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Morphological characterisation and antimicrobial resistance of bacterial isolates from bovine mastitis

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Abstract
The antimicrobial susceptibility of the pathogens is essential for the successful treatment of bovine mastitis. Antimicrobial resistance develops in microbes as a result of indiscriminate use of antibiotics. Therefore, regular screening is required for the selection of appropriate and effective antimicrobials. A study was carried out in 60 crossbred dairy cows in early lactation with the objective to assess the morphological characteristics and antimicrobial resistance of bacterial isolates from bovine mastitis. California mastitis test (CMT) was done for 235 individual quarter milk samples. Isolation of bacteria was done by direct streaking of the individual quarter CMT positive milk samples on to blood agar followed by incubation of the plates at 37 °C for 24 h. On Gram's staining, 32 isolates were found to be Gram positive cocci, three were Gram positive bacilli and ten were Gram negative bacilli. The disc diffusion assay revealed that antimicrobial resistance was highest against tetracycline (71.11 percent), followed by resistance to ampicillin and ceftriaxone-tazobactam (66.67 percent), ceftriaxone (64.44 percent), amoxicillin-sulbactam (62.22 percent), amikacin (57.78 percent), ceftriaxone-sulbactam (55.56 percent), enrofloxacin (46.67 percent), amoxicillin-clavulanate (40 percent), ciprofloxacin (37.78 percent) and gentamicin and co-trimoxazole (28.89 percent).

Keywords: California mastitis test, antimicrobial resistance, mastitis

1. Introduction
Mastitis is one of the most common production diseases affecting the global dairy cattle industry. Its occurrence is linked to both direct and indirect losses and expenses [16]. Mastitis is classified as clinical or subclinical. Clinical mastitis is distinguished by its abrupt onset, changes in milk composition and appearance, decreased milk production and the presence of the cardinal signs of inflammation in infected quarters of mammary glands. Whereas, subclinical mastitis has no visible signs on the udder or in the milk, but milk production decreases and somatic cell count increases [2]. The standard method for identifying mastitis is the bacteriological culture of milk samples. Milk culture detects the presence of mastitis pathogens but does not quantify the degree of inflammation caused by the infection [20]. Routine bacterial identification provides valuable epidemiologic information that can be used to formulate appropriate antibiotic policies or even preventive measures in many situations. With newer pathogens emerging and older ones re-emerging, identifying the agent is critical in implementing such measures [11]. A rising issue in bovine mastitis is antimicrobial resistance. Antimicrobial resistance, which aids in the survival of bacteria after antibiotic therapy, can occur due to the presence of antimicrobial resistance genes, which can spread between bacteria *via* horizontal genetic transfer using mobile genetic elements like plasmids, phages and pathogenicity islands, as well as through random mutations when the bacteria are stressed[8,14]. The indiscriminate use of antibiotics in the treatment of mastitis greatly increases the risk of antibiotic resistance being installed and transmitted to humans [15].

2. Materials and Methods
The present study was carried out in 60 crossbred dairy cows in early lactation from University Livestock Farm (ULF) and Fodder Research and Development Scheme (FRDS), Mannuthy and Cattle Breeding Farm (CBF), Thumburmuzhy. The study was conducted from October 2021 to May 2022.

All the experimental animals under study were maintained as per the 2016 package of practices recommendations of Kerala Veterinary and Animal Sciences University (KVASU). Five quarters from four cows out of the 240 quarters from 60 cows were blind and were excluded from the study. California mastitis test (CMT) was done for the 235 individual quarter milk samples as per procedure described by Schalm *et al.* (1971) [19], and the reaction was scored within 15 seconds as per the interpretation criteria of Ruegg and Reinemann (2002) [18]. California mastitis test positive milk samples were collected aseptically. Isolation of bacteria was done by direct streaking of the individual quarter milk samples on to blood agar followed by incubation of the plates at 37 °C for 24 h. The microscopic morphology of the colonies was examined after Gram's staining. Gram positive bacteria stained purple blue colour and Gram negative took up pink colour of the counter stain.

In-vitro antibiotic sensitivity of the bacterial isolates was tested based on Kirby-Bauer disc diffusion method [6] as per the Clinical and Laboratory Standards Institute guidelines (CLSI, 2021) [9]. Antibiotic discs with known concentration in microgram (mcg) per disc were used in the study, *viz.* Ampicillin, Amoxicillin-sulbactam, Amoxicillin-clavulanate, Amikacin, Enrofloxacin, Ciprofloxacin, Gentamicin, Tetracycline, Ceftriaxone-sulbactam, Ceftriaxone-tazobactam, Ceftriaxone and Co-trimoxazole.

3. Results and Discussion

The California Mastitis Test, developed by Schalm and Noorlander in 1957, is a quick and easy diagnostic for mastitis. The authors stated that the degree of precipitation and gel produced when milk and reagent were combined was a reflection of the cell count in the milk [5]. California mastitis test was done for the 235 individual quarter milk samples in the present study. Among the 235 milk samples, 28 (11.91 percent), 15 (6.38 percent) and 5 (2.13 percent) samples had a CMT score of 1, 2 and 3, respectively (Table 1.), whereas according to Anjali (2018), among the 274 milk samples tested, 61 (22.26 percent), 22 (8.03 percent) and 11 (4.01 percent) had CMT scores of 1, 2 and 3, respectively, which were higher than the numbers reported in the current study [4]. The diagnosis rate of mastitis in the present study using CMT was 22.55 percent on quarter basis, whereas higher incidence of 48.34 percent on quarter basis was reported by Anaina (2022) [3].

Table 1: CMT score of individual quarter milk samples

SL. No.	CMT score	Number of quarter samples	Percent of quarter samples
1.	0	182	77.44
2.	T	5	2.13
3.	1	28	11.91
4.	2	15	6.38
5.	3	5	2.13
Total		235	100

In the present study, 45 samples (84.91 percent) out of 53 individual quarter milk samples which were CMT positive yielded bacterial isolates and the remaining eight samples (15.09 percent) did not produce any growth. On Gram's staining of the 45 pure isolates, 32 were found to be Gram positive cocci. Three isolates were found to be Gram positive bacilli and ten isolates were Gram negative bacilli (Table 2). The present findings were in accordance with Rathish (2014), Krupa (2020) and Anaina (2022) who observed a higher prevalence of Gram positive isolates [17, 12, 3]. According to the study conducted by Olivares-Perez *et al.* (2015) in the dairy farms of the tropical region of Mexico, of all positive isolates, 97.5 percent were Gram-negative bacteria, which was not in accordance with the present findings [13]. Subclinical mastitis is a complex disease with varying prevalence due to management system, environment, age, parity, milk yield, body condition and other factors. These factors may have contributed to the observed differences in subclinical mastitis prevalence [7].

Table 2: List of organisms isolated from CMT positive milk samples

SL No.	Organism isolated	No. of Isolates	Percent
1.	Gram positive cocci	32	71.11
2.	Gram positive bacilli	3	6.66
4.	Gram negative bacilli	10	22.22
Total		45	100

Bacterial identification and antimicrobial susceptibility pattern determination is the foundation of clinical microbiology [10]. The overall antibiotic susceptibility pattern of bacterial isolates is shown in Table 3. In the present study, highest number of Gram positive cocci isolates showed resistance against tetracycline. Twenty-two isolates exhibited resistant to ampicillin, amikacin and ceftriaxone-tazobactam. Twenty-one isolates were resistant to amoxicillin-sulbactam and ceftriaxone, followed by ceftriaxone-sulbactam, whereas the maximum number of isolates were sensitive to cotrimoxazole. Two isolates of Gram positive bacilli were resistant to ampicillin, amoxicillin-sulbactam, ceftriaxone-sulbactam, and ceftriaxone-tazobactam, whereas all the three isolates were susceptible to ciprofloxacin. Among the ten Gram negative bacilli isolates, seven isolates were resistant to tetracycline, ceftriaxone-sulbactam and ceftriaxone. Six isolates exhibited resistance to ampicillin, enrofloxacin and ceftriaxone-tazobactam, followed by five isolates to amoxicillin-sulbactam and four isolates to amoxicillin-clavulanate. Three isolates showed resistance against amikacin and co-trimoxazole, whereas the maximum number of isolates were sensitive to ciprofloxacin and gentamicin.

The disc diffusion assay of all isolates showed highest resistance against tetracycline (71.11 percent), followed by resistance to ampicillin and ceftriaxone-tazobactam (66.67 percent), ceftriaxone (64.44 percent), amoxicillin-sulbactam (62.22 percent), amikacin (57.78 percent), ceftriaxone-sulbactam (55.56 percent), enrofloxacin (46.67 percent), amoxicillin-clavulanate (40 percent), ciprofloxacin (37.78 percent) and gentamicin and co-trimoxazole (28.89 percent). Based on the study conducted by Anjali (2018), antibiotics were ranked according to their resistance towards the mastitis causing organisms as amoxicillin (100 percent), ceftriaxone (89.7 percent), oxytetracycline (87.2 percent), enrofloxacin (82.1 percent), amikacin (56.4 percent) and gentamicin (42.3 percent) [4]. According to the study conducted by Singh *et al.* (2016), among bacterial isolates from 35 bovine milk samples, 88.24 percent were found resistant to ampicillin-sulbactum, 85 percent to ceftriaxone and 83 percent to amoxyclav [21].

Table 3: Antibiotic sensitivity and resistance of isolated organisms

SL No.	Antimicrobial	Gram positive cocci		Gram positive bacilli		Gram negative bacilli		Overall (%)	
		S	R	S	R	S	R	S	R
1.	Tetracycline	8	24	2	1	3	7	28.89	71.11
2.	Ampicillin	10	22	1	2	4	6	33.33	66.67
3.	Ceftriaxone-tazobactam	10	22	1	2	4	6	33.33	66.67
4.	Ceftriaxone	11	21	2	1	3	7	35.56	64.44
5.	Amoxicillin-sulbactam	11	21	1	2	5	5	37.78	62.22
6.	Amikacin	10	22	2	1	7	3	42.22	57.78
7.	Ceftriaxone-sulbactam	16	16	1	2	3	7	44.44	55.56
8.	Enrofloxacin	18	14	2	1	4	6	53.33	46.67
9	Amoxicillin-clavulanate	19	13	2	1	6	4	60	40
10.	Ciprofloxacin	17	15	3	0	8	2	62.22	37.78
11.	Gentamicin	22	10	2	1	8	2	71.11	28.89
12.	Co-trimoxazole	23	9	2	1	7	3	71.11	28.89

S: Sensitive, R: Resistant

Abdi *et al.* (2021) reported that the proportion of resistant isolates was relatively higher in Gram-negatives as compared to Gram-positives, which is in agreement with present findings [1]. According to Pascu *et al*. (2022), except for cephalothin, Gram positive bacteria showed low susceptibility to the majority of the antimicrobials tested. Gram-negative bacteria were fairly susceptible to penicillins and quinolones, but resistant to macrolides, aminoglycosides and tetracyclines. The majority of isolates were multidrug resistant and the resistance pathotypes were resistant to the most commonly used antimicrobials in cow mastitis treatment [15].

4. Conclusion

In the present study, the major pathogens causing bovine mastitis were Gram positive cocci, followed by Gram negative bacilli and Gram positive bacilli. Tetracycline resistance was found in the majority of isolates, followed by resistance to ampicillin and ceftriaxone-tazobactam, ceftriaxone, amoxicillin-sulbactam, amikacin, ceftriaxone-sulbactam, enrofloxacin, amoxicillin-clavulanate, ciprofloxacin, and gentamicin and co-trimoxazole. Regular screening is required for the selection of appropriate and effective antimicrobials to be used.

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