INTERNATIONAL RESEARCH JOURNAL OF PHARMACY
www.irjponline.com
ISSN 2230 – 8407

Research Article

DETECTION OF METHICILLIN RESISTANCE GENE IN *S. AUREUS* BY PCR

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Article Received on: 28/04/19 Approved for publication: 12/05/19

DOI: 10.7897/2230-8407.1005190

ABSTRACT

Background: *Staphylococcus aureus* is a serious pathogen responsible for nosocomial and community acquired infections. It is leading cause of health-care associated infections globally. Methicillin was developed to overcome penicillin resistant *S. aureus* but by 1961 Methicillin resistant *S. aureus* (MRSA) were identified in UK. MRSA strains developed due to integration of 21-67 Kbp mobile element called as staphylococcal cassette chromosome mec (SCCmec) into its genome, which harbours the methicillin resistance (mecA) gene. Material and Methods: Known MRSA and MSSA cultures were received from Dr. Y. Patil Medical College, Pune. These organisms were isolated from patient samples like blood, pus, urine, wound swabs and other body fluids. They were characterized and confirmed to be *S. aureus* based on conventional microbiological methods like Gram staining, coagulase test and growth on mannitol salt agar. The cultures were segregated as MRSA and MSSA. Total 80 PCRs were carried out for detection of mecA gene from MRSA cultures using genomic DNA and 20 from MSSA cultures. Result: All microbiologically identified MRSA gave amplification of 147 bp Amplicon of mecA gene whereas; all MSSA did not show amplification of mecA gene. Conclusion: PCR based tool has advantage over earlier used molecular techniques since Rapid detection of MRSA is critical as it helps in faster patient care and also minimizes the transmission.

Keywords: *Staphylococcus aureus,* MRSA and MSSA, mecA gene

INTRODUCTION

Staphylococcus, the genus composed of nearly 24 species associated with humans or animals. Out of these species, commonly observed commensally bacteria and human pathogen is *Staphylococcus aureus*. It is frequently colonized on the skin and mucous membranes and can be identified by bacteria specific coagulase test. It is a leading cause of bacteremia and Infective endocarditis as well as osteoarticular, skin and soft tissue, pleuropulmonary and device related infections[1]. Extracellular enzymes and toxins produced by different strains of *S. aureus* causes food poisoning, Toxic shock syndrome, Scalded skin syndrome.

Recently, *S. aureus* has been reported to be second only to *Clostridium difficile* as a cause of healthcare associated infections in the United States[2]. Development of antibiotic resistance in *S. aureus* is a serious concern for tissue or organ specific infections. Development of antibiotic resistance in *S. aureus* is a serious concern for tissue or organ specific infections. Numerous anti-staphylococcal agents exist, including linezolid, daptomycin, tetracyclines and fluoroquinolones, but these are rapidly becoming of less value due to the ability of the bacterium to develop efficient mechanisms to neutralize this agents[3]. Designing a strategy for detection and treatment of healthcare associated and community associated Methicillin Resistant *Staphylococcus aureus* (MRSA) is the biggest challenge in medical microbiology.

The development of resistance to many antibiotics by *S. aureus* involves chromosomal or plasmid mediated β-lactamases. Resistance also can emerge by mutations that alter the drug binding sites on molecular targets and by increasing expression of endogenous efflux pumps[4]. In 1940s, penicillin, a β-lactam antibiotic was introduced to treat staphylococcal infections. Mechanism of resistance is the synthesis of β-lactamase enzyme by blaZ gene which is located on transposable element of large plasmid. Due to penicillin resistance in bacteria, methicillin, semi synthetic penicillin was used for the treatment which was followed by reports of Methicillin resistant *Staphylococcus aureus* (MRSA).

The mecA gene (the gene responsible for methicillin resistance) is part of a mobile genetic element found in all MRSA strains. In 2000 a study[5], demonstrated that mec A is part of a genomic island designated staphylococcal cassette chromosome mec (SCCmec). To date, five different SCCmec elements varying in size from 21 to 67 kb have been characterized and they are known to integrate at the same time in the chromosome by a mechanism called site specific recombination. Each SSCmec has different degree of drug resistance. SSCmec II and III are found in many nosocomial MRSA, whereas CA-MRSA harbors SSCmec IV which carries mecA gene. Mec A gene expresses protein called altered penicillin binding protein (PBP 2a). When this protein is changed, the β –lactam antibiotic cannot prevent cell wall formation and hence the resistance to the antibiotic is seen[4].

In contrast to the numerous different strains of methicillin-susceptible *S. aureus* (MSSA) that cause infections, only a limited number of clones are responsible for the epidemic spread of MRSA. This distinction reflects the genetic constraints of horizontal transfer of the mec element from related staphylococcal species into *S. aureus*[3].

Methicillin-resistant *Staphylococcus aureus* (MRSA) is present in the hospitals of most countries. Clinical infections are most common in patients in intensive care units and in high-risk wards.

Colonization frequently occurs in elderly patients in long-term care wards.

In the United States, the National Health Care Safety Network estimates that hospitalized patients acquire 2 million health care-associated infections (HAI) per year, of which MRSA infections are known to make up a large percentage[6]. Established risk factors for MRSA infection includes recent hospitalization or surgery, residence in a long term care facility, dialysis and indwelling percutaneous medical devices and catheters[7]. It can create wide range of complications like septicaemias, bacteraemia, endocarditis, pneumonia and even toxic shock syndrome. Vertebral complications are also reported which include vertebral osteomyelitis, spondlodiscitis and epidural abscess[8].

CA-MRSA (Community acquired) strains tend to be more susceptible to non-ß lactam agents when compared with the hospital-acquired MRSA isolates. Many CA-MRSA have acquired the Panton-Valentine leukocidin (PVL) gene that produces a series of chemicals contributing to the invasiveness of these MRSA strains. PVL is a multi component protein cytotoxin which belongs to the pore-forming toxin family that forms an octameric pore in the affected membranes of human polymorphonuclear neutrophils (PMNs), macrophages and monocytes[9].

Nowadays, medical researchers follow both traditional microbiology methods and molecular methods to determine methicillin resistance. Methicillin resistance of *S. aureus* strains is confirmed using the Kirby–Bauer disc diffusion with 1 mg oxacillin discs[10] [Clinical and Laboratory Standards Institute (CLSI), 2012]. In comparison with previous molecular detection methods, such as Southern blotting and pulsed-field gel electrophoresis (PFGE), PCR assays such as multiplex-PCR (M-PCR), real time PCR, hyper variable region (HVR) and spa-typing techniques can provide a rapid amplification, detection and typing tool for MRSA strains[11]. Staphylococcal cassette chromosome mec (SCCmec) mobile element can be easily detected by PCR into types and subtypes which helps to understand molecular epidemiology of community acquired MRSA[12].

This work was mainly focused on using molecular method (PCR) to detect presence of mecA gene in community acquired MRSA isolated from clinical samples. Rapid detection of resistant bacteria in infected individual of Indian population will definitely direct the revision in treatment strategies.

MATERIAL AND METHODS

Morphological and biochemical confirmation

Morphological and biochemical confirmation of Methicillin resistant and sensitive *S. aureus* cultures isolated from clinical samples provided by medical college was done by Gram Staining, Coagulase test and growth on selective medium like Mannitol salt agar using standard protocols.

Genomic DNA isolation

Overnight grown pure cultures of MRSA and MSSA were subjected to DNA isolation. Genomic DNA from all MRSA (80) and MSSA (20) isolates was isolated by following 2009 a protocol described in 2009[11].

PCR for mecA gene

Primers for mecA gene, designed[12] previously in 2005 were procured from Sigma. 80 ng of isolated genomic DNA was used as template. PCR conditions were also exactly followed and the amplified product was visualised on a 1.8 % agarose. Negative control was set up with same components except the Template DNA was not added.
Forward Primer – 5’ GTG AAG ATA TAC CAA GTG ATT 3’
Reverse Primer - 5’ ATG CGC TAT AGA TTG AAA GGA T 3’

RESULTS

Morphological and biochemical confirmation of the isolates

Gram’s staining showed presence of positive (purple) cocci in clusters. In coagulase test *S. aureus* gave clumping of the plasma whereas negative control which had saline mixed with plasma gave no clumping reaction. Phenol red indicator showed change in color which can be attributed to mannitol fermentation while the growth of these organisms at the high salt concentration present in this agar indicates presence of Gram positive *Staphylococcus aureus*.

Amplification of mecA gene by PCR

Amplicon of mecA gene (147bp) was obtained by using genomic DNA MRSA. As is seen in Figure 2, the amplicon size is right when compared with 100 bp DNA ladder on 1.8 % agarose gel. This amplicon was used subsequently as a reference to compare the amplification of mecA gene from gDNA of MRSA and MSSA cultures isolated from clinical samples.

Confirmation of MRSA by PCR (mecA gene in genomic DNA of MRSA)

When genomic DNA was used as a template for PCR, the mecA gene (147 bp) was detected as the primers used in the PCR were mecA specific. Interestingly, all the MRSA could be also confirmed by molecular characterization using mecA as a marker gene. This band of 147 bp was detected in all MRSA isolates is depicted in Figure 3.

Confirmation of MSSA by PCR: Absence of mecA gene amplification

As seen in all panels of Figure 4, when mecA gene specific primers were used to amplify the gene from genomic DNA of MSSA cultures isolated from clinical samples, the amplicon was not obtained, only primer dimmers were seen. Positive control used in the reaction was control MRSA strain which could give amplicon of right size indicating that the PCR reaction is giving right size amplicon and absence of amplicon when MSSA gDNA is used as template indicates true absence of gene.

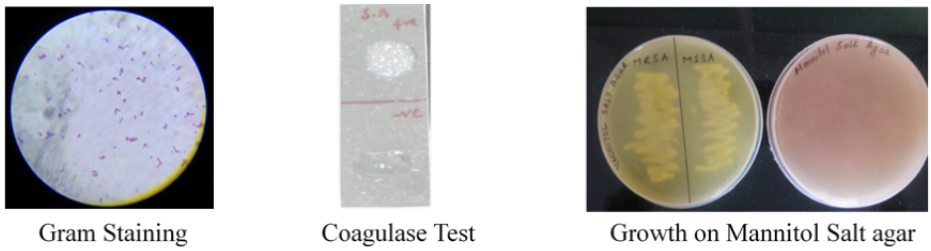

Figure 1: Morphological and biochemical confirmation of the isolates

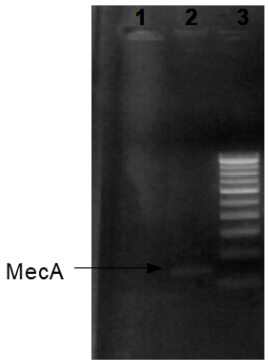

Figure 2: Amplification of *mecA* gene from genomic DNA of MRSA
Lane 1: Non template control, Lane2: MecA amplicon (147bp), Lane3: 100bp Ladder

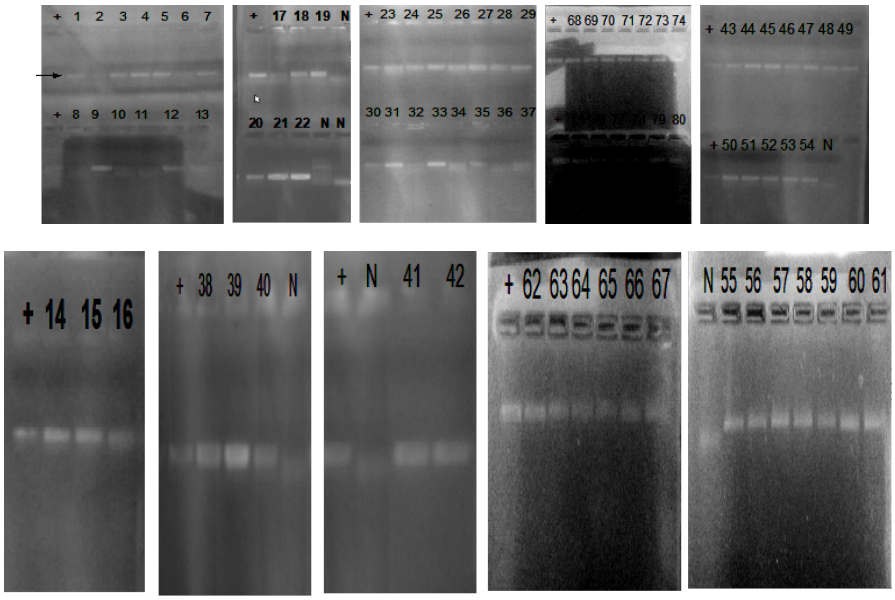

Figure 3: Amplification of MecA gene conferring methicillin resistance
+: MecA amplicon from control MRSA strain, N: Non Template control, 1-80: MecA Amplicons from MRSA (gDNA/colony) isolated from patient samples

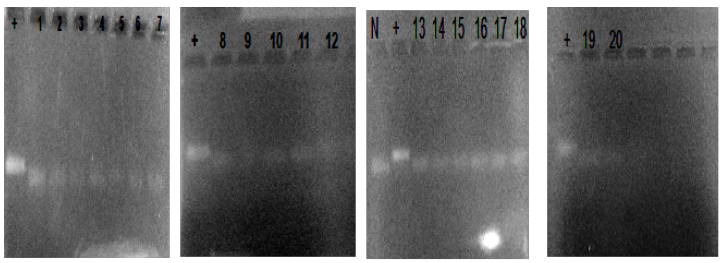

Figure 4: Amplification of *mecA* gene from MSSA cultures isolated from patients
+: *mecA* amplicon from control MRSA strain, N: Non Template control, 1-20: *mecA* PCR reaction loaded where MSSA gDNA is used as template

DISCUSSION

Considering the advancement of antimicrobial therapy, pathogens like *S. aureus, S. pneumoniae, P. aeruginosa, and M. tuberculosis* have emerged as a challenge to clinical physicians. Resistance mechanisms developed by these bacteria had reduced efficacy for antibiotics. Among these group of bacteria *S. aureus* is opportunistic pathogen commonly found on the skin and mucous membrane of human. Epidemiology of pathogenic *S. aureus* has wide range such as bacteremia and infective endocarditis (IE) as well as osteoarticular, skin and soft tissue, pleuropulmonary, and device related infections[1]. Resistance developed by this organism to penicillin in 1940's and then for methicillin in 1960's immediately after its introduction had initiated research work for the diagnosis and treatment for resistant strain.

Researchers have found out the mechanisms of resistance developed by bacteria, for penicillin, it was synthesis of β-lactamase and for methicillin, it was synthesis of altered penicillin binding protein[4]. Current study was focused on Methicillin Resistant *Staphylococcus aureus* (MRSA) with two broad types of bacterial isolates i.e. community-acquired and hospital acquired (CA- MRSA and HA- MRSA). Some key factors were examined to conclude Health care–acquired MRSA cases like MRSA infection identified after 48 hours of admission to a hospital; a history of hospitalization, surgery, dialysis, or residence in a long-term care facility within 1 year of the MRSA culture date; a permanent indwelling catheter or percutaneous medical devices present at the time of culture and a known positive culture for MRSA prior to the study period. For community-acquired cases, absence of above mentioned factors were examined by medical record review of patients[7].

MRSA is a major pathogen involved in both hospital, associated and community acquired infections. Rapid detection of MRSA is critical as it helps in faster patient care and also minimizes the transmission[13]. Various molecular methods for detection of MRSA have been established. PCR based tool could have advantage over earlier used molecular techniques such as southern blotting and Pulse Field Gel Electrophoresis so it was applied in this study[11].

It has been known that MRSA strains have integrated into its genome a 21-67 Kb mobile element called as staphylococcal cassette chromosome mec (SCCmec), which harbours the methicillin resistance (mecA) gene[12]. According to CLSI guidelines MRSA can be confirmed by detection of mecA gene. PCR based amplification of mecA is commonly used for detection of MRSA[14]. FDA approved PCR methods[6] have sensitivity in the range of 82 to 100 %. Molecular detection of mecA gene shortens the detection period as compared to conventional methods but later method is clinically and technically validated.

In the current study, we could get 100 % specificity for mecA based PCR. All the samples which were confirmed by microbiological methods to be MRSA could also give amplification of mecA gene. There were no false positive or negatives observed. Even the cultures which were confirmed by conventional methods to be MSSA did not give mecA amplification confirming the specificity.

This shows that the detection of mecA is robust. However, the study[6] mentions that there are many commercially available kits for molecular detection of MRSA but the specificity is less than 100 %. They have also mentioned that there are some populations where there is mecA drop out from SSCmec and it is been attributed to regional differences. It is hence necessary to study large population.

A study[14] identified a novel multi locus fluorescence based PCR where mecA and nuc gene were detected simultaneously. The specificity of this PCR was shown to be over 96 % if PCR copy number reaches certain threshold. Apart from mecA amplification, scientists have used other genes like PVL, nuc, femA along with mecA in multiplex PCR. It is claimed that this improves the sensitivity and specificity for detection of MRSA. There are many commercially available PCR kits by companies like BD diagnostics, Roche and Cepheid which are approved by FDA[6]. PCR detection of MRSA definitely gives advantage over traditional methods in terms of time and robustness. A group of researchers[12] have even reported a multiplex PCR method to concomitant sub typing of SSCmec in MRSA. They have characterized MRSA into I to V subtypes.

Fast and rapid detection of MRSA is extremely important for patient treatment. Various PCR based diagnostic methods have already been tried by different scientists. Even the commercial kits are available to carry out this detection. The critical point to be considered is use of multi locus amplification for the detection of MRSA[15,16]. MRSA is also showing multiple changes. There are reports of excision of mecA gene from SSCmec. Hence to avoid false negative cases it is important to develop multiplex PCR detecting multiple genes of MRSA as markers which will improve the specificity of this rapid detection.

CONCLUSION

HA-MRSA and CA-MRSA are known to be serious bacterial infections. MRSA presence in different tissues as well as body fluids of human body causes multiple complications in health. Traditional microbiological methods to detect MRSA are well established but are time consuming and hence pose a challenge during patient treatment. Molecular method like PCR for the detection of mecA gene (for methicillin resistance) in bacterial genomic DNA, have been attempted earlier. In the current study the specificity of this method was tested using known cultures of MRSA and MSSA. Optimized PCR conditions for mecA gene amplification gave expected size amplicon of 147bp. The specificity of the method in the current study was found to be 100 % in case of both MRSA and MSSA detection. There were no false positive or negative isolate. The data matched precisely with the conventional microbiological observations. This method has definite time based advantage over traditional diagnosis of MRSA infections. A time gap between symptoms and diagnosis can be reduced which further support for treatment procedures. Application of molecular methods will result in better future in healthcare sector.

ACKNOWLEDGEMENT

Authors are thankful to Dr. D. Y. Patil Arts, Commerce & Science College, Pimpri, Pune, India, for providing facilities available at their laboratory.

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Cite this article as:

Suneeta Panicker *et al*. Detection of methicillin resistance gene in *S. aureus* by PCR. Int. Res. J. Pharm. 2019; 10(5):200-204 http://dx.doi.org/10.7897/2230-8407.1005190

Source of support: Nil, Conflict of interest: None Declared