

Original Research Article

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## ***In vitro* Antibiotic Sensitivity Pattern of *Staphylococcus aureus* and Coagulase Negative Staphylococci Isolated from Bovine Mastitis**

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### ABSTRACT

#### Keywords

*S. aureus*, CoNS, Mastitis, Antibiotic

#### Article Info

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Staphylococcal mastitis is a major and costly problem of dairy cattle all over the world. In the present study, Clinical (n=165) and sub-clinical mastitic milk samples were processed for bacterial isolation. All the 83 isolates recovered on primary isolation were confirmed as *Staphylococcus* spp. based on morphological and biochemical characteristics. Based on the result of tube coagulase test, 55 (66.27 %) isolates were considered as a *Staphylococcus aureus*, whereas 28 (33.73 %) isolates were considered as Coagulase-negative staphylococci (CoNS). Among the 12 antibiotics tested, highest resistance was noticed to penicillin to the extent of 60.00 per cent among *Staphylococcus aureus* and 82.14 per cent in CoNS isolates whereas Aminopenicillins *i.e.*, ampicillin-sulbactam tested in this study showed maximum effectiveness of 98.18 and 96.43 per cent towards *Staphylococcus aureus* and CoNS isolates, respectively.

### Introduction

Mastitis is one of the most crucial diseases of cattle and buffalo because it causes innumerable problems to milk production, milk processing and quality of milk and milk products which results in huge economic losses to the dairy industry. The physical, chemical, bacteriological and other qualities of milk are affected by mastitis. An annual economic loss of over 4300 crore INR due to mastitis is reported (Ranjan, 2010), out of

which 70 percent could be attributed to sub-clinical mastitis (Hamadani *et al.*, 2014). *Staphylococcus aureus* is the most common etiological pathogen of contagious bovine mastitis and it has the potential to develop resistance to almost all the antimicrobial agents (Barkema *et al.*, 2009). These organisms are well known for their tolerance to wide range of adverse circumstances. Staphylococci comprise 45 species and 21 subspecies (Bergeron *et al.*, 2011). In addition to *Staphylococcus aureus*, coagulase-negative

staphylococci (CoNS) are increasingly becoming recognized as etiologic agents associated with intramammary infections (IMI) in most countries. Antimicrobial therapy is one of the measures for controlling staphylococcal mastitis. The determination of antimicrobial susceptibility of clinical isolates is required not only for therapy, but also for monitoring the spread of resistant strains throughout the populations. Although antimicrobial susceptibility testing is a valuable tool in determining the best therapeutic choice against a mastitis pathogen in practice, it is rarely performed and therapy decisions are usually made empirically.

Therefore, susceptibility data for a large number of isolates in an area or region can be useful to veterinarians (Salmon, 2002). The resistance of *Staphylococcus aureus* to antimicrobial agents has been extensively documented and it contributed significantly to the treatment failure (Kumar *et al.*, 2010). Beside *S. aureus*, CoNS isolates can also harbour resistant genes to several antimicrobials (Silva *et al.*, 2013). Hence, present study was designed to evaluate *In vitro* antibiotic sensitivity of *Staphylococcus aureus* and coagulase negative staphylococci bacterial species isolated from cases of mastitis against range of antibiotics potentially useful in mastitis treatment and control programs carried out in field.

## **Materials and Methods**

### **Collection of milk samples**

A total of 165 milk samples from suspected cases of clinical mastitis in cows and buffaloes belonging to North Gujarat were collected aseptically in sterilized vials. In addition, a total of 256 milk samples from cows (n = 198) and buffaloes (n = 58) belonging to North Gujarat were also collected aseptically for screening of sub-clinical mastitis.

### **Screening of sub-clinical mastitis**

All the milk samples (n = 256) were screened for sub-clinical mastitis (SCM) by California Mastitis Test (CMT) as per the procedure described by Schalm and Noorlander (1957).

### **Bacterial isolation, identification and biochemical characterization**

Samples which were detected positive in sub-clinical mastitis and the samples from clinical cases were processed for isolation of *Staphylococcus*. Milk samples were inoculated on the plates of nutrient agar by spreading heavy inoculums of thoroughly mixed milk. The plates were incubated at 37°C for 24 hours. Thereafter, golden yellow pigmented or white colour colony indicative of presumptive *Staphylococcus* were transferred to mannitol salt agar which is considered as selective medium for *Staphylococcus*. The colonies of *Staphylococcus*, forming yellow and red colouration indicative of mannitol fermentation and non-fermentation, respectively were transferred to nutrient Agar slants for further identification. The isolates were identified by morphological characteristics and Biochemical characterization as per the methods described by Buchanan and Gibson (1974) and Cowan and Steel (1974). Different biochemical tests such as catalase, oxidase, phosphatase, maltose fermentation and coagulase test were performed. The coagulase positive isolates were considered as a *Staphylococcus aureus* whereas the isolates, negative by coagulase test, were considered as a coagulase negative staphylococci.

### **Antibiotic sensitivity patterns of isolates**

All the isolates obtained were subjected to *in vitro* antibiotic sensitivity test, as per the method described by Bauer *et al.*, (1966). Each test strain was grown in BHI broth

overnight at 37°C. Sterile plates of Mueller and Hinton agar were seeded with about 1 ml of inoculums and were allowed to dry. Monodiscs (Hi Media, Pvt. Ltd., Mumbai) of antibiotics viz., Penicillin G (10 Units), Ciprofloxacin (30 mcg), Gentamicin (10 mcg), Enrofloxacin (10 mcg), Chloramphenicol (25 mcg), Cephalexin (30 mcg), Ampicillin-Sulbactam (10/10 mcg), Ampicillin (10 mcg), Amoxicillin-Clavulanic acid (30 mcg), Streptomycin (25 mcg) Methicillin (10 mcg), Oxacillin (5 mcg) were then placed in the plate and were incubated aerobically at 37°C overnight. Zones of inhibition were measured and compared with zone size interpretative table furnished by the manufacturer and graded as either sensitive or resistant.

## Results and Discussion

Thirty-four samples which were found positive for sub-clinical mastitis by California mastitis test and 165 samples of clinical mastitis were inoculated on Nutrient Agar for primary isolation. All the 83 isolates, recovered in primary isolation, were inoculated on Mannitol Salt Agar (MSA), wherein 78 isolates showed mannitol fermentation. In the present study, all the 83 presumptive strains of *Staphylococcus* isolates were subjected to Gram's staining and various bio-chemical reactions for further identification. In Gram's stained culture smears under microscope, all the 83 isolates revealed spherical and irregular clusters like bunch of grapes. On bio-chemical characterisation, all the 83 isolates were Catalase positive, Oxidase negative and positive for Maltose fermentation and Phosphatase production. In light of these tests, a total of 83 isolates were confirmed as *Staphylococcus* spp. Similar phenotypic methods were also employed by Kumar *et al.*, (2010); Momtaz *et al.*, (2010); Medeiros *et al.*, (2011) and Rusenova *et al.*, (2013) for the identification of *Staphylococcus* spp.

Bovine mastitis is the most common and the quite damaging disease throughout the world. It is a versatile disease in milch animals and is caused by pathological, genetical, physiological or environmental factors. In spite of the concerted efforts to control or reduce the incidence of mastitis for decades, it still remains a major threat to the dairy industry causing huge economic loss. In India, 50.00 per cent of milch animals are affected with mastitis, out of which clinical mastitis is accounted for 10.00 per cent with annual economic loss of approximately 6000 crores (Dua, 2001). It is a multi-etiological disease, but the prevalence of pathogenic and antibiotic resistant strains of *Staphylococcus aureus* causing bovine mastitis is serious cause of concern in south Asian countries including India as these animals are significant contributor to milk production (El-Jakee *et al.*, 2010).

*Staphylococcus* is usually divided into two groups based on the ability to produce coagulase, an extracellular enzyme that clots blood plasma. The coagulase positive group that includes *S. aureus*, *S. intermedius* and some strains of *Staphylococcus hyicus*, whereas coagulase negative *Staphylococcus* (CoNS) is a large and heterogeneous group that contain more than 40 species and subspecies of *Staphylococcus* (Holt *et al.*, 1994).

Coagulase produced from *Staphylococcus aureus* is a single chain protein with a molecular weight of 61,000 d (Bas *et al.*, 1974). Coagulase forms a complex by binding with protein which has thrombin-like activity that converts fibrinogen to fibrin (McDevitt *et al.*, 1992). Coagulase is of two types, one bound to cell wall responsible for clotting of plasma in slide test and another secreted as free coagulase in the media responsible for clotting of plasma in tube test (Duthie, 1954). Virulence of *Staphylococcus aureus* depends

upon its ability to produce coagulase, but not with its ability to coagulate plasma. It is postulated that coagulase might function in some way to protect organism against the antibacterial activity of plasma which can contribute to its virulence. Coagulase is encoded by *coa* gene and is considered as one of the important criteria for identification of this type of organism. Many of the laboratories have utilised this property along with its combination with other tests like fermentation on MSA and production of Catalase and DNase enzymes (Kateete *et al.*, 2010).

In the present study, all the 83 *Staphylococcus* isolates were subjected to tube coagulase test by using rabbit plasma. Out of 83 isolates, 55 (66.27 %) isolates were found to be positive for coagulase production and were considered as a *Staphylococcus aureus*, whereas 28 (33.73 %) isolates were found negative hence they were tentatively considered as a CoNS.

#### ***In-vitro* antibiotic sensitivity test**

One important reason for therapy failure in the management of mastitis could arise from various factors involving the pathological changes in the udder, etiology, lower efficacy of antimicrobials and improper veterinary services (Adesola, 2012). But above all, antimicrobial susceptibility testing, typically using a disk diffusion assay is often performed as part of the routine laboratory evaluation with an attempt to provide guidelines for the treatment of mastitis, especially the pathogens causing clinical and sub-clinical mastitis and thus forming a crucial part in development of mastitis control program. World Health Organisation for animal health has also recommended monitoring and observation of efficacy of antimicrobials towards pathogenic and commensal bacteria affecting animals (Acar and Rostel, 2001). In a particular region, antibiotic susceptibility/resistance in bacterial

isolates varies due to different strategies and policies in containment programmes of mastitis. The isolation of organism and choice of antimicrobial agent is often considered empirical in the management of mastitis. Thus in the present study, antibiogram of *S. aureus* and CoNS isolates was analysed to find out the status or information on the sensitivity/resistance patterns among the isolates. Antibiogram trend of twelve commonly used antimicrobial drugs for treatment of bovine mastitis is presented in Table 1.

In the present study, only 14 (16.87 %) isolates *viz.*, 12 *Staphylococcus aureus* and 2 CoNS were susceptible to all antibiotics tested, while the remaining 69 (83.13 %) isolates were resistant at least to one of the antibiotics which corroborates the finding of Turutoglu *et al.*, (2006) who reported that 14.64 per cent *Staphylococcus* isolates were susceptible to all antibiotics tested.

However, in contrast to the present study, higher susceptibility rates of staphylococcal isolates were reported in some countries (Gentilini *et al.*, 2002; Kaszanyitzky *et al.*, 2003 and Rajala-Schultz *et al.*, 2004). This might be attributed to misuse of antibacterial agents in India, where these are practically dispensed without a prescription. In cases of mastitis, the wrong or incomplete duration of treatment of animals and indiscriminate and injudicious use of antibiotics also contribute significantly to the development of bacterial resistance against them (Turutoglu *et al.*, 2006).

Among the 12 antibiotics tested, highest resistance was noticed to penicillin to the extent of 60.00 per cent among *Staphylococcus aureus* and 82.14 per cent in CoNS isolates. Similarly, 56.37 per cent of *Staphylococcus aureus* and 71.43 per cent of the CoNS isolates were resistant to ampicillin.

**Table.1** Antibiogram trend of coagulase positive and negative *Staphylococcus* isolates against antibiotics

Sr. No.	Antibiotics	No. of <i>Staphylococcus aureus</i> isolates (n=55)		No. of CoNS isolates(n=28)	
		Sensitive	Resistant	Sensitive	Resistant
1	Penicillin G	22 (40.00 %)	33 (60.00 %)	05 (17.86 %)	23 (82.14 %)
2	Methicillin	48 (87.27 %)	07 (12.73 %)	21 (75.00 %)	07 (25.00 %)
3	Oxacillin	48 (87.27 %)	07 (12.73 %)	21 (75.00 %)	07 (25.00 %)
4	Ampicillin	24 (43.63 %)	31 (56.37 %)	08 (28.57 %)	20 (71.43 %)
5	Ampicillin-Sulbactam	54 (98.18 %)	01 (01.82 %)	27 (96.43 %)	01 (03.57 %)
6	Amoxycillin-Clavulanic acid	38 (69.09 %)	17 (30.91 %)	21 (75.00 %)	07(25.00 %)
7	Streptomycin	45 (81.82 %)	10 (18.18 %)	18 (64.29 %)	10 (35.71 %)
8	Gentamicin	46 (83.64 %)	09 (16.36 %)	27 (96.43 %)	01 (03.57 %)
9	Ciprofloxacin	24 (43.63 %)	31 (56.37 %)	06 (21.43 %)	22 (78.57 %)
10	Enrofloxacin	40 (72.73 %)	15 (27.27 %)	17 (60.71 %)	11 (39.29 %)
11	Chloramphenicol	48 (87.27 %)	07 (12.73 %)	24 (85.71 %)	04 (14.29 %)
12	Ciphalexin	52 (94.55 %)	03 (05.45 %)	22 (78.57 %)	06 (21.43 %)

In accordance to the present study, 87.00, 94.4 and 100.00 per cent resistance to penicillin was reported by Ebrahimi and Taheri (2009); Abera *et al.*, (2013) and Thaker *et al.*, (2013), respectively. Whereas, higher resistance of 83.00 and 73.30 per cent to Ampicillin was also reported by Mekonnen *et al.*, (2005) and Murugaiyah *et al.*, (2014), respectively.

A large number of the isolates were found to be resistant to long ago established antibiotics (penicillin G and ampicillin) compared to susceptibility of the isolates to the more recently developed compounds (ampicillin-sulbactam, amoxycillin-clavulanic acid, enrofloxacin and cephalexin).

In the present study, resistance to penicillin and ampicillin must be of concern because these antibiotics represent the main antibiotic group recommended for the treatment of staphylococcal mastitis. The resistance of

*Staphylococcus* to penicillin and ampicillin might be attributed to the production of  $\beta$ -lactamase, an enzyme that inactivates penicillin and closely related antibiotics (Abera *et al.*, 2010).

Regular use of antibiotics for the treatment of cows and buffaloes may result in the spread of resistant strains. These results suggest that penicillin and ampicillin might not be a proper choice for treatment of mastitis caused by *Staphylococcus*. Similar observations have also been made by Abera *et al.*, (2013), Thaker *et al.*, (2013) and Parth (2016).

Aminopenicillins *i.e.*, ampicillin-sulbactam tested in this study showed maximum effectiveness of 98.18 and 96.43 per cent towards *Staphylococcus aureus* and CoNS isolates, respectively, which corroborate the findings of Turutoglu *et al.*, (2006) and Soares *et al.*, (2012) who reported 82.00 and 100.00 per cent efficacy of ampicillin-

sulbactam against *Staphylococcus* isolates, respectively.

It is quite interesting that when the isolates were tested against ampicillin-sulbactam showed maximum efficacy against *Staphylococcus* isolates, however these isolates showed resistant against ampicillin alone. This may be due to the fact that ampicillin-sulbactam is a combination of a  $\beta$ -lactam antibiotic and a  $\beta$ -lactamase inhibitor in which sulbactam blocks the enzyme which breaks down ampicillin and thereby allows ampicillin to attack and kill the bacteria. This also attributed due to the synergistic effect of antibiotics together. Similar observations were also made by Turutoglu *et al.*, (2006) and Soares *et al.*, (2012).

During the present study, 46 (83.64 %) and 45 (81.82 %) isolates of *Staphylococcus aureus* were found sensitive to Gentamicin and Streptomycin, respectively, whereas, 27 (96.43 %) and 18 (64.29 %) CoNS isolates were found sensitive against Gentamicin and Streptomycin, respectively. This is a very good result on antibiotic susceptibility of organisms against amino glycoside group of antibiotics such as Gentamicin and Streptomycin. This might be due to the introduction of newer antibiotics in the field which have resulted in the less use of these antibiotics. Moreover, mode of development of resistance is different in case of these antibiotics.

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