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Occurrence, epidemiological studies and antibiogram of bovine mastitis bacteria among different geographical areas of Haryana

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ABSTRACT

Mastitis is most prevalent disease in cattle and buffalo, causing huge economic losses to dairy industry due to decreased milk production and cost of treatment. The present study aimed to estimate the occurrence, epidemiology, etiological agents and their antibiogram profiling in four different districts of Haryana, India. In the present study, 77.54% (n = 3,943 out of 5,085) milk samples were detected mastitis positive by CMT, while 98.05% (n = 3,378 out of 3,445) milk samples were tested positive by culture examination. Quarter-wise, occurrence was detected in 67.66% and 55.62% of affected quarters in cattle and buffaloes, respectively. Overall, clinical and subclinical mastitis was found in 79.75% and 52.63% quarter milk samples, respectively. Of the 3445 animals, gram-positive and gram-negative bacteria were seen in 64.56% (n = 2224/3445) and 23.40% (n = 806/3445) cases, respectively. Antibiotic sensitivity assay revealed that gentamicin was most sensitive antibiotic against isolates followed by enrofloxacin, chloramphenicol and cefoperazone among all four districts of Haryana, India. Penicillin was found to be most resistant antibiotic among all four districts. Majority of gram-positive and gram-negative isolates of present study were detected as multidrug resistant (MDR). The findings of present study enhance our understanding of mastitis and factors affecting AMR in bacteria, which could be used for future prevention and control strategies.

Keywords: Antibiotic resistance, Buffalo, Cattle, Mastitis, MDR

Mastitis is defined as the inflammation of mammary gland which causes huge economic losses to dairy sector in terms of treatment, loss of milk production, alteration in milk quality and culling of unproductive animal from the herd (Maity *et al.* 2020). Epidemiologically, bovine mastitis is categorized as contagious and environmental, depending upon involvement of microbes which have their origin either in diseased animals or in environment. Bacteria that are most frequently encountered during mastitis are *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Klebsiella* spp., *Mycoplasma* spp., *Corynebacterium bovis*, and *Streptococcus agalactiae* (Yadav *et al.* 2020, Han *et al.* 2022). On the basis of presence or absence of changes in milk and udder, bovine mastitis is categorized as clinical (CM) and subclinical (SCM).

Both CM and SCM cause USD$1 billion annual loss, of which SCM is responsible for approximately USD$85 million (Singh 2022). Antimicrobials are often used for the treatment of livestock mastitis. Excessive use or misuse or indiscriminate use of antimicrobials for the treatment of mastitis results in the development of antimicrobial resistance (AMR) bacteria (Abdi *et al.* 2021, Ajose *et al.* 2022). Even though National Action Plan (NAP) for AMR had been initiated in India in April, 2017 by the Union Ministry of Health and Family Welfare (Ranjalkar and Chandy 2019), it is still in preliminary stages. Therefore, this study was aimed to estimate the occurrence, epidemiology, etiological agents and their AMR status in different districts of Haryana, India.

MATERIALS AND METHODS

Sample collection and detection of mastitis: Milk samples from cattle and buffalo were processed for detection of mastitis from the period of July 2021 to June 2022 received in sterile container by local dairy farmers as per the standard sampling procedure at Ambala, Bhiwani, Mahendergarh and Rohtak districts of Haryana, India. The climate of Haryana is subtropical, semi-arid to subhumid, continental climate with a monsoon pattern. Animal's history and other information such as age of animal/ lactation number, lactation month and clinical signs, etc. were recorded from livestock's owner. Mastitis detection was performed by California mastitis test (CMT) and culture examination using standard procedure (Quinn *et al.* 2011). Bacterial species were identified as gram-positive and gram-negative isolates on the basis of gram staining and colony morphology. Clinical (CM) and subclinical (SCM) forms of mastitis were identified on the basis of clinical symptoms shown either in milk (blood, flakes,

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wateriness in milk) and udder (redness, pain, fibrosis, udder size abnormalities, etc.) of the animal. Severity of subclinical mastitis was detected in quarter milk samples by performing CMT and analyzed by observing degree of gel formation graded as trace (t), mild (+), moderate (++) and severe (+++) as described by Belay *et al.* (2022).

Antibiotic sensitivity test and detection of multiple antibiotic resistances (MAR) index: A number of 3445 milk samples (1192 cattle and 2253 buffalo) were subjected to antibiotic sensitivity test as per the guidelines described by Bauer (1966) and Quinn *et al.* (2011) against various antibiotics obtained from HiMedia Laboratories (Mumbai, India). The zone of inhibition around each disc was measured and interpreted using clinical and laboratory standards institute (CLSI 2015). There were specific guidelines for particular bacteria species (such as *Staphylococcus* spp. and *Enterobacterales*) not as for gram-positive or gram-negative organisms in both CLSI and EUCAST. Therefore, guidelines of *Staphylococcus* spp. (for gram-positive) and *Enterobacterales* (for gram-negative) were followed for interpretation. This was due to the fact that gram-positive *Staphylococcus* spp. was considered as the major causative organism of mastitis worldwide followed by gram-negative *Enterobacterales* (*E. coli*, *Klebsiella* spp. etc) (Kaur *et al.* 2015, Chhabra *et al.* 2020). 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. Statistical analysis was done by 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.

RESULTS AND DISCUSSION

Detection of mastitis in milk samples of cattle and buffalo: Analysis of various parameters related to mastitis in cattle and buffalo, based on the number of samples processed are presented in Table 1. In the species-wise analysis, results indicated that a higher percentage of cattle samples (91.93%) tested positive for mastitis as compared to the samples from buffalo species (83.31%). However, the difference was statistically non-significant (P>0.05). Although the samples tested in present study were already suspected for mastitis, but culture examination proven high sensitivity and specificity towards detection of mastitis. Species-wise variations in mastitis have been observed by many workers previously (Krishnamoorthy *et al*. 2021). The reason for the high prevalence in the present study might be attributed to poor milking hygiene and other management factors including dirty cowshed and bedding (Han *et al*. 2022). Culture examination was considered as a gold standard for detection of mastitis (Kala *et al*. 2021). The reason of lower detection rate of mastitis by CMT may be explained by the presence of bacteria that do not trigger the immunological response of the infected quarter resulting in a limited increase in the somatic cell count (Damian *et al*. 2021). The difference in quarter-wise occurrence of mastitis probably is due to some predisposing factors like teat injury and sphincters defect, etc. that vary from quarter to quarter (Shaikh *et al*. 2019).

Severity of infection: Detection of mastitis in 25,687 quarters milk samples from cattle (n = 7179) and buffalo (n = 18508) did not have any significant difference (P>0.05) in between them (Table 1). In the present study, clinical mastitis (79.75%) was found in significantly (P<0.05) higher proportion than sub-clinical mastitis (52.63%) in quarter milk samples across both species. Significant variation (P>0.05) was observed in presence of clinical signs in milk and/or udder of the animal (Table 1). Presence of trace infection/intensity (t) by CMT was significantly high (P<0.05) in 19.90% of the quarter milk samples followed by mild (+; 15.94%), severe (+++; 9.35%) and moderate (++; 7.44%) intensity. Severity of infection was statistically significant (P<0.05) in present study (Table 1). Previous reports declared that subclinical mastitis occurs more often (15 to 40 times) than the clinical form and its duration was also longer (Belay *et al.* 2022). Subclinical mastitis is therefore more difficult to detect, and infection serves as a reservoir of pathogens that spreads the udder infection among animals within the herd (Cobirka *et al.* 2020). The difference in occurrence of CM and SCM in both species usually depends on causative organisms, breed, age, immunity, and stage of lactation of the animal. Clinically, Baloch *et al.* (2022) observed flakes in bovine milk was significantly higher (P>0.05) in 41.95% samples followed by watery consistency (7.73%), pus (15.01%) and blood in milk (10.06%). Various clinical symptoms such as redness, swelling, increased heat, and pain, was dependent on involvement of the particular bacteria in mastitis. The severity of infection usually depends upon several factors such as virulence properties of pathogens involved, host immune system, environment and duration of disease (Zhang *et al.* 2016). Baloch *et al.* (2022) found moderate severe (+) infection in 42.73% (359/840) followed by moderate severe (4+) in 12.14% (102/840). Ali *et al.* (2011) revealed that strongly positive intensity (+++) was noted in 64% cases of subclinical mastitis in cattle and buffaloes using CMT.

Effect of age/lactation number and lactation month on occurrence of mastitis: Both, age/ lactation number and lactation month did not have significant (P>0.05) effect on the prevalence of mastitis. Highest occurrence of mastitis was noted in the old age lactation followed by mid age and young age. However, the incidence of mastitis was

Table 1. Occurrence of mastitis in cattle and buffalo

Parameter	Samples processed (n)	Mastitis		Chi-square	*df* (degree of freedom)	P-value
		+ve	%			
Species-wise (N = 8530)						
Cattle	2492	2291	91.93	0.463	1	0.49
Buffalo	6038	5030	83.31			
Total	8530	7321	85.82			
Tests conducted (N = 8530)						
California mastitis test	5085	3943	77.54	2.273	1	0.13
Culture examination	3445	3378	98.05			
Lactation number / age (N = 8530)						
Young age (1-3 years)	6055	5107	84.34	0.370	2	0.83
Mid age (4-7 years)	2296	2050	89.29			
Old age (8-10 years)	179	164	91.62			
Lactation month (N = 8530)						
Early lactation (1-3 month)	5893	4976	84.44	0.146	2	0.93
Mid lactation (4-7 month)	2053	1836	89.43			
Late lactation (8-10 month)	584	509	87.16			
Quarter milk samples (n = 25687)						
Cattle	7179	4857	67.66	1.161	1	0.28
Buffalo	18508	10294	55.62			
Total	25687	15151	58.98			
Types of mastitis (n = 25687 quarter milk samples)						
Clinical mastitis	6016	4798	79.75	5.481	1	0.01*
Sub-clinical mastitis	19671	10353	52.63			
Clinical symptoms (n = 6016 quarter milk samples)						
Flakes in milk	6016	2002	33.28	43.337	5	0.00*
Swelling/fibrosis		1473	24.48			
Blood in milk		706	11.74			
Pain		533	08.86			
Wateriness in milk		472	07.85			
Pus in milk		200	03.32			
Severity (n = 19671 quarter milk samples)						
Trace (t)	19671	3914	19.90	8.462	3	0.03*
Mild (+)		3136	15.94			
Moderate (++)		1464	7.44			
Severe (+++)		1839	9.35			

*Level of significance, P value is significant at the 0.05 level or lesser; *N*, no. of animals; *n*, no. of quarter milk samples.

significantly higher ($P<0.05$ and <0.001) in young age (1-3 years) and early lactation (1-3 month) from both the species (Supplementary Table 1). The interaction between age/ lactation number and lactation month was analysed by bivariate Pearson's correlation with set level of significance at ($P<0.05$) (Supplementary Table 2). The occurrence of mastitis was positively correlated (91.70%) between young age (1-3 years of lactation number) and early lactation (1-3 month). The linear regression line has given the best fit as the value of correlation coefficient (R^2) was high ($R^2=0.841$) as shown in Fig. 1. Contrarily, occurrence of mastitis was negatively correlated (75.60%) between mid-lactation (4-7 months) and late lactation (8-10 months). The linear regression line has given the best fit as the value of correlation coefficient (R^2) was high ($R^2=0.572$) as shown in Fig. 2. Other combinations of age/ lactation number and lactation month were negatively or positively correlated but not significant ($P>0.05$) (Supplementary Table 2). The high rate of infection during young age and early lactation stage might be due to the change in physiological processes which lead to reduced immunity of cow and thus making more susceptible to contagious pathogens. The high rate of mastitis in fourth to fifth lactation may be due to fact the high milk production occurs during that period (Shaikh *et al.* 2019).

Area-wise occurrence of mastitis: Occurrence of mastitis was non-significantly ($P>0.05$) higher in Bhiwani district followed by Ambala, Rohtak and Mahendergarh. Similarly, non-significant difference ($P>0.05$) was found in occurrence of mastitis between cattle and buffalo species from each district (Table 2). Area wise, non-significantly ($P>0.05$) difference in mastitis detection was observed in present study. It may be due to the fact even though the milk samples were collected from four districts but of

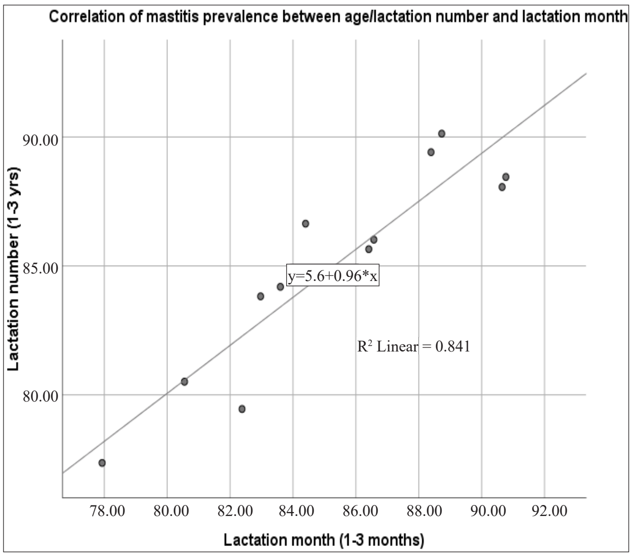

Fig. 1. Linear regression for best fit between young age (1-3 yrs) and early lactation (1-3 months).

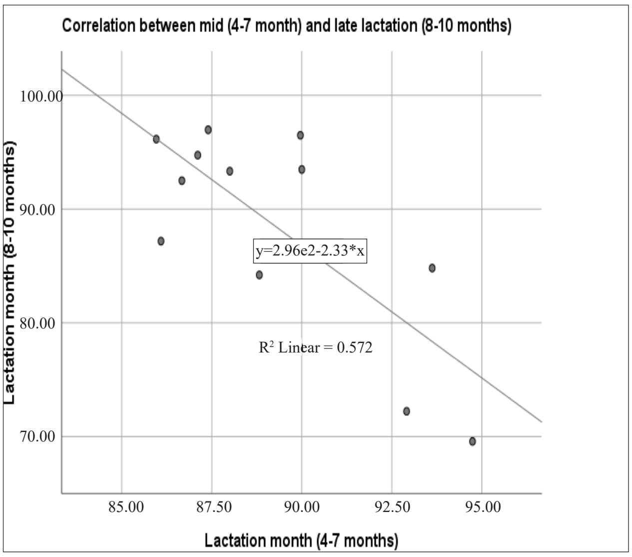

Fig. 2. Linear regression line for best fit between mid-lactation (4-7 months) and late lactation (8-10 months).

Table 2. Area-wise occurrence of mastitis in cattle (n = 2492) & buffaloes (n = 6038)

District	Sample type	Cattle			Buffalo			Statistical significance (between species)		
		n	*+ve*	%	*n*	*+ve*	%	Chi square	*df*	P-value
Ambala	Total Animals	299	289	96.66	241	229	95.02	0.021	1	0.89
	Quarter milk samples	769	743	96.62	638	596	93.42	0.084	1	0.77
Bhiwani	Total Animals	270	270	100	649	649	100	-	-	-
	Quarter milk samples	1080	710	65.74	2564	1659	64.70	0.008	1	0.93
Mahendergarh	Total Animals	1789	1603	89.60	4788	3795	79.26	0.716	1	0.40
	Quarter milk samples	4798	2888	60.19	13866	6619	47.74	1.333	1	0.25
Rohtak	Total Animals	134	129	96.27	360	355	98.61	0.046	1	0.83
	Quarter milk samples	532	516	96.99	1440	1420	98.61	0.020	1	0.89
Total animals		2492	2291	91.93	6038	5028	83.27	0.463	1	0.50
Total quarters		7179	4857	67.66	18508	10294	55.62	1.161	1	0.28
Statistical significance in occurrence of mastitis between different areas										
		Cattle			Buffalo			Aggregate		
Chi square		0.551			3.038			2.128		
df		3			3			3		
P *value*		0.90			0.38			0.54		

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

these three (Bhiwani, Rohtak and Mahendergarh) were not distantly situated and come within same agro climatic zone of Haryana state. Previous reports suggested that hot and humid months of summer were more favourable for mastitis in cattle and buffalo (Ali *et al.* 2021). It creates favourable conditions for bacterial growth by development of coliform in the bedding, potentially leading to higher mastitis rates (Sharma *et al.* 2020). Different geographical area also may have distinct dairy farming practices, including milking techniques, cow housing, cleanliness protocols, and milking equipment maintenance (Ali *et al.* 2021).

Season-wise occurrence of mastitis: Occurrence of mastitis was non-significantly (P>0.05) higher in spring/ autumn season among cattle and in winter season among buffalo (Table 3). Similarly, occurrence of mastitis was non-significant higher in cattle (P>0.05) in each season than buffalo.

Culture examination of milk samples: Overall, 98.05% (N = 3378/3445) milk samples from cattle and buffalo were found positive for mastitis by culture examination from Ambala, Bhiwani, Mahendergarh and Rohtak. Overall, gram-positive and gram-negative bacteria were seen in 64.56% (n = 2224/3445) and 23.40% (n = 806/3445).

Table 3. Season-wise occurrence of mastitis in cattle (n = 2492) and buffaloes (n = 6038)

Season	Cattle (n=2492)			Buffalo (n=6038)			Statistical significance		
	Total samples	Positive (*n*)	%	Total samples	Positive (*n*)	%	Chi square	*df*	P-value
Rainy	835	769	92.10	1843	1458	79.11	0.988	1	0.32
Spring/ Autumn	586	544	92.83	1773	1485	83.76	0.458	1	0.49
Winter	490	446	91.02	1748	1512	86.50	0.090	1	0.76
Summer	581	532	91.57	674	575	85.31	0.277	1	0.59
Statistical significance in occurrence of mastitis (per animals) between different seasons									
	Cattle			Buffalo			Aggregate		
Chi square	0.022			0.415			0.163		
df	3			3			3		
P-value	0.99			0.93			0.98		

*Level of significance, P-value is significant at the 0.05 level or less. Note: Rainy (July, August, September), Spring/Autumn (October, November, March), Winter (December, January, February), Summer (April, May, June).

Statistically significant ($P<0.05$ and $P<0.001$) difference was observed in frequency of gram-positive and gram-negative bacterial isolates by Pearson's chi-square test (χ^2= 20.045; *df*-01). Mixed infection of gram-positive and gram-negative bacteria was found in 10.10% (n = 348/3445). While 01.94% (n = 67/3445) samples were found as culture negative.

Antibiotic sensitivity assay: Antibiotic sensitivity assay revealed that gentamicin was the most sensitive antibiotic against gram-positive and gram-negative isolates followed by enrofloxacin, chloramphenicol and cefoperazone among all four districts (Table 4). However, the sensitivity percentage of majority antibiotics was significantly lower in Ambala ($P<0.05$) districts than others. Penicillin was most resistant antibiotics among all four districts. Area-wise sensitivity and resistance frequency of the antibiotics were shown in Table 4. None of the antimicrobials had 100% sensitivity and resistance towards both gram-positive and gram-negative isolates of any geographical area. Overall, 39.05% gram-positive and 25.55% gram-negative isolates of present study were detected as multidrug resistant (MDR) on the basis of multiple antibiotic resistance (MAR) index value of being more than 0.2 (Supplementary Table 3). Previous reports concluded that gram-positive *Staphylococcus* spp. was considered as the major causative organisms of mastitis followed by gram-negative *Enterobacterales* (*E. coli*, *Klebsiella* spp. etc.) worldwide (Kaur *et al.* 2015, Chhabra *et al.* 2020, Ali *et al.* 2021). In the present study, isolates were primarily identified as gram-positive and gram-negative on the basis of gram staining and colony morphology. Majority of infections was caused by gram-positive organism followed by gram-negative. Contagious mastitis pathogens (*Staphylococcus aureus*, *Streptococcus agalactiae*) were usually spread from infected udders to "clean" udders during the milking process through contaminated teat cup liners, milkers' hands, contaminated cloth towels etc (Yadav *et al.* 2020, Han *et al.* 2022). Coliform infections are usually associated with an unsanitary environment (manure and/or dirty, wet conditions), while *Klebsiella* were found in sawdust that contains bark or soil (Sharma *et al.* 2020, Damian *et al.* 2021). CLSI (2015) have interpretation guidelines of zone of inhibition by specific pathogens (such as *Staphylococcus* spp. and *Enterobacterales* etc.) not as for gram-positive or gram-negative. Therefore, for *Staphylococcus* spp. (for gram-positive) and *Enterobacterales* (for gram-negative) guidelines for interpretation and further discussion were used from findings of present study. In agreement, Kaur *et al.*

Table 4. Antibiogram of organisms (gram-positive and negative) isolated from mastitic milk of aggregately both species

Total sample (N = 3445)	Sensitivity (%)				Resistance (%)			
Antibiotic	Ambala	Bhiwani	Mahendergarh	Rohtak	Ambala	Bhiwani	Mahendergarh	Rohtak
Amikacin (30 mcg)	56.50	8.90	72.37	78.24	43.50	91.10	27.53	21.77
Cefoperazone (75 mcg)	79.45	6.10	74.74	76.00	20.55	93.90	25.11	24.00
Ceftriaxone (30 mcg)	74.15	5.35	35.08	67.33	25.85	94.65	64.87	32.67
Chloramphenicol (30 mcg)	81.10	11.00	76.10	82.35	18.90	89.00	23.85	17.65
Enrofloxacin (5 mcg)	92.10	10.40	72.19	93.76	7.90	89.60	27.76	6.25
Gentamicin (10 mcg)	78.65	18.50	87.97	93.79	21.35	81.50	12.03	6.22
Levofloxacin (05 mcg)	73.50	4.40	56.69	88.38	26.50	95.60	43.31	11.63
Oxytetracycline (30 mcg)	71.10	4.60	39.64	70.18	28.90	95.40	60.36	29.82
Moxifloxacin (05 mcg)	66.50	2.50	41.30	-	33.50	97.50	58.70	-
Penicillin G (10 unit)	18.10	-	22.34	-	81.90	-	77.66	-
Ceftizoxime (30 mcg)	-	4.10	30.93	46.15	-	95.90	69.07	53.85
Kanamycin (30 mcg)	-	3.80	49.99	-	-	96.20	50.01	-
Ampicillin (10 mcg)	-	4.85	-	34.05	-	95.15	-	65.95

(2015) also found that gentamicin (91.21%), was the most sensitive antibiotic for 91.21% isolates (both gram-positive and negative) followed by ciprofloxacin (89.60%) and enrofloxacin (88.28%). Cloxacillin and amoxicillin were resistant to 78.62% and 70.71% of the isolates (both gram-positive and negative) from bovine mastitis milk samples. Similar to our observation, Chhabra *et al.* (2020) reported that majority (>65%) of the *Staphylococcus* and *Streptococcus* species isolated from bovine mastitic milk samples in Hisar, Haryana were sensitivity towards enrofloxacin, moxifloxacin and least (<25%) were sensitive towards Penicillin. Likewise, Verma *et al.* (2022) revealed that gentamicin to be the most effective drug (93.34%), followed by enrofloxacin (66.67%), chloramphenicol (60.00%) against the bacterial isolates from bovine mastitis milk. MAR index an epidemiological tool that signifies potential risk of spreading MDR isolates in the environment. Similar to the present study, Ali *et al.* (2015) also found that most of the *S. aureus* isolates from buffalo milk samples had an MAR index more than 0.28 in Egypt. Yadav *et al.* (2021) also reported high average group MAR index value of 0.53 among *Klebsiella pneumoniae* isolates from bovine milk samples in similar geographical area. The MAR index value of more than 0.20 was considered as multi drug resistance (MDR) strain of bacteria. It signifies that group of bacteria/ individual bacteria were resistant to two or more than two antibiotics or may have encountered several antibiotics (Krumperman 1983, Sharma *et al.* 2020).

Conclusively, bovine mastitis poses a serious threat to livestock owners and general public because of antimicrobial resistance (AMR) and zoonotic implications in country like India. The disease has been reported in different part of the country and the prevalence of the disease varies widely. However, such local or state level studies are important to enhance our understanding of mastitis, but also provides us an insight into factors affecting AMR in bacteria, which could be used for future prevention and control strategies.

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