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Microbiological study of methicillin-resistant *Staphylococcus aureus* isolated from wound samples at a tertiary care hospital, Central India

Sachin Mishra[1], Prashant Peshattiwar[1*], Sonali Mishra[2], Pragati Khanorkar[2]

[1]Department of Microbiology, Index Medical College Hospital and Research Centre, Indore, Madhya Pradesh, India, [2]Department of Biochemistry, Index Medical College Hospital and Research Centre, Indore, Madhya Pradesh, India

ABSTRACT

Background: *Staphylococcus* aureus continues to be a dangerous pathogen for both community-acquired as well as hospital-associated infections. *S. aureus* resistant to methicillin were reported soon after its introduction in October 1960. Methicillin-resistant *S. aureus* (MRSA) is now endemic in India. MRSA has recently emerged as a major nosocomial pathogen worldwide with a significant morbidity and mortality.

Materials and Methods: This study was thus undertaken to characterize MRSA isolates, isolated from wound samples from a tertiary care teaching hospital in Central India and to also probe antibiotic susceptibility of its clinical isolates.

Results: About 120 MRSA isolates from various clinical samples such as pus swabs and aspirates, blood, urine, sputum, and endotracheal tube aspirates were collected and processed in the laboratory for various tests. Of 120 isolates subjected to gelatin liquefaction test with appropriate controls, 104 were found to be positive while 16 were tested negative. Majority of the hospital-associated MRSA (HA-MRSA) patients were in the age group of >50 years of age 42 (35%) followed by the 41–50 years of age group 26 (21.7%). The age in the HA-MRSA group varied from 18 to 75 years with mean age of 41.2 ± 22.29. Majority of the HA-MRSA infections presented as cellulitis (41.9%), followed by abscess (28.9%) and post-operative wound discharge (23.8%), whereas most of the community-associated-MRSA infections presented as abscesses (83.5%), followed by furuncle (9.6%), and cellulitis and carbuncle (2.8% each). Among 120 isolates subjected to alkaline phosphatase test, 114 were found to be phosphatase positive while 6 being phosphatase negative.

Conclusion: Our study is a preamble to enable epidemiologists to understand the nature of MRSA isolates in this part of India. There is a need to study epidemiology of such infections. Robust antimicrobial stewardship and strengthened infection control measures are required to prevent spread and reduce emergence of resistance.

Key words: Biochemical test, methicillin-resistant *Staphylococcus aureus*, microbiological study, prevalence, *Staphylococcus*, wounds

INTRODUCTION

Staphylococcus aureus is a major pathogen associated with serious community-and hospital-acquired infections. It is commonly found in air, dust, water, and as normal flora on skin and in the respiratory tracts of humans. The most common mode of transmission is by skin-to-skin contact from an infected host. *S. aureus* is one of the most harmful species of staphylococci encountered. It is the leading cause of bacteremia, pneumonia, myocarditis, acute endocarditis, pericarditis, osteomyelitis, encephalitis, meningitis, chorioamnionitis, mastitis, and scalded skin syndrome.[1]

Human morbidity and mortality in hospital settings are largely caused by staphylococcal bacteremia.[2,3]

The pathogenic capacity of *S. aureus* is clearly dependent on its production of exoproteins and toxins. The species is identified on the basis of a variety of conventional physiological or biochemical characters. The key characters for *S. aureus* are colony pigment, free coagulase, clumping factor, protein A, heat-stable nuclease, lipase, and acid production from mannitol.[1,3]

Early detection of emerging trends in antimicrobial resistance may facilitate implementation of effective control measures. The antibiotic susceptibility contributes directly to patient care and the expertise of microbiology laboratory can have a powerful influence on antibiotic usage, and hence, on the pressure that facilitates the emergence of antimicrobial drug resistance.

The present work was planned to identify and characterize *S. aureus* isolates collected from post-operative wound swab of male and female patients hospitalized in the various departments of a tertiary care hospital, Central India.

Address for correspondence:
Prashant Peshattiwar, Department of Microbiology, Index Medical College Hospital and Research Centre, Index City, Nemawar Road, NH-59A, Indore-452 016 Madhya Pradesh, India. Phone: +91-9425402197. E-mail: drprashant07@yahoo.co.in

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MATERIALS AND METHODS

A cross-sectional observational study carried out in a 750-bed tertiary care hospital in Central India. The study commenced after obtaining approval from the institutional ethics committee and continued for a span of 10 months. Data were analyzed at the end of study.

Inclusion Criteria

1. Subjects with confirmed methicillin-resistant *S. aureus* (MRSA)-positive wound infections
2. Subjects 18 years of age and older
3. Subject's willing to participate.

Exclusion Criteria

1. Subjects <18 years of age
2. Subjects not agreeing to participate
3. Pregnant females
4. Any condition resulting in severe learning disability (e.g., brain injury), or
5. Those unable to comprehend for other reasons will be excluded from the study.

A pro forma was prepared for recording patient-related information and 120 MRSA isolates from clinical materials which were preserved from October 2013 to July 2014, from various clinical samples such as pus swabs and aspirates, blood, urine, sputum, and endotracheal tube aspirates were included in this study. Consecutive isolates of MRSA from same patients were excluded.

Study Technique

All clinical samples were processed in the laboratory as per standard guidelines. *S. aureus* isolates were identified by standard laboratory procedures. Only strains obtained from a pure culture were included. Only the first strain from each patient was included. All the strains were collected aseptically, transferred into Mannitol salt agar (MSA) media, Hi-Media (Mumbai), and incubated overnight at 37°C.[4]

Processing of Samples and Culture

The sterile swab samples were placed in sterile transport containers immediately. If there was any delay in processing, the swabs were placed in a tube containing Amies transport medium without charcoal in an ice pack box and transported carefully to the laboratory within 12 h and then processed.

Phenotypic Characterization

Samples were plated on 5% sheep blood agar, MacConkey agar, and THIOGLYCOLATE medium, respectively. Plates were incubated at 37°C. After overnight incubation, colony morphology and hemolysis was observed duly. Gram's stain was done on white colony showing lysis on blood agar. Colonies showing Gram-positive cocci arranged in clusters were subjected to coagulase test by slide and tube coagulase. Coagulase-positive strains were subjected to antibiotic susceptibility by the modified Kirby-Bauer method and isolates showing methicillin resistance in the screening by cefoxitin disc diffusion (MRSA) were included in the study. Further, characterization of the MRSA was done using MSA, DNAse agar, phosphatase agar, Christensen's urease slopes, and gelatin medium.[5]

Hemolyses on 5% Sheep Blood Agar

Colonies were inoculated on 5% sheep blood agar and incubated overnight in a 5% CO_2 atmosphere. A narrow zone of clearing around the colonies was looked for after incubation. Any strains showing clearing around the colonies were considered hemolytic and no clearing was considered non-hemolytic.[5]

Gram's Stain

The colonies showed Gram-positive cocci in cultures.

Catalase Test

The breakdown of hydrogen peroxide into oxygen and water is mediated by the catalase. This can be detected when a part of the colony of the organism producing catalase is introduced into hydrogen peroxide by the rapid elaboration of bubbles of oxygen. A drop of 3% hydrogen peroxide was placed onto a glass slide. With a sterile wooden stick, a small amount of pure growth from the agar was transferred into the drop.

Brisk effervescence of bubbles was considered positive and no visible bubbling was considered negative. Positive control - ATCC *S. aureus* strain and Negative control - ATCC *Enterococcus faecalis* strain were included.[5]

Slide Coagulase Test

Bound coagulase which is present only on the cell surface can be detected by emulsifying the colony into saline on a slide and adding plasma onto the suspension.

A colony was emulsified in a drop of saline on a microscopic slide. Similar suspensions were made of positive and negative control strains to confirm the proper reactivity of the plasma. The plasma used was human plasma obtained from the blood bank and that was screened for hepatitis B and HIV infections. A flamed and cooled straight inoculating wire was dipped into the undiluted plasma at room temperature, withdrawn, and the adherent plasma stirred into the *Staphylococcal* suspension. A coarse clumping of bacterial suspension visible to naked eye within 10 s was read as positive result.[5]

Tube Coagulase Test

The test detects free coagulase secreted by *S. aureus* into the culture medium and is negative for all the other *Staphylococci.* A 1 in 6 dilution of human plasma in saline was made and 1 ml of the diluted plasma was placed in a sterile test tube. A colony of the *Staphylococcus* under test was emulsified in the diluted plasma. With each batch of tests, a known coagulase-positive and known coagulase-negative culture and a tube of unseeded dilute plasma were included as controls. The tube was incubated at 37°C for up to 4 h and examined at 1, 2, and 4 h for clot formation by tilting the tube through 90°. Negative tube was left at room temperature overnight and reexamined the next day.[6]

Any degree of clot formation was read as positive. Tubes in which plasma remained wholly liquefied or showed only ropey precipitate were considered negative. Coagulase-positive staphylococci were identified as those which were catalase positive and coagulase positive by tube method.

Alkaline Phosphatase Test

Staphylococci produce phosphatase enzyme. This activity may be constitutive in some species and repressed in others. Mueller-Hinton agar at 5.6–5.8 pH with p-nitrophenyl phosphate 0.495 mg/ml used as reagents. The medium in spot inoculated and read within 18 h of incubation. Bright yellow color under and

around the inoculums was considered as positive test. Isolates not showing bright yellow color under and around the inoculums was considered as negative.[7,8]

Urease Production

Bacteria decompose urea by means of an enzyme urease and produce ammonia, which can be detected by a suitable pH indicator. The strains under test were inoculated heavily over the entire slope of the Christensen's urease medium at pH 6.8 and incubated at 37°C for 18–24 h. With each batch of strains, a known positive and a known negative control was included. Any slant which showed reddening was considered positive and no change in color was considered negative.[7,8]

MSA

This selective and indicator medium contains 1% of mannitol, sodium chloride 7.5% with phenol red as indicator of acid production. *S. aureus* and other *Staphylococcus* turn the indicator yellow. The strains under test were inoculated onto the medium along with appropriate controls and incubated at 37°C for 24 h. Strains producing yellow colonies were taken as mannitol fermenters and strains not producing yellow colonies were considered as non-mannitol fermenter.[7,8]

DNAse Test

Deoxyribonuclease enzyme is produced by *S. aureus*. This enzyme hydrolyzes the DNA in the media and gives clear halo around the colonies. Some strains of *S. aureus* do not hydrolyze the DNA and some coagulase-negative staphylococci give a weak-positive reaction. The strains under the test were inoculated on DNAase agar medium with controls - ATCC *S. aureus* as positive control and ATCC *Escherichia coli* as negative and were incubated at 37°C for 18–24 h. Any strains showing clear halo around the colonies were considered as positive.[8]

Gelatin Liquefaction

Gelatinase is a proteolytic enzyme that breaks down gelatin into amino acid. Gelatinase activity is detected by loss of gelling of gelatin. Inoculate the gelatin deep with 4–5 drops of a 24 h broth culture of tests and controls. Incubate at 37°C up to 14 days. Remove the gelatin tube daily from the incubator and placed at 4°C to check liquefaction. Refrigerate an uninoculated control along with the inoculated tube. Liquefaction is determined only after the control has hardened. Tube showing liquefaction after refrigeration is taken as positive and gelatin tube not showing liquefaction after 14 days is considered negative.[8]

Cefoxitin Disc Diffusion Test

All the isolates were again subjected to cefoxitin disc diffusion test (for reconfirmation) using a 30 µg disc. A 0.5 McFarland standard suspension of the isolate was made and lawn culture done on MHA plate. Plates were incubated at 37°C for 18 h and zone diameters were measured. An inhibition zone diameter of ≤19 mm was reported as oxacillin resistant and ≥20 mm was considered as oxacillin sensitive.[8]

Oxacillin Disc Diffusion Test

All the isolates were subjected to oxacillin disc diffusion test using a 1 µg disc. A 0.5 McFarland standard suspension of the isolate was made and lawn culture done on MHA plate. Plates were incubated at 37°C for 18 h and zone diameters were measured. An inhibition zone diameter of ≤10 mm was reported as oxacillin resistant, 11–12 intermediate resistance and ≥13 mm was considered as oxacillin sensitive.[8]

RESULTS

About 120 MRSA isolates from various clinical samples such as pus swabs and aspirates, blood, urine, sputum, and endotracheal tube aspirates were collected and processed in the laboratory for various tests.

Majority of clinical samples in the present study was pus swabs and aspirates 88 (73.33%) followed by blood 9.17% and ET tube aspirates 6.67% [Table 1].

Majority of the hospital-associated MRSA (HA-MRSA) patients were in the age group of >50 years of age 42 (35%) followed by the 41–50 years of age group 26 (21.7%). The age in the HA-MRSA group varied from 18 to 75 years with mean age of 41.2 ± 22.29. Majority of the HA-MRSA infections presented as cellulitis (41.9%), followed by abscess (28.9%) and post-operative wound discharge (23.8%), whereas most of the community-associated MRSA (CA-MRSA) infections presented as abscesses (83.5%) [Figure 1], followed by furuncle (9.6%), and cellulitis and carbuncle (2.8% each).

Among 120 isolates subjected to alkaline phosphatase test, 114 were found to be phosphatase positive while 6 being phosphatase negative [Table 2].

Among 120 clinical MRSA isolates were processed for urease test, where 116 were found urease positive and 4 were negative [Table 3].

About 120 strains under test were inoculated onto the MSA medium along with appropriate controls and incubated at 37°C for 24 h. 112 were found to be positive while 8 were found to be negative [Table 4]. The 120 isolates under the test were inoculated on DNase agar medium. Of 120 samples, 115 were found to be DNase positive while 5 were DNase negative [Figure 2].

Of 120 isolates subjected to gelatin liquefaction test with appropriate controls, 104 were found to be positive while 16 were tested negative [Table 5]. About 120 isolates were subjected to various tests such as oxacillin disc diffusion test, Cefotoxin disc diffusion test, MIC of oxacillin and oxacillin screening agar test for the detection of MRSA, and methicillin-sensitive *S. aureus*.

S. aureus were identified for *S. aureus* by different biochemical tests. Gram staining, catalase, coagulase, and thermonuclease were important phenotypic identifying markers of *S. aureus*. In this study, we found that 114 (95%), 116 (96.67%),and 112 (93.33%) isolates were positive for alkaline phosphatase, urease, and mannitol salt sugar, respectively [Tables 2-4]. The enzyme gelatinase was secreted by *S. aureus* liquefy gelatin protein. The present study also showed that 104 (86.67%) of *S. aureus* isolates were able to produce gelatinase [Table 5]. In about 81%, 51%, and 48% of isolated strains produced protease, lipase, and non-white pigmented colonies, respectively. The present study showed that 40% of *S. aureus* isolates were able to produce clearing zone surrounding their growth on blood agar media demonstrating that they can produce hemolysin. Our findings showed high prevalence

of MRSA in pus swabs (73.33%) followed by blood (9.17%) and endotracheal tube aspirates (6.67%), respectively. This is in accordance with previous studies where most MRSA were found in pus samples. All *S. aureus* strains isolated in this study were found to be multidrug resistant [Figures 3 and 4].

DISCUSSION

S. aureus is a Gram-positive coccus belonging to the family Micrococcaceae. Isolated colonies of staphylococci are usually large, about 6 to 8 mm in diameter, smooth, with entire edges, slightly raised, and opaque. The colonies of most strains are pigmented with colors ranging from cream-yellow to orange. Rare strains have relatively large capsules, which gives them mucoid appearance. *S. aureus* appears as grape-like clusters when viewed through a microscope and has large, round, golden-yellow colonies, often with beta-hemolysis when grown on sheep blood agar plates. It reproduces asexually by binary fission. The two daughter cells do not fully separate and remain attached to one another. This is why the cells are observed in clusters. *S. aureus* is catalase positive. Catalase enzyme converts hydrogen peroxide to water and oxygen. Catalase-activity tests are sometimes used to distinguish staphylococci from enterococci and streptococci. Previously, *S. aureus* was differentiated from other staphylococci by the coagulase test. However, it is now known that not all *S. aureus* are coagulase positive and that incorrect species identification can impact effective treatment and control measures.[9]

Table 1: Frequency of MRSA in clinical specimens

Clinical specimen	MRSA *n*=120 (%)
Pus swabs and aspirates	88 (73.33)
Blood	11 (9.17)
Urine	6 (5)
Sputum	7 (5.83)
Endotracheal tube aspirates	8 (6.67)

MRSA: Methicillin-resistant *Staphylococcus aureus*

Table 2: Alkaline phosphatase test

Test result	Frequency (%)
Phosphatase positive	114 (95)
Phosphatase negative	06 (5)
Total	120 (100)

Table 3: Urease test

Test result	Frequency (%)
Urease positive	116 (96.67)
Urease negative	04 (3.33)
Total	120 (100)

Table 4: MSA

Test result	Frequency (%)
MSA positive	112 (93.33)
MSA negative	08 (6.67)
Total	120 (100)

MSA: Mannitol salt agar

Table 5: Gelatin liquefaction test

Test result	Frequency (%)
Gelatinase positive	104 (86.67)
Gelatinase negative	16 (13.33)
Total	120 (100)

High prevalence of MRSA is an emerging problem in India. Several authors have reported a substantial increase in the prevalence of MRSA in India. It has increased from 12% in 1992 to 40% in 2009.[10,11] Increasing resistance of MRSA in recent years has had a significant impact on several aspects of patient care and infection control.

Our findings showed high prevalence of MRSA in pus swabs (73.33%) followed by blood (9.17%) and endotracheal tube aspirates (6.67%), respectively. This is in accordance with previous studies where most MRSA were found in pus samples. In our present study, drug resistance patterns of 120 MRSA isolated from clinical specimens were found to be highly variable.

S. aureus was identified as Gram-positive cocci in clusters, β or non-hemolytic golden-yellow colonies on BA, minute lactose fermenting colonies on MA, yellow mannitol fermenting colonies on MSA [Figure 5], catalase positive, coagulase positive, oxidation fermentation (OF) media showing fermentative pattern, urea hydrolyzed, mannitol fermented, Voges–Proskauer (VP) positive, Furazolidone (100 μg) sensitive, bacitracin (0.04 U) resistant, DNase positive, and phosphatase positive. DNase test was

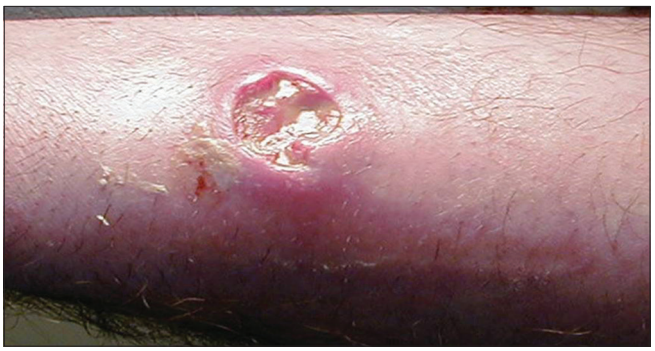

Figure 1: Cutaneous abscess on foot caused by methicillin-resistant *Staphylococcus aureus*

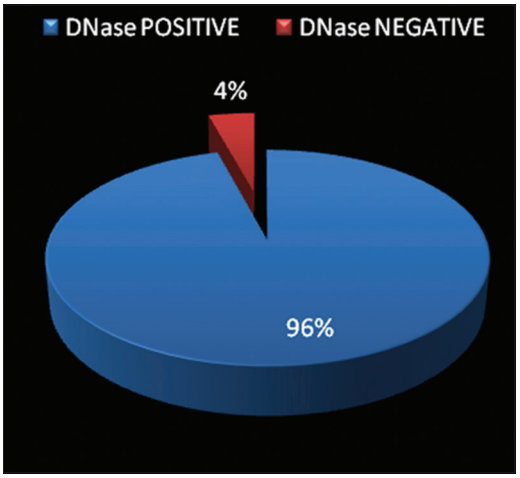

Figure 2: DNase test

interpreted as positive when spot inoculums on DNase agar showed clear uncloudy zones on exposure to hydrochloric acid.[12]

Figure 3: Colonies of *Staphylococcus aureus*, *Staphylococcus albus*, and *Staphylococcus citrus* on nutrient agar showing golden yellow, white, and lemon yellow pigment, respectively

Figure 4: Colonies of methicillin-resistant *Staphylococcus aureus* on MacConkey agar (golden-yellow pigment)

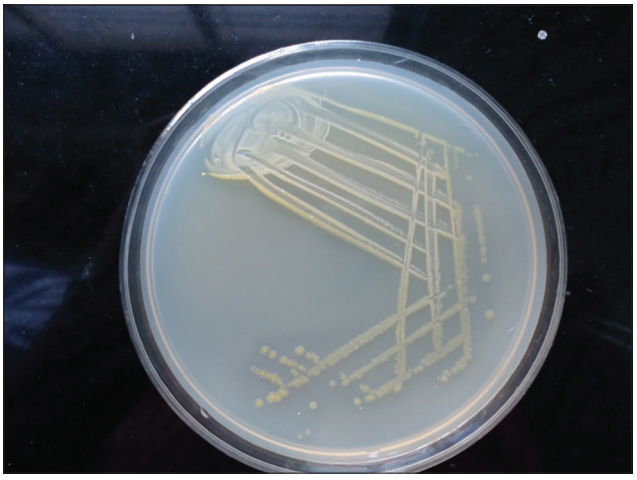

Figure 5: Colonies of methicillin-resistant *Staphylococcus aureus* on nutrient agar (golden-yellow pigment)

Phosphatase producing strains were identified as those which gave a transient pink color on exposure to ammonia vapor.

John *et al*. showed most of the HA-MRSA infections presented as cellulitis (42.6%) followed by abscess (27.8%) and post-operative wound discharge (24.1%).[13] Zervos *et al*. reported that healthcare-associated complicated SSTIs were more likely to present as surgical site infection and ulcer.[14] Majority of the HA-MRSA infections presented as cellulitis (41.9%), followed by abscess (28.9%) and post-operative wound discharge (23.8%), whereas most of the CA-MRSA infections presented as abscesses (83.5%) [Figure 1], followed by furuncle (9.6%), and cellulitis and carbuncle (2.8% each).

Majority of the CA-MRSA infections in John *et al*. presented as abscesses (86.5%). Similar results were obtained in a study on SSTIs due to CA-MRSA by Forcade *et al*. where the predominant clinical presentation was a cutaneous abscess.[15] Literature on the subject reveals that CA-MRSA has a predilection for lower extremities and torso.[16,17]

Bhatt *et al*. showed that out of 100 *S. aureus* isolates 19% isolates were found to be MRSA. Of that, 8 MRSA were isolated from urine, 6 from blood, and 5 from pus.[18] In contrast to our study, Mahmood *et al.* included 265 cases, of which 102 (38.4%) were from sputum, 76 (28.6%) from IV catheters/ETT/suction tips, etc., 65 (24.5%) from pus/wound swabs, 17 (6.4%) from blood, and 5 (1.8%) from urine cultures.[19] Another study conducted by Vaez *et al*., rate of MRSA strains isolation in wound and sputum was higher than other samples with 42.9% and 40%, respectively.[20] Tahnkiwale *et al.* also reported 19.5% of the total isolates of the *S. aureus* were MRSA..[21] Mahmood *et al.* reported that majority of patients 170 (64.1%) were in the age group of 41–80 years.[19]

CONCLUSION

The degree of resistance or sensitivity of MRSA toward commonly used antibiotics is recognized to be diverse from region to region and vancomycin was the only antibiotic found to give uniform sensitivity (100%). The study of the prevalence of MRSA will not only provide the current antimicrobial situation but also help to devise the appropriate treatment of these infections. Hospitals are also contributing to a great extent in spreading antibiotic resistance elevating MRSA. Our study is a preamble to enable epidemiologists to understand the nature of MRSA isolates in this part of India. MRSA showed highest distribution in medical ward as it is a nosocomial pathogen and patients usually acquire it during hospital stay. The treatment of MRSA can become a challenge in the near future. Overuse and misuse of antibiotics along with self-medication should be avoided.

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