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Characterization and antibiogram study of microbes involved in subclinical mastitis at Sylhet region

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Abstract

Subclinical mastitis is a common infectious disease of cow caused by different kinds of bacteria and fungi .In each year the dairy industry is suffered a great economic losses due to subclinical mastitis. So it is essential to investigate about causal agents and which drug works well in subclinical mastitis. This research aims to determine what organisms are responsible for subclinical mastitis as well as antimicrobial susceptibility of subclinical mastitis milk samples. The present study was conducted from January to June, 2017 to determine presence of microbes responsible to subclinical mastitis (*Staphylococcus aureus, Streptococcus agalactiae, Escherichia coli, Aspergillus spp and Candida spp*) in 103 California Mastitis Test (CMT) positive quarter samples collected from 59 dairy cows in 11 different dairy farms located in Sylhet region of Bangladesh. The prevalence of CMT positive quarter samples was 24.75%(103/416).Through culturing on Nutrient agar, Mannitol Salt agar, Eosin Methylene Blue agar, Edward's media, Blood agar, Mac Conkey agar, Sabouraud Dextrose Agar and Indole, Voges-Proskauer, Methyl-Red, Citrate, Triple Sugar Iron test of CMT positive samples, five isolates were showed the positive result, these were:- *S. aureus* (80/103,77.66%), *Strept. agalactiae* (72/103,69.9%), *E. coli* (10/103,9.7%), *Aspergillus spp* (70/103,67.96%), *Candida spp*(15/103,14.56%). Antibiotic susceptibility of the CMT positive samples was investigated by disc diffusion method. Ampicillin, Amoxycillin were resistance to 95% samples. Gentamycin, Ciprofloxacin, Trimethoprim-sulphamethazole, Tetracycline were highly susceptible to above 90% samples. Results of sensitivity to other antibiotics were varied. According this research work, *S. aureus*, *S. agalactiae*, *Aspergillus spp* are mainly responsible for subclinical mastitis and Gentamycin, Ciprofloxacin, Tetracycline will be suggested for treatment of subclinical mastitis since these antibiotics showed higher sensitivity than other antibiotics.

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Introduction

Bovine mastitis is one of important diseases of dairy cows and continues to have major economic impact on the dairy industry through the world (Bachaya *et al.,* 2011).Subclinical mastitis 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).This disease also poses a risk for the transmission of major zoonotic diseases like tuberculosis, brucellosis, leptospirosis and streptococcal sore throat to human beings (Bachaya *et al.,* 2011). Mastitis has therefore become a major area of concern in the field of veterinary clinical practice worldwide. There are several causal agents of subclinical mastitis in dairy cows -bacteria like *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis, E.coli*, *Actinomyces pyogenes, Pseudomonas aeruginosa, Nocardia asteroides, Clostridium perfringens and others like Mycobacterium, Mycoplasma, Pasteurella* and *Prototheca species* and fungi *like Aspergillus spp , Candida spp. Crytococcus neoformans, Saccharomyces* etc.

At present antimicrobial resistance is major concern to human health due to careless use of antibiotics in livestock production in developing countries. Bovine mastitis is the single most common reason for antimicrobial use in lactating cows. In Bangladesh the major antibiotics like ciprofloxacin, ceftraxione, gentamycin and trimethoprim-sulphamethoxazole. used for treatment of mastitis. The haphazard use of these antibiotics leads to antibiotic resistance.

The present study was conducted to determine the presence of major subclinical mastitis casual agent (*Streptococcus agalactiae, Staphylococcus aureus, E.coli, Aspergillus spp, Candida spp)* in dairy cows and investigate the in vitro susceptibility of CMT positive milk samples to several antimicrobial agents.

Materials and methods

Study area and sample

Total 103 CMT positive quarter samples were collected from 59 cows in 11 different dairy farms of Sylhet region of Bangladesh and transported to the laboratory of department of microbiology and immunology (Sylhet Agricultural University) in an ice-cooled box.

California Mastitis Test (CMT), Identification of bacteria and Fungi

In Farm, milk samples from each quarter were tested for subclinical mastitis using CMT reagents. This screening test was conducted according to the standard procedure (Quinn PJ *et.al*., 2002).The CMT positive milk samples were subjected to bacteriological examination according to the standard protocols with slight modifications (Quinn PJ *et.al* ,2002) (NMC,1990) (NCCLS,1997) . A full loop of milk samples was streaked on blood agar base enriched with 5-10% defibriniated sheep blood , then subcultured on selective media for different bacteria using., Mannitol Salt Agar for *Staphylococcus spp* , Eosine Methylene Blue Agar for *E.coli* , Edwards media for *Streptococcus spp*. For differentiation the samples were also cultured on Mac Conkey Agar . All plates were incubated aerobically at 34^{o}c for 24-48 h. The plates were examined for gross colony morphology, pigmentation, and haemolytic characteristics at 24-48h. For further identification of the organisms different biochemicals tests (Gram reaction, the catalase test, haemolysis, TSI test, IMViC tests) were conducted (NMC.1990). For fungal isolation, samples were inoculated in Sabouraud Dextrose Agar (SDA) and incubated at room temperature for 3-5 days. Then subcultured from primary culture (Bilgeham H.1995)

Antimicrobial Susceptibilty Test

Antimicrobial susceptibility test was conducted on 103 CMT positive milk samples and susceptibility test was conducted using Kirby-Bauer disk diffusion method (Quinn PJ *et.al,* 2002). About eight antimicrobials such as Gentamycin (10µg), Ciprofloxacin (5µg), Ampicillin (25µg)

Trimethoprium-Sulphamethoxazole (25µg), Neomycin (30µg), Ceftraxione (30µg), Amoxycillin (30µg) Tetracycline (30µg),(Himedia, India) were selected from main class of antimicrobials and investigated for sensitivity test. The antibiotic discs were placed on the surface of the inoculated agar plates using aspectic technique. The inhibition zone diameters were measured to the nearest millimeter and samples were characterized as susceptible(S), Intermediate (I), or resistant(R). (CLSI, 2008).

Data Management and Statistical Analysis

The data was properly gathered and recorded; The coded data was calculated by using MS Excel. The prevalence of subclinical mastitis quarter samples in the lactating cows was determined as proportion of quarter that showed CMT positivity out of the total quarters examined. The prevalence of different microbes from CMT positive samples were determined by dividing the number of positive samples by the total number of samples examined and multiplied by 100. P-values were calculated using single way ANOVA to determine distribution of antibiotic resistance properties of milk microbes among eleven farms of Sylhet region and also among the antibiotics used. Also P values were calculated to determine the prevalence of different species bacteria and fungi among eleven farms and among them.

Results and discussion

Prevalence of CMT positive quarter samples

According to P value, prevalence of CMT positive samples significantly different among eleven farms (*P<.05) and there is no significant difference of CMT among four quarter (**P>.05)

In present study, the prevalence percentage of CMT positive sample in Fore left quarter was 29.62, Fore right and Hind left quarter were same 22.22 and Hind right quarter was 21.96. (Table. 1).

Table 1. Prevelance of CMT positive quarter samples in the different farms.

Farm No	Number of quarter sample examined				CMT positive quarter sample				Percentage of CMT Positive sample.			
	FL	FR	HL	HR	FL	FR	HL	HR	FL	FR	HL	HR
1	5	5	5	5	0	0	1	1	0	0	20	20
2	10	10	10	10	3	4	3	3	30	40	30	30
3	16	16	16	16	6	1	4	5	37.5	6.25	25	31.25
4	6	6	6	6	3	2	3	3	50	33.33	50	50
5	7	7	7	7	2	2	2	1	28.57	28.57	28.57	14.28
6	3	3	3	3	3	2	1	1	100	66.66	33.33	33.33
7	3	3	3	3	0	2	1	0	0	66.66	33.33	0
8	6	6	6	6	1	1	0	0	16.66	16.66	0	0
9	25	25	25	25	8	7	7	7	32	28	28	28
10	18	18	18	18	4	2	1	1	22.22	11.11	5.55	5.55
11	5	5	5	5	2	1	1	1	40	20	20	20
Total	104	104	104	104	32	24	24	23	29.62	22.22	22.22	21.96

FL=Fore Left quarter,FR=Fore Right quarter,HL=Hind Left quarter,HR= Hind Right quarter.

The similar research was conducted in Nigeria that showed 590 (43.25%) milk samples from the four mammary quarters were CMT positive, while 681 (56.75%) samples were CMT negative.The individual quarter level prevalence of Subclinical Mastitis (SCM) was 50.67%, 43.67%,39.67% and 39.13% for the left fore-quarter (LFQ), right hind-quarter (RHQ), left hind–quarter (LHQ) and right fore-quarter (RFQ), respectively.(Aminu shiottu *et.al,*2012)

Results of Prevalence study of microbes involved in subclinical mastitis in study area

According to P value, prevalence percentage of bacteria were not significantly different among eleven farms (*P>.05) and prevalence percentages were not significantly different between three bacteria spp (**P>.05).

In present study, *Staphylococcus aureus* (77.66%) was found to be most prevalent organism followed by *Streptococcus agalactiae* (69.90%), *Escherichia coli* (9.7%). (Table. 3).

This is consistent with previous findings in India of 24% in clinical mastitis (Sumathi B R *et. al* 2008) and 34.7% in subclinical mastitis (Sharma N *et.al,* 2012). The prevalence of *Streptococcus* spp. was 19.6% and 17.2% for clinical and subclinical mastitis, respectively. Earlier studies have found a prevalence of *Streptococcus* spp. ranging from 5.5% to 16% in clinical mastitis and 31.9% in subclinical mastitis(Sumathi B R *et. al* 2008)(Sharma N *et.al* 2012) Other *Staphylococcus* spp. was the most common cause of clinical mastitis (30.5%) and also occurred frequently in subclinical cases (17.2%) in the present study.

Table 2. Cultural, morphological , staining and biochemical characteristics of isolated bacteria.

Feature	Appearance		
	E. coli	*S. aureus*	*S. agalactiae*
Nutrient Broth	Turbidity, cloudiness	Turbidity, cloudiness	Turbidity, cloudiness
Nutrient Agar	Smooth, circular,white to grayish white colonies	Smooth, circular,white to grayish white colonies	Smooth, circular,white to grayish white colonies
Eosin Methylene Blue agar	black color colonies with metallic sheen were found	---------	----------
MacConkey agar	Rose pink color colonies were formed	---------	----------
Mannitol salt Agar	----------	Round,smooth, glistening, yellow color colonies were produced	----------
Blood agar	----------	β haemolysis occur	β haemolysis occur
Edward's media	----------	----------	Transperant bluisy, grey colored to colorless colonies
Staining property	Gram negative, small rod shaped organism	Gram positive, small round cocci shaped organisms	Gram positive, organism found in chain form , but found in pair or single form
Indole	+	-	+
MR test	+	+	-
VP test	-	+	+
Citrate test	-	+	+
TSI test	Slant-Yellow,Butt- Yellow; gas produce at the bottom	Slant-Yellow,Butt- Yellow, no gas produce in butt.	Slant-Yellow,Butt- Yellow, no gas produce in butt.

+ =positive, - =negative; VP test=Voges-Proskauer test, MR test=Methyl-Red test,TSI test =Triple Sugar Iron test.

The study differed slightly with 16% *S. epidermidis* (CNS) in samples from clinical mastitis (Sumathi B R *et. al* 2008). Also, the prevalence of *Staphylococcus* spp. among subclinical cases in the present study was lower than the 29.3% of CNS (Sharma N *et.al*, 2012).

According to P value, prevalence percentage of fungi were significantly different among eleven farms (*P<.05) and prevalence percentages were significantly different between two fungi spp (**P<.05).

In present study, *Aspergillus spp* (67.96%) and *Candida spp* (14.56%) were found from subclinical mastitis milk samples. (Table. 5).

Table 3. Different bacteria isolated from milk samples of dairy cows in study area.

Farm No	Sample Number (CMT positive)	*E. coli*		*Staphylococcus aureus*		*Streptococcus agalactiae*	
		Number	Percentage	*Number*	*Percentage*	*Number*	*Percentage*
1	3	0	0	3	100	1	33.33
2	13	3	23.07	11	84.61	9	69.23
3	16	3	18.76	13	81.25	11	68.75
4	11	0	0	7	63.63	6	54.54
5	7	0	0	2	28.57	5	71.42
6	7	0	0	3	42.85	4	57.14
7	3	0	0	2	66.66	2	66.66
8	2	1	50	2	100	2	100
9	29	2	6.89	26	89.65	25	86.20
10	8	1	12.5	6	75	5	62.5
11	5	0	0	5	100	2	40
Total	103	10	9.7	80	77.66	72	69.90

Table 4. Cultural characteristics of *Aspergillus spp* and *Candida spp.*

Media used	Colony characteristics	Isolated fungi
Sabouraud Dextrose Agar	Greenish color colonies in SDA media	*Aspergillus spp*
Sabouraud Dextrose Agar	Creamy color colonies in SDA media	*Candida spp*

Fungi as an etiology agent of mastitis have also been reported by various worker. (Gaudie C .M *et. al* 2009) (Türkyılmaz, S. *et. al* 2010). Another study showed that prevalence of *Candida spp* in clinical and subclinical mastitis was 30% and 25% respectively. Prevalance of *Aspergillus spp* in clinical and subclinical mastitis was to be 40% to 37.5% (Pachauri S 2013). Other researchers have also reported *Aspergillus spp* as a causative agent of bovine mastitis (Blowey R *et. al* 2010) (Mdegela R H *et al.* 2005) (Stephan R *et al.,* 2000). As *Aspergillus spp.* is a common pathogen of human skin this high incidence of may be attributed to possible transmission during milking.

A higher percentage of isolation of fungi from clinical cases and subclinical cases reveal that the incidence of fungal mastitis is increasing which may be due to unhygienic conditions of the animal sheds supporting the growth of fungal spores and hyphae in the vicinity of lactating animals.

Thereby, increasing the probability of fungal spores and yeast cells to enter into the udder parenchyma which provides the best environment for the growth of these fungi (Elad D *et. al.,* 1995) (Williamson JH and Di Menna ME 2007) *Antibiogram profile.*

All CMT positive samples around 103 samples were selected for antibiotic sensitivity test. A total of 8 antibiotics were used against bacteria of milk samples.

According to p value, prevalence of susceptibility patterns of microbes were not significantly different among eight antibiotics (**p>0.05). But prevalence of susceptibility patterns of microbes were significantly different among eleven farms (***P< .05).

In present study, CMT positive Milk samples were found sensitive to Gentamycin (100%), Tetracyline (97%), Ciprofloxacin (94%), Trimethoprim-sulphamethoxazole (89%), Ceftraxione (88%), and Neomycin(77%), whereas CMT positive samples resistance to be Ampicillin (96%), Amoxycillin (97%), Ciprofloxacin (5%), Trimethoprim-sulphamethazole (4%), and Ceftraxione (2%).

CMT positive milk samples showed intermediate resistence to Neomycin (23%), Ceftraxione(10%) Trimethoprim-sulphamethoxazole (7%),Tetracycline (3%), Ampicillin (3%), Amoxycillin (2%). (Table.6), (Figure. 1)

The development of antibiotic resistance nearly always has followed the continuous and under dosage use of the antimicrobial agents (Quinn PJ *et. al.*, 2002).CNS isolate were 100% susceptible to Tetracycline, Amoxicillin and Kanamycin however intermediate to Chloramphenicol and Ampicillin (50%) (Bishi AS, 1998).

Table 5. Different fungi isolated from milk sample of dairy cow in study area.

Farm No.	Sample Number(CMT positive)	*Aspergillus spp*		*Candida spp*	
		Number	Percentage	Number	Percentage
1	3	2	66.66	1	33.33
2	13	8	61.54	3	23.07
3	16	12	75	2	12.5
4	11	9	81.81	1	9.09
5	7	5	71.42	2	28.57
6	7	3	42.85	1	14.28
7	3	2	66.66	0	0
8	2	1	50	0	0
9	29	24	82.75	5	17.24
10	8	5	62.5	0	0
11	5	2	40	0	0
Total	103	70	67.96	15	14.56

Table 6. Antimicrobial resistance and susceptibility patterns of microbes of milk sample (CMT positive).

Antimicrobial agent	Susceptibility		Intermediate		Resistence	
	Number	Percentage(%)	Number	Percentage(%)	Number	Percentage(%)
Neomycin	79	77	24	23	0	0
Gentamycin	103	100	0	0	0	0
Trimethoprim-sulphamethoxazole	92	89	7	7	4	4
Tetracyline	100	97	3	3	0	0
Ampicillin	1	1	3	3	99	96
Amoxycillin	1	1	2	2	100	97
Ciprofloxacin	97	94	1	1	5	5
Ceftriaxone	91	88	10	10	2	2

Another study shows among 108 bovine isolates tested the highest antimicrobial resistance patterns against *S. agalactiae* were observed for streptomycin and gentamycin (97.2% and 80.6%, respectively) (Rato MC *et.al* , 2013).

Conclusion

Based on results of the research, it can be concluded that many organisms are responsible for subclinical mastitis. Among these organisms *S. aureus, S. agalactiae, Aspergillus spp,* were requently isolated from CMT positive milk samples. Amoxycillin, Ampicillin showed resistance above 95% of CMT positive milk samples .On the other hand,

Gentamycin showed susceptibility to 100% samples, besides Gentamycin, Tetracycline and Ciprofloxacin showed susceptibility to 97% and 94% samples respectively. For best choice of antibiotics for treatment of subclinical mastitis cases, antimicrobial susceptibility test should be done. Because it reduce the cost of treatment of subclinical mastitis . Well established sanitation system help to reduce incidence of subclinical mastitis.

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