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Research article

CHARACTERIZATION OF HOSPITAL ACQUIRED METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS*

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ABSTRACT : Methicillin Resistant *Staphylococcus aureus* (MRSA) strains have emerged as one of the most important nosocomial pathogens. The MRSA can cause a wide range of diseases, which is associated with its production to large number of extracellular toxins and other virulence factors. The diseases are toxic shock syndrome, scalded skin syndrome and food poisoning. Hospital-acquired MRSA (HA-MRSA) in persons who have had frequent or recent contact with hospitals or healthcare facilities within the previous year, has recently undergone an invasive medical procedure, or is immunocompromised. Mostly HA-MRSA are transmitted most frequently through direct skin-to-skin contact or contact with shared items or surfaces that have come into contact with someone else's colonized or infected skin. Panton Valentine Leukocidin (PVL) is a biocomponent toxin has been shown to induce lysis of host defence cells. The absence of the PVL gene confirms the MRSA as HA-MRSA. Slime layer plays a remarkable role in bacterial colonization of exterior surfaces by adhesion and production of slime factor plays an important role in antibiotic resistance. Beta lactamases render bacteria resistant to beta-lactam antibiotics by hydrolyzing the beta lactam ring of penicillin's and cephalosporins.There is a linear correlation between beta-lactamase activity and the level of resistance of bacteria to penicillins. The phage groups II and III were present in hospital acquired MRSA which colonizes on the normal skin and enter the body through cut/wound or by fracture and cause osteomyelitis and bacterial arthritis. Bacteriophage typing of MRSA strains is an epidemiological marker and is a successful method in strain characterization.

Keywords: Hospital, *Staphylococcus aureus*, Methicillin Resistant

MRSA: Methicillin Resistant *Staphylococcus aureus*; HA-MRSA: Hospital acquired Methicillin Resistant *Staphylococcus aureus*; PVL: Panton Valentine Leukocidin

INTRODUCTION

Methicillin Resistant *Staphylococcus aureus* (MRSA) strains have emerged as one of the most important nosocomial pathogens, in the last decade which was initially described in 1961 (Shazia Parveen and Jyothsna, 2011). As early in 1940s itself the Penicillin-resistant strains of *S. aureus* has been appeared, but for years these strains remained susceptible to beta lactamase, and termed as "Methicillin Resistant *S. aureus*" (MRSA), because methicillin was initially used to detect their resistance to betalactamase stable penicillins (oxacillin, methicillin, nafcillin). The strains of MRSA have the ability to cause a broad variety of diseases, ranging from minor infections of the skin to post-operative wound infections (Deurenberg, et. al., 2011), which is associated with its production to large number of extracellular toxins and other virulence factors. It is to be known that these toxins are involved in producing specific diseases such as toxic shock syndrome, scalded skin syndrome and food poisoning. Staphylococcal Scalded Skin Syndrome causes spectrum of blistering skin diseases induced by the exfoliative (epidermolytic) toxins (ET) of *Staphylococcus aureus*.

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They include Ritter's disease, bullous impetigo, pemphigus neonatorum, and staphylococcal scarlatiniform rash. It is a disease primarily affecting infants and young children, but very few cases have been reported in adults. (Shamez Ladhani and Robert W Evans, 1998). Usually MRSA infections have been acquired almost exclusively in hospitals long-term care facilities, or similar institutional settings. Risk factors for MRSA colonization in the hospitals include prior antibiotic exposure, admission to an intensive care unit, surgery, and exposure to an MRSA-colonized patient (Henry F. Chambers. 2001).

The Centre for Disease Control defines hospital-acquired MRSA (HA-MRSA) in persons who have had frequent or recent contact with hospitals or healthcare facilities (such as nursing homes or dialysis centers) within the previous year, have recently undergone an invasive medical procedure, or are immunocompromised. Although HA-MRSA are transmitted most frequently through direct skin-to-skin contact or contact with shared items or surfaces (such as towels or bandages) that have come into contact with someone else's colonized or infected skin. The Panton Valentine Leukocidin (PVL) infections were strongly associated with human primary necrotizing cutaneous infections such as pneumonia and furuncles (Couppie, et. al., 1994; Loffler, et. al., 2010) and 2% of *S. aureus* strains produce PVL (Prevost, et. al., 1995).

The toxins produced by the MRSA strains can be identified by various methods one which is called as Phage typing. Phage typing is a successful method in strain characterization of MRSA strains, which is widely applied in studying staphylococcal infections origin and outbreak (Blair and Williams, 1961). The existence of a particular strain can be identified by phage typing which is spread and cause several severe outbreaks. MRSA produces an extracellular matrix, called slime layer which is made of carbohydrate and protein molecules. The major part of this layer is called as the polysaccharide intercellular adhesion. Due to the over-production of this component the exo-polysaccharides leads to a decrease in metabolic activities of the cells as it can act as barrier to the penetration and diffusion of the nutrients and oxygen (Henriques, 2006). This glycocalixic layer contributes to antibiotic resistance by limiting or preventing antibiotic diffusion (de Allori, 2006). Beta-lactamases are enzymes produced by some bacteria and are responsible for their resistance to beta-lactam antibiotics like penicillins, cephamycins, and carbapenems (ertapenem).

MATERIALS AND METHODS

Collection of Samples

A total of 50 wound swabs were collected from various laboratories in and around the city. All the swab samples were transported to the laboratory within 24 hrs after collection. The specimens were plated in 5% blood agar and Mannitol Salt agar in duplicates and were incubated at 37ºC for 24-48 hrs.

Identification of the isolates

The identification was done by Gram's staining, catalase, DNAse (Cown and Steel, 1974) and coagulase tests. *S. aureus* ATCC 25923 was used as a standard control strain. Further the isolates were confirmed by 16S rRNA sequencing. PCR was performed to amplify the 16S ribosomal RNA was determined by direct sequencing. Total DNA was isolated by using SoluteReady® Genomic DNA purification kit. Agarose gel (2%) was used to isolate 16S rRNA. 16S rRNA was separated by gel electrophoresis on gel made with 2% acrylamide and bis-acrylamide.

Antibiogram

Standard Discs like Gentamicin (30µg), Kanamycin (30 µg), Imipenem (10 µg), Erythromycin (15µg), Norfloxacin (10µg) were used and diffusion test was carried out by the modified Kirby Bauer method (Baur, 1966).

Confirmation of Methicillin Resistant Staphylococcus aureus (MRSA)

Disc diffusion sensitivity test of the isolates were performed with 5µg of Oxacillin discs. According to CLSI recommendation on Mueller Hinton Agar the complete inhibition zone of diameter ≤ 12mm were considered as resistant, those with inhibition zone of ≥ 13mm were susceptible (Cifti, et. al., 2009).

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Confirmation of Hospital Acquired Methicillin Resistant Staphylococcus aureus (HA-MRSA)

PCR was used to detect the PVL gene. Genomic DNA was extracted. The PCR analysis was carried out with a volume of 50µl. The primer sequence of size 151bp was used (Al-Talib, et. al., 2009).

Forward 5'- CAGGAGGTAATGGTTCATTT 3'

Reverse 5'- ATGTCCAGACATTTTACCTAA 3'

Betalactamase Production

A loopful of heavy inoculum of 24 hours old culture from MH agar was mixed well with 1.0 ml Penicillin solution containing 10000 U per ml. The tubes were left for 60 minutes at room temperature, mixing between every 15 minutes. Then 2 drops of 1% soluble starch solution was added followed by one drop of Iodine solution. The tubes are mixed well and the results are recorded as: Instant discoloration: ++++ Strong positive, Discoloration in 1-5 min: +++ Average positive, Discoloration in 6 to 10 min: ++ Moderately positive, Discoloration in 10-15 min: + Weak positive, No discoloration - Negative All test tubes showing discoloration within 10 minutes after adding iodine solution is taken as positive for beta lactamase production. (Sykes and Mathews, 1979).

Slime Layer Production Test

Slime production was detected by the method as described by Jones, et. al.,1992. Pure cultures of the organisms were grown in 5 ml of trypticase soy broth supplemented with glucose 10% w/v and incubated at 35°C for 18 h in glass test tubes in a slanting position. The broth culture was then decanted and the slime layer adherent to the glass surface was stained with 5 ml of saffranin (0.25 g in 100 ml of ethanol 95%). The tubes were allowed to dry before the results were read. When no stained slime or only a trace of film was observed, the isolate was reported as slime negative. Organisms forming an extensive film of slime were designated slime-positive.

Phage Typing

The isolated strains of MRSA were sent to Staphylococcal Phage Typing at Moulana Azad Medical College in NewDelhi.

RESULTS

Isolation and identification of MRSA strains

Among 50 samples collected from different laboratories, only seven isolates were identified and confirmed based on Gram staining, colony morphology and biochemical characteristics (Fig. 1). The species level identification by 16s rRNA analysis also confirm the strains and the results showed that the isolate was *S.aureus* with an identification of 100% similarity and the sequence has been deposited in the GenBank under the Accession Number JQ958397.

Table.1 . Antimicrobial susceptibility of MRSA strains

S.No	Antibiotics	Disc Content	Sensitivity Pattern						
			022	023	03	N1	P8	P9	P11
1.	Oxacillin	5µg	R	R	R	R	R	R	R
2.	Gentamicin	30µg	R	R	I	R	I	I	R
3.	Imipenem	10µg	S	S	S	S	S	S	S
4.	Kanamycin	5µg	S	R	I	S	S	R	S
5.	Erythromycin	10µg	R	S	I	I	R	I	R
6.	Norflaxcin	10µg	S	S	I	S	S	I	S

R- Resistant; I- Intermediate; S- Sensitive

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Antibiogram

Sensitivity tests showed the strains were resistant to Gentamicin and sensitive to Imipenem and Norfloxacin. The zone of inhibition formed was compared with the standard chart by CLSI, 2006.

Confirmation of MRSA

All the isolates were resistant to oxacillin with the zone formation less than 10mm when compared with standard chart by CLSI, 2006.

Confirmation of HA-MRSA

The absence of Panton Valentine Leukocidin gene confirms the isolate as Hospital Acquired Methicillin Resistant *Staphylococcus aureus.* (Fig. 4)

Beta lactamase Production

Three out of seven strains shows positive reaction for beta lactamase production. (Fig.2).

Slime Layer Production Test.

All the strains of MRSA produced a slime layer in tube test. (Fig. 3)

Phage Typing

Only three strains of 7 MRSA strains have turned positive for phage typing. In those, 2 strains belong to group II of phage type 55 and 71. Another strain belongs to group III of type 47 and 84 respectively.

Fig. 1 *S.aureus* on MSA plate

Fig. 2 Beta Lactamase Production

Fig. 3 Slime Layer Production

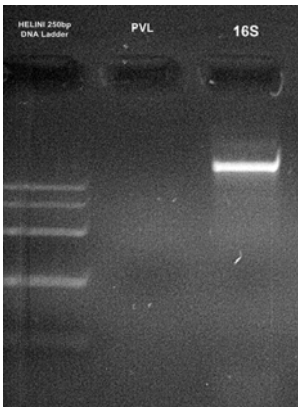

Fig. 4 PVL Gene

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DISCUSSION

Isolation and identification of MRSA strains

The strains of *S. aureus* obtained in this study were coagulase positive. The coagulase is an extracellular substance and not an enzyme (Monica, 2002). *S. aureus* is a major human pathogen which causes wide variety of diseases ranging from minor skin infection to life threatening septicemia, meningitis and toxic shock syndrome (Lowy, 1998). The organism possesses an ability to colonize and exploit the host functions thereby becoming a highly successful and opportunistic pathogen which is difficult to eradicate (Uziel, et. al., 2004).

Antibiogram

Staphylococci are known to have a remarkable genetic versatility, such that many strains of *S. aureus* can be multiresistant to several classes of drugs (Akindele, et. al., 2010). In the field of medicines antibiotics play the role of active controller and occupy an important status in the treatment of infections and diseases caused (Syed Kashif, et. al., 2009)

Confirmation of MRSA

The strains of *S. aureus* tend to be more resistant to antimicrobial agents especially to methicillin (Hussain, et. al., 2000). The strains acquired from the hospitals have become resistant to various other antimicrobial agents (Diekema, et. al., 2001). In the present study, we found clinical isolates were resistant to oxacillin. The epidemiology of MRSA shows the gradual changing since its emergence was reported. Earlier, there were some occasional reports but now it has become one of the established hospital acquired pathogen.

Confirmation of HA-MRSA

The toxin has a cytolytic effect has been described as a crucial marker of virulence (Nathalie and Maria de Lourdes, 2010), since PVL is not found in the isolated strains that cause hospital acquired MRSA infections (Gillet, et. al., 2002).

Beta lactamase Production

Staphylococcus in respect to beta lactamase production is used guide the clinician as a choice of antimicrobial agents (Akindele, et. al., 2010). The increased beta-lactamase activity is seen in the anaerobically grown culture, than the cultures grown under aerobic conditions. There is a linear correlation between beta lactamase activity and the level of resistance of bacteria to penicillins (Richmond, et. al., 1973 and Sykes and Matthew, 1976).

Slime layer production

Slime layer production, is one of the major part of biofilm formation, and this slime layer plays a remarkable role in bacterial colonization of exterior surfaces (O'Gara, et. al., 2001). Since determining slime layer production can lead to the recognition of the biofilm producing staphylococci (de Allori, et. al., 2006 and Stevens, et. al., 2008), evaluating slime production was considered as a key focus (Petrelli, et. al., 2006). The antibiotic resistance may therefore increase the pathogenic potential of opportunist bacteria.

Phage typing

The phage type 55 and 71 which belongs to group II produces staphylococcal bacteriocin to the toxin which is responsible for Staphylococcal scalded skin syndrome, which causes disease newborn mice related to human disease. (Admans S. Dajani, 1972). The phage type 84 which belongs group III causes osteomyelitis. Both these phage groups II and III were present in hospital acquired MRSA which colonizes on the normal skin and enter the body through any cut on the skin/ wound or by fracture and cause osteomyelitis and bacterial arthritis (Bhattacharya, et. al., 1972 and Sourek, et .al., 1979).

Bacteriophage typing of MRSA strains is an epidemiological marker. The existence of particular epidemic strain spread and outbreak can be identified only by Phage-typing (Hanifah, 1991). Phage-typing is a useful technique and is one of the successful method in strain characterization of S. aureus other the antibiogram, coagulase typing and plasmid typing (Hanifah, 1990).

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CONCLUSION

The *S. aureus* which was initially isolated in early 1880s, have became a threat in the last decade, as some strains have become a multi-drug resistant and named as Methicillin Resistant *Staphylococcus aureus* which have been isolated in 1960s, has the capability to resist the activity to broad range of antibiotics including penicillin. Various antibiotics have been used to confirm the isolates for their Methicillin resistance activity. The slime layer producing ability and production of betalactamases the organisms shows that the strains have the capacity to produce infection which increases the mortality rate of humans and animals widely, which was again confirmed by the phage typing test. Currently the infection caused by MRSA has been increased gradually and the mortality rate increases if left untreated.

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