Prevalence and Antimicrobial Resistance of Methicillin Resistant *Staphylococcus aureus (MRSA)* in Cow Milk from Gangtok, East Sikkim

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Abstract

This study was conducted to estimate the prevalence and antibiotic resistance of Methicillin Resistant Staphylococcus aureus in cow milk and to determine its antimicrobial resistance pattern in Gangtok, East Sikkim. A total of 100 milk samples were collected aseptically and analysed. Further phenotypic characterization of *Staphylococcus aureus* was done and its susceptibility to 5 antibiotics was evaluated using Kirby-Bauer disc diffusion method. Out of 54 pure isolates of *S.aureus*, Resistance was detected for Amoxyclav (40.74%) Penicillin (92.59%), Erythromycin (5.56%), Clindamycin (3.70%), Oxacillin(7.4%).

The MRSA strains showed 100% resistant to Oxacillin , Penicillin, and Amoxyclav , 50% resistance to Erythromycin and showed no resistance to Clindamycin. The MSSA strains showed 36% resistance to Amoxyclav, 92% resistance to Penicillin 2% resistance to Clindamycin and showed no resistance to Oxacillin and Erythromycin. This study identifies S aureus as a potential pathogen in cow milk and its antibiotic resistance raises concern in this region where dairy farming is a keen topic of interest.

KEYWORDS: Antibiotic resistance, *S. aureus*, MRSA,

Introduction

One of the major causal bacterium in contagious bovine mastitis is *Staphylococcus aureus* (Piepers et al., 2007). In bovine mastitis the lactating mammary gland is the primary reservoir of *Staphylococcus aureus*. Thus, Resistance of *Staphylococcus aureus* to antimicrobial agents can complicate treatment of its infections **(**Lowy., 2003).MRSA isolated from bovine mastitis was first reported in 1972 (Devriese et al., 1972). It has recently been reported that MRSA can be transferred from humans to animals and vice versa and both can act as reservoirs once colonised by MRSA **(**Graveland H., 2010; Huber H ., 2009;). A major concern is the use of antimicrobials for the treatment of MRSA leading to a probable increase in its antimicrobial resistance, with a consequent effect on the transmission from animals to humans in an immunocompromised state , Although the prevalence of MRSA in mastitis is generally low, and this view can be supported by the fact that MRSA has been described in mastitis only occasionally. (Kwon et al., 2005 ; Lee, 2006; Juhász- Kaszanyitzky et al., 2007; Vanderhaeghen et al., 2010). However, Animal and public health, is still a major concern due to the emergence of MRSA infection on dairy farms. MRSA-contaminated livestock products, including bovine milk, could be the causal agents for human MRSA infection .

Therefore, screening for MRSA from bovine milk is the dire need of the situation. Economically, milk production is of great importance in the agricultural sector. Economic losses due to mastitis include the cost of reduced milk production, discarded milk due to clinical episodes, antibiotic treatment, veterinary fees, therapy, and cow replacement cost(Owens *et al*., 1990). Sikkim like other parts of India has a great prospect for livestock raising, and in the present scenario Mastitis has emerged as a challenge to the veterinarians and farmers.The main objective of this study was to estimate the prevalence of MRSA in Cow milk from East Sikkim, N.E India, to isolate, identify MRSA from mastitic milk and to conduct antimicrobial susceptibility test on the isolates.

Materials and Methods:

The present study was conducted in Gangtok, East Sikkim .A total of 100 Milk Samples were randomly collected from different regions of East Sikkim. The milk was collected from lactating cows in morning hours. Information on age, lactating stage, and previous history of mastitis were gathered. Clinical inspection of the udder for the presence of any swelling or lesion, and the quality, colour and consistency of the milk was also checked.

Collection of Milk samples:

For the bacteriological identification milk samples were collected aseptically as described by (Zutic et al., 2012) Prior to sampling the first streams of milk were discarded and the teat ends were washed with water and dried. Further disinfection of the teat ends were carried out with cotton swabs that contained 70% alcohol. 5 ml of milk was then collected ,in a clean, sterile, 50 ml falcon tube. The cow number, name of the farm and date was marked on the falcon tube with a marker for identity purposes. All of the milk samples were transported to the laboratory of Department of Microbiology, Sikkim University in ice cooled containers (Vanderhaeghen et al., 2010) where they were stored at 4°C until cultured on the media.

Analysis of Milk Samples:

The standard protocols of National Committee for Clinical Laboratory Standards (NCCLS) (1997) were followed for the bacteriological identification of the milk sample. An aliquot of 100 µl of aseptically collected milk samples was spread over mannitol salt agar,(Blair et al., 1967; Chapman et al.,1945; Koneman et al., 1992)and streaked over blood agar and incubated at 37° C for 24-48 hours. The plates were further examined for colony morphology, pigmentation and hemolytic characteristics at 24 - 48 h.

Phenotypic Characterization:

The organisms that were isolated were further identified as *S aureus* by Gram's staining, microscopic observation, catalase test, Latex agglutination test, slide coagulase test, and Tube coagulase test. According to the standard methods of (Cruickshank et al., 1975), the isolates have been identified.

Antimicrobial susceptibility Testing

The Antibiotic Susceptibility Testing was performed on Muller-Hinton Agar by Kirby Bauer disk diffusion technique (Cheesbrough ., 2002). The Muellor Hinton and antibiotic discs were obtained from Hi-media Mumbai. Inocula were adjusted at 0.5

McFarland standard and each streaked uniformly with swabsticks in Mueller-Hinton agar plates containing 2% sodium chloride (NaCl) to obtain confluent growth .Oxacillin (1µg) discs, Amoxyclav (30 mcg), Clindamycin (2 mcg) , Penicillin(10 units) ,Erythromycin (15 mcg) discs were placed in the plates which were then incubated aerobically at 37°C for 24h . Zone diameters of the isolates were measured in millimetres with a measuring scale. Isolates were classified as resistant, or sensitive based on the interpretative criteria updated according to the current CLSI standards (Performance Standards for Antimicrobial Disk Susceptibility Tests, CLSI.

RESULT

Out of the 54 isolates of *Staphylococcus aureus* four MRSA strains were found. Out of the four, three MRSA strains were obtained from healthy cow's milk sample and one MRSA strain was obtained from mastitic milk sample. From 100 cows tested in this analysis, 12 (12%) suffered from clinical mastitis. *Staphylococcus aureus* strains were isolated in milk samples from 54 (54%) cows. MRSA strains were isolated from 4 (7.4%) cows as shown in (Figure1). All of the four cows harboured MRSA in their udder. In the present study 54 *Staphylococcus aureus* and 46 Coagulase negative Staphylococci were identified from both the healthy and mastitic milk samples. From where Staphylococcus aureus showed 40.74% resistance to Amoxyclav, 92.59% resistance to Penicillin, 5.56% to erythromycin, 3.70% to Clindamycin, and finally 7.4% resistance to oxacillin (Figure 3). The MRSA strains showed 100% resistant to Oxacillin , Penicillin, and Amoxyclav , 50% resistance to Erythromycin and showed no resistance to Clindamycin (Figure 2). The MSSA strains showed 36% resistance to Amoxyclav, 92% resistance to Penicillin 2% resistance to Clindamycin and showed no resistance to Oxacillin and Erythromycin (Figure 4).

Antibiogram profile:

The antibiogram profile generated for MRSA showed two types of MRSA (Table 2) Type 1 showed Resistance to Oxacillin, Amoxyclav, Erythromycin, Penicillin and Sensitivity towards Cindamycin. Type 2 showed Resistance to Oxacillin, Amoxyclav and Penicillin and Sensitivity towards Erythromycin and Clindamycin (Figure 6).The antibiogram profile generated for MSSA showed five types of MSSA (Table 1) , Type 1 showed Resistance to Penicillin, and Sensitivity towards Oxacillin, Amoxyclav, Erythromycin, Clindamycin., Type 2 showed Resistance to Amoxyclav, Penicillin, and Sensitivity to Erythromycin Clindamycin, and Oxacillin., Type 3 showed Resistance to Amoxyclav, Penicillin, Clindamycin and Sensitivity to Erythromycin and Oxacillin, Type 4 showed Resistance to Erythromycin and Sensitivity to Amoxyclav, Penicillin, Clindamycin, Erythromycin, and Oxacillin., Type 5 showed Sensitivity to Erythromycin, Amoxyclav, Penicillin, Clindamycin, and Oxacillin (Figure 5).

Discussion

In the present study MRSA was detected in 4 (7.4%) of the 54 Staphylococcal isolates, which is quite similar to the reports from Turkey where MRSA was found from 18 out of 103 (17.5%) *S. aureus* isolates from mastitis milk samples. In this study, 100 milk samples were taken from 10 different villages in East Sikkim. Out of the four MRSA, one

was detected from clinical mastitis and three were detected from healthy cows. In the present study, the prevalence of methicillin resistance in *S. aureus* isolated from both the healthy and clinical mastitis cows within three villages is 7.4% which is quite high in comparison to the number of bacteria isolated from Serbia that showed 5.9% methicillin resistance with subclinical mastitis within two Serbian dairy farms (Zutic et al., 2012). In comparison , to the recent reports of increased MRSA prevalence in milk samples from India (29%) (Spohr et al., 2011; Graveland et al., 2010) the present study here in Sikkim revealed 7.4% of MRSA. In the present study 54 *Staphylococcus aureus* and 46 Coagulase negative Staphylococccal species were identified from both the healthy and mastitic milk samples.

In this study the highest level of resistance was observed in Amoxyclav, Oxacillin and penicillin the high resistance of about 54% of *Staphylococcus aureus* to penicillin(92.59%) is in agreement with results reported by Aarestrup et al. (1995). In their study, 75% of the strains isolated from bovine mastitis in Denmark were resistant to penicillin. Two of the MRSA strains showed multiresistance patterns (resistant to 3 or more antibiotics other than β-lactam) on the basis of the multiresistance definition by (Coombs et al., 2004). Which is quite similar to the study made by (Moon et al., 2007). Unlike the studies by (Kwon et al., 2005; Lee ., 2003) .Among all the MRSA strains (n=4), all four strains showed resistance to Amoxyclav(100%) and Penicillin (100%) and all four stains showed susceptibility to Clindamycin . Two of the strains showed resistance to Erythromycin and the other two were susceptible to Erythromycin.

Conclusion

This study was primarily undertaken to assess the prevalence of Methicillin Resistant *Staphylococcus aureus* in the udder of cows. Concerns about MRSA in animals are practical enough and require careful study of various aspects to better understand the emergence and distribution of MRSA in different species, including cows. Further studies are still required to determine the importance of other body sites and the environment as a possible reservoir for MRSA in dairy herds by using Phenotypic and Genotypic tools. Considering the importance of *S. aureus* as a human infectious agent, its highly contagious nature among dairy cows, and the current gaps in information about the potential human-bovine connections, the epidemiology of MRSA in the dairy industry ought to represent a future area of attention by the scientific community.

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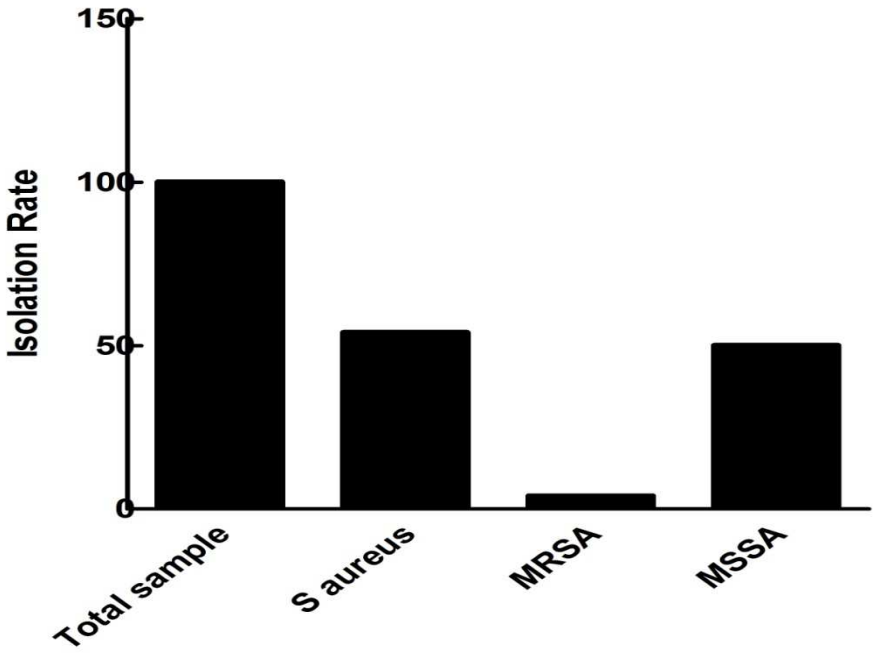

Figure 1 : MRSA isolation from milk.

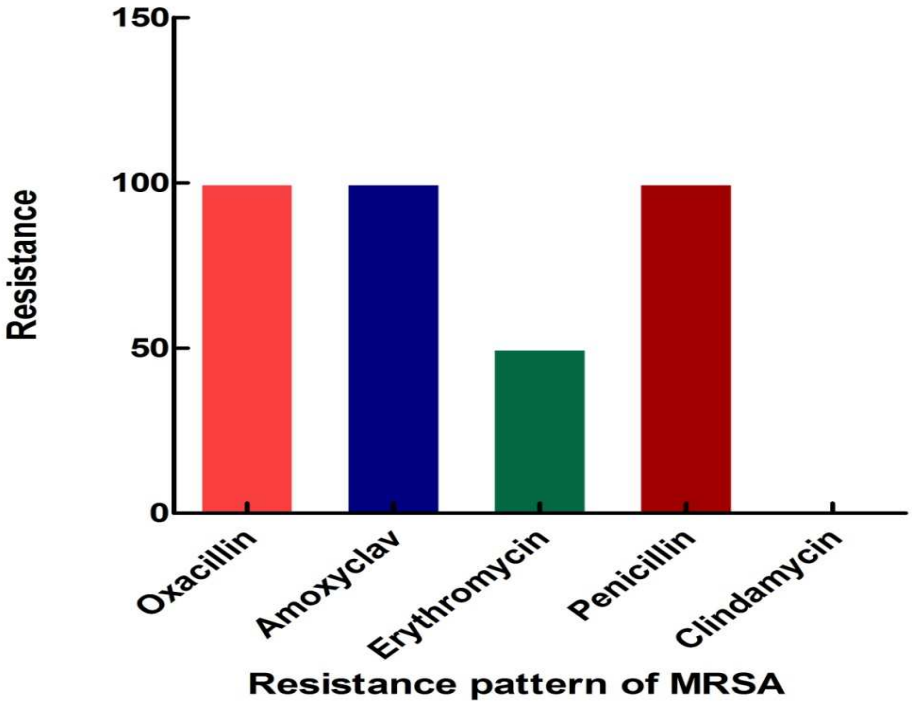

Figure 2

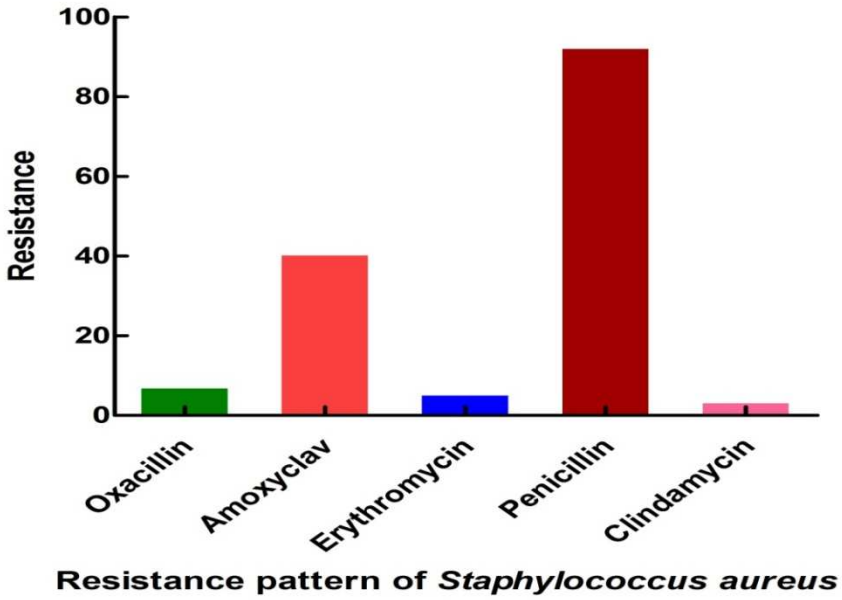

Figure 3

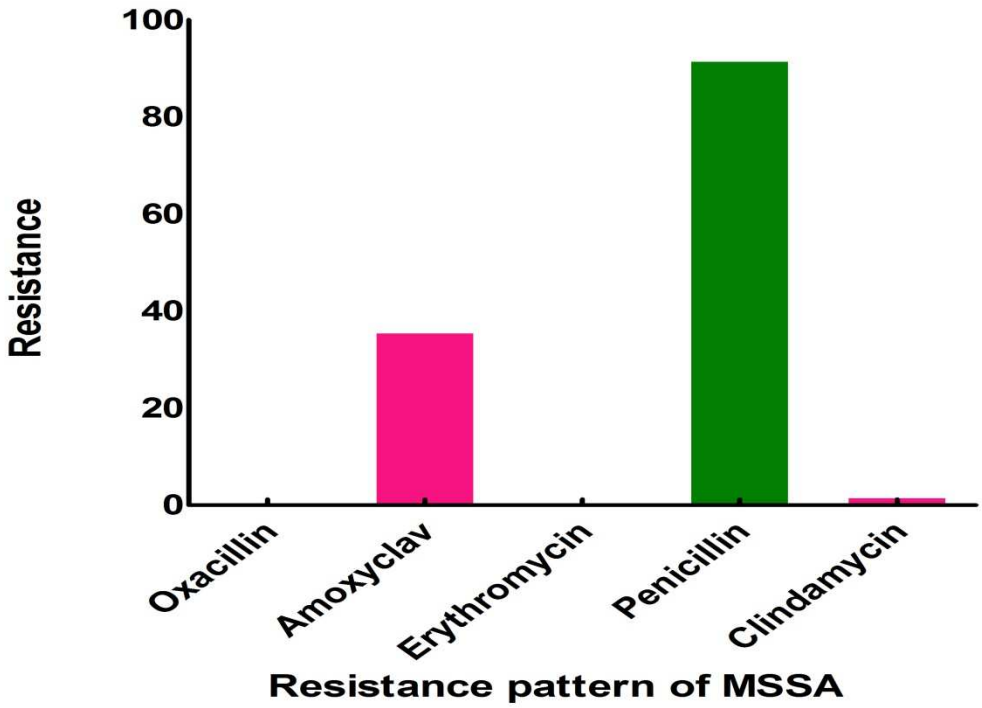

Figure 4

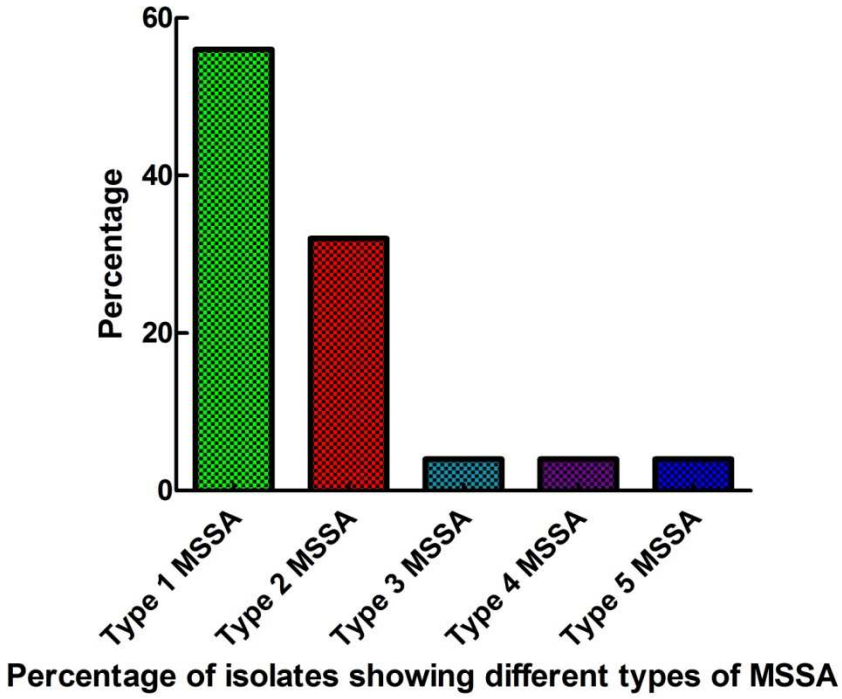

Figure 5

Table 1. **Antibiogram Profiling Of MSSA**

Antibiotic	Oxacillin	Amoxyclav	Erythromycin	Penicillin	Clindamycin
Susceptibility (S) Resistance (R)	S	S	S	R	S
Isolates of MSSA **TYPE 1:**	MSA 1, MSA2, MSA4, MSA5, MSA7, MSA16, MSA18, MSA20,MSA21,MSA22,MSA23,MSA25,MSA26,MSA27,MSA28,MSA30,MSA31,MSA32,MSA33,MSA34,MSA35,MSA40,MSA41,MSA54,MSA6,MSA17, MSA15, MSA46.				
Antibiotic	Oxacillin	Amoxyclav	Erythromycin	Penicillin	Clindamycin
Susceptibility (S) Resistance	S	R	S	R	S

(R)					
Isolates of MSSA **TYPE 2:**	MSA12,MSA14,MSA24, MSA36, MSA38,MSA42,MSA3,MSA8,MSA9,MSA10, MSA 13, MSA11,MSA 29,MSA43, MSA45, MSA37.				
Antibiotic	Oxacillin	Amoxyclav	Erythromycin	Penicillin	Clindamycin
Susceptibility (S) Resistance (R)	S	R	S	R	R
Isolates of MSSA **TYPE 3:**	MSA44,MSA53.				
Antibiotic	Oxacillin	Amoxyclav	Erythromycin	Penicillin	Clindamycin
Susceptibility (S) Resistance (R)	S	S	R	S	S
Isolates of MSSA **TYPE 4**	MSA48,MSA50.				
Antibiotic	Oxacillin	Amoxyclav	Erythromycin	Penicillin	Clindamycin
Susceptibility (S) Resistance	S	S	S	S	S

(R)					
Isolates of MSSA **TYPE 5**	MSA 51, MSA52.				

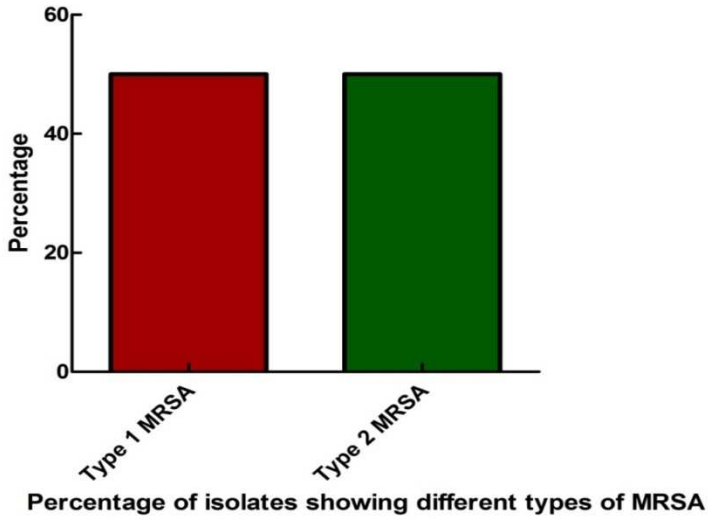

Figure 6

Table 2. Antibiogram profiling of MRSA

Antibiotic	Oxacillin	Amoxyclav	Erythromycin	Penicillin	Clindamycin
Susceptibility (S) Resistance (R)	R	R	R	R	S
Isolates of MRSA **TYPE 1**	MSA19, MSA39.				
Antibiotic	Oxacillin	Amoxyclav	Erythromycin	Penicillin	Clindamycin

Susceptibility (S) Resistance (R)	R	R	S	R	S
Isolates of MRSA **TYPE 2**	MSA47, MSA49.				

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