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Analysis of antibiotic resistance in *Staphyloccocus aureus* present in various environmental sources of Ambur Town, Tamil Nadu.

Arun Bennet Samuel*, and Arabi Mohammed Saleh MA.

*School of Biosciences and Technology, Vellore Institute of Technology, Vellore 632014, Tamil Nadu.
Assistant Professor (Senior), School of Biosciences and Technology, Vellore Institute of Technology, Vellore 632014, Tamil Nadu.

ABSTRACT

Antibiotic resistant varieties of *Staphylococcus aureus*, especially Methicillin Resistant *S.aureus* (MRSA), present in the environment can pose hazard to the community through exposure, leading to spread of the same in healthy persons, resulting in disease. In order to assess the degree of antibiotic resistance present in this organism in various environments of Ambur town, Tamil Nadu, India, the present study was conducted. Kirby Bauer method, targeted PCR and MLST typing was used to determine the antibiotic resistance and molecular profile of various strains isolated from different sources. From 250 environmental samples, 233 strains were obtained. Of these, 132 were CA-MSSA strains and 1 CA-MRSA strain. The CA-MSSA strains were showed resistance to only pencillin whereas the single MRSA strain was resistant to azithromycin, erythromycin, clarithromycin, lomefloxacin, pristinomycin and linezolid; intermediate resistance to moxifloxacin, gatifloxacin and ciprofloxacin was also observed. All the strains were sensitive to vancomycin. Molecular typing revealed the following - All CA-SA strains possessed PVL gene. The MRSA strain was SCCmec type I. MLST types of MRSA strain was ST30 whereas all the pencillin resistant strains were ST 772 type. The current study necessitates the need for additional regular screening of environmental sources and implementation of prevention and control measures to prevent spread of antibiotic resistant varieties of *S.aureus* in the community.
Keywords: *Staphylococcus aureus;* environment; antibiotic resistance; MRSA; India.

Corresponding author

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INTRODUCTION

The growing antimicrobial resistance in commensal microflora of the human body is a major concern for the healthcare field today. The spread of the resistance factors and organisms both on national and global scale has been studied and documented [1,2].

Among the pathogenic micro-organisms of concern is *Staphyloccocus aureus*. It is an opportunistic pathogen that is part of the human microflora and is capable of causing mild to serious symptoms [3]. During the last five decades, S.aureus has gained resistance to many class of antibiotics, especially β-lactams, rendering it multi drug resistant (MDR). The most well known MDR variant is Methicillin Resistant S.aureus (MRSA). There are two variants of the MRSA strain which are Community Acquired (CA-MRSA) and Healthcare Acquired (HA-MRSA) [4]. Several methods of typing the resistance in *S.aureus*, through conventional and molecular approaches, have been used to characterize and trace the epidemiology of various strains around the world [5].

The environment has been determined as a factor in the transmission of resistant strains of *S.aureus*, especially via air and via fomites. Fomites refer to any object capable of carrying infectious organisms which vary from dead skin cells and hair to inanimate organisms like door knobs and paper money [6].

The ability of *S.aureus* to survive in various environments for extended periods of time without loss in viability or virulence enables it to spread to other sources and also to the community [7].

Several fomites for *S.aureus* transmission have been identified. Drug resistant *S.aureus* strains have been found in milk [8], carried through bovine staphylococcal mastitis or contamination of milk through environment [9] and in meat, through contamination via handling and processing [10] and in currency notes [11]. It was also found in wastewater sources, including treated water from water treatment plants [12-13]. Other studies report on finding drug resistant S.aureus strains, including MRSA on surfaces with frequent human contact which are not subjected to regular cleaning such as hospital furniture [14] and athletes training equipment [15].

In view of the paucity of research with regards to environmental assessment of resistant strains in India, the present study was conducted. In this study, we assessed the presence of *S.aureus* in air, water, soil and surfaces of various locations frequented by large groups of people. We also investigated the antibiotic resistance pattern of the isolated strains and determined the molecular profile of the resistant strains to determine epidemiological patterns of spread.

MATERIALS & METHODS:

Design and Sampling

This cross sectional study was performed on environmental samples taken from various locations in Ambur. Environmental samples were collected from air, wastewater, soil (in proxmimity to wastewater) and surfaces (frequent contact locations such as vending/cash counters) and in the following locations – transport hubs (bus stand/railway station), markets (fish and meat market), cineplexes, eateries (restaurants and roadside stalls) and hospitals where there was movement of large numbers of human population. The entire study was carried out from January 2014 to June 2014.

Isolates Testing

Standard methods were used for isolation, identification and confirmation were used of *S.aureus* isolates. Air samples were collected via settle plate method [16] using Mannitol Salt Agar (MSA) (Himedia, India) plates; soil and water samples were collected in sterile containers; surface samples collected through sterile swabs which were swabbed several times over test surface after dipping in sterile saline. After appropriate dilution (for soil and water samples), these and surface samples were swabbed on MSA plates. All plates were incubated at 35±2ºC for 24 hours. All the plates were inspected for growth and isolates were identified initially through colony morphology; *S.aureus* colonies are small, golden yellow colonies with yellow zone of hydrolysis around them. Further confirmation of *S.aureus* was done by performing the following tests:

Gram staining (grape like clusters of Gram positive small cocci), catalase test (evolution of bubbles when colonies are mixed in 3% hydrogen peroxide on slide), plasma coagulation test (equal volumes of log phase culture and fresh plasma are mixed in a clean tube and incubated at 35±2ºC overnight, *S.aureus* causes plasma coagulation resulting in clumping of plasma at the bottom of the tube) and hemolysis test using Blood Agar (Himedia, India) (test colonies are streak plated on blood agar plates and incubated overnight at 35±2ºC; *S.aureus* colonies appear golden yellow and have clear zone of beta hydrolysis around them).

Primary antibiotic susceptibility testing was performed using Kirby– Bauer disk diffusion method on Mueller–Hinton agar (Himedia, India) [Bauer and Kirby] using following antibiotics disc obtained from Himedia, India: Penicillin 10µg; and Cefoxitin 30µg. Temperature was maintained at 35±2ºC with an incubation period of 24 hours to accurately determine resistance. Isolates that were resistant to cefoxitin are considered as MRSA (as per CLSI guidelines 2012) and resistance to more than one antibiotic are considered Multi Drug Resistant (MDR) [17]. Secondary antibiotic susceptibility testing was performed using the previous method using multi-antibiotic disc sets Dodeca Staphylococci 1 and 2 (Himedia, India). Minimum Inhibitory Concentration (MIC) testing to determine inhibitory concentrations of Oxacillin and Vancomycin for the resistant strains was performed by the previous method using Ezy MIC Oxa-Van strips (Himedia, India). Strip concentration of oxacillin ranged from 0.064 – 8.0 µg/ml; vancomycin concentration ranged from 0.19 – 16.0 µg/ml. The interception of the inhibitory zone with the reading on the strip was determined to be the MIC for the strain. The ranges for determing resistance were – for oxacillin: ≤2 (Sensitive), ≥4 (Resistant); for vancomycin: ≤2 (Sensitive), 4-8 (Intermediate), ≥16 (Resistant).

Molecular Analysis

To perform molecular assessment of the resistant strains, DNA templates were isolated from freshly cultured strains using microwave lysis method [18]. Presence of mecA, femA and PVL was verified by multiplex PCR method [19]; *S.aureus* ATCC 25923 was used as positive control. Absence of mecA gene means that strain is Methicillin Sensitive *S.aureus* (MSSA). Determination of SCCmec type was done by multiplex PCR method as described previously [20]. Multi Locus Sequence Typing (MLST) of seven housekeeping genes (arcC, aroE, glpF, gmk, pta, tpi and yqiL) was carried out as described previously [21]. MLST web site (http://saureus.mlst.net/) was used to assign allelic profiles and sequence types (ST). eBurst algorithm, available on the same website was used for phylogenetic analysis.

RESULTS

Strains Isolated from Sources

In this study, a total of 50 air samples, 50 soil samples, 50 water samples and 100 surface samples were collected for environmental assessment. The chosen locations for sampling were – transport hubs (bus stand and railway station), markets (fish and meat), cineplexes, eateries (restaurants and roadside stalls) and hospitals. In 250 samples, 233 contained *S.aureus*. All air, water, and surface samples from the five locations contained *S.aureus*. Lower levels of *S.aureus* was present in soil samples. Table 3.1 shows the presence of *S.aureus* in the various samples in their locations.

This is similar to those found in a study which found 100% prevalence of *S.aureus* in food and wastewater samples [22]. Also, the high levels of *S.aureus* found in surface samples taken from markets is similar to a previous study which found prevalence rates of S.aureus at 73.6% in meat [23]. High levels of *S.aureus* in wastewater samples from the locations is also similar to previous studies reporting 100% prevalence in municipal wastewater [22] and hospital wastewater samples [24]. As *S.aureus* is spread primarily through air, being present high concentrations in the anterior nares, all air samples contained *S.aureus* [25].

Antibiotic Susceptibility

Table 1 shows primary antibiotic susceptibility test results showing the presence of antibiotic resistant strains in water, soil, air and surface samples. Most number of antibiotic resistant strains was found in surface samples with highest numbers from hospitals followed by markets. This was followed by air samples with highest numbers in markets and hospitals followed by transport hubs. Overall, from strains isolated from 233 samples, 133 were drug resistant; highest number of resistant strains were found in surface samples (68%),

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followed by air samples (56%), water samples (48%) and soil samples (39%). A single MRSA strain was isolated from a hospital cash counter; All other 132 strains were resistant towards pencillin only.

Table 1. *S.aureus* strains present in various environmental samples

Location	Source							
	Air (n=50)		Water (n=50)		Soil (n=50)		Surface (n=100)	
	TSF	RS	TSF	RS	TSF	RS	TSF	RS
Transport Hubs	10	7	10	5	6	2	20	12
Markets	10	8	10	6	8	3	20	15
Cineplexes	10	2	10	3	5	2	20	11
Eateries	10	3	10	3	5	2	20	13
Hospitals	10	8	10	7	9	4	20	17
Overall Total	50	28	50	24	33	13	100	68

Key: TSF – Total Strains Found
RS – Resistant Strains

Secondary antibiotic testing using multi antibiotic disc sets showed that all the 132 strains were resistant to penicillin G only. The single surface MRSA strain resistance to six antibiotics which are azithromycin, erythromycin, clarithromycin, lomefloxacin, pristinomycin and linezolid and intermediate susceptibility to ciprofloxacin, gatifloxacin and tigecycline. All the strains were sensitive to vancomycin.

MIC testing showed that except the single MRSA strain, all other strains were sensitive to Oxacillin; all of the 133 strains were sensitive to vancomycin. These are represented in Table 2.

Table 2. MIC and molecular profile of resistant *S.aureus* strains.

Strains	MIC	PVL	SCCmec	MLST
A1-A28, W1-W24, G1-G13, S1-S67	Sensitive to Oxacillin and Vancomycin	Positive	Negative	ST 772
S68	Resistant to Oxacillin; Sensitive to Vancomycin	Positive	Positive	ST 30

Key: A1-A28 – Air samples strains
W1-W24 – Water samples strains
G1-G13– Soil samples strains
S1-S68 – Surface samples strains

Resistance to pencillin by 100% of strains has been reported in other studies which state that pencillin is the most commonly dispensed medication for animals and humans. The antibiotic is only partially metabolized by the diseased individual or animal and the rest is discharged in feces or urine which enters the water systems leading to exposure of antibiotic to the microbes present therein resulting in resistance development [26].

Molecular Profile

Molecular testing using quadriplex multiplex PCR showed that 132 strains were CA-MSSA type as they contained PVL gene but did not have mecA gene; the MRSA strain possessed mecA and PVL gene making it CA-MRSA type. SCCmec typing showed that the MRSA strain was SCCmec type I.

MLST typing showed that the CA-MRSA strain had allelic profile 2,2,2,2,6,3,2 indicating ST30 type; and, all the other 132 CA-MSSA penicillin only resistant strains had same allelic profiles which is 2,2,2,2,6,3,1 indicating ST772 type. These are represented in Table 2.

The global spread of ST30 strains to India has been documented previously and the results of this study are concurrent with the reports [27]. The increased number of ST772 type CA-MSSA with high penicillin resistance is reported in other studies regarding the spread of ST772 strains worldwide, particularly from Asian countries [28].

CONCLUSION

From the present study, we conclude that although MRSA prevalence is low, the high numbers of pencillin resistant CA-MSSA strains in the various environments is a cause for concern. This necessitates increased frequency of surveillance of the various environments and implementation of programmes to enforce regular cleaning and disinfection of the sources of contamination in order to reduce the spread of resistant *S.aureus* strains in the environment and thereby, to the people living/working in these locations.

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