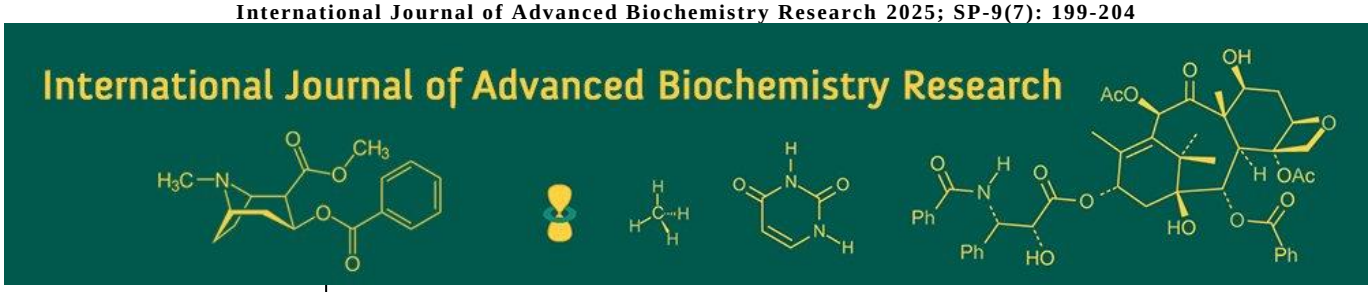

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A study on *in-vitro* anti microbial sensitivity pattern of the bacterial isolates obtained from bovine mastitis

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Abstract
A study was under taken to estimate the prevalence of bacterial agents associated with bovine mastitis in and around Shivamogga and Hassan of Karnataka State, India and to know their invitro susceptibility and resistance patterns to the commonly used antimicrobial drugs in the treatment of mastitis. A total of 117 milk samples were collected and subjected to bacteriological studies and in-vitro antimicrobial sensitivity test. In all, 117 milk samples subjected for isolation of bacteria yielded 132 bacterial isolates. The most predominant bacteria isolated were *Staphylococcus aureus* (27.3%) followed by *Streptococcus* spp. (26.5%), *E. coli* (18.2%), *Klebsiella pneumoniae* (12.1%), *Pseudomonas aeruginosa* (5.3%), *Proteus mirabilis* (0.76%) and *Trueperella pyogenes* (0.76%). The antibiogram studies revealed that most of the bacterial isolates were sensitive to chloramphenicol (56.8%) followed by enrofloxacin (36.36%) and cotrimoxazole (33.3%). However, due to the concerns about drug residues with chloramphenicol causing potential human health risks, the use of drugs like enrofloxacin and cotrimoxazole is proposed for the treatment of bovine mastitis in the study area.

Keywords: Antimicrobial sensitivity, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, drug residues, enrofloxacin

Introduction
Bovine mastitis is an inflammatory condition of the mammary gland in dairy cattle, primarily caused by bacterial infections. The infection develops when the organisms' defense mechanisms are weakened. Mechanical trauma, thermal trauma, and chemical insult also predispose the gland to intramammary infection (IMI). Occurrence of mastitis depends on the interaction of host, agent, and environmental factors. It is an important production related disease of dairy animals as it is directly or indirectly affecting the economy of the farmers and ultimately the country (Miller *et al.*, 1993) [9]. It is estimated that mastitis alone accounts for about 70 percent of all avoidable losses incurred during milk production. At least 137 species of microorganisms from a broad phylogenetic spectrum, including bacteria, yeast, fungi and algae, are associated with bovine mastitis (NAAS, 2013) [11]. In Asia, major mastitis causing organisms are *Staphylococcus aureus*, *Streptococcus* species, *Escherichia coli*, *Corynebacterium* species and *Klebsiella* species. Mastitis can be caused by contagious pathogens like *Staphylococcus aureus*, *Streptococcus agalactiae*, *Mycoplasma bovis* and *Corynebacterium bovis* that reside primarily in the mammary gland of cows and multiply enormously in infected mammary gland and by environmental pathogens like *E. coli*, *Streptococcus uberis*, *Klebsiella* species, *Pseudomonas aeruginosa* (through contaminated intramam-mary tubes) that are present in the environment which enter the udder through contamination of teat ends (*Quin et al.*, 2011) [13].
A knowledge of the predominant bacterial species associated with bovine mastitis in a particular geographical area is required for its effective treatment and control. To prevent the development of antimicrobial resistance and indiscriminate use of antimicrobial drugs, a knowledge of the antimicrobial profile of the bacterial species associated with mastitis is of paramount importance. Hence a study was under taken to know the etiological agents associated with mastitis and to know their invitro susceptibility and resistance patterns.

Materials and Methods

A total of 117 milk samples collected from clinical and subclinical cases of bovine and bubaline mastitis, presented to the veterinary clinical complexes (VCC) of the veterinary colleges at Shivamogga and Hassan during the period of 2013-2015 and 2021-2023 respectively and maintained in the Department of Veterinary Microbiology were used in the present study.

Culture and biochemical tests

The milk samples were inoculated into brain heart infusion broth (BHI) and tryptose phosphate broth (TPB) and thioglycolate broth (TGB) and incubated at 37º C for 12-18 hours under aerobic conditions and in anaerobic gas jar respectively. After the preliminary identification of the bacterial species by the staining morphology of the broth culture they were plated on to BHI agar, Mannitol salt agar (MSA), Mc-Conkey agar, EMB agar, Edward's agar and blood agar. Identification of bacteria on primary culture was done based on hemolytic characteristics, colony morphology, Gram-stain reaction including shape and arrangements of the bacteria. Staphylococci were identified using growth characteristics, tube coagulase tests, and catalase tests. Identification of *Streptococcus* isolates was made on the basis of colony morphology, staining morphology and catalase tests. Gram-negative bacteria grown on MacConkey agar were differentiated according to their growth characteristics, catalase test, oxidase reaction, triple sugar-iron agar test, urease test and the IMViC (indole, methyl red, Voges-Proskauer, citrate) test. Gram positive rods (pleomorphic) by their staining morphology, haemolytic pattern and catalase test.

Antibiogram studies

All the bacterial isolates obtained were subjected to antibiogram studies by employing the Kirby-Bauer disk diffusion method. A total of 13 different antimicrobial drugs commonly used in the treatment of mastitis *viz*., ampicillin cloxacillin (A), amoxycillin with clavulanic acid (AMC), amikacin (AK), co-trimoxazole (sulfamethoxazole with trimethoprim) (COT), ceftriaxone with tazobactum (CIT), cefotaxime (CTX), cefoxitin (CX), ceftriaxone (CTR), cefoperazone (CPZ), chloramphenicol (C), enrofloxacin (EX), gentamicin (GEN) and tetracycline (TE) were used in the present study. The interpretations were carried out as per CLSI standards, 2013.

Results

Out of the 117 milk samples subjected to culture for isolation of bacteria, 11 samples did not yield any growth, 22 samples revealed two bacteria and 4 samples yielded three bacteria (mixed infection) and the remaining 80 samples yielded single bacteria. Seven other isolates (gram positive rods) of the present study could not be assigned any species/genus. The most predominant bacteria isolated were *Staphylococcus aureus* (27.3%) followed by *Streptococcus* spp (26.5%), *E. coli* (18.2%), *Klebsiella pneumoniae* (12.1%), *Pseudomonas aeruginosa* (5.3%), *Proteus mirabilis* (0.76%) and *Trueperella pyogenes* (0.76%) (Table 1, Fig.1). In studies conducted in Asia, *Staphylococcus* species have been reported to be the predominant etiological agents of mastitis in cattle and buffaloes capable of causing peracute, acute, subacute, chronic, gangrenous and subclinical types of mastitis (Sharma *et al.*, 2012) [15]. Bhat *et al.* (2017) [3] reported *Staphylococcus aureus* (61%) as the most predominant bacteria isolated from the mastitic cattle of Jammu and Kashmir, followed by *E. coli* (13%), coagulase-negative staphylococci (13.04%), *Streptococcus uberis* (4.35%), and *Streptococcus dysgalactiae* (8.69%). The etiological differences in mastitis could be attributable to varied topographical and animal management conditions in addition to the usage of varied antimicrobials.

Of the 41 staphylococcal isolates subjected to antibiogram studies using 13 antimicrobial drugs, eight isolates were found to be resistant to all the drugs under study and were categorized as multidrug resistant (MDR). Most of the isolates were found to be sensitive to chloramphenicol (63.4%) followed by enrofloxacin (46.3%), cotrimoxazole (46.3%) and ceftriaxone (39%) (Table.2, Fig.2). Similar sensitivity pattern of staphylococci to chloramphenicol and ceftriaxone has been reported by many workers (Bhati *et al.*, 2013) [4]; Yadav *et al.* (2015b)) [16]. Staphylococci are potent pathogens and control is complicated by high antibiotic resistance (Livermore, 2000) [8] and the carriage of a variety of virulence determinants including several toxins and virulent factors which evade immune responses and biofilm formation (Zecconi and Scali, 2013) [17].

Most of the streptococcal isolates obtained in the present study were also found to be sensitive to chloramphenicol (57.1%) followed by amoxycillin with clavulinic acid (37.1%) and cefotaxime (25.7%) and five of them were resistant to all the drugs under study. However, this is in contrary to the findings of another antibiogram study on bovine mastitis, where the sensitivity pattern of *Streptococcus* isolates showed highest sensitivity for ceftiofur (68%), followed by enrofloxacin (50%), gentamicin (48%), tetaracycline (46%), ampicillin (40%), and ceftrioxone (40%) (Muley *et al.*, 2018) [10]. None of the streptococcal isolates were sensitive to gentamycin and amikacin in the present study (Table 2), contrary to the findings of another study on bovine mastitis in Jordan where the *S. agalactiae* isolates have been reported to show highest susceptibility to gentamycin (40%) (Alekish *et al.*, 2013) [1]. This variation in susceptibility patterns might be due varied environmental conditions, animal husbandry practices and use of different drugs in the treatment of mastitis in different geographical areas that can indeed put significant pressure on organisms resulting in evolution of drug-resistant organisms.

E. coli, the third predominant bacteria isolated in the present study, has been reported to be one of the most significant causes of clinical mastitis in dairy animals, affecting high producing cows as wells as cows in the early lactation period with low somatic cell counts and its endotoxin is potential health threat at consumer end (Liu *et al.*, 2018) [7]. The antibiogram studies on *Enterobacteriaceae* in the present study revealed that 58.5% of the isolates were sensitive to chloramphenicol followed by sensitivity to both ceftriaxone and cotrimoxazole (39%) and three, resistant to all the drugs under study (Fig.2). Least sensitivity was reported to ampicillin cloxacillin followed by cefoxitin. Similar observations were made in a study on antimicrobial resistance of *Enterobacteriaceae* isolated from healthy, clinical and subclinical mastitis udders of cows (Alves *et al.*, 2024) [2].

All the seven isolates of *Pseudomonas aeruginosa* in the preset study were found to be sensitive to only two to three antimicrobial drugs and were MDR exhibiting resistance to

at least three different classes of antimicrobials with most of them exhibiting sensitivity to enrofloxacin followed by ceftriaxone (Table.2). This bacterial pathogen is reported to be a potential environmental pathogen causing mastitis, frequently associated with wet bedding, water used in milking parlor, intramammary tubes used for the treatment and is extremely resistant to commonly used antimicrobials with the potential to form biofilms (Park *et al.*, 2014) [12].

T. pyogenes, isolated from a case of mastitis in the present study (based on phenotypic characters) is a rare pathogen reported from cases of mastitis in India (Fig.3, Fig.4). It is a Gram-positive, catalase negative, facultatively anaerobic, fastidious, coryneform bacteria with enhanced growth on blood agar producing pinpoint, convex, slightly translucent, circular colonies surrounded by a zone of β-hemolysis (Quin *et al.*, 2011)) [13]. In the present study, the milk sample was obtained from a heifer with swelling of the udder, with the milk emanating a foul smell, akin to the clinical signs of summer mastitis caused by *T. pyogenes* as reported by Kibebew (2017) [6]. This bacterial isolate was found to be resistant to all the antimicrobials used in the present study (Table 2). Antimicrobial resistance, including multidrug resistance among *T. pyogenes* isolates, has been reported by various researchers. Rezanejad *et al.* (2019) [14] reported that *T. pyogenes* bacteria isolated from the mastitic milk samples harbored the highest prevalence of resistance toward gentamicin (100%), penicillin (100%), ampicillin (90.62%), amoxicillin (87.50%), trimethoprim-sulfamethoxazole (87.50%), cefalexin (84.37%) and streptomycin (81.25%) antibiotic agents.

Most of the bacterial isolates in the present study exhibited highest susceptibility to chloramphenicol (56.8%) followed by enrofloxacin (36.36) and cotrimoxazole (33.3%) (Fig.2). This might be because of lesser usage of the drug which is usually used as last alternative in mastitis, because of the concerns about drug residues causing potential human health risks. Based on the findings of the present study, the use of enrofloxacin and cotrimoxazole is proposed in the study area for the treatment of bovine mastitis.

Table 1: Bacterial isolates obtained from the milk samples

S. No	Name of the bacteria	Species	No. of isolates
1	Staphylococci	*S. aureus*	36
		CoNS (Non aureus staphylococci)	5
2	Streptococci	*Streptococcal* spp	35
3	Enterobacteria	*K. pneumoniae*	16
		E. coli	24
		P. mirabilis	1
4	*Pseudomonas*	*Pseudomonas aeruginosa*	7
5	*Trueperella*	*Trueperella pyogenes*	1
5	Other Gram-positive bacilli		7
	Total		132

Table 2: Phenotypic pattern of sensitivity of the bacterial isolates to the antimicrobial drugs under study

Species/family assigned (No of isolates)	Ceftriaxone	Cefotaxime	Ampicillin Cloxacillin	Amikacin	Cefoperazone	Cefoxitin	Chloramphenicol	Amoxycillin with clavulanic acid	Cotrimoxazole	Enrofloxacin	Gentamicin	Ceftrioxone with Tazobactum	oxytetracycline
Staphylococcus spp. (41)	16	14	9	15	13	13	26	14	19	19	12	10	4
Streptococcus spp. (35)	1	10	9	-	7	7	20	13	5	7	-	8	1
Enterobacteriaceae (41)	16	11	1	4	4	2	24	4	16	12	5	7	4
Pseudomonas aeruginosa (7)	4	1	-	2	-	-	1	1	-	5	1	1	-
Trueperella pyogenes (1)	-	-	-	-	-	-	-	-	-	-	-	-	-
Others (7)	1	2	2	3	2	4	4	2	4	5	4	3	-
Total (132)	38	38	21	24	26	26	75	34	44	48	22	29	9

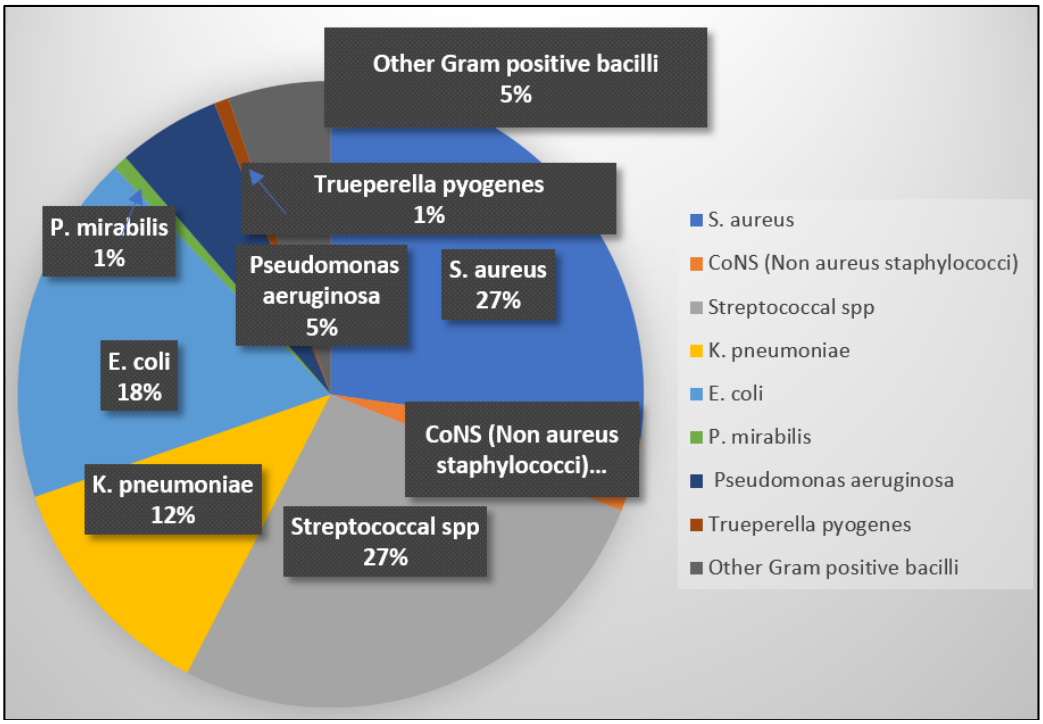

Fig 1: Bacterial isolates obtained in the study

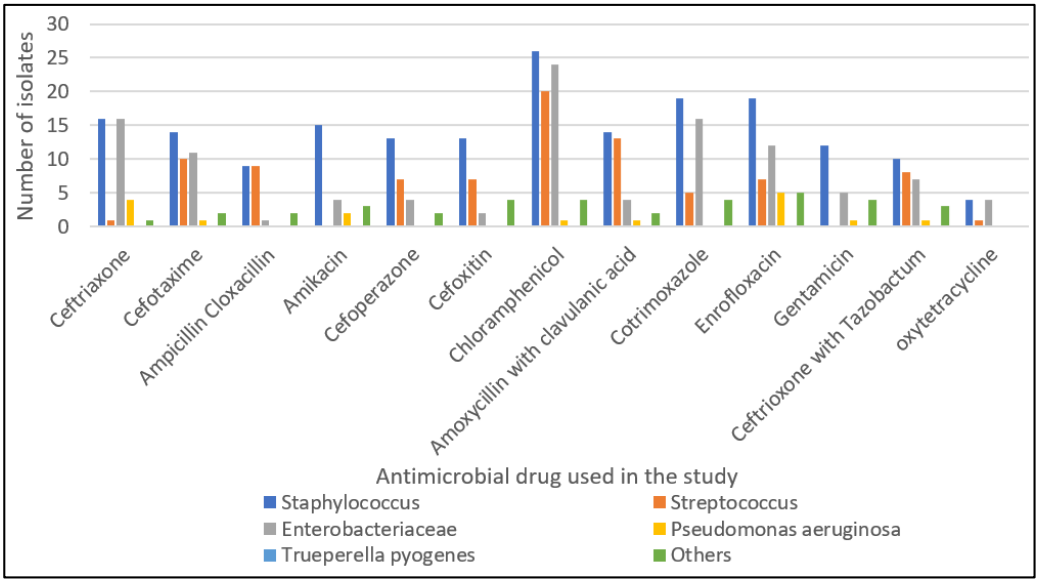

Fig 2: Phenotypic pattern of sensitivity of the bacterial isolates to the antimicrobial drugs under study

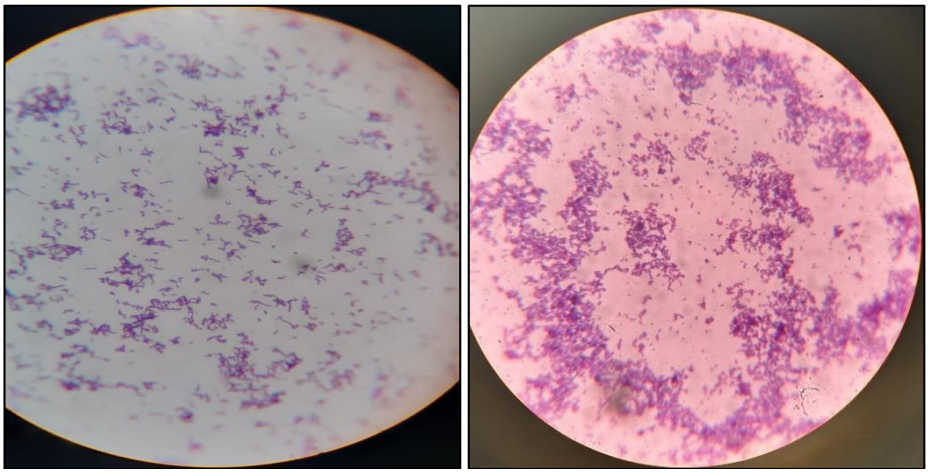

Fig 3: Trueperella pyogenes staining morphology-Gram's stain

Fig 4: Trueperella pyogenes colony morphology-complete haemolysis on blood agar

Conclusion

The study highlights the predominant bacterial species and the antibiogram of the isolates associated with bovine mastitis in the study area. The most predominant bacteria isolated were *Staphylococcus aureus* followed by *Streptococcus* spp. and *E. coli*. Highest sensitivity was observed among all the bacterial isolates to chloramphenicol, followed by enrofloxacin and cotrimoxazole. 17 bacterial isolates were found to be resistant to all the antimicrobial drugs used and several MDR bacteria were also reported. In the present study, though most of the bacterial isolates were found to be sensitive to chloramphenicol, due to the concerns about drug residues causing potential human health risks the use of drugs like enrofloxacin and cotrimoxazole is recommended for the treatment of bovine mastitis in the study area. In addition, for effective control of mastitis, continuous monitoring of mastitis, early detection, careful management and treatment of the mastitis infection which should be preceded with identification of the causative agent and susceptibility test. Such an initiative would prevent, the development of antimicrobial-resistant bacteria (AMR) with a potential to spread resistance genes to previously susceptible bacteria as AMR is a problem that affects animal, human and environmental health and should be evaluated within the one-health concept.

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