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Antibiogram of bacterial pathogens causing mastitis among cattle

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ABSTRACT

The present study was carried out to detect mastitis in cattle milk samples and sensitive antibiotics for therapeutic uses. Milk samples from cattle (n = 1649) were tested for detection of mastitis by white side test (n = 1314) and culture examination (n = 335). Overall, 79.8% and 96.72% milk samples were positive by white side test and culture examination, respectively. Clinical mastitis and sub clinical mastitis were detected in 16.55% and 83.44% samples, respectively on the basis of presence of any clinical signs in milk or udder of animal. Gram-positive and gram-negative organisms were detected in 99.38% and 45.34% of the samples, respectively. Mixed infection of both was detected in 38.88% samples. More than 74% of both gram-positive and gram-negative isolates were found sensitive against enrofloxacin and chloramphenicol followed by amikacin (69.44%), gentamicin (68.73%), moxifloxacin (67.87%) and levofloxacin (61.73%). Organisms were detected as multidrug resistant (MDR) organisms depending upon their MAR index value.

Keywords: Antibiogram, Cattle, Mastitis, Pathogens

Mastitis is characterized by inflammation of the mammary gland due to various infectious pathogens like bacteria, mycoplasma, virus, yeast, fungi, etc. However, bacterial mastitis is the most common in dairy industries followed by mycotic mastitis (Sharun *et al.* 2021). In India, annual economic losses due to mastitis have been estimated to be ₹71.655 billion (Bhat *et al.* 2017). The losses were mainly due to decreased milk production, treatment and management cost. The disease is primarily caused by gram-positive (*Staphylococcus aureus*, *Streptococcus* spp. etc.) and gram-negative bacteria (*Escherichia coli*, *Klebsiella* spp. etc.) (Ganguly *et al.* 2015). Environmental hygiene plays an important role in infection where animal's environment such as bedding material, soil, manure, feces, and stagnant water acts as source of infection (Klaas and Zadoks 2018). Nutritional status of animal and other physiological factors such as age of animal/lactation number, lactation month, milk yield, etc. also have impact on mastitis prevalence (Ali *et al.* 2021). In the current scenario, antibiotics are the only reliable therapeutic management of mastitis. However, success rate is poor due to indiscriminate as well as improper use of antibiotics leading to development of multiple drug resistant pathogens (Arya *et al.* 2020). The present study was aimed to identify causative agents in mastitis milk samples from cattle along with their antibiotic sensitivity patterns.

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MATERIALS AND METHODS

Sample collection: Milk samples from cattle (n = 1649) for diagnosis of mastitis from the period of 1st July, 2019 to 30th June, 2020 were received in sterile container by local dairy farmers at Disease Investigation Laboratory, Mahendergarh (28.2734°N, 76.1401°E) (LUVAS, Hisar), Haryana, India. Farmers were properly guided for sampling procedure while visiting laboratory. In April 2020, none of the milk samples could be received due to COVID-19 Lockdown in India. Animal's history and other information such as age of animal/lactation number, lactation month and clinical signs, etc. were recorded from livestock owners' for further studies. The age of animal was measured using lactation serial number and categorized into three groups (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). Clinical and subclinical forms of mastitis were identified on the basis of clinical symptoms shown either in milk (blood, flakes, wateriness in milk) and udder (redness, pain, fibrosis, udder size abnormalities etc.) of the animal.

Detection of mastitis by white side test (WST): Occurrence of mastitis and its severity was detected in milk samples (n = 1314) (both clinical and subclinical samples) by performing white side test (WST) as described by Ali *et al.* (2011) and Badiuzzaman *et al.* (2015). Severity of infection was observed by degree of gel formation graded as trace (+), mild (++), moderate (+++) and severe (++++).

Culture examination: Detection of various bacterial isolates was carried out by culture examinations of the samples (Quinn *et al.* 2011). Bacterial species were identified as gram-positive and gram-negative isolates on the basis of gram staining and colony morphology. *Candida* spp. were identified as budding yeast cells like structure (large size than bacteria) under the microscope (100×) on gram staining and all antibiotics were found resistant while antibiotic sensitivity testing.

Antibiotic sensitivity test and detection of multiple antibiotic resistance (MAR) index: In our study, isolates were primarily identified gram-positive and gram-negative on the basis of gram staining and colony morphology. Isolates obtained from 335 milk samples were subjected to antibiotic sensitivity test as per the guidelines described by Bauer (1966) and Quinn *et al.* (2011) against 16 antibiotics (HiMedia). Zone of inhibition around each disc was measured and interpreted using clinical and laboratory standards institute (CLSI 2015) and European committee on antimicrobial susceptibility testing (EUCAST) guidelines (2015). Guidelines of *Staphylococcus* spp. and *Enterobacterales* were followed for interpretation as there are specific guidelines for particular bacteria species (such as *Staphylococcus* spp. and *Enterobacterales*) not as gram-positive or gram-negative organisms. However, bacteria belong to same order such as *Enterobacterales* (*E. coli* and *Klebsiella* spp. etc.) have common interpretation guidelines. Both gram-positive and gram-negative isolates were evaluated for their multiple antibiotic resistance (MAR) index value. MAR index value higher than 0.2 signifies high risk potential source of spread of multi-drug resistant (MDR) isolates (Krumperman 1983).

Statistical analysis: The occurrence of mastitis data was calculated using simple percentage values and frequencies in Microsoft Excel Version 2010. The interaction between age/ lactation number and lactation month was analyzed by bivariate Pearson's correlation with set level of significance at (p<0.01). Statistical analysis was done by Statistical Package for Social Sciences (SPSS) Version 26 Software (George and Mallery 2019).

RESULTS AND DISCUSSION

Detection of mastitis in cattle: A total of 1649 milk samples from cattle were tested for detection of mastitis by white side test (n = 1314) and culture examination (n = 335). Of them, 71.84% (n=944) and 96.72% (n=324) milk samples were positive by white side test (WST) and culture examination, respectively. The overall mastitis detection rate was found in 76.90% (n =1268) samples. Both the method for detection of mastitis doesn't had a significant (p<0.05) effect on the occurrence of mastitis (Table 1). Other researchers have used various terms such as occurrence, prevalence or incidence for mastitis. In our study, we use 'occurrence' as tested samples tested were already suspected for mastitis instead of random sampling.

Table 1. Prevalence of mastitis in cattle

Parameter	Samples processed	Mastitis positive		Chi-square	*df* (degree of freedom)	*p*-value
		n	%			
White side test	1314	944	71.84			
Culture examination	335	324	96.72	3.69	1	0.054
Total	1649	1268	76.90			
Clinical mastitis	1649	273	16.55	43.56	1	0.000*
Sub-clinical mastitis		1376	83.44			
Flakes in milk		136	08.24			
Wateriness in milk	1649	108	06.54	0.56	2	0.756
Blood in milk		169	10.24			
Severity						
Trace (+)		1011	76.94			
Mild (++)	1314	475	36.14	25.83	3	0.000*
Moderate (+++)		458	34.85			
Severe (++++)		524	39.87			
Lactation number / age						
Young age (1-3 years)	1196	891	74.50			
Mid age (4-7 years)	402	340	84.58	1.06	2	0.587
Old age (8-10 years)	51	37	72.55			
Lactation month						
Early lactation (1-3 month)	1181	894	75.70			
Mid lactation (4-7 month)	351	283	80.63	0.16	2	0.922
Late lactation (8-10 month)	117	91	77.78			

*Level of significance: *p*-value is significant at the 0.05 level or lesser.

Our results were in agreement with Verbeke *et al.* (2014) and Tiwari *et al.* (2008) who have reported higher prevalence (more than 70%) of mastitis in cattle. Contrary to our findings, Bhat *et al.* (2017), Dabele *et al.* (2021) and Tolosa *et al.* (2013) detected lower prevalence of clinical mastitis in cows 11.5%, 30% and 4.77%, respectively.

Effect of age/lactation number and lactation month on mastitis: Both, age/lactation number and lactation month did not have significant ($p<0.05$) effect on mastitis (Table 1). The highest occurrence (84.58%) was noted in the mid age (lactation number 4-7 years), followed by young age (74.50%; lactation number 1-3 years) and old age lactation (72.55%; lactation number 8-10 years). However, the difference between them was statistically not significant ($p>0.05$). Our findings were supported by Kathiriya *et al.* (2014), who found highest prevalence of mastitis in crossbred cattle with age/ lactation number (5-8 years). The mid lactation month (4-7 months) had highest prevalence (80.63%) followed by late lactation (77.78%; 8-10 months) and early lactation (75.70%; 1-3 months). Contrarily, Kathiriya *et al.* (2014) reported least prevalence

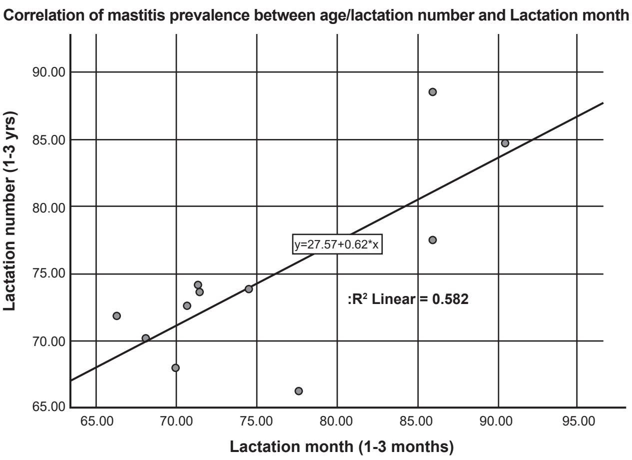

Fig. 1. Linear regression line for best fit between young age (1-3 yrs) and early lactation (1-3 months).

of mastitis in mid lactation (91-180 days) among cattle. The incidence may be influenced by milk yield of animals at young age. The higher milk yielding cattle may have lesser immunity as most of the micronutrients imparting role in immunity loose out in milk (Dabele *et al.* 2021). The interaction between age/ lactation number and lactation month was analysed by bivariate Pearson's correlation (Table 2). Mastitis occurrence was positively correlated (76.3%) only for young age (1-3 years of lactation number) at early lactation (1-3 month) with correlation coefficient ($R^2 = 0.582$) and linear regression line as shown in Fig. 1. Other combinations of age/lactation number and lactation month were negatively or positively correlated and but are non-significant ($p>0.05$) (Table 2).

Severity of infection: Based on any clinical signs present in milk or udder of the animal, clinical mastitis was found as 16.55% (n = 273). While, subclinical mastitis (without any clinical signs) was found in 83.44% (n =1376) of the isolates. Significant variation ($p<0.05$) was found in occurrence of clinical and subclinical mastitis. Similar to our observation, Sarba and Tola (2016), Zeryehun and Abera (2017) and Krishnamoorthy *et al.* (2021) also detected clinical mastitis in only 9.9%, 10.0% and 18% of the milk samples, respectively. Of the 1649 samples processed, blood was found in milk (10.24%) followed by flakes (8.24%) and wateriness (6.54%) in milk. Presence of any clinical signs did not have significant variations ($p>0.05$) in between them (Table 1). Two or more clinical signs were also present in different quarters of an individual animal, simultaneously.

Severity of mastitis was measured by scores of white side test (WST). Presence of trace infection/intensity (+) was significantly high ($p<0.05$) in 76.94% of the quarter samples followed by severe (++++; 39.87%), mild (++; 36.14%), and moderate (+++; 34.85%) intensity. The principal of white side test and california mastitis test (CMT) was same as both reflects presence of excessive leukocytes in milk as an indicator of inflammation.

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

		Age/ Lactation number			Lactation month		
		Young age/L. No. (1-3 yrs)	Mid age/L. No. (4-7 yrs)	Old age/ L.no. (8-10 yrs)	Early Lactation (1-3 months)	Mid lactation (4-7 months)	Late lactation (8-10 months)
Young age/L. No. (1-3 yrs)	Pearson Correlation						
	Sig. (2-tailed)	-					
Mid age/L.No. (4-7 yrs)	Pearson Correlation	-.484					
	Sig. (2-tailed)	.131					
Old age/ L.no. (8-10 yrs)	Pearson Correlation	-.178	-.268				
	Sig. (2-tailed)	.600	.426				
Early Lactation (1-3 months)	Pearson Correlation	.763**	-.095	-.232			
	Sig. (2-tailed)	.006	.781	.492			
Mid lactation (4-7 months)	Pearson Correlation	-.070	-.088	.497	-.533		
	Sig. (2-tailed)	.839	.796	.120	.092		
Late lactation (8-10 months)	Pearson Correlation	-.502	.273	-.143	-.399	-.189	
	Sig. (2-tailed)	.115	.417	.675	.224	.577	-

**, Correlation is significant at the 0.01 level (2-tailed).

Table 3. Month/Season-wise prevalence of mastitis in cattle

Month	Samples processed	Mastitis positive		Chi-square	*df* (degree of freedom)	*p*-value
		n	%			
January	120	91	75.83			
February	112	83	74.11			
March	99	72	72.73			
May	150	123	82.00			
June	191	141	73.82			
July	208	153	73.56	2.35	10	0.993
August	234	174	74.36			
September	200	168	84.00			
October	138	104	75.36			
November	113	95	84.07			
December	84	64	76.19			
Season						
Rainy *(July, August, September)*	642	495	77.10			
Spring/Autumn (*October, November, March)*	350	271	77.43	0.39	3	0.998
Winter *(December, January, February)*	316	238	75.32			
Summer *(May, June)*	341	264	77.42			

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

However, WST is quicker and cheaper than CMT (Kahir *et al.* 2008). Similar to our observation, Ali *et al.* (2021) also found high prevalence (68.6%) of severe intensity mastitis in cattle using CMT. Contrarily, lesser prevalence of mastitis was reported by them for traces intensity (1.4%), mild intensity (14.4%) and moderate intensity (15.6%). In agreement of our findings, Badiuzzaman *et al.* (2015) found higher prevalence of trace infection (+) and mild infection (++) in 54.17% and (36.11%) of the cattle milk samples tested by white side test, respectively.

Month/season-wise occurrence of mastitis: Mastitis was recorded highest in month of November (84.07%), September (84.00%) and May (82.00%) as shown in Table 3. Variations in mastitis occurrence between different months/seasons were not statistically significant (p>0.05). However, number of milk samples processed was highest in the rainy season (July, August and September). Ali *et al.* (2021) also noted highest prevalence of mastitis in the hot and humid months of July, August, and September in cattle. Contrarily, they found least prevalence of mastitic in May and November.

Culture examination of milk samples: Of the 335 mastitis milk samples from cattle, a total of 324 (96.72%) milk samples were found positive for mastitis by culture examination. Isolates were primarily identified gram-positive and gram-negative on the basis of gram staining and colony morphology. CLSI and EUCAST have guidelines of specific pathogens for interpretation of zone of inhibition. However, we only grouped isolates as gram-positive and gram-negative as molecular confirmation was lacking in the present study. Therefore, we followed guidelines of *Staphylococcus* spp. and *Enterobacterales* for interpretation. Overall, gram-positive and gram-negative organism were detected in 99.38% (n = 322) and 45.34% (n = 146) of the samples, respectively. Significant difference (p<0.05) was observed by Pearson's chi square test (value 20.25; *df*-01) for identification of gram-positive and gram-

Table 4. Pearson correlation between antibiotic sensitivity and resistance patterns of gram's negative and positive organisms

		Gram's negative organisms	Gram's positive organisms
Correlation of antibiotics sensitivity (%)			
Gram's negative organisms	Pearson Correlation	1	.657**
	Sig. (2-tailed)	-	.000
Correlation of antibiotics resistance (%)			
Gram's negative organisms	Pearson Correlation	1	.815**
	Sig. (2-tailed)	-	.000

**Correlation is significant at the 0.01 level (2-tailed).

negative isolates. Budding yeast like fungal organisms were also seen under the microscope (100×) in 1.23% (n = 04) of the samples. In agreement with the previous reports by Amit *et al.* (2010) and Verbeke *et al.* (2014), the present study also found predominated infections of gram-positive organism followed by gram-negative. Mixed infection of gram-positive and gram-negative isolates was detected in 126 (38.88%) of the samples in present study. Similar to our findings, Kaur *et al.* (2021) found that gram-positive, gram negative and *Candida* spp. in 53% (n = 471), 38% (n = 341) and 2% (24) bovine milk samples, respectively. In our study we only identified organisms as gram-positive and gram-negative. Previous reports reported that among gram-positive infection, *Staphyloccoccus* spp. was found as the predominating bacteria causing mastitis worlds wide (Amit *et al.* 2010, Dabele *et al.* 2021). The higher incidence of gram-positive such as *Staphylococcus* spp. indicates poor hygienic practices mainly during milking and handling. It is predominantly found on skin, hands, inner lining of milking machine tubes, uncleaned containers etc., and can easily propagate into udder after milking (Verma *et al.* 2018).

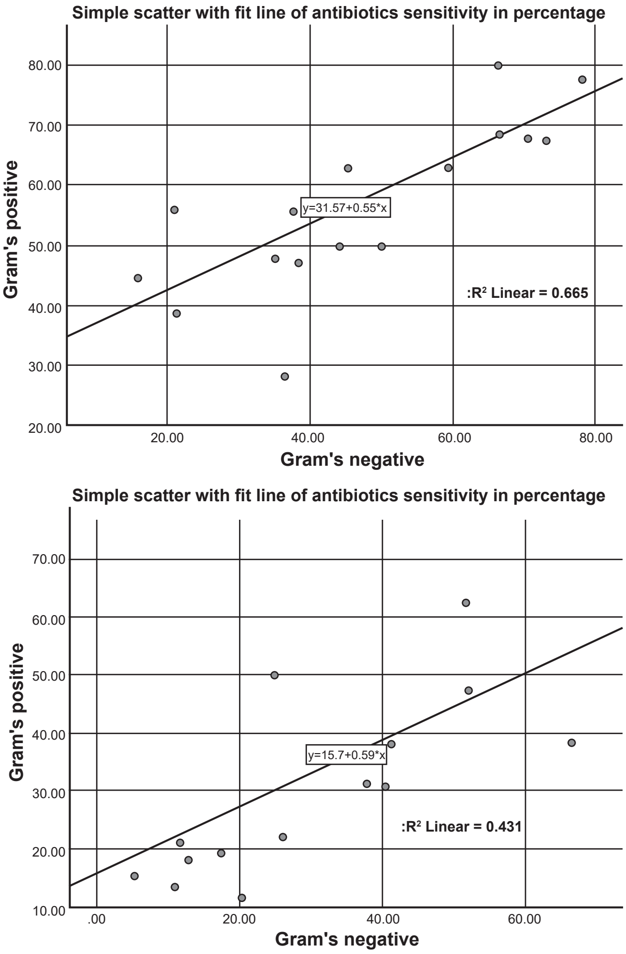

Fig. 2. Linear regression line for best fit between antibiotics sensitivity and resistance patterns of gram's negative and positive organisms.

Antibiotic sensitivity test and detection of multiple antibiotics resistance (MAR) index: Antibiotic sensitivity and resistance patterns of both gram-negative and gram-positive isolates had significant positive correlation (p<0.01) with each other by bivariate Pearson's correlation and regression analysis (table 4). Antibiotic sensitivity patterns of gram-negative and gram-positive organisms were 65.70% positively correlated with high value correlation coefficient (R^2 = 0.665) and linear regression line as shown in Fig. 2. Similarly, Antibiotic resistance patterns among them was 81.50% positively correlated with correlation coefficient (R^2=0.431). Most of the isolates (both gram-positive and gram-negative) were found sensitive towards enrofloxacin (77.78%), chloramphenicol (74.92%), amikacin (69.44%), gentamicin (68.73%), moxifloxacin (67.87%), and levofloxacin (61.73%) as depicted in Table 5. More than 50% of the isolates were found resistant

Table 5. Species-wise antibiogram of organisms isolated from mastitis milk samples of cattle (n = 324)

Antibiotic Discs (Conc. in mcg/disc)	Gm -ve organism		Gm +ve organism		Both organism	
	Sensitive %	Resistance %	Sensitive %	Resistance %	Sensitive %	Resistance %
Amikacin (30 mcg)	73.11	11.76	67.32	20.98	69.44	17.59
Amoxicillin+cloxacillin (20/10 mcg)	15.97	66.39	44.39	38.54	33.95	48.77
Cefoperazone (75 mcg)	37.93	37.93	55.84	30.96	50.35	33.10
Ceftizoxime (30 mcg)	44.16	36.36	49.66	34.90	47.79	35.40
Ceftriaxone (30 mcg)	35.29	41.18	47.80	38.05	43.21	39.20
Chloramphenicol (30 mcg)	66.39	20.17	79.90	11.76	74.92	14.86
Enrofloxacin (5 mcg)	78.15	10.92	77.56	13.66	77.78	12.65
Gentamicin (10 mcg)	70.59	17.65	67.65	19.12	68.73	18.58
Levofloxacin (05 mcg)	59.42	26.09	62.99	22.05	61.73	23.47
Moxifloxacin (05 mcg)	66.67	12.82	68.53	18.18	67.87	16.29
Neomycin (10 mcg)	36.56	51.61	28.13	62.50	32.28	57.14
Norfloxacin (10 mcg)	38.54	52.08	47.12	47.12	43.00	49.50
Oxytetracycline (30 mcg)	45.38	40.34	62.93	30.73	56.48	34.26
Kanamycin (30 mcg)	50.00	25.00	50.00	50.00	50.00	37.50
Cephotaxime (30 mcg)	21.05	5.26	55.93	15.25	47.44	12.82
Ampicillin (10 mcg)	21.43	0.00	38.89	0.00	31.25	0.00

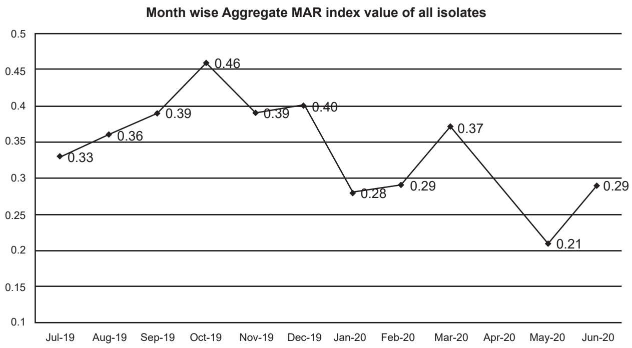

Fig. 3. Month-wise MAR index value of both gram-positive and gram-negative organisms.

towards only neomycin, followed by norfloxacin (49.50%), amoxicillin+cloxacillin (48.77%), ceftriaxone (39.20%) and kanamycin followed by ceftioxime (46.63%), and cefoperazone (43.49%). In agreement with the present findings, Kaur *et al.* (2015) also found that gentamicin (91.21%), ciprofloxacin (89.60%) and enrofloxacin (88.28%) were found to be highly effective antibiotics, while, penicillin (86%), colistin (83.30%), cloxacillin (78.62%), amoxycillin (70.71%) and ampicillin (62.51%) showed least effect against both gram-positive and negative bacteria isolated from bovine mastitis milk samples.

Antimicrobial resistance of isolates against two or more antibiotics signals to become multidrug resistance (MDR) isolates. This can be well evaluated by multiple antibiotics resistance (MAR) index. It is an epidemiological tool that signifies potential risk of spreading MDR isolates in the environment. 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). Level of significance for MAR index value (above 0.2) was calculated by one-sample T test. Majority of the isolates from present study were multidrug resistant (MDR) significantly (p=0.000 at 95% confidence interval) on the basis of their multiple antibiotic resistance (MAR) index value (more than 0.2). The test value was taken as 0.2 MAR index for one-sample T test. The aggregate MAR value of all months was detected as 0.38. Highest number of MDR isolates was recorded in the month of October 2019 followed by December 2019 and September/November 2019 on the basis of their aggregate MAR value (Fig. 3). Numbers of MDR isolates were reduced in the month of May 2020. This may be due to COVID-19 lockdown effect (restricted movements, adopting hygienic measures, hot ambient temperature, etc.) in the entire country.

From findings of the present study it can be concluded that subclinical mastitis with trace (+) score of white side test have more frequency of occurrence in cattle. These types of cases remain undiagnosed and become chronic. Cattle of mid age (4-7 years) during mid-lactation (4-7 months) were more susceptible to mastitis. Therefore, frequent screening of milk samples in interval of 15 days should be followed for early detection of mastitis. Although, enrofloxacin, chloramphenicol, amikacin and gentamicin were most effective antibiotics *in vitro* against most of the bacterial isolates in present study, random use of antimicrobials may lead to emergence of multidrug resistant strains.

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