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Medicine

PREVALENCE OF METHICILLIN RESISTANCE IN STAPHYLOCOCCUS AUREUS FROM HUMAN SAMPLES BY PCR METHOD FOR DETECTION OF NUC GENE AND MECA GENE

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ABSTRACT *Staphylococcus aureus* is an important pathogen causing skin and soft-tissue infections, systemic infections and toxemic syndromes. In order to have adequate information for treatment of *S. aureus* infections, it is important to understand trends in the antibiotic-resistance patterns as well as clonal identities across geographical regions. A total of 20 non-duplicate *S. aureus* isolates (10 clinical, 4 carrier and 6 Methicillin-resistant *S. aureus* (MRSA) strains were resistant to at least 10 antibiotics including all penicillins, penicillin/penicillinase inhibitor combinations, carbapenem and cephalosporins. Methicillin-resistant Staphylococcus aureus (MRSA) is a bacterium responsible for several difficult-to-treat infections in humans. It is also called multidrug-resistant Staphylococcus aureus and oxacillin-resistant Staphylococcus aureus (ORSA). MRSA is any strain of Staphylococcus aureus that has developed, through the process of natural selection, resistance to beta-lactam antibiotics, which include the penicillins (methicillin, dicloxacillin, nafcillin, oxacillin, etc.) and the cephalosporins. Strains unable to resist these antibiotics are classified as Methicillin-sensitive Staphylococcus aureus, or MSSA. The evolution of such resistance does not cause the organism to be more intrinsically virulent than strains of Staphylococcus aureus that have no antibiotic resistance, but resistance does make MRSA infection more difficult to treat with standard types of antibiotics and thus more dangerous. MRSA is especially troublesome in hospitals, prisons and nursing homes, where patients with open wounds, invasive devices, and weakened immune systems are at greater risk of infection than the general public.

KEYWORDS : MRSA, Antibiotics, Infections, Resistance

INTRODUCTION

Staphylococcus aureus is a Gram-positive bacterium belonging to the family Staphylococaceae and is often found as a commensal on the skin, skin glands and mucous membranes particularly in the nose of healthy individuals [1]. It is a versatile human pathogen causing infections ranging from relatively mild skin and soft tissue infections to life threatening sepsis, pneumonia, osteomyelitis, endocarditis as well as toxin mediated syndromes such as toxic shock syndrome and food poisoning [2,3]. *Staphylococcus aureus* is usually a harmless colonizer of about one third of healthy humans and is most likely found in the nares. Nasal carriage of *S. aureus* has been closely associated with staphylococcal disease [4]. Colonization increases the risk of subsequent infection since those with *S. aureus* infections are usually infected with their colonizing strain [5]. Infection may occur when there is a breach of the skin or mucosal barrier that allows the organism access to adjoining tissues or the blood stream [6]. *Staphylococcus aureus* is able to cause a large diversity of both benign and lethal infections in humans and animals because of a wide range of virulence factors that include various toxins and enzymes [7]. It has emerged as one of the most important human pathogens and has become a leading cause of hospital and community acquired infections [8]. Prior to the introduction of penicillin for the treatment of *S. aureus* infections in the 1940s, the mortality rate of individuals with staphylococcal infections was about 80 [9]. However, within two years of the introduction of penicillin to medical use, penicillin-resistant strains were discovered. By 1960, about 80% of all *S. aureus* strains were found to be resistant to penicillin. Methicillin was introduced in 1959 [10] to treat infections caused by penicillin resistant *S. aureus* [11] but by 1961 there were reports of methicillin-resistant *S. aureus* from hospitals [12]. Methicillin-resistant *S. aureus* (MRSA) has become a leading cause of hospital-acquired infections worldwide accounting for more than 60% of *S. aureus* isolates in hospitals in the United states [13]. Established risk factors for hospital-acquired methicillin resistant *S. aureus* (HA-MRSA) infection include recent hospitalization or surgery, residence in a long-term care facility, dialysis and indwelling percutaneous medical devices and catheters [14]. Cases of MRSA infections have been documented among healthy community-dwelling persons without the established risk factors for MRSA infections. These infections are referred to as community acquired or community associated MRSA infection CA-MRSA. The emergence of CA-MRSA became a cause for concern because it differs from HA-MRSA in that it does not generally belong to the major clonal groups of epidemic MRSA, is susceptible to most non β-lactam antibiotics, contains the type IV staphylococcal cassette chromosome *mec* and frequently carries genes responsible for the production of Panton-Valentine leucocidin (PVL) [15]. The PVL toxin is associated with deep skin and soft tissue infection and necrotizing pneumonia [16]. In contrast HA-MRSAs are generally multidrug resistant and contain SCC *mec* types I, II or III.

AIM AND OBJECTIVES OF THE RESEARCH

The aim of this research was to investigate the phenotypic and genotypic characteristics of *S. aureus* isolates from R.S. Pura and Govt Medical Hospital, Gandhinagar, Jammu in order to gain new insights into the short-term and long-term evolution of *S. aureus* strains. The specific objectives of this research were:

1. To determine the antibiotic susceptibility profile of *S. aureus* strains isolated from Clinical samples.
2. To investigate the prevalence of methicillin resistant *S. aureus* strains and subsequently determine their genetic characteristics.

MATERIALS AND METHODS

Bacterial isolates

A total of 20 staphylococcal strains used in this study were obtained from eight medical centres and comprised of isolates from clinical infections. Presumptive identification was done in the Microbiology laboratory of the Department of Immunology, SKUAST-J FVSCH.

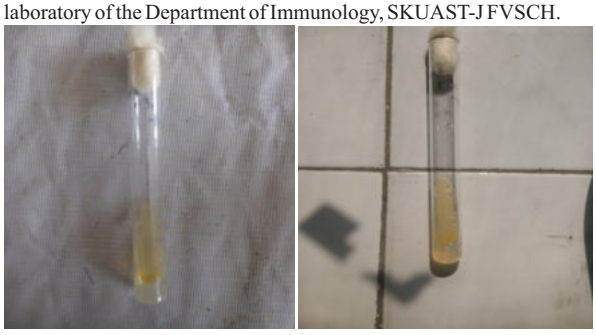


PLATE-1 Isolated Samples from different sites

Isolation and Identification:

The sterile culture media plates of Mannitol Salt Agar (MSA), Blood agar (BA) were dried to remove water of condensation in the plates as well as on the surface of the culture media, by slightly exposing them inside the 37 °C incubation before use. The swab sample was rubbed over one quarter of each of the different agar plates (i.e. MSA) the rest part of the plates was streaked with a sterile wire loop to obtain discrete colonies. The inoculated culture media were incubated at 37 °C in an incubator for 24-48 hrs. Suspected discrete colonies of *Staphylococcus aureus* were sub-cultured on Nutrient Agar plates to obtain pure

culture and for further analysis. Each organism was identified according to Cowan and Steel (2003) method of bacteria identification, by their colonial appearance such as size, shape, consistency, colour, elevation and its differential characteristics such as pigmentation, and Gram Staining were done to further identify the isolates.

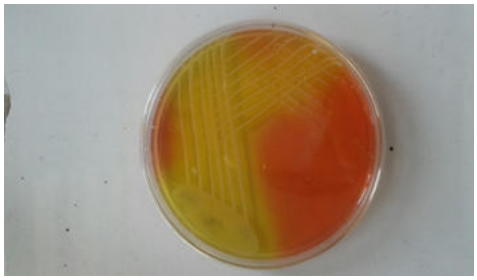


PLATE 2-Mannitol salt agar Media-Golden yellow colonies

Polymerase chain reaction (PCR) primers

The oligonucleotide primers used in the detection of *nuc* and *mecA* were obtained from Integrated DNA technology.

Table 1: The sequence of the primers for PCR amplification specific to different genes of *Staphylococcus aureus*

Primer name nuc gene	Primer sequence 5′ - 3′	Amplicon size
Forward	GCGATTGATGGTGATA CGGTT	270 bp
Reverse	AGCCAAGCCTTGACG AACTAAAGC	

Biochemical Identification Gram Staining Technique

A smear of the suspected colony from the culture plate was made on clean, grease – free slide. The smear was heat-fixed on slide by passing the slide over Bunsen burner flame briefly. The slide was then covered with Crystal violet stain and allowed to stain for 1 minute. The stain was decanted, rinsed with tap water and stained with Lugol's iodine for 1 minute the stain was decanted and the film (smear) decolorized with acetone for few seconds. The slide was quickly washed with distilled water and counter stained with Safranin for 1 minute. The slide was finally washed with water dried and examined under the microscope using the oil-immersion objective. Suspected *Staphylococcus aureus* isolates were Gram-positive cocci (appearing purples) and were arranged in clusters.

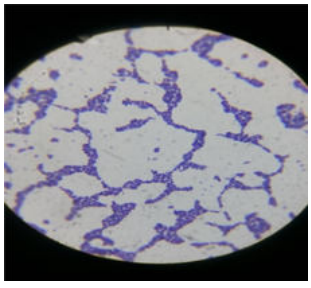


Plate 3 - *Staphylococcus aureus* shows grape-like clusters under microscopy

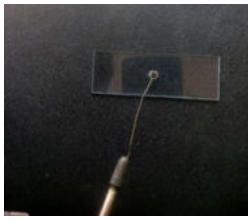
Catalase test:

A drop of 3% hydrogen peroxide solution was placed on a clean, grease-free glass slide; the edge of another clean slide was used to pick the test organisms and was dipped into the hydrogen peroxide. Observed bubble formation was regarded positive.

Oxidase test:

Colonies were taken from the petri plate and placed on a disc the color of disc doesn't change then it shows the presence of *S. aureus.*

Oxidase test –Negative



Catalase Test-Positive

Gel electrophoresis:

Electrophoresis of the DNA was carried out on a 0.8% agarose gel in a 0.5X concentration of Tris-Borate-EDTA (TBE) buffer. Agarose gel was prepared by boiling 0.8g of agarose powder in 100mls of 0.5X TBE buffer. After boiling, the solution was allowed to cool and 10µl of ethidium bromide was added to the cooled agarose solution. This was poured into a casting tray with a comb placed across its rim to form wells. The gel was allowed to set for 30 minutes and the comb was removed. 20 µl of bromophenol blue. A DNA molecular weight marker was also loaded into one of the wells. The gel was there after electrophoresis in a horizontal tank at a constant voltage of 60V for about 1 hour 30 minutes. After electrophoresis, plasmid DNA bands were viewed by fluorescence of bound ethidium bromide under a short wave ultraviolet light transilluminator and the photograph were taken using a digital camera.

Amplification of the *mecA gene*

Methicillin resistance was determined by *mecA* PCR as described (Murakami *et al.,* 1991). The 25µl volume of PCR reaction mixture contained 1µl of genomic DNA, 12.5µl of Red Taq Master mix, 7.5µl PCR H2O and 2µl each of *mecA primers. mecA* gene was amplified with the following primers: *mecA-f:(5'-*AAAATCGATGGTAAAGGTTGGC-3'); *mecA-r:(5'-AGTTCTGCAGTACCGGATTTGC*-3'). DNA amplification was carried out for 40 cycles according to the following protocol: Denaturation at 94° C for 30 s, annealing at 55° C for 30 s, and extension at 72° C for 1 min with a final extension at 72° C for 5 min. The PCR products were analyzed in 2% (w/v) agarose gels stained with ethidium bromide and visualized under UV light. 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

1000

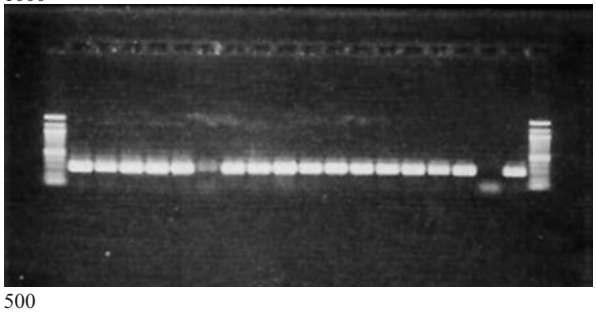


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Plate 4.1. Amplification of the *nuc* gene. Lane 1: 1kb molecular weight marker; lane 2: Y1; lane 3: Y4; lane 4: Y8; lane 5: Y9; lane 6: Y13; lane 7: Y14; lane 8: Y15; lane 9: Y16; lane 10: Y17; lane 11: Y19; lane 12: Y25; lane 13: Y26; lane 14: Y27; lane 15: Y28; lane 16: Y29; lane 17: Y32; lane 18: ATCC 12228 *S. epidermidis* (negative control); lane 19: ATCC 49230) R9/2 (positive control); lane 20: 1kb molecular weight marker.

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Plate 4.2. Amplification of *mecA* gene. Lane 1: 1kb molecular weight marker; lane 2: Y42; lane 3: Y45; lane 4: Y46; lane 5: Y59; lane 6: Y108; lane 7: 1kb molecular weight marker *mecA* positive amplicon 533 bp

RESULTS

Out of the 20 bacteria isolated from clinical specimens, 66 (44%) were

identified to be S*taphylococcus species* (Table 1). Only 23 (34.8%) were identified biochemically to be S*taphylococcus aureus*, out of this, 30.4% wounds swab, 13.0% ear swab and 4.3% from nasal swab. *Staphylococcus aureus* is the leading cause of gram-positive bacterial infections and produces a wide spectrum of diseases, ranging from minor skin infections to fatal necrotizing pneumonia. A prevalence rate of 30.4% was recorded for MRSA.

DISCUSSION

This study presents the comparative analysis of genotypes and virulence profiles of 297 *S. aureus* isolates representing three major phenotypic groups of *S. aureus*—nasal carriage, clinical, and environmental strains. *Staphylococcus aureus* is an important human pathogen and is implicated in a wide variety of infections such as skin and soft-tissue infections (SSTIs), endovascular infections, pneumonia, septic arthritis, endocarditis, osteomyelitis, foreign-body infections, and sepsis (David and Daum, 2010). In Nigeria, *S. aureus* causes significant epidemiologic and therapeutic challenges as in many studies, identification and antibiotic susceptibility testing of *S. aureus* isolates have been based on phenotypic methods and few data exists on the characterization of *S. aureus* isolates using molecular methods (Adesida *et al.,* 2005; Esan *et al.,* 2009). Over the past 20 years, the incidences of both community-acquired (CA) and hospital-acquired (HA) *S. aureus* infections have increased, while antibiotic treatment is increasingly hampered by the spread of *S. aureus* strains that are resistant to multiple antibiotics, including methicillin [18,19,20,21]

Detection of antibiotic resistance genes

The accurate detection of beta-lactam and *mecA*-mediated resistance in *S. aureus* is essential for the treatment of overt infections and the implementation of infection control practices. Resistance to penicillin in *S. aureus* is mediated by production of a penicillinase, encoded by *blaZ* gene. Infections caused by MRSA strains have become one of the most commonly acquired types of nosocomial infections, resulting in increased morbidity, mortality, length of hospital stay, and health care costs [22]. Consequently, there is a need for rapid, reliable, and cost-effective methods for the detection of MRSA. Because many of the MRSA clones exhibit a heteroresistance phenotype, with only a few staphylococcal cells of the population expressing methicillin resistance [23], detection of the *mecA* gene by molecular methods has become the reference method for confirmation of MRSA strains. There are strains of *S. aureus* that hyper produce beta lactamase known as Borderline Oxacillin Resistant *S. Aureus* (BORSA) and while they appear oxacillin resistant, they do not possess the usual genetic mechanism for such resistance. There are also strains of *S. aureus* known as Modified *S. Aureus* (MODSA) which possess a modification of existing penicillin binding proteins rather than the acquisition of a new PBP as is the mechanism for classical MRSA. There are many phenotypic methods available for detection of MRSA. Cefoxitin, a cephamycin, is a potent inducer of the *mecA* regulatory system than penicillin. This study is a comparison of the phenotypic methods with the gold standard which as of now is by the detection of *mecA* gene and *femA* gene by polymerase chain reaction (PCR) [24,25]. Of the 297 strains of *S. aureus* tested, 8 of them were found to be *mecA* positive representing 2.7% of the strain. Despite the low MRSA rate reported in this study, it highlights the occurrence of multiresistant MRSA in R.S. PURA. Methicillin-resistant staphylococci are resistant to all other penicillins, carbapenems, cephems and beta-lactam/beta-lactamase inhibitor combinations. It is therefore advisable that these antibiotics should not be used for treating of methicillin-resistant staphylococci infections. The antimicrobial susceptibility pattern showed that all the MRSA strains were resistant to at least ten antibiotics including penicillins, penicillin/beta-lactamase inhibitor combinations, oxacillin, cephalosporins and carbapenem. An MRSA strain (Y46) was the only strain resistant to teicoplanin, tigecycline and Fosfomycin; this points to an emerging resistance to these drugs. It is therefore, important that this resistance be monitored to prevent the spread. The SCC*mec* typing of the MRSA strains in this study detected only SCC*mec* types I and IV. SCC*mec* types I and II, have historically been associated with multiresistance (resistance to more than three antimicrobials) but all MRSA strains were resistant to 10 or more antibiotics. One of the MRSA strains (Y59) had the characteristics of a CA-MRSA because it possessed the type IV SCC *mec*, was susceptible to antimicrobials other than β-lactams and also harboured the genes responsible for the production of Panton-Valentine leukocidin (PVL).

The R.S. PURA prevalence of MRSA still does not seem to be high and this restricts the need for broad spectrum chemotherapy for the treatment of staphylococcal infections. There is, however, a continuing need for accurate surveillance and control measures in order to keep track and establish the true picture of MRSA infections in R.S. PURA.

CONCLUSION

S. aureus has a remarkable ability to develop antibiotic resistance, leading to four distinct resistance waves that have occurred in the past sixty years. The advent of PRSA, then MRSA and now vancomycin resistance has resulted in a steady decline in the efficacy of these valuable antibiotics. The MRSA first emerged as a nosocomial pathogen (HA-MRSA; in the 1960s), then further surfaced as a community-based infection (CA-MRSA; in the 1990s) and has subsequently increased the staphylococcal disease burden. It is a global public health problem and represents the most commonly identified antibiotic-resistant pathogen. The incidence of HA- and CA-MRSA infections as well as the prevalence of different MRSA clones varies considerably among countries. Some MRSA clonal lineages are more frequently isolated than others owing to their superior survival and transmissibility. The HAMRSA is endemic in many hospitals worldwide. The CA-MRSA has a smaller fitness burden, higher transmissibility and virulence compared to HA-MRSA and is epidemic in many countries. In addition, the distinction between CA- and HA-MRSA is gradually fading, owing to the emergence of *pvl* negative and/or MDRCA-MRSA clones and its invasion into hospitals as well. The MRSA has markedly influenced the empirical therapy for staphylococcal infections. Limited therapeutic options are available for the management of these infections. Most β-lactam antibiotics are ineffective against both HA- and CA-MRSA. The HA-MRSA is usually MDR but CA-MRSA is often susceptible to non-β-lactams. Resistance to non-β-lactam drugs varies geographically and may change over time. The CA-MRSA associated skin and soft-tissue infections are treated with oral antibiotics including doxycycline, minocycline, clindamycin, trimethoprim sulfamethoxazole, rifampicin, and fusidic acid. Severe CA-MRSA infections and HA-MRSA demand intravenous vancomycin therapy. Transmission may be prevented by following universal infection control strategies and decolonization therapy.

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