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Antimicrobial profiling of mastitis causing bacteria in buffalo milk

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ABSTRACT

Mastitis is causing huge economic losses to the dairy industry in India. The losses were mainly due to decreased milk production and treatment cost. This study was aimed to detect occurrence of mastitis in buffaloes. A total of 5,665 milk samples from buffaloes were processed for detection of mastitis by white side test (n=4,884) and culture examination (n=781). Overall, 2,808 (57.49%) milk samples were positive by white side test and 96.79% (n=756) samples were found positive by culture examination. Gram-positive and gram-negative bacteria were detected in 54.16% (n=423) and 31.11% (n=243) of the milk samples, respectively. Mixed infection of both was found in 10.62% (n=83) samples. While, *Candida spp.* was detected in 0.89% (n=7) samples only. Antibiotic sensitivity assay revealed that chloramphenicol was the most sensitive antibiotic against 71.61% samples in both gram-positive and negative bacterial isolates followed by enroloxacin, amikacin, levofloxacin, moxifloxacin and gentamicin in 71.09%, 61.77%, 58.85%, 58.59% and 55.99% samples, respectively. Kanamycin was most resistant against all isolates followed by neomycin (76.36%), norfloxacin (60.99%), amoxicillin+cloxacillin (52.78%), and ceftizoxime (51.85%).

Keywords: Antibiogram, Buffalo, Etiology, Mastitis, MDR

Mastitis is the most important disease of dairy industries from economic point of view. It is characterized by physical, chemical and bacteriological changes in the milk or inflammation in underlying parenchyma of the udder. It is a devastating disease of dairy animals that occurs in clinical and subclinical forms. Clinical mastitis is characterized by overt changes in the udder or milk; however, in sub-clinical mastitis (SCM) such changes are not obvious (Dixit *et al.* 2021). An economic loss of 6000 to 7615.51 crores has been estimated annually in term of decreased milk production and cost of treatment in India (Jeykumar *et al.* 2013, Bansal *et al.* 2019). The estimated loss of 2646 crores per annum is reported in India due to subclinical mastitis; however, actual loss is very difficult to quantify (Dua 2001, Sankar 2016). Various species of bacterial (gram-positive and gram-negative) and fungal agents are responsible for clinical and subclinical forms of mastitis. Other than etiological pathogen, various physiological, genetic, pathological or environmental factors also play major role in emergence of mastitis in dairy livestock's (Manasa *et al.* 2019). Proper treatment of mastitis in buffaloes may be carried out by selection of appropriate antibiotic against the infectious pathogens. Regular monitoring and time to time evaluation of area specific antibiogram could improve therapeutic efficacy and animal welfare (Singh *et al.* 2021). Thus, the present study was aimed to identify various gram-positive and gram-negative bacterial isolates in mastitis milk samples of buffalo along with their antibiotic sensitivity patterns. Occurrence of mastitis and its relationship with other parameter was detected in this study.

MATERIALS AND METHODS

Sample collection, processing and detection of mastitis: Buffalo's milk samples from in and around Mahendragarh, (28.2734°N, 76.1401°E) Haryana were collected in a sterile container by local farmers/livestock's owners and transported to Disease Investigation laboratory, Mahendragarh for detection of mastitis. Farmers were properly guided for sampling procedure while visiting laboratory. Milk samples (n=5,665) received for the period of one year (July 2019 to June 2020) except April 2020 (COVID-19 lockdown), were taken into consideration for present study. A semi-structured questionnaire such as age of animal/lactation number, lactation month and clinical signs, etc. was presented to the farmers/livestock owners for evaluation of risk parameters associated with occurrence of mastitis. Any of the clinical signs identified either in (blood, flakes, wateriness in milk) or udder (redness, pain, fibrosis, udder size abnormalities, etc.) of the animal was considered as clinical mastitic sample. Whereas, in sub-clinical mastitis sample, no clinical attributes were found

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in milk or udder of the animal. Animals were arranged into three groups for age parameter (young age 1-3 years of lactation number, mid age 4-7 years, and old age 8-10 years). Similarly, month of lactation was categorized into three groups (early lactation 1-3 months, mid 4-7 months, and late 8-10 months). Both clinical and subclinical samples (n=4,884) were initially screened for occurrence of mastitis by performing white side test (WST) (Ali *et al.* 2011). Briefly, equal quantity of 1N sodium hydroxide solution (NaOH) and milk samples (5 drop each) was mixed and stirred with an applicator for 10 s. The immediate gel formation after mixing was considered as positive and no gel formation as negative. Severity of infection was observed by degree of gel formation graded as trace (+), mild (++), moderate (+++) and severe (++++).

Culture examination and antibiotic sensitivity assay: Detection of various bacterial isolates was carried out by culture examinations of the samples (Quinn et al. 2011). Briefly, loop full inoculum of milk sample was streaked on to nutrient agar (Himedia) and MacConkey agar (Himedia) plate and incubated for 16-24 h aerobically in bacteriological incubator. Bacterial species were identified as gram-positive and gram-negative based on gram's staining and colony morphology (Charaya *et al.* 2014). *Candida spp.* were identified as budding yeast cells like structure (large size than bacteria) under the microscope (100×) on gram's staining and all antibiotics were found resistant while antibiotic sensitivity testing. All of them were subjected to antibiotic sensitivity test as per the guidelines (Bauer 1966) against 16 antibiotics (Himedia) of various classes and generations. The zone of inhibition around each disc was measured and interpreted using guidelines of clinical and laboratory standards institute guidelines (CLSI 2015) and European committee on antimicrobial susceptibility testing guidelines (EUCAST 2015).

Detection of multiple antibiotic resistance (MAR) index: Multiple antibiotic resistance (MAR) index value of gram-positive and gram-negative isolates was evaluated for assessment of being multi drug resistant (MDR) isolates (Krumperman 1983; Sharma *et al.* 2020). MAR index value higher than 0.2 signifies high risk potential source of spread of MDR.

Statistical analysis and interpretation: Descriptive analysis of data was carried out by Microsoft Excel Version 2010 and Statistical Package for Social Sciences (SPSS) Version 26 Software (George and Mallery 2019). Pearson's chi square test was used for analysis of various parameters on prevalence of mastitis. Interaction of lactation number/age and lactation month for mastitis prevalence, antibiotics sensitivity and resistance of gram-positive and gram-negative isolates, etc. were statistically analyzed by bivariate Pearson's correlation and regression tool of SPSS 26. Level of significance for MAR index value (above 0.2) was calculated by one-sample T test.

RESULTS AND DISCUSSION

Detection of mastitis in milk samples of buffalo: Milk samples (n=5,665) from buffalo were processed for

Table 1. Occurrence of mastitis in buffaloes

Parameter	Samples processed	Mastitis positive		Chi-square	*df* (degree of freedom)	P-value
		n	%			
White side test	4884	2808	57.49			
Culture examination	781	756	96.80	10.39	1	0.001*
Total	5665	3564	62.91			
Clinical mastitis	5665	384	06.78	73.96	1	0.000*
Sub-clinical mastitis		5281	93.22			
Flakes in milk		235	04.15			
Wateriness in milk	5665	193	03.41	0.20	2	0.905
Blood in milk		184	03.25			
Severity						
Trace (+)		3457	70.78			
Mild (++)	4884	980	20.07	58.66	3	0.000*
Moderate (+++)		1252	25.63			
Severe (++++)		797	16.32			
Lactation number/age						
Young age (1-3 years)	4329	2778	64.17			
Mid age (4-7 years)	1288	747	58.00	4.20	2	0.122
Old age (8-10 years)	48	39	81.25			
Lactation month						
Early lactation (1-3 month)	4203	2728	64.91			
Mid lactation (4-7 month)	1159	643	55.48	0.98	2	0.610
Late lactation (8-10 month)	303	193	63.70			

*Level of significance, P-value is significant at the 0.05 level or lesser.

detection of mastitis by white side test (n=4,884) and culture examination (n=781). Overall, 2808 (57.49%) and 756 (96.79%) milk samples were found positive by white side test (WST) and culture examination, respectively. Both the method for detection of mastitis had a significant (P<0.05) effect on the prevalence of mastitis (Table 1).

Severity of infection: Clinical mastitis and subclinical mastitis was found in 06.78% and 93.22% of the samples as depicted in Table 1. Occurrence of clinical and subclinical mastitis has significant variation (P<0.05) in our study. Flakes in milk was found in 04.15% (n=235) followed by wateriness (03.41%) and blood (03.25%) in milk. Presence of all clinical signs didn't have significant variations (P>0.05) in between them. Two or more clinical signs may be present in different quarters of an individual animal, at the same time. Severity of mastitis was measured by scores of white side test (WST) in this study. Presence of trace infection/intensity (+) was significantly high (P<0.05) in 70.78% of the quarter milk samples followed by moderate (+++, 25.63%), mild (++, 20.07%), and severe (++++, 16.32%) intensity. Severity of infection was found statistically significant (P<0.05) in present study.

Effect of different parameters on occurrence of mastitis: Both, age/ lactation number and lactation month had no significant (P<0.05) effect on the prevalence of mastitis (Table 1). The highest occurrence of mastitis was noted in the old age lactation followed by young age and mid age. Likewise, the early lactation month had highest occurrence of mastitis followed by late lactation and mid lactation. The incidence may be influenced by milk yield of animals at young age. The interaction between age/ lactation number and lactation month was analysed by bivariate Pearson's correlation with set level of significance at (P<0.05) as shown in Table 2. The occurrence of mastitis was positively correlated (62.60%) between young age (1-3 years of lactation number) and mid lactation (4-7 month). The linear regression line has given the best fit as the value of correlation coefficient (R^2) was high (R^2 =0.404) as shown

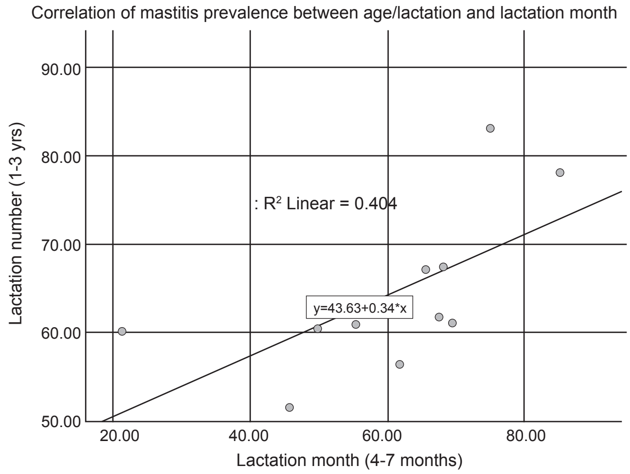

Fig. 1. Linear regression line for best fit between young age (1-3 years) and mid lactation (4-7 months).

in Fig. 1. Other combinations of age/ lactation number and lactation month were negatively or positively correlated but not significant (P>0.05) (Table 2). Occurrence of mastitis was highest in month of September (77.85%) and May (68.92%) as shown in Table 3. Frequency of mastitis occurrence between different months and seasons were not statistically significant (P>0.05) as shown in Table 3. Although, number of milk samples tested was highest in the autumn and winter season (October to March), but mastitis was more prevalent in rainy season (July, august, September). Frequency of mastitis between different months and season was statistically non-significant (P>0.05).

In agreement to the present study, Thakur *et al.* (2020) detected mastitis with higher frequency by culture examination (87.09% quarter milk sample) than WST (59.35% quarter milk sample) among buffaloes (n=135). Contrarily, Islam *et al.* (2019) found lesser occurrence of mastitis in buffaloes (27.50%) by WST. Sharma *et al.* (2018) found lesser occurrence (18.17%) of clinical

Table 2. Pearson Correlation between age/ lactation number and lactation month for mastitis prevalence

Variable		Age/ lactation number			Lactation month		
		Young age/L. No. (1-3 years)	Mid age/ L. No. (4-7 years)	Old age/ L.no. (8-10 years)	Early Lactation (1-3 months)	Mid lactation (4-7 months)	Late lactation (8-10 months)
Young age/L. No. (1-3 years)	Pearson Correlation	1					
	Sig. (2-tailed)						
Mid age/L.No. (4-7 years)	Pearson Correlation	-.060					
	Sig. (2-tailed)	.862					
Old age/ L.no. (8-10 years)	Pearson Correlation	.198	.357				
	Sig. (2-tailed)	.559	.282				
Early Lactation (1-3 months)	Pearson Correlation	.403	.268	.024			
	Sig. (2-tailed)	.219	.425	.944			
Mid lactation (4-7 months)	Pearson Correlation	.626*	.085	.358	-.324		
	Sig. (2-tailed)	.035	.804	.228	.331		
Late lactation (8-10 months)	Pearson Correlation	.012	.318	.136	-.573	.549	
	Sig. (2-tailed)	.973	.341	.689	.065	.080	-

*Correlation is significant at the 0.05 level (2-tailed).

Table 3. Month/ season-wise prevalence of mastitis in buffaloes

Parameter	Samples processed	Mastitis positive		Chi-square	*df* (degree of freedom)	P-value
		n	%			
Month						
January	700	470	67.14			
February	552	331	59.96			
March	250	166	66.40			
May	222	153	68.92			
June	305	178	58.36			
July	324	165	50.93	8.61	10	0.569
August	509	299	58.74			
September	799	622	77.85			
October	696	389	55.89			
November	760	460	60.53			
December	548	331	60.40			
Season						
Rainy (July, August, September)	1632	1086	66.54			
Spring/Autumn (October, November, March)	1706	1015	59.50			
Winter (December, January, February)	1800	1132	62.89	0.39	3	0.942
Summer (May, June)	527	331	62.81			

Level of significance, P-value is significant at the 0.05 level or lesser. *Samples were not received and processed in April 2020 due to COVID-19 lockdown in India.

mastitis in buffaloes. Subclinical form of mastitis may be 15 to 40 times more prevalent than clinical form in dairy animals. Detection of subclinical mastitis at initial stage is very important to initiate proper treatment, control and preventive measures. Contrarily, Baloch *et al.* (2018) observed significantly higher frequency of clinical mastitis (39.95%) than subclinical mastitis (26.95) in buffaloes. Contrary to present observation, Ali *et al.* (2021) found that traces intensity (4.5%) and severe intensity (54.50%) of mastitis was found in buffalo milk samples (n=312) using california mastitis test (CMT). The mild intensity (20.8%) and moderate intensity (20.2%) of infections was similar to the present study. Similar to present study, Ali *et al.* (2021) also noted highest prevalence of mastitis in the hot and humid months of July, August, and September in buffalo. Contrarily, they found least prevalence of mastitic in November.

Culture examination of milk samples: Overall, 96.72% (n = 756) milk samples were found positive for mastitis by culture examination. Gram-positive bacteria were seen in 54.16% (n=423) samples as blue coloured cocci in bunches of grapes like structure, whereas, gram-negative bacteria were seen in 31.11% (n=243) as pink colored rods under microscope (100×). Mixed infection of both was detected in 10.62% (n=83) samples. Frequency of gram-positive and gram-negative bacterial isolates was statistically significant ($P<0.05$) by Pearson's chi square test (χ^2=6.224; *df*-01). Budding yeast like fungal organisms (*Candida spp*.) were also seen under the microscope (100×) in 0.89% (n=07) of the samples only.

Bacterial species were categorised as gram-positive and gram-negative isolates based on gram's staining and colony morphology on nutrient agar plate and macConkey agar plate (Quinn *et al.* 2011). Culture examination was considered as the most suitable, accurate and reliable method to confirm the presence of the causative organisms and many investigations have assured it as the gold standard for identifying intra-mammary infections and for developing a specific mastitis control program for a dairy herd (Kaur *et al.* 2018; Singh *et al.* 2018). Similar to present findings, Thakur *et al.* (2020), revealed 67.78% of mastitis infections were due to gram-positive bacteria (mainly *Staphylococcus spp.*) while 32.22% were caused by gram-negative bacteria (*E. coli*). Singh *et al.* (2018) also found highest prevalence of *Streptococcus agalactiae* and *Staphylococcus aureus* followed by *E. coli* and *Klebsiella pneumonia* in bovine mastitic milk samples. Verma *et al.* (2018) found that major prevalent pathogens isolated from bovine mastitis were *Staphylococcus spp.* (42.55%) followed by *E. coli* (21.28%). The mixed infection was prevalent in 18.26% samples. As *Staphylococcus spp.* are predominantly found on skin, hands, inner lining of milking machine tubes, uncleaned containers, etc. it can easily propagate into udder after milking.

Antibiotic sensitivity assay: There are specific guidelines for particular bacteria (such as *Staphylococcus spp. Pseudomonas spp.*, etc) not as gram-positive or gram-negative organisms in CLSI. However, bacteria belongs to same order such as *Enterobacterales* includes *E. coli*, *Klebsiella spp.*, etc. have common interpretation

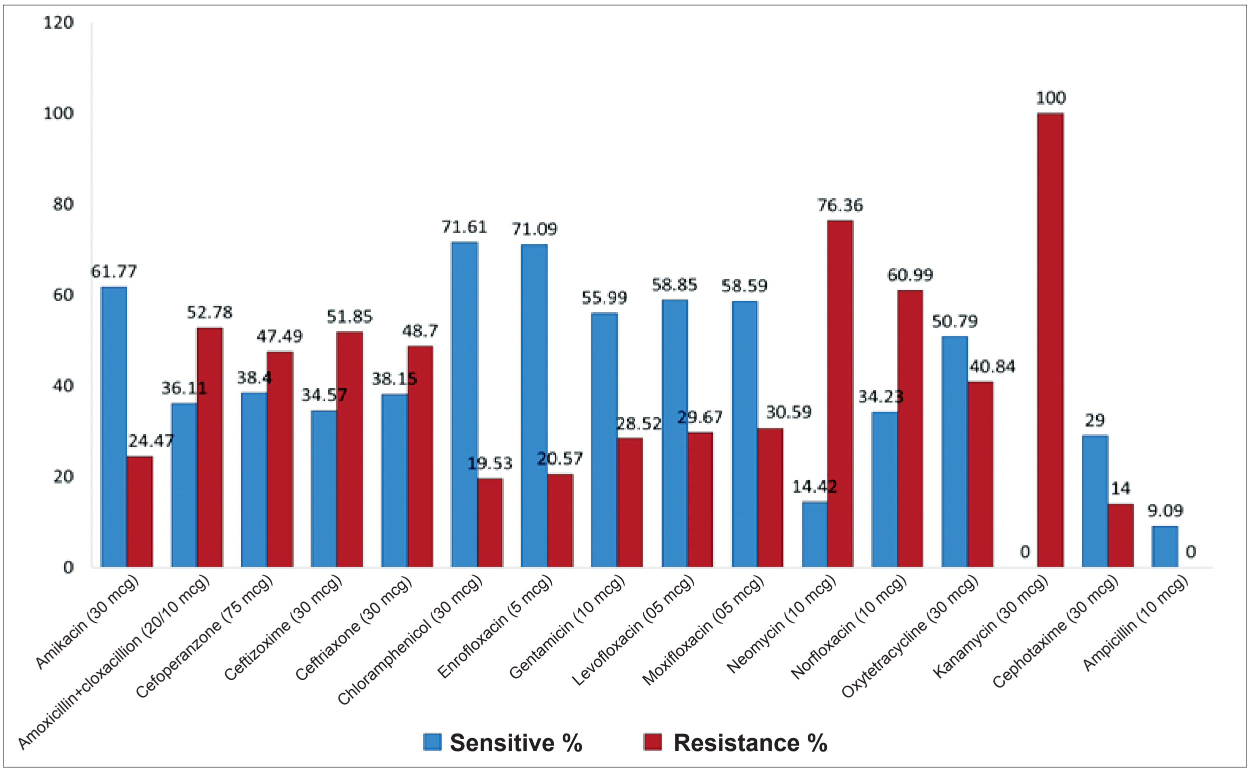

Fig. 2. Antibiogram of both gram-positive and negative organisms isolated from mastitic milk of buffaloes (n=756).

guidelines. In current study, isolates were primarily identified as *Staphylococcus spp.* on the basis of gram staining and colony morphology were categorized as gram-positive. Guidelines of *Staphylococcus spp.* was followed for interpretation. Likewise, gram-negative isolates were primarily identified as *E. coli*, *Klebsiella spp.*, *Pseudomonas spp.* on the basis of same gram's staining and colony characteristics. Guidelines of *Enterobacterales* and *Pseudomonas spp.* were followed for interpretation. Antibiotic sensitivity assay revealed that chloramphenicol was the most sensitive antibiotic against 71.61% isolates (gram-positive and gram-negative). Enrofloxacin, amikacin, levofloxacin, moxifloxacin and gentamicin was found sensitive against 71.09%, 61.77%, 58.85%, 58.59% and 55.99% of the isolates, respectively (Fig. 2). Antibiotic sensitivity patterns of gram-positive and gram-negative isolates had statistically significant positive correlation ($P<0.01$) with correlation coefficient ($R^2=0.764$) by bivariate Pearson's correlation and regression analysis. Antibiotic sensitivity patterns of gram-positive and gram-negative isolates were 87.40% positively correlated and had linear regression line as shown in Fig. 2. In the present study, kanamycin was most resistant antibiotics against all isolates followed by neomycin (76.36%), norfloxacin (60.99%), amoxicillin+cloxacillin (52.78%), and ceftizoxime (51.85%).

Multiple antibiotics resistance (MAR) index: Both gram-positive and gram-negative isolates (combined) of present study were detected as multidrug resistant (MDR). Level of significance ($P<0.05$) for MAR index value (above 0.2) was calculated by one sample-t test. The

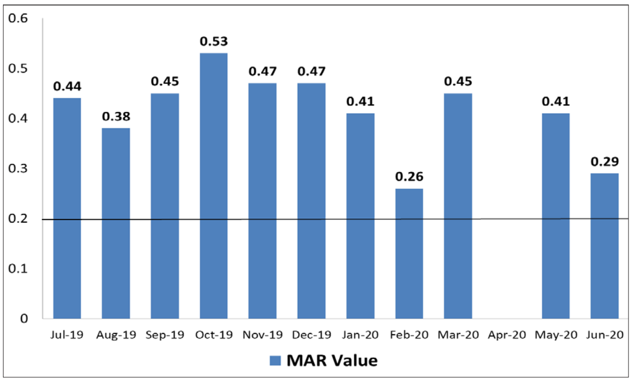

Fig. 3. Month wise MAR index value of both gram-positive and gram-negative organisms.

results were statistically significant ($P<0.05$). The value of t-test (8.946) was significant ($P<0.05$) at 95% confidence interval (Lower: 0.1611; Upper: 0.2680) and 10 degree of freedom (*df*). Month wise aggregate MAR value of isolates was ranged 0.26 to 0.53 as depicted in Fig. 3. The aggregate MAR value of all months was detected as 0.41.

In agreement, Singh *et al.* (2021) found that both *Staphylococcus aureus* and *E. coli* isolates were found sensitive to enrofloxacin and gentamicin and resistant to penicillin, amoxicillin, and streptomycin, vancomycin and methicillin, oxacillin, and ceftriaxone. Similarly, Dar *et al.* (2014) reported that enrofloxacin, gentamicin, tetracycline and amoxicillin clavulanic acid were effective against the whole milk cultures positive. The resistance towards ceftriaxone is in concordance with most of the recent reports of drug resistance pattern in investigations conducted on mastitis pathogens (Kumar *et al.* 2021, Singh *et al.* 2021). Contrarily, Amit *et al.* (2010) found that amikacin was the least susceptible antibiotics against pathogens isolated from bovine mastitis. MAR value of more than 0.2 signifies that group of bacteria/ individual bacteria were resistant to two or more than two antibiotics or may have encountered several antibiotics (Sharma *et al.* 2020). This emergence of multidrug resistance is due to indiscriminate use of antibiotics by vets and para-vets without knowing their antibiotic sensitivity patterns (Imran *et al.* 2021). MAR index an epidemiological tool that signifies potential risk of spreading MDR isolates in the environment.

High occurrence of mastitis was found in present study. Majority of the infections were caused by gram-positive bacteria followed by and gram-negative. Enrofloxacin, chloramphenicol, amikacin and gentamicin were most effective antibiotics *in vitro* against most of the bacterial strains detected in current research. Organisms were detected as multidrug resistant (MDR) organisms depending upon their MAR index value. Therefore, effective therapeutics management of mastitis could be achieved *in vitro* antimicrobial sensitivity testing.

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