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Microbiological and Biochemical Analysis of Methicillin-resistant *Staphylococcus aureus* Isolated from Patients Admitted in RIMS, Ranchi

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Abstract

Background: Since methicillin-resistant *Staphylococcus aureus* strains are resistant to multiple antibiotics, there is a possibility of extensive outbreaks which may be difficult to control. Early detection of methicillin-resistant *Staphylococcus* is important from patients and hospitals point of view. **Materials and Methods:** The present study was carried out in the Department of Microbiology, Rajendra Institute of Medical Sciences, Ranchi, clinical isolates of methicillin-resistant *S. aureus* strains were obtained from admitted patients of Rajendra Institute of Medical Sciences, Ranchi. The sources of isolate included pus from infected surgical wounds, infected burn wounds, conjunctival swab, aural swab, throat swab, vaginal swab, and urine for microbiological and biochemical analysis of methicillin-resistant *S. aureus*. **Results:** All the 264 cases of staphylococcal species isolated from different clinical specimens were subjected to coagulase test. It was observed that out of 264 strains of staphylococci isolated from different sites, 165 strains (62.5%) were coagulase positive and 99 strains (37.5%) were coagulase negative by tube method. Out of the 165 strains of coagulase-positive staphylococci, maximum isolation was obtained from pus 74 followed by throat swab 55, aural swab 21, vaginal 4, conjunctival swab 9, and urine 2. All the 165 cases of coagulase-positive *Staphylococcus* isolated from different clinical specimens were studied for hemolysis, mannitol fermentation, pigment production, and phosphatase production. Out of these 165 strains, 162 (98%) strains produced β-hemolysis on blood agar medium. Pigment production was noted in 160 (97%) of cases. Majority of strains produced characteristic golden yellow pigment on nutrient agar plate. A total of 155 (94%) strains of staphylococci fermented mannitol with the production of acid only. Phosphatase production was observed in 157 (95%) strains of pathogenic staphylococci. **Conclusion:** Considering the above-mentioned pathogenicity test, it was observed that coagulase test was the single most reliable test, though coagulase-negative staphylococci are sometimes pathogenic too.

Keywords: Methicillin-resistant *Staphylococcus aureus*, microbiological and biochemical analysis, Staphylococci

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Introduction

Staphylococci are Gram-positive cocci belonging to the family Micrococcaceae of which *Staphylococcus aureus* is the only coagulase-positive species capable of causing infection in humans. It is one of the major causes of hospital cross infection. In the 1930s, sulfonamides were used in the treatment of staphylococcal infection, but they failed to gain popularity because of their decreased activity in the presence of pus and rapid acquisition of antibiotic resistance by the bacteria.[1,2]

Methicillin-resistant *S. aureus* has emerged in the past decade as one of the most important nosocomial pathogens. Infected and colonized patients provide the primary reservoir and transmission is mainly through hospital staff. Risk factors which contribute to methicillin-resistant *S. aureus* are excessive antibiotic usage, prolonged hospitalization, intravascular catheterization, and hospitalization in intensive care unit. Life-threatening sepsis, endocarditis, and osteomyelitis caused by methicillin-resistant *S. aureus* have been reported.[3,4]

Since methicillin-resistant *S. aureus* strains are resistant to multiple antibiotics, there is a possibility of extensive outbreaks which may be difficult to control. Early detection of methicillin-resistant *Staphylococcus* is important from patients and hospitals point of view. Hence, knowledge of methicillin-resistant *S. aureus* strain and their microbiological and biochemical identification is necessary in selection of appropriate treatment for methicillin-resistant *S. aureus* infection.

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Materials and Methods

The present study was carried out in the Department of Microbiology, Rajendra Institute of Medical Sciences, Ranchi,

clinical isolates of methicillin-resistant *S. aureus* strains were obtained from admitted patients of Rajendra Institute of Medical Sciences, Ranchi. The sources of isolate included pus from infected surgical wounds, infected burn wounds, conjunctival swab, aural swab, throat swab, vaginal swab, and urine. The patients were at first explained the object of the study and the method of obtaining the specimen so that their full cooperation could be obtained and written informed consent was taken.

Collection of Specimen

Pus, conjunctival, aural throat, and vaginal swab were collected by means of sterile cotton swab sticks. The sterile cotton swab sticks were moistened with normal saline and rubbed over the infected area taking care not to touch anything outside so as to prevent contamination. Swabs were then aseptically replaced in sterilized test tubes to avoid drying of the material. Efforts were made to inoculate the specimen within 2 h of collection. Primary inoculation was done on blood agar. The plates after inoculation were incubated at 37°C for 24 h. Midstream samples of urine were received in a sterilized vial and inoculated on MacConkey agar. The plates were incubated at 37°C for 24 h.

On the next day, colonies of staphylococci were identified on blood agar plate by its characteristics appearance. The colonies of staphylococci were of small size, opaque, and surrounded by a zone of β-hemolysis. Colonies of staphylococci on MacConkey agar were smaller in size, pink in color due to lactose fermentation. In general, mixed cultures were obtained from samples collected from different infected sites. To get pure *Staphylococcus* species, suspected colony from the mixed culture was subcultured onto the nutrient agar and incubated at 37°C for 24 h.

The colonies of *Staphylococcus* on nutrient agar were 2–3 mm in diameter, with a smooth glistening surface, entire edge, butyrous in consistency, opaque, and pigmented appearance. Most of them were golden yellow in color but some of them were white and lemon yellow in color.

Smears were prepared from discrete colonies on nutrient agar with normal saline. The air-dried and heat fixed smears were stained by Gram staining method and examined microscopically. Gram-positive cocci arranged in grape-like clusters were identified as *Staphylococcus*.

Isolated colony was picked up from an 18 to 24 h culture on nutrient agar with the help of a sterile platinum wire loop and placed on a clean glass slide. A drop of 3% hydrogen peroxide was added over the colony on the slide. Immediate bubbling was recorded as a positive result. This test differentiates between the catalase-positive Micrococcaceae species are catalase positive.

The organisms were inoculated on blood agar plate, incubated at 37°C for 24 h. Then, it was looked for hemolysis. Usually, there was beta (β) type of hemolysis, that is, a clear zone of hemolysis around the colonies.

On nutrient agar plate, the organism was inoculated and incubated overnight, thus colonies obtained were observed for the color of pigment produced by staphylococci. Usually, golden yellow pigment was produced by pathogenic strains of staphylococci. Pigment production was enhanced when 1% glycerol monoacetate or mild was incorporated in the medium or when the temperature was kept at 22°C.

Organisms were inoculated in Mannitol salt agar containing nutrient agar with 1% mannitol and 7.5% sodium chloride and incubated at 37°C for 24 h. *S. aureus* produces yellowish colonies on the medium due to fermentation of mannitol with the production of acid. Other staphylococcal species produces pink colonies.

Phenolphthalein-diphosphate was added to nutrient agar plate and organisms were inoculated in this plate and allowed to grow by incubating at 37°C for 24 h. Then, 0.1 ml of ammonia solution was placed in the lid of Petri dish and the dish with culture was placed over it for a minute. The pathogenic strains of staphylococci produce the enzyme phosphatase which liberate free phenolphthalein from phenolphthalein-diphosphate which were detected by exposing the growth with ammonia vapor, turning the growth bright pink, indicating a positive test.

Results

All the 264 cases of staphylococcal species isolated from different clinical specimens were subjected to coagulase test [Table 1 and Figure 1]. It was observed that out of 264 strains of staphylococci isolated from different sites, 165 strains (62.5%) were coagulase

Table 1: Number of isolation of *Staphylococcus* sp. from different clinical specimens

Specimens	*Number*
Pus and wound	84
Throat swab	78
Aural swab	39
Conjunctival swab	32
Urine	18
Vaginal swab	13

Table 2: Results of coagulase test of 264 strains of *staphylococci* isolated from different clinical specimens

Specimens	*Coagulase +ve staph.*	*Coagulase –ve staph.*
Pus and wound	74	10
Throat swab	55	23
Aural swab	21	18
Conjunctival swab	9	23
Urine	2	16
Vaginal swab	4	9

Table 3: Results of hemolysis on blood agar, mannitol fermentation, pigment production, and phosphatase production of 165 strains of coagulase-positive *Staphylococcus*

Specimens	*Positive strains*	*Negative strains*
Hemolysis	162	3
Mannitol fermentation	155	10
Pigment production	160	5
Phosphatase production	157	8

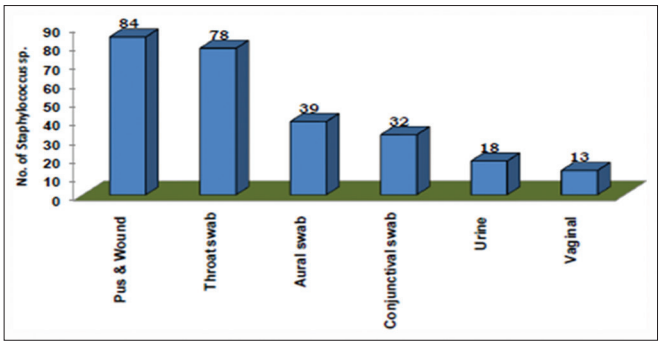

Figure 1: Number of isolation of Staphylococcus sp. from different clinical specimens

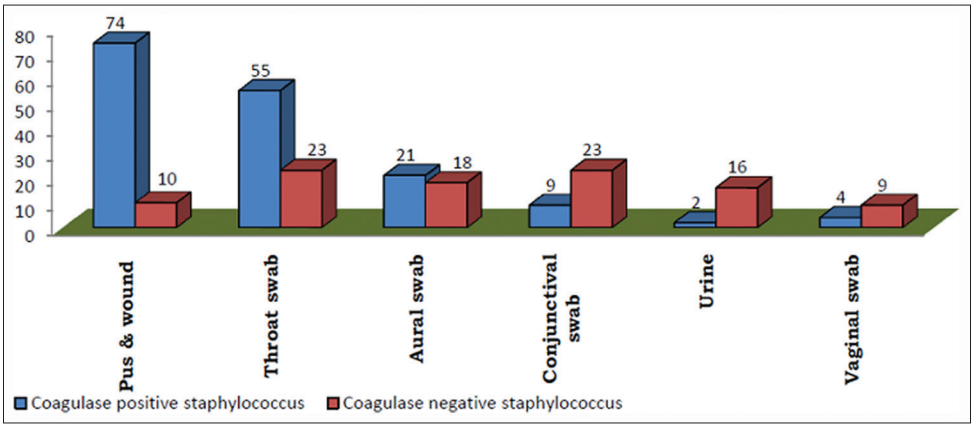

Figure 2: Results of coagulase test of 264 strains of staphylococci isolated from different clinical specimens

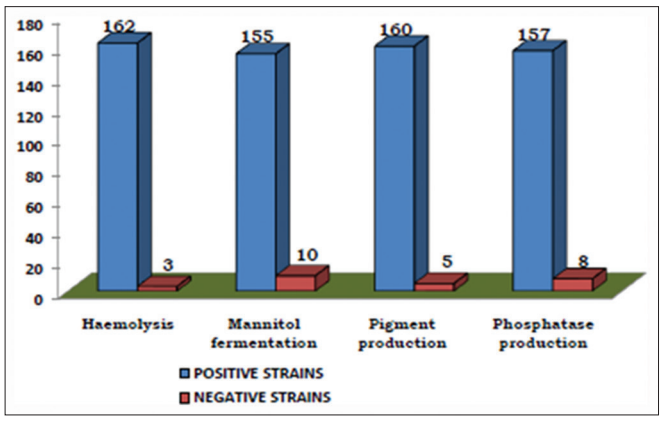

Figure 3: Results of hemolysis on blood agar, mannitol fermentation, pigment production, and phosphatase production of 165 strains of coagulase-positive *Staphylococcus*

positive and 99 strains (37.5%) were coagulase negative by tube method. Out of the 165 strains of coagulase-positive staphylococci, maximum isolation was obtained from pus 74 followed by throat swab 55, aural swab 21, vaginal 4, conjunctival swab 9, and urine 2 [Table 2 and Figure 2].

All the 165 cases of coagulase-positive *Staphylococcus* isolated from different clinical specimens were studied for hemolysis, mannitol fermentation, pigment production, and phosphatase production. Out of these 165 strains, 162 (98%) strains produced β-hemolysis on blood agar medium. Pigment production was noted in 160 (97%) of cases. Majority of strains produced characteristic golden yellow pigment on nutrient agar plate. A total of 155 (94%) strains of staphylococci fermented mannitol with the production of acid only. Phosphatase production was observed in 157 (95%) strains of pathogenic staphylococci [Table 3 and Figure 3].

Discussion

The present work is "study of methicillin resistance *S. aureus* isolated from patient admitted in RIMS and testing their sensitivity to antimicrobial drugs." Samples were collected from different sources such as pus, throat, ear, conjunctiva, vagina, and urine. Various criteria have been proposed to differentiate pathogenic from non-pathogenic strains of staphylococcus. However, coagulase and phosphatase production, ability to hemolyse red blood cell, fermentation of mannitol, and pigment production have been commonly used.[5,6]

The pathogenic strains of staphylococcus were studied for their resistance to methicillin on Mueller-Hinton agar supplemented with 5% sodium chloride using oxacillin or methicillin disc. Recent sensitivity pattern of methicillin resistance *S. aureus* was studied against the available newer antibiotics. Hence, knowledge of the methicillin-resistant *S. aureus* strains and their sensitivity pattern will help in proper treatment of such patients.

Identification of pathogenicity was of prime importance because of the fact that staphylococci are ubiquitous and normal inhabitant and multiply in the anterior nares, throat, and skin of healthy people in addition to their presence in pyogenic lesion. It is also noted that many strains are relatively harmless in comparison to those isolated from infective lesions.[7]

In the present study, 264 strains of staphylococci isolated from different clinical samples were subjected to coagulase test, out of which 165 strains (62.5%) were coagulase-positive staphylococci. The study of coagulase-positive staphylococci is being compared here.

Workers	*Year*	*Coagulase-positive staphylococci (%)*
Raymond and Traub *et al.*[8]	1970	83.20
Deepak *et al.*[9]	1999	80.3
Majumdar *et al.*[10]	2001	71.2
Rajput *et al.*[11]	2008	60.35
Present study	2009	62.5

From the above observation, it is apparent that in the present study, 165 strains (62.5%) produced coagulase enzyme and remaining 99 strains (37.5%) were coagulase negative by tube method, 160 strains (60.6%) were coagulase positive by slide method. This figure correlated will with the positive staphylococci and 39.64% coagulase-negative staphylococci were observed.

The study of coagulase-positive *S. aureus* in different clinical samples is being compared.

Year	*1999*	*2008*	*2009*
Specimen	Deepak *et al.*[9]	Anuradha *et al.*[11]	Present study
Pus	88.19%	72%	88%
Throat swab	70.5%	73.12%	70%
Aural swab	78%	55.56%	54%
Conjunctival swab	33%	27.27%	28%
Urine	12.5%	15.6%	11%
Vaginal swab	33%	28.57%	31%

In the present study, the rate of occurrence of *S. aureus* in pus was 88%, in urine (11%), and in vaginal swab (31%); this figure correlated will with the study of pathogenic staphylococci by Deepak *et al*.[9] showing rate of occurrence of *S. aureus* in pus (88.19%), in urine (12.5%), and in vaginal swab (33%).

In the present study, the rate of occurrence of *S. aureus* in throat swab was 70%, in conjunctival swab (28%), and in aural swab (54%), this figure correlated with the study of pathogenic staphylococci (2008)[11] showing rate of occurrence of *S. aureus* in throat swab (73.12%), in conjunctival swab (27.27%), and in aural swab (55.56%). Among the procedures available to detect coagulase-positive staphylococci, slide coagulase test advocated by William and Harper[12] is very simple, economical, and easy to perform in comparison to tube coagulase test.[13]

Although simple and in general useful, the slide coagulase test should not be used as a sole technique in the diagnostic laboratory. False-positive results are caused by citrate utilizing bacteria such as enterococci and pseudomonas. Therefore, in doubtful cases, the tube coagulase test should be done and those strains which appear to be pathogenic staphylococci and fails to give slide test positive should be subjected to tube method.[14] Hence, it was concluded that coagulase production should be the single test to distinguish pathogenic strains of staphylococci. However, nowadays, coagulase-negative strains are also found to be responsible for disease production. Breckinridge and Bergdoll described an outbreak of acute gastroenteritis in which responsible organism was enterotoxin producing coagulase-negative staphylococcus.[15]

Mannitol Fermentation

It has been used by many workers to distinguish the pathogenic strains of staphylococci from non-pathogenic one. This test has long been considered an important test in the classification of pathogenic staphylococci. In the present work out of 165 strains of coagulase-positive staphylococci studied, 155 strains (94%) were mannitol fermenter producing acid only and 10 strains (6%) were non-fermenter of mannitol. This figure correlated well to the study of Raymond and Traub[8] who observed 83.20% coagulase-positive mannitol fermenter. Mannitol fermentation is now not considered an important test to differentiate non-pathogenic staphylococci from pathogenic staphylococci because good percentage coagulase-negative staphylococci were found as mannitol fermenter.

Pigment Production

Isolated strain of staphylococci was also observed for pigment production. The study of pigment production by coagulase-positive staphylococci is being compared hereby.

Workers	*Year*	*Pigment-producing staphylococci (%)*
Goyle *et al.*[16]	2002	83
Anuradha *et al.*[11]	2004	93.33
Present study	2009	97

In the present study out 165 strains of coagulase-positive staphylococci studied, 160 (97%) strains of staphylococci produced golden yellow and yellow pigment, 5 strains (3%) did not produce pigment. The present study correlates well with the study of pathogenic staphylococci by Anuradha *et al.*[11] who observed 93.33% of coagulase-positive staphylococci producing golden yellow and yellow pigment and rest 6.67% strains did not produce pigment.

However, pigment production is dependent on many factors. Oxygen is important for pigment production; under anaerobic condition, the growth is colorless. An agar medium containing 33% milk is recommended for its study. The pigment production is optimal at 22 degree C than at 37°C. Thus, it is obvious that optimum temperature for pigment production does not coincide with optimum temperature for growth.[12]

β-Hemolysis on Blood Agar

The study of β-hemolysis on blood agar by coagulase-positive staphylococci is being compared hereby.

Workers	*Year*	*β-Hemolysis on blood agar (%)*
Christie and Keogh[17]	1940	90
Anuradha *et al.*[11]	2004	96.67
Present study	2009	98

In the present study, out of 165 strains of staphylococci studied, 162 (98%) strains of staphylococci produced β-hemolysis on sheep blood agar medium and remaining 3 (2%) strains of staphylococci did not produce clear-cut hemolysis on blood agar plate. The above result correlated well with the study of pathogenic staphylococci by Anuradha *et al*. who observed 96.67% of coagulase-positive staphylococci producing β-hemolysis on blood agar medium. The above result also correlates with a study of Christie and Keogh's[17] who observed that 90% of pathogenic strains produced β-hemolysis on blood agar.

Abramson and Friedman[18] observed on 60 coagulase in hospital environment and noted that 50% of these strains produced hemolysis on sheep blood agar plate. This observation gives clear-cut indication that there is intimate relationship between pathogenicity of staphylococci and hemolysis of sheep RBC. Alpha-toxin has been playing a great role in producing lesions of pathogenic staphylococci. Its main significance in pathogenicity is that of producing tissue damage after the establishment of a focus infection. Schwabacher *et al*.[19] reported that 92.1% coagulase-positive staphylococci produced alpha-toxin. Thus, it was suggested that alpha-lysin production is confined to pathogenic staphylococci which is conveniently demonstrable with rabbit's red blood corpuscles. Barber[20] concluded that alpha-toxin, fibrinolysin, and pigment production probably all play an important part, it is difficult to single out any one of them as essential for pathogenicity and quantitative estimation of any one these cannot give much indication of virulence of the organism. There is evidence to suggest that broad spectrum of toxin production is of more significance than high production of any one.[20]

Phosphatase Production

The study of phosphatase production by pathogenic staphylococci is being compared hereby.

Workers	*Year*	*Phosphatase production by pathogenic staphylococci (%)*
Barber and Kuper[21]	1951	53.15
Anuradha *et al.*[11]	2004	83.5
Present study	2009	95

In the present study, out of 165 strains of staphylococci studied, 157 (95%) strains of pathogenic staphylococci produced phosphatase in the phenolphthalein agar medium and rest 8 (5%) strains did not produce phosphatase. The present study correlates

well with the study of pathogenic staphylococci showing 83.5% strains producing phosphatase followed by the study of Barber and Kuper[21] showing 53.15% of *S. aureus* producing phosphatase.

Gelatin liquefaction test, egg yolk reaction, and serological typing could not be performed due to certain difficulties. However, it has been reported in literature that these tests have no advantage over coagulase test. Similarly, animal pathogenicity test is also not satisfactory and not of much help because human being and laboratory animals react differently to staphylococcal infection.[20] Hence, bacteriology is in favor of accepting coagulase test as the most important indicator of distinguishing pathogenic staphylococci from non-pathogenic strains.

Staphylococcus occurs on the body surface of many species of mammals and appears to be their normal habitat. *S. aureus* is carried in the nasopharynx of some 30% of persons, many of whom are also skin carriers. Majority of staphylococci live harmlessly on the surface of body, some penetrate the skin and cause local damage, and few may invade blood stream and give rise to serious manifestation, especially when resistance of the host is impaired. Skin is an excellent barrier to bacterial infection. Invasion of skin and subcutaneous tissue by staphylococci occur most commonly when the continuity of the epithelium is disrupted. Thus, abrasions, wounds, burns, etc., are particularly prone to become infected by staphylococci.[22] When parenteral injection is performed without attention to aseptic techniques, serious local staphylococcal infection not uncommonly followed by bacteremia and often metastatic abscess occurs.

Conclusion

A total of 500 samples were collected from patients admitted in RIMS out of which in 264 cases (52.8%), staphylococcal species were isolated. All the staphylococcal strains were studied for their morphological and cultural characters, pigment production, beta-hemolysis, mannitol fermentation, coagulase, and phosphatase production.

All the 264 cases of staphylococcal species isolated from different clinical specimens were subjected to coagulase test. It was observed that out of 264 strains of staphylococci isolated from different sites, 165 strains (62.5%) were coagulase positive and 99 strains (37.5%) were coagulase negative by tube method. Out of the 165 strains of coagulase-positive staphylococci, maximum isolation was obtained from pus 74 followed by throat swab 55, aural swab 21, vaginal 4, conjunctival swab 9, and urine 2.

All the 165 cases of coagulase-positive *Staphylococcus* isolated from different clinical specimens were studied for hemolysis, mannitol fermentation, pigment production, and phosphatase production. Out of these 165 strains, 162 (98%) strains produced β-hemolysis on blood agar medium. Pigment production was noted in 160 (97%) of cases. Majority of strains produced characteristic golden yellow pigment on nutrient agar plate. A total of 155 (94%) strains of staphylococci fermented mannitol with the production of acid only. Phosphatase production was observed in 157 (95%) strains of pathogenic staphylococci.

Considering the above-mentioned pathogenicity test, it was observed that coagulase test was the single most reliable test, though coagulase-negative staphylococci are sometimes pathogenic too.

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