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PREVALENCE AND ANTIBIOGRAM PROFILE OF *Staphylococcus aureus* ISOLATED FROM MILK SAMPLES OF LACTATING COWS WITH SUBCLINICAL MASTITIS IN GAZIPUR, BANGLADESH

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Abstract

Subclinical mastitis (SCM) has a prominent place amongst the factors that reduce milk production. The detection of disease at subclinical stage is much more effective to prevent the occurrence of clinical mastitis. The purposes of the study were to determine the prevalence of SCM, its causal bacterium *Staphylococcus aureus* and the most appropriate antimicrobial agent(s). A total of 416 quarters milk samples from apparently healthy dairy cows were examined by California Mastitis Test (CMT) to diagnose SCM. A cow was considered as positive to SCM if at least one quarter was CMT-positive. SCM positive quarter milk samples were subjected to bacteriological examination for isolation and identification of *S. aureus*. Kirby-Bauer disk diffusion susceptibility test was done to determine the antibiotic sensitivity pattern of *S. aureus* against seven commonly used antibiotics (penicillin, amoxacillin, ampicillin, oxacillin, ciprofloxacin, gentamicin and streptomycin). The overall prevalence of SCM in dairy cows of Gazipur district was 30.77% and of which 38.46% were found to be infected with *S. aureus*. Prevalence of SCM and isolation of *S. aureus* were comparatively higher in farm with large (21-30) cattle populations. Antibiogram profile revealed that *S. aureus* isolates were highly susceptible to gentamicin (100%), streptomycin (100%) and ciprofloxacin (80%) and were resistant to penicillin (100%), ampicillin (100%), and amoxiciilin (100%). All isolates were moderately (60%) to highly susceptible (40%) to oxacillin. The results concluded that SCM is common among dairy cows of Gazipur region, of which majority is caused by *S. aureus*. The identified bacterium is sensitive to commonly used antibiotics including β-lactam.

Keywords: California mastitis test, susceptibility, disk diffusion, bacterium, antibiotics.

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 (International Dairy Federation, 1987). Different pathogens can cause chronic, subclinical, subacute, acute and peracute forms of the disease (Radostis *et al.,* 2007). In subclinical mastitis, there are no obvious clinical signs such as abnormal milk, udder swelling or tenderness, or systemic signs such as fever, depression. Instead there is an increase in somatic cell count (SCC) of the milk (Radostis *et al.,* 2007). Subclinical mastitis (SCM) is 3–40 times more common than clinical mastitis and causes the greatest

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overall losses in most dairy herds (Bachaya *et al.,* 2011). In Bangladesh, the annual economic losses occur due to reduced milk production alone caused by SCM have been estimated to be Tk. 122.6 (US $ 2.11) million (Kader *et al*., 2003).

Milk is a major component in human diet all over the world and is rich in carbohydrate, proteins, fats, vitamins and minerals. However, health risk to consumers can be associated with milk, due to the presence of zoonotic pathogens and antimicrobial drug residues. Pathogenic organisms in milk can be derived from the cow itself, the human hand or the environment (Bradely, 2002). Mastitis caused by contagious pathogens is transmitted directly from cow to cow and consumption of such milk from affected cows may lead to food poisoning or in rare cases provide mechanisms for spread of disease to humans (Radostits *et al.,* 2007). *Staphylococcus aureus* is an important contagious pathogen that causes both nosocomial and community-acquired infections in people and is also responsible for disease in animals specially clinical and subclinical mastitis in dairy cattle worldwide (Petrovski *et al.,* 2009; Sampimon *et al.,* 2009).

Following the introduction of penicillin in the 1940's, staphylococcal infections were treated using penicillin. But over the years, strains of *S. aureus* producing penicillinase were detected, hence resistance developed against penicillin. Subsequently, new β-lactam antibiotics such as methicillin, oxacillin and cephalosporins that were resistant to the action of penicillinase, were developed. Shortly following the introduction of these drugs, the first emergence of methicillin resistant *S. aureus* (MRSA) was reported in human in the UK in 1960s (Jevons *et al.,* 1961). High-level resistance to β-lactams is conferred by a *mecA* gene encoding a modified penicillin binding protein (PBP2a or PBP'). However, in Bangladesh, cattle are the most commonly reared livestock and major source of milk. SCM is the prominent factor reducing milk production and causes greater economic losses in the dairy industry but most of the cases remain unnoticed by the farmer. *S. aureus* is one of the most common major pathogens causing SCM and treatment of staphylococcal mastitis become difficult due to resistant development against various antimicrobials. Prolonged and indiscriminate usage of antibiotics often leads to possible resistance development in the animals. Considering these facts, the present research work was undertaken to determine the prevalence of SCM, its causal bacterium *S. aureus* from milk samples and the most appropriate antimicrobial agent(s) for treatment.

Materials and Methods

Study population and area

A cross-sectional study was conducted in 104 randomly selected apparently healthy lactating cows from BSMRAU dairy farm and 16 smallholder dairy farms of Gazipur district. All cows at the farms were physically examined, and cows with signs of clinical mastitis were excluded. The minimum sample size (n=384) was calculated using the formula, $n=Z^2P(1-P)/d^2$ considering the average expected prevalence of 50%, absolute desired precision of 5% and confidence level of 95% (Thrusfield, 2007). A total of 416 quarter milk samples were examined by California

mastitis test (CMT) and CMT positive milk samples were collected and transported to the laboratory.

Detection of subclinical mastitis

California mastitis test (CMT) was carried out to detect SCM as per manufacturer's instruction (ImmuCell CMT test kit®, ImmuCell Corporation, USA). First few stream of milk was discarded and foremilk from each quarter was drawn into corresponding cup of testing paddle. Excess milk was discarded by tilting the testing paddle to level with the outside (largest) circle in each cup. Testing paddle was tilted back until milk was halfway between the inner and outer circles. CMT working solution was slowly added into each cup until mixture was even with the inside circle of each cup. Mixing was accomplished by gentle rotation of the paddle in a horizontal plane for few seconds. Positive samples showed gel formation within a few seconds and no gel formation indicated negative. The result was scored as negative (-), trace (±), weak (+), distinct (++) and strong (+++) based on the tendency toward gel formation. CMT-positive milk samples (CMT ±, +, ++, +++) of each examined quarter was suspected as samples of subclinical mastitis and analyzed bacteriologically. A cow was considered as positive to SCM if at least one quarter was CMT-positive and a farm was considered positive if one cow of the herd had SCM.

Collection and transportation of samples

CMT positive milk samples was collected and transported to the laboratory according to the procedure described earlier by Sumon *et al.* (2017). In brief, grossly dirty teats and udders were thoroughly washed and dried before proceeding with sample collection. The teat end and orifice were 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 since their cells and bacterial counts reflect the situation within the teat rather than that of the udder as a whole. The collection tube was held at a 45° angle to keep debris (hair, manure, dirt) from accidently falling into the tube. A volume of 10 ml of milk per udder quarter was collected in a sterile plastic tube and marked as front right (FR), front left (FL), rear right (RR) and rear left (RL). Then the samples were transported immediately to the laboratory in ice-box at 4°C.

Bacteriological examination of samples

Isolation and identification of the *Staphylococcus aureus* was performed on the basis of culture characteristics, staining reactions and biochemical tests as described by Quinn *et al.* (2002). About 100 µl of CMT positive milk sample was inoculated into nutrient broth (NB) and incubated at 37^{o} C for 24 hours. Then inoculums from nutrient broth were streaked on blood agar (BA) and Mannitol salt agar (MSA) media and incubated at 37^{o} C for overnight. The characteristic single colony of *S. aureus* was further subjected to Gram staining and biochemical tests (catalase, coagulase and oxidase tests) for confirmation.

Antimicrobial sensitivity test

Antimicrobial sensitivity test was done by Kirby-Bauer disk diffusion susceptibility test

(NCCLS, 2015). Each of the well isolated *S. aureus* was inoculated into nutrient broth and incubated at 37°C overnight. On the following day, freshly growing broth culture was uniformly spread on Muller-Hinton agar plate with the help of sterile cotton swab. Then antibiotic discs were placed apart onto the surface of the inoculated plates aseptically with the help of a sterile forceps and incubated at 37°C for 24 hours. After incubation, the plates were examined and the diameters of the zone of inhibitions were measured. The isolates were defined as resistant, intermediate and susceptible (Table 1) according to the standard guidelines (NCCLS, 2002). The following antimicrobial discs (Oxoid Ltd., Hampshire, UK) with their corresponding concentrations were used: Penicillin (10 µg), Amoxacillin (10 µg), Ampicillin (10 µg), Oxacillin (1µg), Ciprofloxacin (5µg), Gentamicin (10 µg) and Streptomycine (10 µg).

Statistical analysis

All farms and laboratory examinations related data were entered into Microsoft Excel spread sheet and then transferred into Statistical Package for the Social Sciences (SPSS 20.0). Chi-square test with Z-test for column proportion was done to identify the significant differences of the percentage values of SCM and isolation of *S. aureus.*

Results and Discussion

Prevalence of subclinical mastitis

Based on CMT screening, the overall prevalence of SCM in lactating cows in Gazipur district was 30.77%. The quarter level prevalence of SCM was 12.50% (Table 2). The result is in agreement with that in a previous study by Islam *et al.* (2011). However, the reported prevalence of SCM based on CMT was 20-43% in various regions of Bangladesh (Sarker *et al.,* 2013; Islam *et al.,* 2011 and Rabbani and Samad, 2010). Studies from other countries using similar criteria to characterize a cow as SCM positive or not, reported that prevalence rates varied from 25.2 to 55.2 % at cow level (Bitew *et al.,* 2010; Harouna *et al.,* 2009; Mdegela *et al.,* 2009 and Joshi and Gokhale, 2006). However, comparative higher prevalence of SCM indicates lack of awareness among farmers to use different screening tools to detect SCM in timely fashion and maintain the udder and teat hygiene. Besides, milking practices in Bangladesh are also poor, only

Table 1. Disk diffusion zone diameter (mm) interpretative chart of seven commonly used antibiotics against *S. aureus* (NCCLS, 2002)

Antibiotic (mcg/disc)	Resistant	Intermediate	Susceptible
Penicillin (10)	≤ 28	-	≥ 29
Amoxacillin (10)	≤ 19	-	≥ 20
Ampicillin (10)	≤ 28	-	≥ 29
Oxacillin (1)	≤ 10	11-12	≥ 13
Ciprofloxacin (5)	≤ 15	16-20	≥ 21
Gentamicin (10)	≤ 12	13-14	≥ 15
Streptomycine (10)	≤ 14	15-20	≥ 21

Table 2. CMT screening results and status of *S. aureus* isolated from CMT positive quarter milk samples

Parameters	No. of dairy cows (%)	No. of quarters (%)
CMT performed	104	416
SCM positive	32 (30.77)	52 (12.50)
S. aureus isolates		20 (38.46)

fewer farmers are practicing of pre-dipping and drying the teats before milking as well as post-dipping is usually not adapted in most of the farms because calves stay with the cows after milking to suck residual milk (Sumon *et al.,* 2017). Therefore cows are more prone to get infection by environmental pathogens.

According to the location of mammary quarters, the prevalence of SCM (15.38%) and isolation of *S. aureus* (50%) were higher for rare quarters than front quarters (Table 3). Among four quarters, rear left (RL) quarters were more commonly infected with *S. aureu* (66.67%) and rear right (RR) quarters had the highest prevalence of SCM (19.23%). Although mammary quarter location had significant effect on SCM but the effect on percentage of pathogen was not significant. A study conducted by Rabbani and Samad (2010) stated that quarter-wise prevalence of SCM was found comparatively higher for right and left hind quarters. Rear quarters might be more susceptible to infections than front quarters, as published previously (Pearson and Mackie, 1979), because of larger capacity and mass, greater vulnerability to direct trauma 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.

The farm was grouped into four on the basis of cattle population (Table 4). The majority of the dairy farms in the study area had 1-5 lactating cows and there was a considerable variation in the prevalence of mastitis depending on the farm size (40.00 to 100%). The farm with large herd size (21-30) was more prone to SCM than smaller farm. However, the effect of herd size on the prevalence of mastitis was not significant.

Considering herd size, the cow level prevalence of SCM (24.14 to 36.36%) and *S. aureus* isolation (42.86 to 75.00%) were also

Table 3. Prevalence of SCM and *S. aureus* causing SCM according to the mammary quarter location

Quarter location	No. of quarter	SCM positive (%)	*S. aureaus* positive (%)
Rear right (RR)	104	20 (19.23)^{a}	8 (40.00)^{a}
Rear left (RL)	104	12 (11.54)^{ab}	8 (66.67)^{a}
Front right (FR)	104	8 (7.69)^{b}	0 (0.00)
Front left (FL)	104	12 (11.54)^{ab}	4 (33.33)^{a}
Rear quarters	208	32 (15.38)^{a}	16 (50.00)^{a}
Front quarters	208	20 (9.62)^{a}	4 (20.00)^{b}

Values having different letter within a column differ significantly *($p \leq 0.05$)*

Table 4. Herd level prevalence of SCM in study area

Herd size	Farm studied	SCM positive herd (%)	p-value
1-5	10	4 (40.00)	0.34 (NS)*
6-10	04	2 (50.00)	
11-20	02	2 (100.00)	
21-30	01	1 (100.00)	

*NS: Non-significant, $p > 0.05$

higher for dairy farm with large population than smaller one although statistically not significant (Table 5). Almost similar to these findings, Rahman *et al.* (2009) reported that the prevalence of SCM in farm with large cattle population was comparatively high and ranged from 46.40 to 100%. This may be due to the poor hygienic management of large number of cows and cow itself may harbor organisms which may be the potential source of infection to others. Additionally, contagious nature of *S. aureus* may potentiate the transmission of SCM within and between farm populations.

Bacteriological results

All SCM positive quarter milk samples were subjected to bacteriological examination for isolation and identification of *S. aureus.* Colonies of *S. aureus* were golden yellow with beta (β) hemolysis on BA media (Fig. 1) and yellow colonies with yellow zones on MSA media (Fig. 2). They were Gram-positive cocci in clusters (Fig. 3); catalase and coagulase tests positive and oxidase test negative (Fig. 4). Overall, 38.46% of SCM positive quarters were found to be infected with *S. aureus* (Table 2). Almost similar to this finding, *S. aureus* was reported as the most frequently isolated pathogen of bovine mastitis in some other countries such as Algeria (40%), Ethiopia (43.3%), Zimbabwe and Australia (34.2-38.5%) and Brazil (30.8%) (Saidi *et al.,*

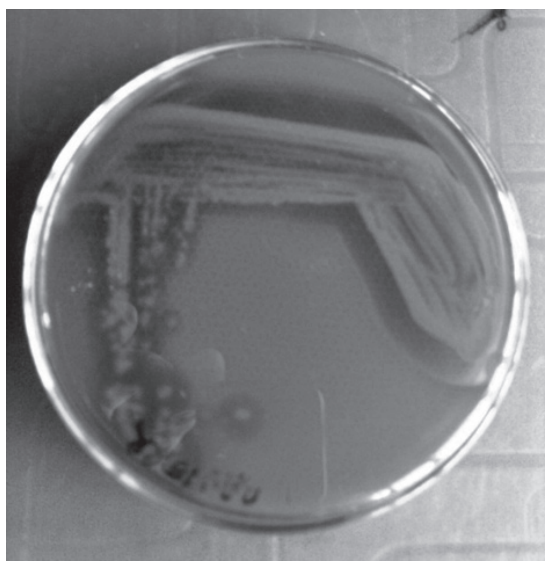

Fig. 1. Beta (β) hemolytic colony of *Staphylococcus aureus* on BA.

Table 5. Cow level prevalence of SCM and its causal agent *S. aureus* considering herd size

Herd size	No. of cows	SCM positive (%)	p-value	Isolation of S. aureus (%)	p-value
1-5	29	7 (24.14)	0.800 (NS)*	3(42.86)	0.625 (NS)*
6-10	27	9 (33.33)		6 (66.67)	
11-20	26	8 (30.77)		5 (62.50)	
21-30	22	8 (36.36)		6 (75.00)	

*NS: Non-significant, $p > 0.05$

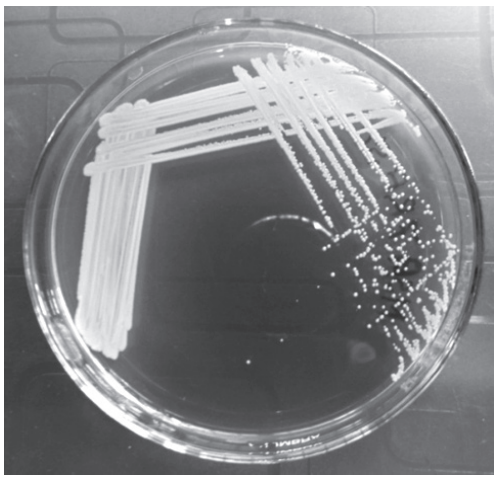

Fig. 2. Yellow colony with yellow zone of *Staphylococcus aureus* on MSA.

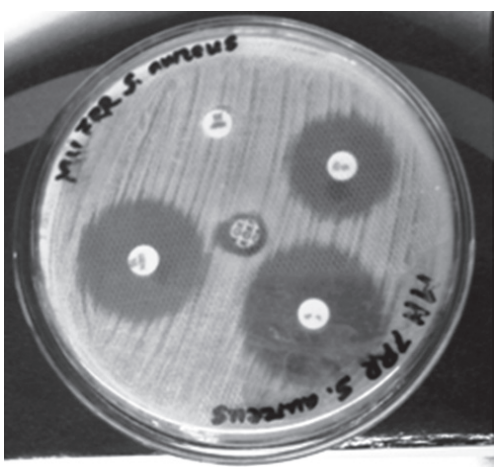

Fig. 3. Diameter of zone of inhibition of *S. aureus* to different antibiotics.

2013; Duguma *et al.,* 2013; Malek dos Reis *et al.,* 2011 and Kudinha and Simango, 2002). Higher prevalence of isolation of *S. aureus* as causal agent of SCM in this study indicated that the prevention of spread of contagious bacteria during milking was not effective (Sumon *et al.,* 2017). In addition, *S. aureus* was still the most prevalent pathogen in clinically healthy animals and associated with both environmental and contagious forms of mastitis (Rall *et al.,* 2013; Barkema *et al.,* 2009).

Antimicrobial susceptibility pattern

Antimicrobial susceptibility test was performed on all isolated *S. aureus* in this study. It was observed that *S. aureus* isolates were found to be highly susceptible towards Gentamicin, Streptomycin and Ciprofloxacin exhibiting 100%, 100% and 80% susceptibility, respectively (Table 6 and Fig. 5). These finding are almost in line with the previous study where *S. aureus* were highly susceptible towards Gentamicin (78.6-91.7%), Streptomycin (86.1%) and Ciprofloxacin (94.4%) (Sharma and Brintya, 2014; Islam *et al.,* 2014; Abera *et al.,* 2013).

In this study about 60% of *S. aureus* isolates were moderately susceptible to Oxaciilin and

Table 6. Susceptibility pattern of *S. aureus* to different antimicrobials

Antibiotics (mcg/disc)	Resistance (%)	Intermediate (%)	Susceptible (%)
P* (10)	20 (100)	0 (0.00)	0 (0.00)
AMP (10)	20 (100)	0 (0.00)	0 (0.00)
AMX (10)	20 (100)	0 (0.00)	0 (0.00)
OX (1)	0 (0.00)	12 (60)	8 (40)
CIP (5)	0 (0.00)	4 (20)	16 (80)
GEN (10)	0 (0.00)	0 (0.00)	20 (100)
S (10)	0 (0.00)	0 (0.00)	20 (100)

* P: Penicillin, AMP: Ampicillin, AMX: Amoxicillin, OX: Oxacillin, CIP: Ciprofloxacin, GEN: Gentamicin; S: Streptomycin

40% were highly susceptible. This indicates all isolates of *S. aureus* from milk samples were devoid of *mecA* gene, a gene responsible for resistance to betalactam antibiotics like methicillin, oxacillin and cephalosporins. However, in a Korean study, only a small percentage (1.3%) of *S. aureus* isolates from SCM milk samples were *mecA* positive that was methicillin resistanc (Lee, 2003). The findings of this study also revealed that all isolates (100%) were resistant to Penicillin, Ampicillin and Amoxicillin. This result is comparable with the reported resistant of isolates to penicillin (94.4%) and ampicillin (83%) as described by Abera *et al.* (2013) and Mekonnen *et al.* (2005), respectively. The resistance of *S. aureus* to penicillin, amoxicillin and ampicillin may be attributed to the production of penicillinase, an enzyme that inactivates penicillin and others closely related antibiotics. It is believed that around 50% of mastitis causing *S. aureus* strains produce betalactamase (Green and Bradely, 2004).

Conclusion

Subclinical mastitis is one of the most prevalent diseases (30.77%) in Gazipur district and *S. aureus* was associated with 38.46% cases of SCM. *S. aureus* isolates were found to be highly susceptible towards gentamicin, streptomycin and ciprofloxacin and moderately susceptible towards oxacillin. This group of antibiotics could be used more effectively for the treatment of staphylococcal mastitis. As the organisms resistant to penicillin, ampicillin and amoxiciilin, it would be better to avoid these antibiotics to reduce the misusages as well as to ensure cost effective treatment for SCM. However, further research is needed for the molecular detection of *S. aureus* and *mecA* gene from milk samples of dairy cows in Bangladesh.

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