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Multidrug Resistant *Staphylococci* Isolates from Bovine Mastitis in Wayanad District

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ABSTRACT

The phenotypic resistance pattern of staphylococci isolates from bovine mastitis was studied. The phenotypic resistance percentages obtained for the antibiotics tetracycline (17.90 %), sulpha- trimethoprim (15.40 %), ceftriaxone- tazobactum (14.19%), gentamicin and amoxycillin- clavulanate (11.11 %) each, enrofloxacin (10.49%) and cefoperazone (8.64 %) was assessed to identify multi-drug resistance (MDR) in the isolates. The MDR bacterial isolates were identified as *Staphylococcus* spp (12.34%). Seven MDR coagulase positive isolates and 13 coagulase negative isolates were selected for the multi-drug resistance profiling. The MAR index was also calculated for each isolate. The highest MAR index was noticed for the isolate which showed resistance against all the antibiotics studied. The occurrence of multi-drug resistance in mastitis pathogens results in recurrent mastitis in dairy cattle and to the related economic losses for the livestock owners along with serious implications in public health.

HIGHLIGHTS

- High occurrence of MDR *Staphylococcus* spp. was reported from recurrent bovine mastitis.
- Emergence of coagulase negative staphylococci as a major pathogen for subclinical mastitis in cattle

Keywords: *Staphylococci,* Phenotypic resistance, MDR, MAR index

Staphylococci are recognized as the leading etiological agents for clinical mastitis worldwide. *Staphylococcus aureus*, notorious for its antimicrobial resistance and unresponsiveness to therapy has been studied extensively by many scientists (Salaluddin *et al.,* 2020; Tegegne *et al*., 2021; Mbindyo *et al.,* 2021). Pathogenesis and persistence of staphylococcal mastitis is determined by coagulase secretion and is used to distinguish *S. aureus* from other staphylococci. The emergence of multi-drug resistance in coagulase positive staphylococci (CPS) has been documented by various authors (Marami *et al*., 2022; Lopes *et al*., 2022). Coagulase negative staphylococci (CNS) were usually considered as teat skin opportunistic pathogens. The CNS staphylococci such as *S. chromogenes*, S. *simulans, S. xylosus, S haemolyticus and S. equorum* were identified as the causative agents of clinical and subclinical mastitis by many scientists (Condas *et al*., 2017; De Visscher *et al*., 2017). The endogenous mechanisms for multidrug resistance of *S. aureus* are decrease in outer membrane permeability, the active efflux of antibiotics and excessive production of beta lactamase

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enzyme. Acquired antibiotic resistance is mainly through resistance by mutations, acquisition of resistance genes and biofilm mediated resistance (Guo *et al.,* 2020).

The practice of random usage of antibiotics and inadequate therapy has resulted in multidrug resistance in the mastitis causing bacteria and also their entry in to the food chain. The present study was carried out in the Department of Veterinary Epidemiology and Preventive Medicine during the period from January 2021 to July 2022 to assess the phenotypic profile of multidrug resistance in staphylococci isolates from bovine clinical and subclinical mastitis.

MATERIALS AND METHODS

Isolation and identification of the staphylococci isolates

A total of 110 dairy cows affected with mastitis and 105 cows with subclinical mastitis were selected for the study. Milk samples collected from mastitis cases were streaked on to Brain heart infusion agar for primary culturing. Among the 110 samples 101 samples revealed bacterial growth and nine samples did not produce colonies.. Out of the 101 bacterial isolates from clinical mastitis obtained in brain heart infusion agar, eighty-six were Gram- positive isolates and fifteen were Gram- negative isolates. In the 197 quarter samples from 105 subclinical mastitis cases, milk samples from eighty animals revealed bacterial growth and milk samples from 25 animals did not produce colonies. Among the eighty bacterial isolates from subclinical mastitis cases, all were Gram- positive isolates. The staphylococci isolates were identified based on colony morphology, Gram-staining and biochemical tests.

Identification of the MDR isolates

In-vitro antibiotic sensitivity of the staphylococci isolates was studied using the Kirby Bauer disc diffusion method (Bauer *et al.,* 1966) as per the Clinical and Laboratory Standards Institute guidelines (CLSI, 2019). The spread of antibiotic resistant bacteria was also found by calculation of multiple antibiotic resistance (MAR) index (Krumperman, 1983). Antibiotic discs amoxicillin-clavulanate (20/10 mcg), trimethoprim/sulphamethoxazole (1.25/23.75 mcg), enrofloxacin (10 mcg), cetriaxone-tazobactum (80/10 mcg), cefoperazone (75 mcg), gentamicin (10 mcg) and tetracycline (30 mcg) were used in this study. Isolates showing phenotypic resistance to minimum of the three different class of antibiotics were considered as multi drug resistant isolates (MDR) (Fig. 1). Twenty staphylococci isolates were identified as multi-drug resistant isolates and were selected for characterization. Methicillin resistance of the MDR staphylococci were studied using the antibiotic discs cefoxitin (30 mcg) and oxacillin (1 mcg).

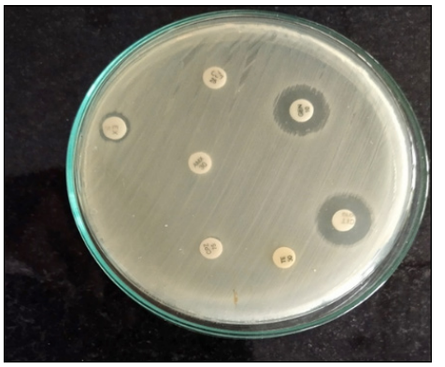

Fig. 1: MDR *S. aureus* in MHA plate

Characterization of the MDR isolates

Cultural characterization and colony morphology of MDR coagulase positive staphylococci isolates in Mannitol salt agar, Baird Parker agar, DNase agar, purple agar with 1% maltose, and VP test revealed seven CPS and 13 CNS isolates. Along with the above tests, sugar fermentation tests, urease test and nitrate tests were used to identify the isolates (Fig. 2).

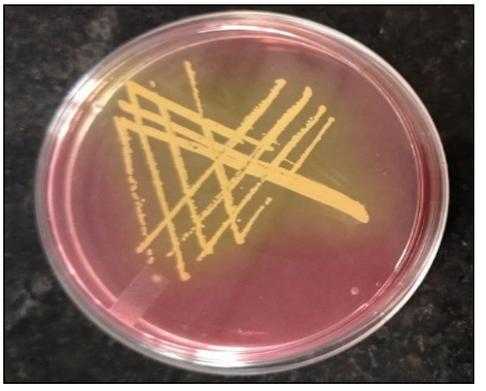

Fig. 2(A): *Staphylococcus aureus* in Mannitol salt agar

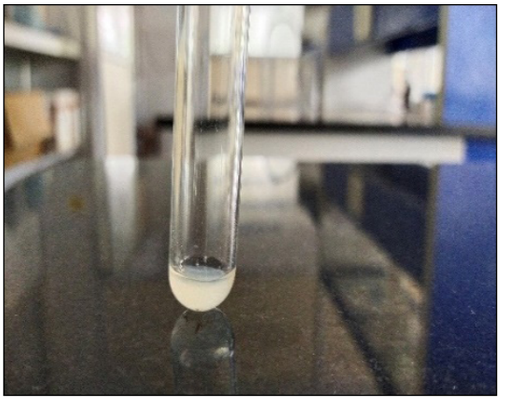

Fig. 2(B): Coagulase positive *Staphylococcus spp*

Fig. 2(C): *S. aureus* in BP agar

RESULTS AND DISCUSSION

Occurrence of multi-drug resistant isolates in bovine mastitis

Among 110 clinical mastitis cases screened, 78.18 per cent were *Staphylococcus* spp, 13.63 per cent were Gram-negative isolates, 8.18 per cent showed no growth. Among the 105 subclinical mastitis cases, 76.19 per cent were *Staphylococcus* spp., 23.80 per cent showed no growth. Among the 162 staphylococci isolates 20 (12.34 per cent) were identified as multi-drug resistant. The MDR coagulase positive isolates (n=7) were classified into six *S. aureus* and one *S. pseudointermedius* isolate. The MDR coagulase negative staphylococci isolates (n=13) were classified into five *S. chromogenes,* four *S. epidermidis*, two *S. saprophyticus*, two *S.simulans* each (Fig. 3).

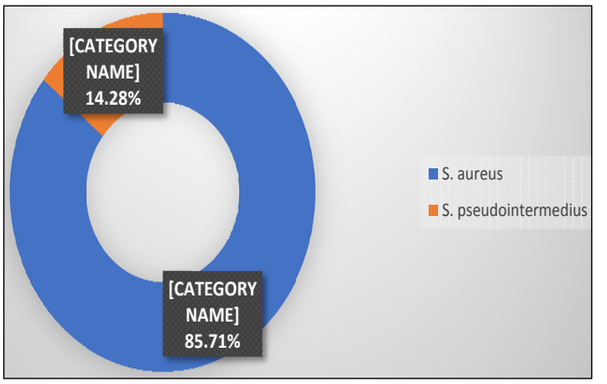

Fig. 3(A): Classification of MDR CPS isolates from bovine mastitis

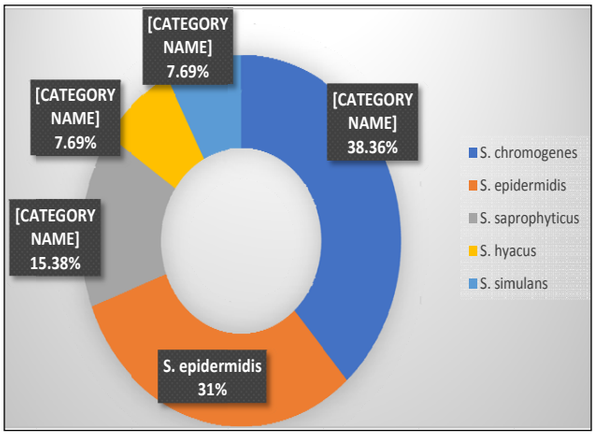

Fig. 3(B): Classification of MDR CNS isolates from bovine mastitis

Antibiotic resistance profile

Among the 162 staphylococci isolates, 29 isolates were resistant to tetracycline (17.90 5), 25 isolates were resistant to sulpha- trimethoprim (15.40 %), 23 isolates were resistant to ceftriaxone- tazobactum (14.19 %), 18 isolates were resistant to gentamicin and amoxycillin- clavulanate (11.11 %), 17 isolates were resistant to enrofloxacin (10.49%) and 14 isolates were resistant to cefoperazone (8.64 %) (Table 1).

Table 1: Antibiotic resistance profile of the *Staphylococcus* spp isolates from clinical mastitis (%)

Sl. No	Name of antibiotic	Resistant isolates (No)	Per cent (%)
1	Tetracycline	29	17.90
2	Ceftriaxone -Tazobactum	23	14.19
3	Sulpha -Trimethoprim	25	15.43
4	Cefoperazone	14	8.64
5	Gentamicin	18	11.11
6	Amoxycillin clavulanate	18	11.11
7	Enrofloxacin	17	10.49
	Total	162	100

A higher resistance to tetracycline, amoxicillin-clavulanic acid and methicillin in staphylococci isolates in clinical and subclinical mastitis were earlier reported by Phophi *et al*. (2019), Pascu *et al*. (2022) and Freitas *et al.* (2018).

Multidrug resistance pattern of the isolates

The multidrug resistance pattern and MAR index of the seven CPS isolates is given in table 2. Among the seven isolates, five isolates showed resistance to four different classes of antibiotics (71.40 %), whereas two isolates showed resistance to three different classes of antibiotics (28.57 %). The MAR index was calculated for each isolate. The highest MAR index was noticed for the isolate which showed resistance against all the antibiotics studied.

Among the seven MDR coagulase positive isolates, the present study revealed six *S. aureus* isolates and one *S. pseudointermedius* isolate. The findings of Marami *et al.* (2022) are also similar who reported a high percentage of MDR coagulase positive staphylococci in dairy farms in Ethopia. Mbindyo *et al.* (2021) also reported 29.67 per cent MDR *S. aureus* isolates and 16.3 per cent MDR CNS. In India, Brahma *et al*. (2022) reported 57.20 per cent *S. aureus* isolates as MDR.

In the present study the characterization of the methicillin resistant MDR CPS isolates was performed based on cefoxitin (42.85 per cent) and oxacillin (57.14 per cent), which revealed that high prevalence of methicillin resistant staphylococci exist among the MDR staphylococci which is in accordance with the findings of Brahma *et al.,* 2022. Mahanti *et al*. (2020) also reported multidrug resistance in all the MRSA isolated. Co resistance to βlactam antibiotics, tetracycline and aminoglycocides in MRSA isolates was earlier reported by Carfora *et al*. (2015). *S. pseudointermedius* a commensal bacteria seen in skin and mucous membrane might have been transmitted through the close contact between farm animals, human beings and pets.

The multidrug resistance pattern of the 13 CNS isolates are given in table 3. Among the 13 isolates, one isolate showed resistance to five different classes of antibiotics (7.69 per cent). Five isolates showed resistance to four different classes of antibiotics (38.46 per cent). Seven isolates showed resistance to three different classes of antibiotics (53.84 per cent).

Initially CNS were considered as minor pathogens with less clinical importance but recent studies (Freitas *et al*., 2018) have reported higher prevalence of CNS in clinical mastitis and the emergence of multidrug resistance in CNS isolates. This is in accordance with the present findings

Table 2: Multidrug resistance profile of the CPS isolates

Sl. No	Antibiotics								MDR pattern to different classes of antibiotics	MAR index
	TET	ENR	GEN	CPZ	CFT-TZ	AMXCLV	MET	SUT		
1	1				1	1	1	1	TET + BL + SUT (3)	0.625
2	1	1			1	1		1	TET + ENR + BL + SUT (4)	0.625
3	1	1			1	1		1	TET + ENR + BL + SUT (4)	0.625
4	1	1			1			1	TET + ENR + BL + SUT (4)	0.50
5		1		1	1	1	1	1	ENR + BL + SUT (3)	0.75
6	1	1	1		1		1		TET + ENR + GN + BL (4)	0.625
7	1	1	1		1	1	1	1	TET + ENR + GN + BL (4)	0.875

TET- Tetracycline ENR- Enrofloxacin BL- β lactams SUT- Sulpha -trimethoprim GN- Gentamicin.

Table 3: Multidrug resistance profile of the CNS isolates

Sl. No.	Antibiotics								MDR pattern	MAR index
	TET	ENR	GEN	CPZ	CFT-TZ	AMXCLV	MET	SUT		
1	1			1				1	TET + BL + SUT (3)	0.375
2	1			1	1	1	1	1	TET + BL + SUT (3)	0.75
3		1		1	1			1	ENR + BL + SUT (3)	0.50
4	1	1			1				TET + ENR + BL (3)	0.375
5	1				1	1		1	TET + BL + SUT (3)	0.50
6	1		1		1	1	1		TET + ENR + GN + BL (4)	0.625
7	1		1	1	1		1		TET + GN + BL (3)	0.625
8	1	1	1		1		1		TET + ENR + GN + BL (4)	0.625
9	1	1	1	1			1	1	TET + ENR + GN + BL + SUL(5)	0.75
10	1	1	1		1				TET + ENR + GN + BL (4)	0.50
11	1				1	1		1	TET + BL + SUT (3)	0.50
12	1		1		1		1	1	TET + GN + BL + SUT (4)	0.625
13	1		1	1			1	1	TET + GN + BL + SUT (4)	0.625

TET- Tetracycline ENR- Enrofloxacin BL- β lactams SUT- Sulpha -trimethoprim GN- Gentamicin.

which showed an occurrence of 65 % of MDR CNS. The predominant multidrug resistant CNS species recovered from the milk samples was *S. chromogenes* followed by *S. epidermidis*, *S. saprophyticus* and *S. simulans* which accounted for 38.46 %, 30.76 %, 15.38 %. This is in agreement with Lopes *et al*. (2022) who also reported a higher presence of MDR *S. chromogenes* than *S. aureus*. The occurrence of multidrug resistance is higher in clinical mastitis than subclinical mastitis and the reason might be due to the repeated usage of antibiotics for the treatment of clinical mastitis and subclinical mastitis are usually under diagnosed (Bansal *et al*., 2015).

CONCLUSION

The present study studied the phenotypic resistance pattern of the staphylococci isolates from clinical and subclinical mastitis in Wayanad district. Based on the phenotypic resistance pattern of the isolates twenty staphylococci isolates were selected as multidrug resistant (12.34 %). Morphological and biochemical charecterisation of the isolates revealed six *S. aureus* and one *S. pseudointermedius* isolate among the CNS isolates and The predominant multidrug resistant CNS species recovered from the milk samples was *S. chromogenes* followed by *S. epidermidis*, *S. saprophyticus* and *S. simulans*. The multidrug resistance pattern and MAR index of the isolates were assessed. The significantly higher percentage of multidrug resistance warrants formulation of future strategies for combating multidrug resistance in mastitis pathogens.

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