Journal of Animal Research: v.5 n.3, p. 539-545. September 2015

DOI: 10.5958/2277-940X.2015.00091.1

Prevalence of *Staphylococcus aureus* and Methicillin Resistant *Staphylococcus aureus* (MRSA) in Clinical Setting and Dairy Farm Environment of Jammu

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Received: 28 May, 2015 **Accepted:** 24 August, 2015

ABSTRACT

A study was carried out to assess the occurrence of *Staphylococcus aureus* and the methicillin resistant *Staphylococcus aureus* (MRSA) in the dairy farm environment of Jammu district and the clinical setting in the SKUAST- J, R. S. Pura *vis-à-vis* their phenotypic and genotypic characterization for the antimicrobial pattern and the *mecA* gene producing ability. A total of 60 samples of environment comprising of dust and clinical setting swabs were collected and analysed using standard bacteriological methods. The *S. aureus* isolates were further processed to identify and characterize MRSA, with an aim to ascertain the characteristics that might be associated with the virulence and resistance of MRSA, making use of conventional and molecular methods. Polymerase chain reaction was employed to identify 533bp *mecA* gene from MRSA isolates. The results revealed a prevalence of *S. aureus* 14/30 (46.7%) and 18/30 (60%) in dairy farm environment and clinical settings, respectively. From 32 positive *S. aureus* dust swabs, 6 (18.75%) were found to be MRSA. This study revealed resistance of *S. aureus* to Penicillin G, Ampicillin and Streptomycin. However, sensitivity to Cephalothin, Amikacin and Gentamicin was found. The present study revealed that *Staphylococcus aureus* and MRSA transmission can occur from infected farms, dairy and clinical environments to animals. However, future studies are required to elucidate the transmission potential and magnitude of the problem.

Keywords: *Staphylococcus aureus,* MRSA, Prevalence, Antimicrobial resistance, Jammu.

Staphylococcus aureus, a Gram positive bacterium is the causative agent of cellulitis, impetigo, folliculitis, scalded skin syndrome, furuncles, acute food poisoning episodes and toxic shock syndrome in humans. *S. aureus* is also responsible for many infective and systemic infections in the health care setting, nosocomial infections (Soomro *et al.,* 2003). Methicillin-resistant *Staphylococcus aureus* (MRSA) is drug resistant strain of *S. aureus* and public health experts are alarmed by the spread of such strains which are hard to treat, and therefore MRSA is sometimes called a “super bug”. (Anonymous, 2013). Isolation of MRSA from animals was first reported in 1972 following its detection in milk from mastitic cows (Devreise *et al.,* 1972). Pigs are reported to be major reservoirs for MRSA multilocus sequence type 398 (ST398) and this sequence type has also been isolated from other animal species. The clone of MRSA associated with livestock is referred to as livestock-associated MRSA (LA-MRSA) (Leonard and Markey, 2008).

In India, the prevalence of human nosocomial infection caused by MRSA varies between 20-40 per cent (Mehta *et al.,* 1998). MRSA isolates have reportedly reached phenomenal proportions in Indian hospitals, with some cities reporting 70 per cent of strains being resistant to methicillin (Anupurba *et al.,* 2003). The worst feature of MRSA has been simultaneous multi drug resistance to many antibiotics due to production of a supplemental penicillin binding protein 2’ or PBP2a and high plasmid transfer between strains in hospital environment and

chronic carrier stage. The *S. aureus* and MRSA associated infections impose a serious burden in terms of medical and socio-economic costs and cause a significant morbidity and mortality (Marples and Cooke, 1988).

The incidence of hospital acquired methicillin resistant *Staphylococcus aureus* (HA-MRSA) continues to rise globally. Most transmission of MRSA from patient to patient is thought to be mediated by transiently colonized health care workers, although air borne dispersal and contacts with contaminated surfaces are also important mode of transmission.

The occurrence of *S. aureus* and MRSA in the foods of animal origin, humans, animals and in the environment poses a serious threat to the well-being and sustenance of the living beings. It has a lot of difficult to treat clinical implications and thus the study was undertaken to investigate the *S. aureus* and MRSA in dairy farm environment and in clinical settings.

MATERIAL AND METHODS

The sampling areas were selected purposively and the samples were collected from the dairy farm of F.V.Sc & A.H., R. S. Pura block of Jammu district and two other unorganised dairy farms located in the central city areas of Jammu city viz; Talab Khati Ka and Bazaar Qasaba. Chemicals, reagents and antibiotics used in the study were of analytical and/or molecular biology grade procured from Sigma, Bangalore Genie, Hi-Media, India and other firms of national and international repute. Each sample was enriched in peptone water and incubated at 37 ºC for 24 hours. Each inoculum was cultured on Baird Parker agar and incubated at 37 ºC for 48 hours. Then, a single isolated colony was picked and streaked on mannitol salt agar and incubated at 37 ºC for 24-48 hours and further the organisms were identified on the basis of their cultural, morphological, staining and various biochemical characteristics. All positive samples were subjected to coagulase test for confirmation of *Staphylococcus aureus* as described by Monica (1991). Biochemical characterization of the presumptive *S. aureus* isolates was performed as described by Bennett and Lancette, 2001. Thus, confirmed *S. aureus* isolates were inoculated and cultured on Oxacillin resistance screening agar (ORSA) for detection of Methicillin Resistant *Staphylococcus aureus* (Pereira *et al.*, 2009). The presumptive blue coloured colonies of MRSA on Oxacillin resistance screening agar were further inoculated in MRSA alert test kit for Methicillin Resistant *Staphylococcus aureus* confirmation. Detection of *mecA* gene by the polymerase chain reaction which is considered as the "Gold standard" for diagnosis of MRSA was carried out, to reveal the 533bp bands. The primers used in the study are shown in table 1.

Table 1. Details of the primers used for *mecA* gene detection (Pereira *et al.*, 2009)

Oligo-nucleotide Primers	Primer Sequence	Product Size
Forward Primer	5' AAA ATC GAT GGT AAA GGT TGGC 3'	533bp
Reverse Primer	5'AGT TCT GCA GTA CCG GAT TTGC 3'	

DNA from the colonies was extracted by the routinely used snap and chill method in the laboratory. The reaction mixture used in PCR for the detection of *mecA* gene for methicillin resistance of *S. aureus* was prepared from the stock solution of the various reagents. Titration of $MgCl_2$ (25mM) was carried out at 1.5, 2, 2.5 and 3mM to obtain the clear bands of 533bp on agarose gel electrophoresis. Final reaction mixture of 25µl was prepared as depicted in Table 2.

Table 2. Reaction mixture used for PCR detection of *mec A* gene for MRSA

S. No	Contents	Volume	Final Concentration
1.	5X PCR buffer	5 µl	1X
2.	MgCl2 (25mM)	3µl	3mM
3.	dNTP mix(10mM each)	0.5µl	0.2mM
4.	Forward primer (25µM)	0.5µl	0.5µM
5.	Reverse primer (25µM)	0.5µl	0.5µM
6.	Template DNA	1µl	1.5 U
7.	Taq DNA polymerase (3U/µl)	0.5µl	---
8.	Nuclease free water	14µl	
	Total	25 µl	

Amplification parameters were chosen as described for *mecA* PCR (Murakami *et al.*, 1991). The amplification cycle consisted of initial denaturation at 94ºC for 5 min, followed by 40 cycles, each consisting of initial denaturation at 94ºC for 1 min, annealing at 58ºC for 1 min and extension at 72ºC for 2 min which was followed by final extension at 72ºC for 5 min.

RESULTS

S. aureus in environmental samples

The *S. aureus* revealed higher prevalence in the environmental samples, whereas that of MRSA was considerably low. The clinical settings recorded a higher prevalence of *S. aureus* 18/30 (60%) whereas, 14/30 (46.7%) *S. aureus* were reported from dairy farm environments.

The isolation and identification of the organism was carried out on the basis of their colonial, morphological, cultural and biochemical characteristics. *S. aureus* showed typical pale yellow, friable, large opaque colonies on mannitol salt agar. On Baird Parker agar media jet black colonies surrounded by a light halo were considered as presumptive *Staphylococcus.* On microscopic examination all the *S. aureus* isolates were found to be Gram positive, non spore forming, non-motile cocci giving a clustered bunch of grape like irregular appearance. *S. aureus* were found to be catalase and coagulase positive and showed a ++++ pattern on IMV$_i$C biochemical testing (Table 4).

Table 3. Prevalence of *S. aureus* and MRSA in environmental samples.

Sample		No. (n)	*S. aureus* +ve (%)	MRSA +ve (%)
Dust/floor swabs	Dairy farm environment	30	14 (46.7%)	2 (6.7%)
	Clinical setting	30	18 (60%)	4 (13.3%)

MRSA in environmental samples

The MRSA reported from the clinical settings was also higher (4/18, 13.3%) compared to the MRSA from dairy farm environment which was recorded as 2/14 (6.7%) (Table 3).

The MRSA were detected by the agar screening test using Oxacillin Resistance Screening Agar. (National Committee for Clinical Laboratory Standards, 1999). The confirmed *S. aureus* isolates were further inoculated on the Oxacillin Resistance Screening Agar. Presumptive MRSA colonies appeared as deep blue colored colonies (Fig.1).

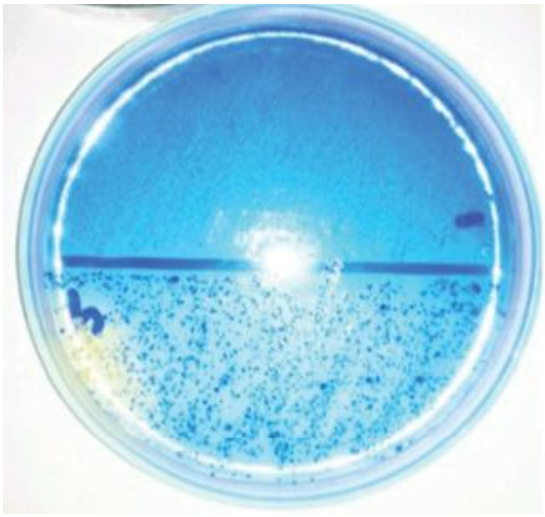

Figure 1: Characteristic deep blue colonies of Methicillin Resistant *S. aureus* (MRSA) on Oxacillin resistance screening agar media.

Table 4. Biochemical characteristics of bacterial isolates:

S. No.	No. of isolates	Gram's Staining	Motility	Coagulase	0xidase	Catalase	Mannitol Fermentation	Indole production	Methyl red test	VP-test	Citrate utilization	Urease Production	Haemolysis on Blood Agar	MRSA alert test kit	Organism
1.	32	+	-	+	-	+	+	+	+	+	+	+	+	±	S.aureus
2.	6	+	-	+	-	+	+	+	+	+	+	+	+	+	MRSA

The presumptive deep blue MRSA colonies growing on the oxacillin resistance screening agar were further processed using MRSA Alert Kit. The alert kit results showed a change in colour from red to orange- pale yellow and clot formation on incubation at 37ºC for 24 hrs confirming the MRSA (Figure 2). The amplification of the *mecA* gene generated a product of approximate molecular band size of 533bp on agarose gel electrophoresis (Figure 3).

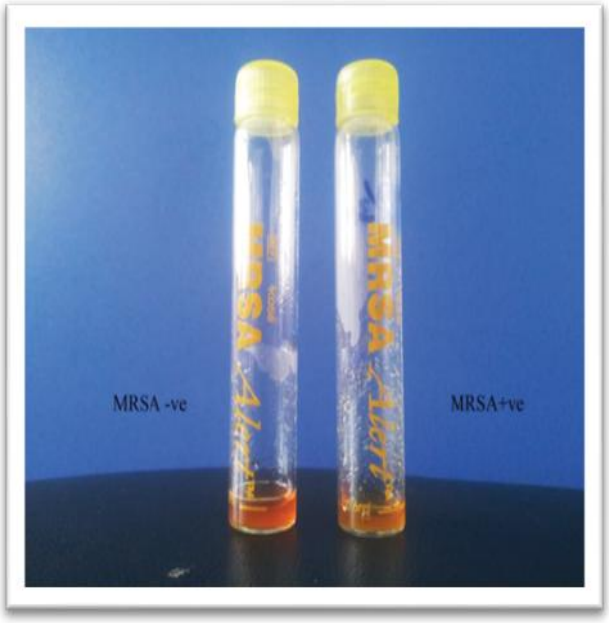

Figure 2: Typical colour change of the MRSA alert test kit from red to orange-pale yellow.

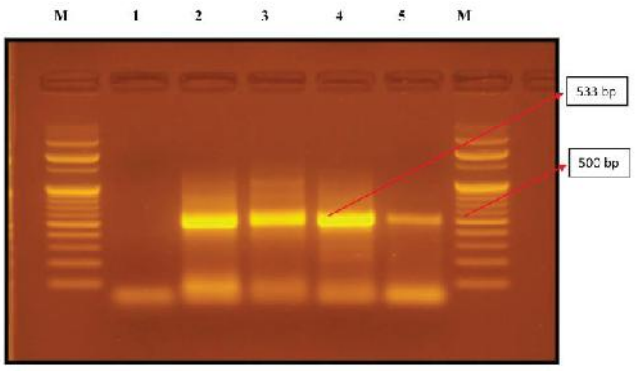

Figure 3: Agarose gel electrophoresis showing amplified PCR product of *mecA* gene of MRSA

Lane M: 100bp molecular weight marker.

Lane 1: Negative.

Lane 2, 3, 4 and 5: Amplified product of 533bp of mecA gene.

All the positive *S. aureus* isolates were subjected to antibiotic susceptibility testing against 14 commonly used antimicrobials/ antibiotics against Gram positive organisms by disc diffusion technique of Bauer *et al.*, 1966 (Table 5).

Table 5. Antibiogram of the *S. aureus* isolates against commonly used antimicrobials

S. No.	Antibiotic	Sensitive	Inter-mediate	Resistant
1.	Penicillin G	8 (25%)	2 (6.25%)	22 (68.75%)
2.	Ampicillin	2 (6.25%)	9 (28.125%)	21 (65.625%)
3.	Streptomycin	6 (18.75%)	7 (21.875%)	19 (59.375%)
4.	Ceftriaxone	7 (21.875%)	15 (46.875%)	10 (31.25%)
5.	Cefexime	13 (40.625%)	10 (31.25%)	9 (28.125%)
6.	Tetracycline	14 (43.75%)	9 (28.125%)	9 (28.125%)
7.	Amikacin	19 (59.375%)	5 (15.625%)	8 (25%)
8.	Ciprofloxacin	15 (46.875%)	9 (28.125%)	8 (25%)
9.	Cefuroxime	11 (34.375%)	13 (40.625%)	8 (25%)
10.	Enrofloxacin	17 (53.125%)	7 (21.875%)	8 (25%)
11.	Cephalothin	20 (62.5%)	5 (15.625%)	7 (21.875%)
12.	Amoxacillin/ Salbactum	16 (50%)	9 (28.125%)	7 (21.875%)
13.	Gentamicin	19 (59.375%)	6 (18.75%)	7 (21.875%)
14.	Gatifloxacin	9 (28.125%)	18 (56.25%)	5 (15.625%)

The study revealed high percentage of resistance to Penicillin G (68.75%), Ampicillin (65.625%) and Streptomycin (59.375%). On the other hand, *Staphylococcus aureus* isolates were found to be most

sensitive to Cephalothin (62.5%), Amikacin, Gentamicin (both 59.375%) followed by Enrofloxacin (53.125%). Intermediate resistance was shown by Gatifloxacin (56.25%) followed by ceftriaxone (46.875%) and cefuroxime (46.625%) as shown in Table 5.

DISCUSSION

Out of the 60 dust/ floor swabs from clinical setting, 32 (53.33%) were positive for *S. aureus*, of which 6 (18.75%) were MRSA. The component of dust swabs was studied to test the hypothesis that *S. aureus*, including antibiotic-resistant and multidrug-resistant *S. aureus*, are present in the bioaerosol of the environment. Study findings support the hypothesis and show corroboration with the findings of Gandara *et al*. (2006), who found resistant strains of air-borne culturable *S. aureus* in higher concentrations 71.4 per cent (n=5) inside the study homes than outside (28.6%, n=2) in Penssylvania, U.S.A. The respirable *S. aureus* recovered from inside each home observed 54.59 per cent resistance to Ampicillin and 60.46 per cent to Penicillin, which suggests the presence of an indoor source of these organisms as well. On circumstantial analysis of such incidences Das *et al*. (2002) and Roberts *et al*. (2006) reported that human activities involving the movement of dry fabrics, such as bed making and curtain movement, are associated with elevated concentrations of airborne bacteria, it clearly demonstrates that elevated concentrations of *S. aureus* may exist indoors compared to outdoors. The results thus obtained indicate that strict control, preventive measures and regulated movement of personnels have to be ensured to prevent the spread and contamination of the environment for good health of all the living beings. Since, not many studies have been carried out on the prevalence of *S. aureus* and MRSA in the dust swabs and environmental samples, no definite comparison and conclusions could be drawn regarding the occurrence and transmission of *S. aureus* and MRSA in environment.

Presently, antimicrobial therapy is one of the primary control measures in reducing the *Staphylococcal* morbidity and mortality. However, the indiscriminate use of antimicrobials has led to the development of resistance against many such antimicrobials. The resistance of *S. aureus* can be attributed to the presence of *mecA* gene that leads to synthesis of an altered penicillin binding protein PBP2a. Drug sensitivity trial of *S. aureus* against 14 commonly used antibiotics in this study revealed high percentage of resistance to Penicillin G, Ampicillin and Streptomycin, but the isolates were found to be most sensitive to Cephalothin, Amikacin, Gentamicin and Enrofloxacin. However intermediate resistance was shown to Gatifloxacin, Ceftriaxone and Cefuroxime. The results however, differed from the observations of Corti *et al*. (2003) who reported only 9 per cent of *S. aureus* isolates had resistance to Penicillin G. The results also showed divergence from the findings of Adejuwon *et al*. (2011) wherein great resistance was showed by *S. aureus* isolates to Gentamicin and Amoxicillin in Nigeria. However, our results are nearly comparable to those of Sabra and Farag (2012), in terms of resistance of *S. aureus* to Penicillin and sensitivity to the floroquinolone group (Norfloxacin and Enrofloxacin). The findings of our study were partly in consonance with the findings of Mahony *et al*. (2005) who on the basis of antibiotic susceptibility, reported the isolates in two different groups. Group one isolates were resistant to tetracyclines (Tetracycline) and fluoroquinolones (Gatifloxacin, Enrofloxacin, Ciprofloxacin), while the isolates of group two being resistant to aminoglycosides (Gentamicin, Streptomycin, Amikacin), tetracyclines (Tetracycline) and trimethoprim/ sulphamethoxazole and variably resistant to fluoroquinolones. Some of our results were in agreement with the finding of Ombui *et al*. (2000), wherein among the isolates, resistance observed for Penicillin was 60 per cent. However, resistance to Ampicillin was about 22.7 per cent, which is not in agreement to our results. This may be postulated here that, the indiscriminate use of Ampicillin over time, in our state may have lead to the increased resistance of the organism towards the drug.

Several phenotypic measures have evolved for the detection of methicillin resistant *S. aureus*, including the oxacillin resistance screening agar test and cefoxitin test, commercial automated assays such as MRSA latex agglutination tests, the Vitek 2 system (GPS-SA card) and Microscan (Felten *et al*., 2002). However, such methods are not very often sufficiently sensitive or specific, thus, PCR detection of the *mec*A gene is being frequently employed to check the resistance against methicillin and related synthetic penicillins (Chambers, 1997). In our study, all 32 *Staphylococcus* positive isolates were subjected to PCR, to detect the presence of 533bp *mec*A gene. The result of PCR showed an agreement with the

phenotypic method of identification and isolation. Out of 32, 6 (18.75%) *Staphylococcus* isolates were tested as *mecA* gene positive. Gene *mecA* was also targeted by Normanno *et al*. (2007), in 6 MRSA isolates derived from bovine milk (4) and pecorino and mozzarella cheese (2) in Italy. The positivity observed was 3.5 per cent. Detection of the *mecA* gene by PCR was also carried out by Pereira *et al*. (2009), wherein PCR revealed the presence of the *mecA* gene in 18 (18%) isolates from the neonatal and paediatric units of a Brazilian teaching hospital. The confirmation of the presence of the *mecA* gene, has until now been the "Gold standard" for detection of MRSA worldwide. However, this has been changed as a new *mecA* homologue gene, *mecC* (formerly named $mecA_{LGA251}$) has been described in *S. aureus* from humans and cattle. The methods of detection of *mecC* gene were first described by Stegger *et al*. (2012), that identified *S. aureus* by amplification of the *spa* gene, *PVL* or *Luk*F PV encoding gene and also multiplex PCR method which could be used for confirmation of Methicillin Resistance by amplification of both *mecA* and *mecC*. These findings brought fore a concern regarding a possible animal origin of the isolates harbouring the *mecC* gene and also the need to update the methods for detection of methicillin resistance that need to be supplemented with further testing to identify the *mecC* gene successfully.

CONCLUSIONS

The present study indicates the prevalence of *S. aureus* and MRSA in dairy farm environment and clinical settings. The resistance to commonly used antibiotics is a matter of veterinary public health concern that needs constant monitoring and surveillance using molecular tools at larger scale for better understanding its epidemiology. The presence of *Staphylococcus aureus* in significant counts, in environment poses a potential threat to the public health. MRSA can further complicate and aggravate the situation. There is an urgent need for public enlightenment and awareness *vis-à-vis* safety. This can be achieved by reduction in flaring up of the nasal commensal due to the breakdown of immune system. Moreover, avoiding the indiscriminate use of antibiotics, leading to high risk of plasmid transfer to mediate resistance can help, until the corrective actions of prevention and cure have been put in place to assure public safety. Molecular methods of surveillance should be used at a larger scale for understanding the epidemiology in a more efficient way.

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