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RESEARCH ARTICLE

Comparative antibiogram analysis of bacterial isolates from mastitic milk of cattle and buffalo in Haryana

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Abstract: Mastitis causes huge economic losses to dairy industries worldwide. It is caused by various microorganisms, of which bacterial etiology is primarily important. Both Gram-positive and Gram-negative bacteria are involved in causation of disease. In the present study, 8561 milk samples from both cattle and buffalo combined were tested and occurrence of mastitis was recorded in 72.73 % (n = 6227/8561) milk samples. Occurrence of mastitis was non–significantly (p>0.05) higher in cattle (86.31%) than buffaloes (66.97%). California Mastitis Test (CMT) showed significantly (p<0.05) lower prevalence of mastitis compared to the culture examination statistically. By CMT analysis, buffalo had a significantly (p<0.05) lower percentage of mastitis positivity than cattle. Whereas, culture examination revealed that both cattle and buffalo exhibited a high prevalence of mastitis, with 97.75% and 98.15% positive samples, respectively. Gram-positive bacteria were found as the predominating etiological agents causing mastitis in 62.62% samples followed by Gram-negative bacteria (24.54%) from milk samples of cattle and buffalo combined. Mixed infection of both Gram-positive and Gram-negative bacteria was found in 10.50% milk samples. Over all 2970 samples from cattle (n = 1071) and buffalo (n = 1899) were subjected for culture examination and antibiotic sensitivity assay. The findings from the present study revealed variations in antibiotic sensitivity across different districts. The district of Bhiwani consistently showed lower sensitivity rates for most antibiotics compared to the other districts. Overall, chloramphenicol, enrofloxacin, gentamicin, ciprofloxacin, cefoperazone and levofloxacin were most sensitive antibiotic across all districts. Amoxicillin + Clavulanic acid and ampicillin were most resistant antibiotics in all districts.

Keywords: Antibiotic resistance; Buffalo; Cattle; Mastitis

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Introduction

Mastitis is characterised by an inflammation of the mammary gland in dairy animals such as cattle and buffalo. It causes significant economic losses to national economy and compromises animal welfare (El-Ashker et al. 2020; Yadav et al. 2020). Mastitis can be classified into sub-clinical, clinical and chronic mastitis, depending upon causative organisms, breed, age, immunity, and stage of lactation of the animal (Maity et al. 2020). It is caused by a variety of microorganisms; where bacterial infections are the primarily causative agents. Both Gram-positive and Gram-negative bacteria are involved in bovine mastitis (Algammal et al. 2020; Chhabra et al. 2020). Mastitis results in decreased quality and quantity of milk production. Such infected milk or milk products, usually enters into the food chain and humans can also acquire the infection through the consumption of contaminated milk (Krishnamoorthy et al. 2021). Antimicrobial resistance is also serious concern for both human and animal health. To effectively manage and treat mastitis, it is crucial to understand the bacterial pathogens involved and their antibiotic susceptibility patterns. Appropriate selection of antibiotics can be done on the basis of antibiotic susceptibility profiles of causative agents (Yadav et al. 2020; Ali et al. 2021). Furthermore, understanding the antibiotic resistance patterns of these bacteria is essential for implementing appropriate control measures and preventing the spread of multidrug-resistant strains among human and animal population (Yadav et al. 2021; Singh, 2022). Therefore, the present study seeks to bridge this knowledge gap and contribute to the current understanding of mastitis management in these important livestock species. The aim of present study is to investigate the antibiogram of bacterial isolates obtained from mastitis milk in cattle and buffalo by comprehensive approach, involving bacterial isolation and susceptibility testing, to generate a detailed antibiogram. This study may have practical implications for veterinarians, farmers, and dairy industry stakeholders. It may also contribute to develop the prevention and control strategies for mastitis and generate awareness about the emergence of antimicrobial resistance.

Material s and Methods

Sample collection and detection of mastitis

Milk samples from cattle and buffalo were carefully collected using aseptic techniques at different districts of Haryana (Ambala, Bhiwani, Mahendergarh and Rohtak). Farmers were properly guided for aseptic collection of samples in sterilized container. Samples were processed for detection of mastitis during the period of July 2020 to June 2021 at various Disease Investigation Labs i.e Ambala, Bhiwani, Mahendergarh and Rohtak, Lala Lajpat Rai University of Veterinary and Animal Sciences, (LUVAS), Haryana, India. Mastitis detection was performed by California mastitis test (CMT) and culture examination. Severity of mastitis was detected in quarter milk samples (subclinical) by performing CMT and analysed by observing degree of gel formation graded as trace (t), mild (+), moderate (++) and severe (+++) as described by Belay et al. (2022).

Bacterial isolation and identification

Detection of various bacterial isolates was carried out by culture examinations of the samples using standard procedure (Quinn et al. 2011). Briefly, milk samples were inoculated onto Nutrient agar and MacConkey agar. The plates were then incubated at the optimal temperature for bacterial growth, typically 37^{o}C, for a period of 24 to 48 hours. Colony morphology, including shape, size, color, and other distinguishing characteristics, were observed and recorded. Gram staining was performed to differentiate the bacterial isolates as Gram-positive or Gram-negative.

Antibiotic Susceptibility Testing

For antibiotic susceptibility testing, standard guidelines were followed as per the described by Bauer (1966) and (Quinn et al. 2011) for various antibiotics (Himedia). Briefly, bacterial suspension was prepared from pure cultures of the bacterial isolates. Mueller-Hinton agar (Himedia) plates were inoculated with the bacterial suspension using sterilized swab to obtain lawn culture (Bauer, 1966). Antibiotics discs were placed on the surface of agar and plates were then incubated at the appropriate temperature and duration as recommended for each antibiotic. Following incubation, the zones of inhibition around the antibiotic discs were measured and recorded. Interpretation of zone of inhibition was carried out according to the guidelines provided by the Clinical and laboratory standards institute (CLSI, 2015) and European committee on antimicrobial susceptibility testing (EUCAST, 2015). The collected data were compiled to create an antibiogram representing the antibiotic susceptibility patterns of the bacterial isolates.

Statistical analysis

To analyze the data, the prevalence of antibiotic resistance among the bacterial isolates from mastitic milk in cattle and buffalo was determined. The frequency of resistance for each antibiotic was calculated in Microsoft Excel Version 2010, and statistical analysis, such as chi-square was done by Statistical Package for Social Sciences (SPSS) Version 26 Software (George and Mallery, 2019) to evaluate any significant differences in antibiotic resistance patterns in cattle and buffalo from different geographical areas.

Results and Discussion

Occurrence of mastitis

A total of 8561 milk samples from both species combined were tested for detection of mastitis. A comprehensive analysis of mastitis and related parameters among cattle and buffalo were shown in table 1. Overall, the occurrence of mastitis was recorded in 72.73% (n = 6227/8561) milk samples from both species combined. The species-wise analysis indicates that the occurrence of mastitis was non–significantly (p>0.05) higher in cattle (86.31%) than in buffaloes (66.97%). Contrary to our study, Dabele et al. (2021) found that the prevalence of mastitis in lactating Zebu cows of Ethiopia was found to be lesser than our findings 30.5% (95% CI: 26.0–35.2%). The slightly higher occurrence of mastitis in cattle compared to buffaloes (86.31% vs. 66.97%) could be attributed to factors such as anatomical differences between the udders of cattle and buffaloes, which might contribute to variations in mastitis susceptibility. Cattle have four quarters, while buffaloes generally have two larger lobes. The structural differences in mammary glands may influence the efficiency of milk let-down, milking procedures, and the ability to clear infections (Hughes and Watson, 2018). The non-significant difference in mastitis occurrence between cattle and buffaloes suggests that similar approaches can be used for mastitis prevention and control in both species. Implementing good husbandry practices, such as udder hygiene, proper milking techniques, and regular monitoring of udder health, can help reduce the incidence of mastitis in both cattle and buffaloes (Sah et al. 2020). Variation in management practices between cattle and buffalo farms might explain the observed differences in mastitis occurrence (Sharun et al. 2021). Al-Zurgani and Mohammed (2021) stated that (CMT) is a quick and distinguished field and laboratory test to detect mastitis in farm animals, including buffaloes. Hokmabad et al. (2011) and Ali and Dahl (2022) also found that, CMT has acceptable sensitivity and specificity in diagnosis of mastitis among buffaloes.

Of the 8561 milk samples, 5591 and 2970 milk samples were tested CMT and culture examination, respectively. CMT showed significantly (p<0.05) lower prevalence of mastitis compared to the culture examination with chi-square value (9.688) and the p-

value (0.002). By CMT analysis, buffalo had significantly (p<0.05) lower percentage of mastitis positivity than cattle. Whereas, culture examination revealed that both cattle and buffalo exhibited a high prevalence of mastitis, with 97.75% and 98.15% positive samples, respectively. The etiological agents causing mastitis were also investigated. Gram-positive bacteria were found as the predominating etiological agents causing mastitis in 62.62% samples followed by Gram-negative bacteria (24.54%) from milk samples of cattle and buffalo combined. Mixed infections of Gram-positive and Gram-negative bacteria were found in 10.50% samples from both species. Similar to our observation Verma et al. (2022) found that Gram-positive bacteria was found as the major cause of bovine mastitis 46.67% samples followed by Gram-negative bacteria (36.67%) and mixed infection of both (16.67 %). The high frequency of Gram-positive bacterial infections indicates unhygienic and poor management practices at farms. The contamination usually occurs from the external surface of the udder and teats, milker's hand and from the surface of the milking equipment and utensils etc. (Ali et al. 2021). While Gram-negative infections such as coliform mastitis were usually occurs due to poor environmental hygiene, contaminated water, fecal contamination, inadequate refrigeration etc (Deddefo et al. 2023).

The significant difference in detection of mastitis by CMT and culture examination showed variations in sensitivity and specificity between these diagnostic methods. Although, CMT being a quick and cost-effective screening tool, might underestimate the true prevalence of mastitis compared to culture examination, which provides a more accurate identification of causative agents (Mbindyo et al. 2020). The lower percentage of mastitis positivity observed in buffaloes compared to cattle by CMT analysis, although not statistically significant, could be attributed to differences in udder anatomy and physiological characteristics (Hughes and Watson, 2018; Diwakar et al. 2020). Further studies are needed to explore these factors in more detail and understand the potential implications for mastitis diagnosis in buffaloes. Gram-positive bacteria were identified as the major etiological agents, consistent with findings from Girma and Tamir (2022), who did a meta-analysis of mastitis data between year 2005-2022 in Ethiopia and concluded that Gram-positive bacteria (84.70%) were the most prevalent mastitis causing agents compared with Gram-negative bacteria (15.30%). Additionally, mixed infections (10-30%) of both Gram-positive and Gram-negative organisms were also reported by previous reporters, highlighting the complexity of mastitis and emphasising the need for targeted treatment approaches (Steele et al. 2020; Saleh et al. 2022).

Mastitis occurrence in different areas among cattle and buffalo populations was shown in Table 2. A notable variation in mastitis prevalence was observed only in Mahendergarh district. The occurrence of mastitis was non-significantly (p>0.05) higher in cattle (86.31%) than buffalo (66.97%). Both cattle and buffalo exhibited similar high percentages of positive samples in Ambala, Bhiwani and Rohtak districts. The chi-square test indicates no significant difference between the two species as the p-value (>0.05) was above the significance threshold. Looking at the individual areas, it is observed that mastitis prevalence varies across locations. In cattle, occurrence of mastitis was non-significantly (p>0.05) higher in Rohtak (100%) followed by

Table 1: Occurrence of mastitis in cattle and buffalo

Parameters	Samples processed (n)	Mastitis		Chi-square	*df* (degree of freedom)	*p* value
		+ve	%			
Species wise (n = 8561)						
Cattle	2550	2201	86.31	2.359	1	0.125
Buffalo	6011	4026	66.97			
Total	8561	6227	72.73			
California mastitis test (n = 5591)						
Cattle	1479	1154	78.02	4.771	1	0.029*
Buffalo	4112	2162	52.57			
Total	5591	3316	59.30			
Culture examination (n = 2970)						
Cattle	1071	1047	97.75	0.0	1	1.000
Buffalo	1899	1864	98.15			
Total	2970	2911	98.01			
Organisms isolated						
Gram-positive		1860	62.62			
Gram-negative		729	24.54			
Candida spp.	2970	10	0.03	85.891	3	0.000*
No Growth		59	1.9			
Mixed infection		312	10.50			

*Level of significance: *p* value is significant at the 0.05 level or lesser.
n= no. of animals; *df:* degree of freedom

Table 2: Area wise occurrence of mastitis in cattle (n = 2550) & buffaloes (n = 6011)

Area	Cattle			Buffalo			Statistical significance (between species)		
	n	*+ve*	%	*n*	*+ve*	%	Chi square	*df*	*p value*
Ambala	345	330	95.65	228	217	95.18	0.005	1	0.942
Bhiwani	225	223	99.11	737	728	98.78	0.000	1	1.000
Mahendergarh	1871	1539	82.25	4760	2799	58.80	3.752	1	0.053
Rohtak	109	109	100	286	282	98.60	0.005	1	0.943
Grand Total	2550	2201	86.31	6011	4026	66.97	2.359	1	0.125

Statistical significance in occurrence of mastitis between different areas

	Cattle	Buffalo	Aggregate
Chi square	2.215	12.864	5.978
df	3	3	3
p value	0.529	0.005*	0.113

*Level of significance: *p value is significant at the 0.05 level or less*
df: degree of freedom

Table 3: Season wise occurrence of mastitis in cattle (n = 1979) & buffaloes (n = 5036)

Season	Cattle			Buffalo			Statistical significance		
	Total samples	Positive (*n*)	%	Total samples	Positive (*n*)	%	Chi square	*df*	*p value*
Rainy	987	845	85.61	2011	1350	67.13	2.359	1	0.125
Spring/ Autumn	515	450	87.38	1514	1025	67.70	2.329	1	0.127
Winter	474	386	81.43	1855	1175	63.34	2.250	1	0.134
Summer	574	520	90.59	631	476	75.44	1.542	1	0.214
Grand Total	2550	2201	72.74	6011	4026	86.31	2.359	1	0.125

Statistical significance in occurrence of mastitis (per animals) between different seasons

	Cattle	Buffalo	Aggregate
Chi square	0.588	1.095	0.812
df	3	3	3
p value	0.899	0.778	0.847

*Level of significance: *p value is significant at the 0.05 level or less, df:* degree of freedom

Note: Rainy (*July, August, September*), Spring/Autumn (*October, November, March*), Winter (*December, January, February*), Summer (*April, May, June*)

Bhiwani (99.11%), Ambala (95.65%) and Mahendergarh (82.25%). In buffaloes, occurrence of mastitis was significantly ($p<0.05$) higher in Bhiwani (98.78%) followed by Rohtak (98.60%), Ambala (95.18%) and Mahendergarh (58.80%). The variation in the studies with respect to season and prevalence of mastitis is due to the varying agro-climatic conditions and geographical areas.

These include climate, weather conditions, environmental hygiene, management practices and breed characteristics (Easaw and Vijayakumar, 2022). Differences in management practices, including milking routines, udder hygiene, and housing conditions, also affect mastitis occurrence. Additionally, certain breeds may be more susceptible to mastitis, and variations in breed dominance across regions can lead to differences in mastitis prevalence. Mastitis was most common in Jersey breeds (78.6%), than in Holstein Friesian and indigenous zebu cow crossbreeds (51.9%), and least common in indigenous zebu breeds (16.7%). Moreover, the availability and accessibility of veterinary services play a role in mastitis control and delay in mastitis treatment could amplify the number of cases or complicate the mastitis (Caneschi et al. 2023). Also, the credibility/education status of veterinary practitioners reaching to the doorsteps of the owners (if the owners not visiting the veterinary clinics or if the villages do not have the veterinary dispensaries), misuse and overuse of antibiotics, underdosing of antibiotics, not following the proper treatment regimen by owners, over-reliance on home-recipes for mastitis controls are some other factors which affect the fate of mastitis affected glands.

Season wise occurrence of mastitis in cattle and buffalo was shown in table 3. It was observed that occurrence of mastitis varies with different seasons across all regions. However, the difference was not statistically significant (p>0.05) in between same species of different areas and different species of same area. Overall, occurrence of mastitis was non-significantly (p>0.05) higher in summer followed by spring, rainy and winter season in case of cattle and buffalo. Season to season variation was observed in the occurrence of mastitis due to variation in growth of pathogenic organism. For example, hot and humid climates can create favourable conditions for bacterial growth, while poor sanitation practices or limited access to clean water can also contribute to higher mastitis rates (Singh et al. 2021). Previous reports in India, showed highest prevalence of mastitis during summer and rainy season in as compared to winter season (Easaw and Vijayakumar, 2022). This could be associated with increased multiplication of organisms and environmental stress, which altered the immune system, thereby making animals prone of infection/mastitis. The high prevalence during monsoon season could be attributed to the temperature and humidity conditions. This was contradictory to the findings by Ranjan et al. (2011) who found least prevalence during raining season (7.37%). OldeRiekerink et al. (2007) found that clinical mastitis was found in high frequency (increasing somatic cell count) during winter season in USA. Therefore, the Understanding these agro climatic conditions in different geographical areas are crucial for implementing targeted control strategies and interventions to reduce mastitis incidence and improve udder health in specific areas (Chen et al. 2023).

Antibiotic Susceptibility Testing

Overall, 2970 samples from cattle (n = 1071) and buffalo (n = 1899) were subjected for culture examination and antibiotic sensitivity assay (Table 4). The findings from present study revealed variations in antibiotic sensitivity across different districts. The district of Bhiwani consistently showed lower sensitivity rates for most antibiotics compared to the other districts. In terms of individual antibiotics, amikacin showed relatively highest sensitivity in Mahendergarh (84.44%) and Rohtak (81.51%), while lower sensitivity rates in Ambala (57.49%) and Bhiwani (32.70%).

Table 4 Antibiogram of organisms isolated from bovine mastitic milk samples

Total	Sensitivity (%)				Resistance (%)			
Antibiotics	Ambala	Bhiwani	Mahendergarh	Rohtak	Ambala	Bhiwani	Mahendergarh	Rohtak
Amikacin (30 mcg)	57.49	32.70	84.44	81.51	42.52	67.30	13.50	18.50
Amoxiclav 30 [Amoxicillin (20mcg)+ Clavulanic acid (10 mcg)]	67.36	23.10	27.72	67.59	32.64	76.90	71.89	32.41
Ampicillin (10 mcg)	-	-	15.70	15.70	-	-	84.11	84.30
Cefoperazone (75 mcg)	62.85	28.70	75.69	62.79	37.16	71.30	20.96	37.21
Ceftizoxime (30 mcg)	57.62	13.30	43.66	86.35	42.38	86.70	55.85	13.65
Ceftriaxone (30 mcg)	65.51	24.00	42.93	63.26	34.49	76.00	56.23	36.74
Chloramphenicol (30 mcg)	58.85	33.80	83.79	77.19	41.15	66.20	15.91	22.81
Ciprofloxacin (30 mcg)	-	23.20	80.62	63.37	-	76.80	18.18	36.64
Enrofloxacin (5 mcg)	86.71	27.80	83.97	94.08	13.30	72.20	15.15	5.92
Gentamicin (10 mcg)	60.56	34.40	95.20	95.84	39.45	65.60	4.22	4.16
Levofloxacin (05 mcg)	75.82	24.40	70.74	84.41	24.19	75.60	28.28	15.59
Moxifloxacin (05 mcg)	-	23.10	64.35	-	-	76.90	33.59	-
Oxytetracycline (30 mcg)	-	20.10	55.38	80.01	-	79.90	43.84	20.00

Similarly, enrofloxacin was high sensitivity in Rohtak (94.08%) but relatively low sensitivity in Bhiwani (27.80%). Amoxicillin + Clavulanic acid have higher sensitivity in Rohtak (67.59%) and Ambala (67.36%) than Mahendergarh (27.72%) and Bhiwani (23.10%). Regarding antibiotic resistance, the combination of amoxiclav shows high resistance in Mahendergarh (71.89%) and Bhiwani (76.90%), while Rohtak has relatively lower resistance (32.41%). Additionally, gentamicin exhibited high resistance in Ambala (95.17%) and Rohtak (95.84%), but much lower resistance in Bhiwani (34.40%). Overall, chloramphenicol, enrofloxacin, gentamicin, ciprofloxacin, cefoperazone and levofloxacin were most sensitive antibiotic across all districts. Amoxiclav and ampicillin were most resistant antibiotics in all districts. Similar to our findings, Ranjan et al. (2011) found high sensitivity towards enrofloxacin (91.67%). Pankaj et al. (2012), studied the antibiogram of isolates of mastitis and revealed high (90.90-100%) sensitivity to cefoperazone, enrofloxacin and gentamicin. Serdal and Funda (2021) found that ampicillin and streptomycin were the least effective antimicrobial agents, while the most effective antibiotics were amikacin and kanamycin.

Conclusion

In conclusion, findings of present study highlighted the importance of regional differences in antibiotic sensitivity and resistance patterns among mastitis-causing bacteria. Understanding these variations is crucial for the selection and proper use of antibiotics in mastitis treatment and control strategies. It is essential to consider local antibiotic sensitivity profiles and regularly monitor for changes in resistance patterns to ensure effective management of mastitis in cattle and buffaloes across different districts.

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