony was examined macroscopically (colony morphology) and microscopically (Gram's stain) as described by Merchant and Packer (1967). Identification of all isolates was performed by using standard biochemical tests (Buchnan and Gibbon, 1984).

Antibiotic sensitivity tests

Antibiotic sensitivity test of 20 bacterial isolates of single infection was performed to seven different antibiotics by the disc diffusion method (Oxioid Ltd., UK) as described by Bauer (1966) and Ellner (1978). The disc concentration of antibiotics included ampicillin (10 µg), amoxicillin (10 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), enrofloxacin (5 µg), gentamicin (30 µg) and streptomycin (10 µg). The procedure in brief was nutrient and blood agar cultures (24 h at 37°C) for each isolated organism were evenly spread over plates. About 2.5 ml of different bacterial suspensions were poured over the plates. Then the plates were tipped to one side and the surplus fluids were removed by suction. Cultures were allowed to dry for one hour at 37°C after which different antibiotics discs were carefully placed over the surface of the plate with the help of alcohol-flamed, fine pointed forceps. The discs were so placed that there was enough space around each disc for diffusion of the antibiotic. Plates were incubated for 48 h at 37°C and the zone of inhibition around each disc was measured. The inhibition of the growth was demonstrated by a clear zone of growth around the discs due to the result of two processes viz. (a) diffusion of the antibiotics and (b) growth of the bacteria. Sensitivity was expressed as '3+', '2+', '1+' and '–', expressing high, moderate, sensitive and resistant levels of susceptibility, respectively.

Statistical analysis

Results were analyzed by Chi-square test to observe the significant influence of age, parity, lactation stage and milk yield on SCM of lactating buffalo cows and Cochran's test for sensitivity and specificity of different diagnostic tests using Statistical Package for Social Science (SPSS) Version 13.0 (Coakes *et al*., 2006).

RESULTS AND DISCUSSION

The comparative diagnostic values of indirect tests and bacteriological methods were evaluated on 120 mammary quarter milk samples of 30 apparently healthy lactating cross-bred (Nili-Ravi × Murrah and Nili-Ravi × local) buffalo cows in an organized Government Buffalo Breeding and Development Farm, Bagerhat, Bangladesh.

Animal and quarter-wise prevalence of SCM

Animal-wise prevalence of SCM was 53.30%, 50.0% and 56.66%, while quarter-wise prevalence of SCM was 29.16%, 26.66% and 32.50% by using WST, SFMT and CMT, respectively (Table 1). These results support the findings of Sharma *et al.* (2007) who reported 66.00%, 68.60% and 72.0% animal-wise, and 38.99%, 42.0% and 45.0% quarter-wise prevalence of SCM by using modified WST, modified CMT and somatic cell count (SCC). In addition, Said and Abd-el-Mlik (1968) reported 38.07% SCM in buffaloes using WST and CMT. Anwar and Chaudhary (1983) reported an overall 47.5% prevalence of SCM in buffaloes using the Strip Cup test, pH test and WST. Rehman *et al.* (1983) reported prevalence of SCM 59.2% in cows and 36.8% in buffaloes using direct and indirect tests. Hussain *et al.* (1984) reported SCM 33% in cows and 8% in buffaloes using WST. Bachaya *et al.* (2005) reported 77.98% animal-wise and 58.75% quarter-wise prevalence of SCM in buffaloes using

SFMT. Sharif and Ahmad (2007) reported an overall 51.0% animal-wise and 37.75% quarter-wise prevalence of SCM by using SFMT in buffaloes. These differences in the prevalence of SCM might be due to differences in management practices, methods of detection, breeds of animals, immune response of animals and climatic conditions.

Comparative evaluation of indirect tests

The comparative evaluation of indirect tests (WST, SFMT, CMT) for the detection of SCM in lactating buffaloes is presented in Tables 1 and 2. The positive reaction of these indirect tests seem to depend on the presence of somatic cells (leukocytes) in the milk (Sharma *et al*., 2008). and the number of the somatic cells is mainly associated with the severity of intra-mammary infections (IMI). The principle of these indirect tests is that the reagents (detergents) dissolve or disrupt the outer cell wall and the nuclear cell wall of leukocytes (somatic cells), which are primarily fat (detergent dissolves fat). DNA is released from the nuclei of somatic cells and it strings or gels together to form a stringy mass. As the number of leukocytes in a quarter increases due to IMI, the amount of formed-gel increases parallel linearly (Sharma *et al*., 2010). In addition, the accuracy, sensitivity and specificity of these indirect tests are varied due to different chemicals the detergents contain.

Of the 120 milk samples, 32 (26.70%) were positive and 81 (67.5%) were negative for SCM by all the three tests (Table 2). Three (2.5%) samples were positive by WST and CMT but not by SFMT. Four (3.3%) samples were positive by CMT alone. The Qa for Table 2 was 10.57, which exceeds the critical value and indicates that the tests differ significantly from each other in the diagnosis of SCM as positive or negative. The results of the comparative evaluation of these three indirect tests showed highest diagnostic value of CMT in both the animal-wise (56.66%) and quarter-wise (32.50%) in comparison to WST (50.0% & 26.66%) and SFMT (50.0% & 26.66). It appears that CMT had significantly higher diagnostic value in comparison to WST and SFMT. These findings support Sharma *et al.* (2007) who reported 66.0%, 68.60% and 72.0% prevalence of SCM in buffaloes using Modified

WST, Modified CMT and SCC, respectively.

Quarter side-wise prevalence of SCM

The respective quarter side-wise prevalence of SCM in LF (left front), LH (left hind), RF (right front) and RH (right hind) using WST (30.0%, 26.66%, 20.0% & 40.0%), SFMT (23.33%, 30.0%, 20.0% & 33.33%) and CMT (33.33%, 30.0%, 23.33% & 43.33%) are presented in Table 3. It appears that the highest prevalence of SCM was recorded in the RH quarter by all the tests: WST (40.0%), SFMT (33.33%) and CMT (43.33%), whereas lowest prevalence was in the RF quarter represented as 20%, 20% and 23.33% by WST, SFMT and CMT, respectively. These observations support the report of Sharif and Ahmad (2007) who reported highest prevalence of SCM in RH quarter (30.46%) in comparison to LF (24.51%), LH (21.19%) and RF (23.84%) quarters. However, Khan and Muhammad (2005) reported a high prevalence of SCM in LH quarter (37%) in comparison to LF (18.5%), RF (14.8%) and RH (29.6%) quarters. Saini *et al*. (1994) also reported higher prevalence of SCM in hind quarters in comparison to front quarters but the highest in left hind quarters. Dhakal (2006) reported insignificant ($p > 0.05$) differences in the prevalence of SCM among LF (8.0%), LH (6.0%), RF (10.0%) and RH (8.0%) quarters. Kavitha *et al.* (2009) also did not find any significant ($p > 0.05$) difference on the prevalence of SCM in buffaloes among the LF (7.03%), RF (10.1%), LH (10.93%) and RH (10.93%) quarters. It may be concluded from these reports that the prevalence of SCM was higher in hind-quarters than fore-quarters, which may be due to the greater chances of hind-quarters being soiled with urine or contaminated from the tail.

Prevalence of SCM associated with host risk factors

It appears from Table 5 that the highest prevalence of SCM in buffaloes was recorded in the > 9 to 12 year (23.33%) age group in comparison to the > 6 to 9 year (20.00%), the > 12 year (06.67%) and the 3 to 6 years (06.67%) groups. Kumar and Sharma (2002) reported the highest prevalence of SCM in buffaloes between 5 and 7 years of age. Sharma *et al.* (2007) reported the highest prevalence of SCM in animals 5 to 9 years of age.

The maximum prevalence of SCM in buffaloes was recorded during the 4th parity (16.67%), followed by 1st (13.33%), 2nd and 3rd (10.0%) and the lowest during the 5th and 6th (03.33%) parity (Table 5). These results support the findings Sharma *et al*. (2007) who reported maximum prevalence of SCM during the 3rd and 4th parity. Kumar and Sharma (2002) also recorded majority of SCM cases during the 3rd parity. Kavitha *et al*. (2009) reported increased prevalence of mastitis with increase of parity in buffaloes.

An obvious trend of increasing prevalence of SCM was observed with the increased of stage of lactation. The highest prevalence of SCM was recorded in late lactation (30.0%) in comparison to early (20.00%) and mid (13.33%) lactation (Table 5). These observations are in conformity with the findings of Sharma *et al*. (2007) who reported maximum prevalence of SCM in the late lactation followed by early and mid lactation. Patil *et al.* (1995) also reported the highest prevalence of SCM during late lactation period as compared to early and mid lactation. Higher prevalence of SCM during late lactation might be due to fact that during this period buffaloes are more vulnerable to usher infection.

Effects of SCM on milk production

The daily average milk production in lactating buffaloes that had intramammary infection (IMI) showed an insignificant ($p > 0.05$) decreased (4.5 ± 0.72 liter / day) in comparison to buffaloes (4.8 ± 0.88 liter / day) that had no IMI (Table 4). This finding supports the observation of Moroni *et al*. (2006) who reported no drastic decrease in milk yield among the SCM affected buffaloes compared to healthy contemporaries. However, Dua (2001) has estimated Rs 17,233.2 million due to SCM in buffaloes as compared to Rs 6,962.9 million due to clinical mastitis in India. Munro *et al.* (1984) reported effects of mastitis on milk production, milk composition and quality of milk products.

Bacterial pathogens

The major agents involved in bacterial intramammary infection (IMI) isolated from milk samples were *Staphylococcus* spp. (30.77%), followed by *Streptococcus* spp. (20.51%), *Bacillus* spp. (15.39%), *E. coli* (12.82%) and mixed (12.82%) species (Table 6). These results support the report of Khan and Muhammad (2005) who reported *Staphylococcus aureus* was found with the highest frequency (45%), followed by *Streptococcus* spp. (23%), *E. coli* (18%) and *Bacillus* spp. (14%) in buffaloes. Similar results have also been observed by Memon *et al*. (1999) who reported *Staph. aureus* as the major pathogen (38%), followed by *Str. uberis* (13%), *E. coli* (11%) and *Klebsiella pneumoniae* (11%). Bhalerao *et al*. (2000) also reported *Staph. aureus* as the major pathogen (54.55%), followed by streptococci (36.36%), *E. coli* (4.55%) and Klebsiella (2.27%). Khan *et al.* (2004) reported *Staph. aureus* (45%) as the major pathogen, followed by *Streptococcus* spp. (23%), *E. coli* (18%) and *Bacillus* spp. (14%). Staphyococci are usually spread during the milking process (Harmon, 1993). Therefore, hygiene at milking is of paramount important in the control of IMI in lactating animals. Streptococci and *E. coli* are environmental pathogens, and their occurrences in mastitis are mainly associated with type of bedding and wallowing habits of buffaloes.

Antibiotic sensitivity

The emergence of drug resistant organisms causing mastitis due to indiscriminate use of antibiotics is well established in bovine mastitis in Bangladesh (Kader *et al*., 2002). Moreover due to lack of prophylactic agents, chemotherapy continues to play a major role in the therapeutic management of mastitis. For the success of the treatment, sensitivity testing plays a pivotal role. Recently newer antibiotics have been introduced for the treatment of both SCM and clinical mastitis. Thus, it has become imperative to control this dreaded disease with most effective antibiotic therapy. Hence, the present study was also designed to probe into *in vitro* sensitivity of isolated bacterial species from cases of SCM against a range of traditional as well as newly introduced antibiotics potentially useful in mastitis treatment and control programs.

The antibiotic sensitivity of randomly selected 20 different single culture isolates of *Staphylococcus* spp. (5 isolates), *Streptococcus* spp. (5 isolates), *Bacillus* spp. (5 isolates) and *E. coli* (5 isolates) were tested with seven different antibiotics (Table 7). It appears from the results of the antibiotic sensitivity profiles that all the tested four isolates of staphylococci, streptococci, bacilli and *E. coli* were found moderately (2+ / 20%) to highly (3+ / 80%) sensitive to gentamicin, ciprofloxacin, endrofloxacin and chloramphenicol, whereas all the bacterial isolates showed resistance (- / 0%) to less sensitivity (1+ / 20%) against ampicillin, amoxycillin and streptomycin (Table 7). These

Table 1. Prevalence (animal-wise and quarter-wise) and severity of sub-clinical mastitis in lactating buffaloes.

S/N	Test used	Types	Total number tested	Number positive (%)			Total No. (%)
				1+	2+	3+	
1	White Side Test	Animal-wise Quarter-wise	30 120	09 (30.00) 23 (19.16)	04 (13.33) 07 (05.83)	03 (10.00) 05 (04.16)	16 (53.30) 35 (29.16)
2	Surf Field Mastitis Test	Animal-wise Quarter-wise	30 120	08 (26.60) 20 (16.66)	05 (16.66) 08 (06.66)	02 (06.66) 04 (03.33)	15 (50.00) 32 (26.66)
3	California Mastitis Test	Animal-wise Quarter-wise	30 120	09 (30.00) 25 (20.83)	05 (16.66) 09 (07.50)	03 (10.00) 05 (04.16)	17 (56.66) 39 (32.50)

Table 2. Comparison of three indirect tests to detect sub-clinical mastitis in lactating buffaloes.

White Side Test (WST)	Surf Field Mastitis Test (SFMT)	California Mastitis Test (CMT)	Samples No. (%)	Test of Significance
+	+	+	32 (26.70)	Cochran's Q value (Qa) was 10.57 for 2 df at p = 0.00
-	-	-	81 (67.50)	
-	-	+	04 (03.30)	
+	-	+	03 (02.50)	

Table 3. Quarter-side-wise prevalence of sub-clinical mastitis in lactating buffaloes.

S/N	Test used	Quarter side	No. of samples tested	Number positive (%)			Total No. (%)
				1+	2+	3+	
1	White Side	LF	30	07 (23.33)	01 (03.33)	01 (03.33)	09 (30.00)
	Test (WST)	LH	30	05 (16.66)	02 (06.66)	01 (03.33)	08 (26.66)
		RF	30	03 (10.00)	02 (06.66)	01 (03.33)	06 (20.00)
		RH	30	08 (26.66)	02 (06.66)	02 (06.66)	12 (40.00)*
		Total	120	23 (19.16)	07 (05.83)	05 (04.16)	35 (29.16)
2	Surf Field	LF	30	05 (16.66)	01 (03.33)	01 (03.33)	07 (23.33)
	Mastitis Test	LH	30	05 (16.66)	03 (10.00)	01 (03.33)	09 (30.00)
	(SFMT)	RF	30	03 (10.00)	02 (06.66)	01 (03.33)	06 (20.00)
		RH	30	07 (23.33)	02 (06.66)	01 (03.33)	10 (33.33)*
		Total	120	20 (16.66)	08 (06.66)	04 (03.33)	32 (26.66)
3	California	LF	30	06 (20.00)	03 (10.00)	01 (03.33)	10 (33.33)
	Mastitis Test	LH	30	06 (20.00)	02 (06.66)	01 (03.33)	09 (30.00)
	(CMT)	RF	30	05 (16.66)	01 (03.33)	01 (03.33)	07 (23.33)
		RH	30	08 (26.66)	03 (10.00)	02 (06.66)	13 (43.33)*
		Total	120	25 (20.83)	09 (07.50)	05 (04.16)	39 (32.50)

LF = Left front quarter, LH = Left hind quarter, RF = Right front quarter, RH = Right hind quarter,
*Insignificantly ($p > 0.05$) higher prevalence.

Table 4. Comparison of milk production between sub-clinical mastitis negative (n = 13) and positive (n = 17) lactating buffaloes.

Animal No.	Milk production (l/d)		Animal No.	Milk production (l/d)		Animal No.	Milk production (l/d)	
	Normal	SCM		Normal	SCM		Normal	SCM
1	6.0	5.5	8	4.0	3.0	15	-	5.0
2	4.0	4.0	9	4.0	3.5	16	-	4.5
3	5.0	5.0	10	5.0	4.0	17	-	5.5
4	5.5	4.0	11	4.0	4.5			
5	5.5	4.5	12	4.0	5.0	Total	62	77
6	4.0	4.0	13	4.5	5.5	Mean	4.8	4.5*
7	6.5	5.0	14	-	4.5	± SD	± 0.88	± 0.88

SCM = Sub-clinical mastitis l/d = Liter / day n= No. of animals *Decreased insignificantly ($p > 0.5$)

Table 5. Host risk factors associated with sub-clinical mastitis in lactating buffaloes detected by California Mastitis Test (CMT).

S/N	Risk factors	No. of Buffaloes tested	Positive	Nagative
			No. (%)	No. (%)
1	Age (years)			
	3 to 6	05	02 (06.67)	03 (10.00)
	> 6 to 9	11	06 (20.00)	05 (16.67)
	> 9 to 12	10	07 (23.33)*	03 (10.00)
	> 12	04	02 (06.67)	02 (06.67)
	Total	30	17 (56.67)	13 (43.33)
2	Parity			
	1st	08	04 (13.33)	04 (13.33)
	2nd	05	03 (10.00)	02 (06.67)
	3rd	05	03 (10.00)	02 (06.67)
	4th	07	05 (16.67)*	02 (06.67)
	5th	03	01 (03.33)	02 (06.67)
	6th	02	01 (03.33)	01 (03.33)
	Total	30	17 (56.67)	13 (43.33)
3	Lactation period			
	Early (0 to 10 weeks)	11	05 (16.67)	06 (20.00)
	Mid (>10 to 20 weeks)	07	03 (10.00)	04 (13.33)
	Late (>20 to 24 weeks)	12	09 (30.00)*	03 (10.00)
	Total	30	17 (56.67)	13 (43.33)

*Highest insignificant ($p > 0.05$) values.

Table 6. Frequency distribution of bacteria isolated from milk samples (n = 39) of lactating buffaloes.

S/N	Bacterial species	+ ve, No. (%)	S/N	Bacterial species	+ve, No. (%)
1	*Staphylococcus* spp.	12 (30.77)	5	*Staphylococcus* spp.+*Streptococcus* spp.	02 (05.13)
2	*Streptococcus* spp.	08 (20.51)	6	*Bacillus* spp. + *Staphylococcus* spp.	02 (05.13)
3	*Bacillus* spp.	06 (15.39)	7	*Escherichia coli* + *Streptococcus* spp.	01 (02.56)
4	*Escherichia coli*	05 (12.82)		**Mixed infection**	05 (12.82)
	Single infection	31 (79.49)	8	Unclassified bacterial growth	03 (07.69)

n = 39 CMT positive mammary quarter milk samples

Table 7. Percentage *in vitro* sensitivity of different bacterial isolates to different antibiotics.

S/N	Antibiotics	Status	*Staphalococcus* spp.	*Streptococcus* spp.	*Bacillus* spp.	*E. coli*
1	Gentamicin	R	00.00	00.00	00.00	00.00
		LS	00.00	00.00	00.00	00.00
		MS	20.00	20.00	20.00	40.80
		HS	80.00	80.00	80.00	60.00
2	Ciprofloxacin	R	00.00	00.00	00.00	00.00
		LS	00.00	00.00	00.00	00.00
		MS	40.00	20.00	20.00	20.00
		HS	60.00	80.00	80.00	80.00
3	Enrofloxacin	R	00.00	00.00	00.00	00.00
		LS	00.00	00.00	00.00	00.00
		MS	40.00	40.00	20.00	60.00
		HS	60.00	60.00	80.00	40.00
4	Chloramphenicol	R	00.00	00.00	00.00	00.00
		LS	00.00	00.00	00.00	00.00
		MS	60.00	40.00	20.00	80.00
		HS	40.00	60.00	80.00	20.00
5	Amoxicillin	R	60.00	60.00	60.00	20.00
		LS	40.00	40.00	40.00	80.00
		MS	00.00	00.00	00.00	00.00
		HS	00.00	00.00	00.00	00.00
6	Ampicillin	R	40.00	40.00	60.00	40.00
		LS	60.00	60.00	40.00	60.00
		MS	00.00	00.00	00.00	00.00
		HS	00.00	00.00	00.00	00.00
7	Streptomycin	R	80.00	60.00	80.00	40.00
		LS	20.00	40.00	20.00	60.00
		MS	00.00	00.00	00.00	00.00
		HS	00.00	00.00	00.00	00.00

R = Resistance LS = Less sensitive MS = Moderately sensitive HS = Highly sensitive antibiotic sensitivity results support the earlier similar reports made on bovine and bubaline mastitis causing pathogens (Hussain *et al*., 1984; Chanda *et al*., 1989; Pal *et al*., 1989; Kader *et al*., 2002; Khan *et al*., 2004; Hussain *et al*., 2007).; however, results varied somewhat due to development of drug resistance due to the indiscriminate use of antibiotics in the treatment of mastitis in Bangladesh.

The high (56.66%) prevalence of SCM in the lactating buffaloes of the Government Buffalo Breeding and Development Farm - ultimately indicates the bad quality of milk available to the consumers. This is mainly due to the non-use of regular screening tests for SCM and the unhygienic management of animals and milking system. The CMT was found more sensitive than WST and SFMT, thus it could be used for regular screening of SCM in lactating buffaloes; however, SFMT can be used as a cheaper and easily available animal side SCM diagnostic test in poor countries like Bangladesh. In case of *in vitro* antibiotic sensitivity, the possible effective antibiotics are gentamicin, ciprofloxacin, enrofloxacin and chloramphenicol. Therefore, it is recommended that regular screening of SCM by using any available indirect test and the tested effective antibiotics be evaluated *in vivo* under field conditions.

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