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Characterization and AST pattern of bacterial pathogens of bovine mastitis milk

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Abstract

Bovine mastitis is a global problem in the dairy sector. In this study, seven different types of bacteria were isolated from bovine mastitis milk. The most sensitive antibiotic for the main *Bacterial* isolates was Fosfomycin and Cefotaxime. Sensitive drugs were *Ampicillin*, *Ceftriaxone*, *Ampicillin sulbactam*, *Neomycin* and *Doxycycline*. Drugs with intermediary action were Gentamicin, Bacitracin, Neomycin and resistant drugs were Penicillin-G, Streptomycin and Erythromycin. *Bacterial* isolates were multidrug resistant.

Keywords: SCC, AST, MDR, Resistance %, bovine mastitis, pathogenic *Bacterial* isolates

Introduction

Bovine mastitis is one of the most prevalent and economically significant diseases affecting dairy cows worldwide. It is characterized by inflammation of the parenchyma of mammary gland. Mastitis occurs in both clinical and subclinical forms. SCM often goes unnoticed but contributes to treatment and substantial hidden losses (Parasana *et al.*, 2021) ^[14]. It is a multi-etiological (Hawari and Fawzi, 2008) ^[10] caused by a plethora of microorganisms (Vakkamaki *et al.*, 2017) ^[17] and multi-factorial disease as well (Deb *et al.*, 2013) ^[4]. Bovine mastitis is a complicated disease that is difficult to prevent by vaccination. Global surveys indicate that Subclinical Mastitis (SCM) affects about 40-42% of dairy animals and Clinical Mastitis (CM) accounts for about 25% of cases. Pathogens like *Staphylococcus aureus*, coagulase-negative staphylococci (CoNS), *Escherichia coli*, *Klebsiella* and *Pseudomonas* spp. are the most common pathogens. So dairymen rely heavily on antibiotics for mastitis treatment. This overuse and irrational application leads to development of antibiotic resistant bacteria (Acar and Rostel, 2001) ^[1]. Which compromises therapeutic success and poses public health risks.

Study objective

Detection of antibiotic resistance in *Bacterial* pathogens of bovine mastitis

Study area

A total of 153 bovine milk samples were collected from dairy herds in Chengalpattu, Chennai and Thiruvallur districts (North Eastern zone of Tamil Nadu). Briefly, milk samples were collected, from every udder quarter (1sample per quarter) and diagnosed on the basis of visual abnormality and inflammation of the udder. Samples were collected in sterile bottles (5-10 ml) and transported on ice to the laboratory within 6 hours.

Materials and Methods

Mastitis diagnosis was performed on bovine milk for the Somatic Cell Count (SCC) using TANUCHECK SCC kit based on an enzymatic reaction, which is directly related to the number of somatic cell present. This is a cow side kit that helps to screen and grade the quality of milk as good, mild, moderate, severe for mastitis followed by poor or bad quality milk. Electrical Conductivity (EC) was also done for all the milk samples.

Sample processing for phenotypic characterization

Bovine milk was divided as sub-clinical (mild to moderate) and clinical mastitis as per screening by TANUCHECK SCC kit and Electrical conductivity (Rana *et al.*, 2023) ^[15]. For *Bacterial* isolation, conventional microbiological methods are considered as gold standard and are routinely employed for confirmation of the causative agents (Javia *et al.*, 2018) ^[11]. About 20µl of mastitis milk was inoculated into 3ml of Tryptic soya broth and incubated overnight at 37°C for 16-18 hr.

Cultural characterization

Bacterial cultures were stained by Gram's method and then streaked on various selective media like, Mannitol Salt, Baird Parker, MacConkey, Nutrient and 5% Sheep blood agar. Biochemical test were done for differential identification of *Bacterial* isolates of Enterobacteriaceae family (IMViC).

Antimicrobial Sensitivity Test was done for all *Bacterial* isolates using 16 different antibiotics and the pattern was recorded (Forbes *et al.*, 2007) ^[7].

Antimicrobial Susceptibility Test (AST)

Single pure colony from selective agar plate of each *Bacterial* isolate was inoculated in 5ml of Tryptic soya broth and incubated at 37°C for 6 hours till a turbidity of 10^{5-6} cfu per ml was obtained. Culture was centrifuged and the pellet was suspended in Phosphate buffer saline and adjusted to 0.5 scale McFarland turbidity. About 20µl of re-suspended culture was added on Muller Hinton agar and swabbed to form a lawn. AST was done as per the guidelines of CLSI (Wayne, 2013) ^[18]. All cultures were tested against sixteen antibiotics as listed in Table 1 Himedia (Mumbai). The zone of inhibitions were measured and interpreted.

Table 1: Details of antibiotic discs used for AST

No	Code	Antibiotic & Conc. (µg)	No	Code	Antibiotic & Conc. (µg)
1	AMC	Amoxicillin 10	1	CLX	Cloxacillin 10
2	A/S	Ampicillin sulbactam 10/10	2	DoHcl	Doxycycline 15
3	AMC	Ampicillin 30	3	E	Erythromycin 5
4	B	Bacitracin 10	4	ETP	Ertapenem 5
5	CIP	Ciprofloxacin 30	5	MRP	Meropenem 5
6	CTR	Ceftriaxone 30	6	N	Neomycin 10
7	CTX	Cefotaxime 30	7	OTC	Oxytetracycline 30
8	COT	Co-trimazole 10/5	8	OX	Oxacillin 5

Results

Out of the total bovine milk (N=153) screened, 33% (N=32) were of good quality milk and 79.08 % were found to be Mastitis positive (N=121) with 71 subclinical (59%), 50 (41.3%) clinical by TANUCHECK kit as detailed in Table.2. The SCC values ranged from 1-7 lakh cells/ml, correlating with increasing EC scores. SCC and EC are used as indicators for detecting bovine mastitis and show a positive correlation. An increase in SCC causes damage to udder tissue leading to changes in milk composition, like elevated sodium and

chloride ion concentrations and increasing EC of the milk (Tommasoni *et al.*, 2024) ^[16].

The milk samples identified as SCM using the TANUCHECK SCC kit revealed 62% of the samples had SCC in the range of 1-1.5 lakhs graded as Good quality and 72% samples had a SCC range of 2-3.5 lakhs graded as Mild. In CM milk, 42% had a SCC range of 3.5-4.2 lakhs graded as Moderate and 58% had a SCC range of 5.5-6.5 lakh and was graded as Poor quality. Further, 8% milk was unfit with SCC range of 7 to 8lakhs and was discarded.

Table 2: Screening by somatic cell count, grading and electrical conductivity of milk samples

No	SCM tested	SCC (lakhs/ml) range	Grade	EC millisiemens/cm
1	34 (62%)	1.00 (≤1 lakh to 1.5 lakh)	Hygienic	0.65-1.48
2	37 (72%)	3.00 (2 lakh and ≥3.0 lakh)	Moderately hygienic	0.93-2.32
3	21 (42%)	Upto 5 (3.5 lakh to 4.2lakh)	Unhygienic	1.43-3.52
4	29 (58%)	5.5-6.5 (4.5-≥ 6 lakh)	Poor	4.11-4.85
5	09 (18%)	7.00 (6 lakh and ≥ 7 lakh)	Unfit	5.0-5.8
	121/153	71-SCM & 50-CM 09-Unfit & Good milk-32		

The mastitis milk samples yielded a total of 113 isolates inclusive of gram positive as well as gram negative organisms belonging eight different genres. *Bacterial* isolation confirmed the isolates as *Staphylococcus* spp in 72% and 22% and *Streptococcus* spp. in 4.2% and 8% of the SCM and CM milk respectively. *Escherichia coli* was 28% and 33%; *Klebsiella* spp was 15.5% and 26% and *Pseudomonas* spp was 10% and 18% from SCM and CM milk respectively as mentioned in Table 3 which is in alignment with the findings of Nathawat *et al.*, 2013 ^[14]. The antibiotic susceptibility for the pathogens involved in mastitis milk revealed resistance to multiple antibiotics tested. Multidrug resistance (MDR) is

defined as resistance to ≥ 3 antimicrobials (Magiorakos *et al.*, 2012) ^[12]. The number of samples showing sensitivity or resistance was expressed in percentage. Milk of cows in the third or higher lactation stages showed resistance to more number of antimicrobial classes than mastitis isolates from younger cows.

The *Bacterial* isolates obtained from the milk samples were found to be highly sensitive to Fosfomycin, cefotaxime and *Ampicillin* sulbactam followed by gentamicin, ciprofloxacin and intermediate or resistant to the other tested antibiotics as given in Table 4.

Table 3: Bacterial strains obtained by cultural isolation (Harini and Sumathi 2011) ^[9]

No	Bacterial isolates	SCM (N=71) 59%		CM (N=50) 41.3%		Overall Total	% of isolates
		Total	%	Total	%		
1	<i>Staphylococcus</i> spp	51	72	11	22	62	51
1a	CoPS (coa +ve) <i>Staphylococcus</i> spp	07	14	03	06	10	8.2
1b	CoNS (coa-ve) <i>Staphylococcus</i> spp	44	86	08	36	52	43
2	<i>Escherichia coli</i>	20	28	33	66	53	44
3	<i>Streptococcus</i> spp	03	4.2	04	08	07	6
4	<i>Klebsiella</i> spp	11	15.5	13	26	24	20
5	<i>Pseudomonas</i> spp	07	10	09	18	16	13.2
6	<i>Bacillus</i> spp	03	4.2	02	04	05	4.1
7	<i>Enterobacter</i> spp	13	18.3	05	10	18	15
8	<i>Clostridium</i> spp	05	7	Nil	Nil	05	4.1
Total Bacterial isolates		113		77		190	

Table 4: AST pattern for the Bacterial isolates from Bovine Mastitis Milk

No	Bacteria from mastitis milk	Very Sensitive	Sensitive	Intermediary	Resistant	Indicator drugs
1	<i>Staphylococcus</i> (N=62)	FOS,CTX	AMC-A/S/EX	CTR/B /CT	P/S/ E/AMP/	CLX/OX
2	<i>E.coli</i> (N=53)	FOS/CIP	N /DoHCl	GEN/AMC/CT/S	B/CTX/COT	ETP,IMP
3	<i>Streptococcus</i> (N=07)	AMC/CTR	FOS/NX	E/S/COT	P-G/E/S	OX
4	<i>Klebsiella</i> spp (N=24)	AMP/OF/ FOS	Do/AMP/OTC	GEN/COT	B/CTX	MRP
5	<i>Pseudomonas</i> (N=16)	GEN/CTX/CIP	CTR/CIP	GEN/EX/COT	DoHcl/S	IMP/MRP
6	<i>Bacillus</i> spp (N=5)	AMP/EX	B/S	NIL	P-G/AMP	NIL
7	Enterobacters (N=18)	P-G	AMP/OF/COT	NIL	AMC/S	NIL
8	<i>Clostridium</i> (N=5)	Streptopenicillin	CIP	NIL	NIL	NIL

The overall antibiogram profile for different *Bacterial* types (Gao *et al.*, 2017) ^[8] of the Gram positive family are as indicated in Table 5. For *Staphylococcus* spp the most sensitive drug was Fosfomycin (42%) and Cefotaxime (50%), while Ampicillin (34%) was sensitive. The drugs with intermediary action were Bacitracin (31%), Gentamicin (19.3%) Ceftriaxone (15%). The resistant drugs were Penicillin G (85%) Streptomycin (31%) and Ampicillin (24%). They were resistant to Cloxacillin (53%).

The *Streptococcus* spp isolates were most sensitive to Ampicillin (100%); Ampicillin sulbactam (86%) and Ceftriaxone (71%) while sensitive to Fosfomycin (57%) and

Norfloracin (43%) whereas Intermediary reaction against Co-trimoxazole (43%) Streptomycin and Erythromycin (29% each) and resistant to Penicillin-G (100%) Erythromycin (71%) and Oxacillin (43%).

The *Bacillus* spp showed 100% sensitivity to Ampicillin and Enrofloxacin, 80% sensitivity to Streptomycin and 60% to Bacitracin whereas completely resistant to Penicillin G (100%) and 80% to Ampicillin. *Clostridium* spp could be successfully isolated from gangrenous cases which were sensitive to Strep to or Benzyl penicillin and to Ciprofloxacin (100%) and Cefotaxime (80%).

Table 5: Percentage of drug action in different Gram +ve bacteria of mastitis milk

No	Bacterial spp	<i>Staphylococcus</i> spp (N=62)	<i>Streptococcus</i> spp (N=07)	<i>Bacillus</i> spp (N=05)	<i>Clostridium</i> spp (N=05)
1	Most Sensitive	FOS-26 (42) CTX-31 (50)	AMC-7 (100) A/s-6 (86) CTR-5 (71)	AMP & EX-5 (100)	Strepto or Benzyl Penicillin
2	Sensitive	AMC-21(34) A/s-7 (11)	FOS-4 (57) NX-3 (43)	S-4 (80); B-3 (60)	CIP-5 (100) CTX-4 (80)
3	Intermediary	B-19 (31)/CTR-9 (15) GEN-12 (19.3)	COT-3 (43) S & E (2) each (29)	NIL	S-4 (80)
4	Resistant	PG-53 (85); S-19 (31) ; AMP-15(24)	PG-7 (100) E-5 (71)	PG-5 (100); AMP-4 (80)	N-2 (40); B-4 (80)
5	Indicator drugs	CLX-33 (53)	OX-3 (43)	NIL	NIL

The gram negative *Bacterial* isolates belonging to *E.coli* (79%) and *Klebsiella* spp (38%) were most sensitive to Fosfomycin and followed by Ciprofloxacin (56%) and Ampicillin (29%) respectively. Sensitive drugs were Doxycycline (58%) and Neomycin (46%) for *E. coli* whereas for *Klebsiella* it was Ampicillin (38%) and Oxytetracycline (21%). Intermediary drug was Ampicillin (63%) for *E. coli* along with Gentamicin (56%) and (71%) for *Klebsiella* spp. Co-trimoxazole was intermediary by (42%) and (29%) for *E. coli* and *Klebsiella* respectively. Resistant drugs were Bacitracin (63%) and (54%); Cefotaxime (23%) and (46%)

for *E.coli* and *Klebsiella* spp respectively. *Pseudomonas* spp was most sensitive to Gentamicin (56%) Cefotaxime (44%) and Sensitive to Ceftriaxone (56%) and Ciprofloxacin (31%). Intermediary drugs were Co-trimoxazole (69%) and Doxycycline (75%). Resistant drugs were Bacitracin (69%) and Streptomycin (50%) for *Pseudomonas* spp. Enterobacters are considered opportunistic pathogens arising from the bedding and environment of the cattle shed. The AST results for Gram negative bacteria is given in Table.6 and described herein.

Table 6: Percentage of sensitivity in Gram-ve *Bacterial* isolates of mastitis milk

No	AST Pattern	<i>Escherichia coli</i> (N=53)	<i>Klebsiella spp</i> (N=24)	<i>Pseudomonas spp</i> (N=16)	Enterobacters (N=18)
1	Most Sensitive	FOS-41 (79) CIP-29 (56)	FOS-9 (38) AMP-7 (29)	GEN-9 (56) CTX-7 (44)	PG-13 (72) AMP-9 (50)
2	Sensitive	DoHcl-30 (58) N-24 (46)	AMP-9 (38) OTC-5 (21)	CTR-9 (56) CIP-5 (31)	AMC-11 (61) COT-7 (39)
3	Intermediary	AMC-33 (63) GEN-29 (56) COT-22 (42)	GEN-17 (71) COT-7 (29)	COT-11 (69) DoHcl-12 (75)	OF-6 (33)
4	Resistant	B-33 (63) COT-14 (27) CTX-12 (23)	B-13 (54) CTX-11 (46)	B-11 (69) S-8 (50)	GEN-12 (67) S-11 (61) E-9 (50)
5	Indicator drugs	ETP-33 (63) IMP-19 (37)	MRP-14 (58)	IMP-7 (44) MRP-4 (25)	NIL

Note: Bold numerical in bracket is the % values of sensitivity & resistance to antibiotics

Discussion

In India, the burden of bovine mastitis is much higher, with SCM reported at 65% and CM at 35% (Dua K 2001) [5] making it a persistent challenge to milk production and herd health. Most of the bovine intra-mammary infections are *Bacterial* in origin (Bradley AJ, 2002) [2]. It can effectively be cured by early detection and judicious use of antimicrobials. This study used antimicrobial susceptibility test to screen and select effective drugs for early treatment of mastitis. Multiple drug resistance in mastitis bacteria has been reported by several researchers (Das *et al.*, 2017) [3] and can be attributed to the injudicious use of antibiotics or selection pressure of pathogens to antimicrobials. Isolates in this study, showed resistance to third generation cephalosporin's viz. cefotaxime and ceftriaxone which is similar with the recent reports of drug resistance pattern conducted on mastitis pathogens (Fair and Tor 2014) [6].

Conclusion

The study highlights a high prevalence of mastitis and significant antimicrobial resistance (Acar and Rostel, 2001) [1] among the pathogens causing bovine mastitis in the study region. This increase in antimicrobial resistance is a new hurdle-in treatment options. *Bacterial* species, managemental practices, region and preferences of antibiotics used in the study gave variation in anti-biogram patterns between the three districts (Deb *et al.*, 2013) [4]. So screening mastitis milk by AST will be useful for selecting an appropriate antibiotic and also to know the changing trends of antibiotic resistance. This may limit the indiscriminate use of many types of antibiotics. Proper hygiene and milking methods helps to prevent and control mastitis.

Conflict of Interest

Not available

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Not available

Reference

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