

Amplification Based Detection of Four Target Genes of Staphylococcus Isolates by Multiplex PCR

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Abstract

Original Research Article

Background: MRSA infections have become a global health problem. The community acquired MRSA has been increasingly reported. Hence characterization of these strains is important to prevent the spread of infections in hospitals and community. **Aim:** The present study evaluated the amplification based detection of four target genes of Staphylococcus isolates by multiplex PCR. **Method:** A total of 100 exudate samples from suspected cases of Orthopaedic infections were collected. Majority of the isolates were Staphylococcus, which were identified and speciated based on cultural characteristics and biochemical reactions and coagulase test. Antibiogram was done by Kirby Bauer disc diffusion and Methicillin resistance by cefoxitin disc diffusion method. Amplification based detection of four target genes of Staphylococcus isolates was done by multiplex PCR. **Result:** Out of total 100 samples processed. 27 Gram positive cocci were isolated. Out of 27, 23 were Staphylococcus aureus, 4 were CONS. Out of all staphylococci isolated (27), 20 Staphylococcus aureus and 1 out of 4 CONS showed methicillin resistance. Multiplex PCR confirmed the results as Genus staphylococcus by the presence of 16SrRNA (100%), species by the presence of femA (95.8%), methicillin resistance by the presence of mecA (87.5%) and community acquired MRSA by the presence of luk PVL (40%). **Conclusion:** Multiplex PCR assay was found to be rapid and accurate procedure for confirmation of genus, species, methicillin resistance and community acquired strains. Hence multiplex PCR is considered as Gold standard.

Keywords: MRSA, CA-MRSA, Multiplex PCR, Staphylococcus aureus.

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INTRODUCTION

Infection is a major problem in orthopaedic cases particularly in developing countries. Bones and joints are normally sterile areas. Bacteria may reach the sites by haematogenous spread or endogenous contiguous focus of infection [1].

The incidence of nosocomial infections caused by MRSA continues to increase worldwide, and therefore the importance of their detection, especially for therapeutic and epidemiological purposes arises [2] Methicillin resistant *S. aureus* (MRSA) is now endemic in India. The incidence of MRSA varies from 25 per cent in western part of India [3] to 50 per cent in South India [4]. Community acquired MRSA (CA-MRSA) has been increasingly reported from India [5]. In India, high rates of MRSA have been reported in clinical isolates from various studies with rates as high as 54.8%

(ranging between 32% and 80% among the *S. aureus* pool).

MATERIALS AND METHODS

A total of 100 exudate samples were collected from orthopaedic patients

CLINICAL ISOLATES

Ethical clearance was obtained prior to the start of the study. After obtaining an informed consent clinical samples were collected from patients attending orthopaedic department, Osmania general hospital, Telangana, India.

SAMPLE COLLECTION

1. Pus samples collected from skin and soft tissue infections, non traumatic infective cases, Post operative infections.

2. Aspirated fluid.
3. Sequestrum from Osteomyelitis case.

3 swabs were collected from each patient. One for Gram stain, another for culture onto Nutrient agar, 5% sheep blood agar and MacConkey agar. The third swab was inoculated into BHI broth. The plates were incubated at 37⁰ C for 18-24 hours aerobically and examined for the growth of bacteria. All positive cultures were identified by their characteristic morphological appearance on their respective media. Gram staining was done. Identification of the pathogen was done by standard biochemical reactions. If no growth was observed on the plates, subcultures from the Brain Heart infusion broth were inoculated onto 5% sheep

Samples showed the following colony characters

On Blood agar: colonies are large (2-4mm) in diameter circular, convex smooth and opaque colonies were observed with β -haemolysis.

On Nutrient agar: The colonies are similar to those on blood agar with golden yellow pigmentation.

On MacConkey agar

Small pink coloured colonies due to lactose fermentation were observed.

These colonies were tested for coagulase production, Mannitol fermentation, Phenolphthalein phosphatase production, β haemolysis on blood agar and golden yellow pigment on Nutrient agar



Golden Yellow Pigmentation on NA

Antibiotic susceptibility test

Done on Mueller -Hinton agar using cefoxitin 30mcg disc incubated overnight at 35⁰ C for detection of MRSA according to the CLSI guidelines.

Detection of four target genes by multiplex PCR

All the isolated Gram positive cocci were subjected for genotyping at SRRITCD (Fever hospital), Nallakunta, Hyderabad, Telangana, India blood agar and MacConkey agar.

Bacterial Genomic DNA Extraction

HiPurA[®] Bacterial Genomic DNA Purification Kit. The isolation of DNA from bacteria is done by the spin-column procedure. Bacterial cells are grown in a nutrient broth till they reach log phase (8 hours after subculture) and are harvested by centrifugation. After harvesting, the bacterial cell wall is degraded by lysozyme and Proteinase K. (provided in the kit). Following lysis, the DNA is bound to the silica-gel membrane of the HiElute Miniprep Spin Column (Capped) to yield approximately upto 20 μ g of pure DNA. Two rapid wash steps done to remove traces of salt and protein contaminants resulting in the elution of pure DNA in the Elution Buffer.



Tube Coagulase



Mannitol Fermenting Colonies on MSA

HiElute Miniprep Spin Column

It is based on the advanced silica binding principle presented in a micro spin format. The system efficiently couples the reversible nucleic acid binding properties of the advanced silica gel membrane and the speed plus versatility of spin column technology to yield high quantity of DNA. The use of spin column facilitates the binding, washing, and elution steps thus enabling multiple samples to be processed simultaneously. This column eliminates the need for alcohol precipitation, expensive resins, and harmful organic compounds such as phenol and chloroform,

otherwise employed in traditional DNA isolation techniques. DNA binds specifically to the advanced silica-gel membrane while contaminants pass through. PCR inhibitors such as divalent cations and proteins are completely removed in two efficient wash steps, leaving pure nucleic acid to be eluted in the buffer provided with the kit. The purified DNA is upto 20-30 kb in length and can be used for further downstream applications.

GENOTYPIC CHARACTERISATION

Genotypic confirmation of S.aureus and methicillin resistant S.aureus was done by Multiplex PCR

Methicillin Resistant Staphylococcus aureus (MRSA) detection kit (Multiplex) (HIMEDIA) Principle

The Methicillin Resistant Staphylococcus aureus (MRSA) PCR Detection kit (Multiplex) is used for simultaneous detection of four targets

,Staphylococcus genus (16S rRNA gene), discrimination between S. aureus and Coagulase negative Staphylococci-CoNS (femA gene) methicillin resistant staphylococci (mecA gene) and community acquired MRSA (lukPVL gene).

PCR Procedure

1. 25 ul of 2X PCR Master mix added in a PCR tube.
2. In the same tube 16 ul of MRSA primer mix and
3. 3-5 ul of template DNA (upto 50 ng of extracted DNA) was added.
4. The nuclease free water is added to make the final volume to 50ul.
5. Then tube was centrifuged briefly at 6000rpm for about 1 sec.
6. Then tubes were placed in PCR machine and PCR program was adjusted.

Table-1: Distribution of Gram positive isolates

Step	Temperature (0c)	Time (minute :second)	No of cycles
Initial denaturation	94	03:00	1
Denaturation	94	00:30	30
annealing	56	00:30	30
extension	72	00:30	30
Extra Annealing	60	00:30	1
Final extension	72	05:00	1
Post run	4	Hold	-

Positive control: This is a control reaction using a known template (target pathogen). A positive control is usually used to check that the primers have been designed properly and the PCR conditions have been set up correctly.

Polymerase Chain Reaction (PCR) is a very sensitive and specific method for amplification based detection of target genes. The three steps of a successful PCR reaction include Denaturation, Annealing and Extension. The double-stranded DNA melts and forms single stranded DNA at high temperature (Denaturation). Sequence-specific primers bind to the target sequence on single-stranded DNA at lower temperature (Annealing). Taq DNA Polymerase adds dNTPs onto the single stranded DNA at intermediate temperature (Extension). These 3 steps of PCR are usually repeated upto 30 to 40 times in each PCR assay.

PCR products were visualized in gel electrophoresis and documented in Gel documentation system.

RESULTS

The present study was a cross sectional study conducted over a period of 2 years from August 2017 to September 2019. The total samples studied were 100.

Staphylococcus were isolated and antibiotic sensitivity was done by Kirby bauer disc diffusion method and phenotypic resistance was determined by cefoxitin disc (30mcg) diffusion method genotyping was done by multiplex PCR.

Of the 100 samples, 27 Staphylococcus were isolated. 23 were Staphylococcus aureus and 4 were CONS. 20 isolates were MRSA (inhibition zone of <21mm).

Antibiotic sensitivity pattern showed all the MRSA were 100 % sensitive to vancomycin and Linezolid.

Table: Distribution of Gram Positive Isolates (27)

Staphylococcus aureus	23
Coagulase negative staphylococci	4

NUMBER OF MRSA AND MSSA ISOLATES AMONG STAPHYLOCOCCUS AUREUS BY CEFOXITIN DISC DIFFUSION TEST

MRSA	20
MSSA	3

AMPLIFICATION BASED DETECTION OF 4 TARGET GENES OF STAPHYLOCOCCUS BY MULTIPLEX PCR

The kit used in the study is a Multiplex PCR Detection kit which is a qualitative conventional PCR which simultaneously amplifies four targets.

16SrRNA-universally present in all Staphylococcus genus (genus specific)

fem A -The fem A determinant is a unique feature of S. aureus .Not found in other Staphylococci . This gene is present in Staphylococcus aureus and absent in CONS.

mec A -molecular marker of methicillin resistance in all Staphylococci. mec A gene is present in MRSA and absent in MSSA.

lukPVL gene -present in Community acquired-MRSA(CA-MRSA) and absent in Hospital acquired MRSA(HA-MRSA).

The amplified target is confirmed by using 1.8% agarose gel electrophoresis. kit also contained the positivecontrol. GEL ELECTROPHORESIS IMAGE OF AMPLIFIED PRODUCTS ladder samples 1-11, NC, PC, genes in order from top are 16SrRNA,femA,mecA,lukPVL gene.

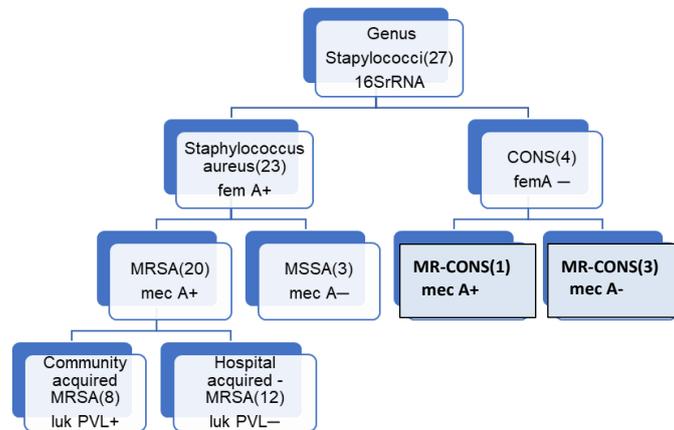
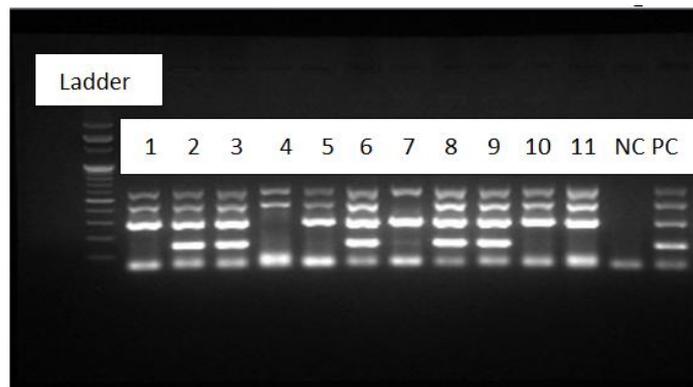


TABLE 2: RESULTS OF CEFOXITIN DISC DIFFUSION AND MULTIPLEX PCR AMPLIFICATION OF mec A AND femA

Organisms	Cefoxitin DDT	No of isolates tested	mecA Positive	mecA Negative	fem A Positive	fem A Negative
S. aureus	S	3	NIL	3	3	
	R	20	20	NIL	20	
CONS	R	1	1			1

S-susceptible , RResistance

TABLE 3: DISTRIBUTION OF lukPVL GENE IN PCR :

ORGANISM	lukPVL positive	lukPVLnegative	TOTAL
<i>S.aureus</i> (20)	8	12	20

In the present study, out of 20 MRSA, 8 samples showed presence of

lukPVL gene i.e Community acquired MRSA

DISCUSSION

Infections are known to occur inspite of aseptic precautions by the Orthopaedicians.

A total of 100 samples were collected from clinically infected Orthopaedic cases and were processed according to the standard operating procedures.

Staphylococcus were isolated and antibiotic sensitivity was done by Kirby bauer disc diffusion method and phenotypic resistance was determined by cefoxitin disc (30mcg) diffusion method.

Amplification based detection of four target genes specific for genus of Staphylococcus, species, resistance and identification of community acquired MRSA (CA-MRSA) were done by Multiplex PCR.

The Results of the Study Are Discussed Below

In the present study, 20 MRSA (86.9%) were isolated out of 23 Staphylococcus aureus which is close to the study done by Gupta *et al.*, [6] (75%), Mahesh *et al.*, (75%), Golia *et al.*, (75%), Preist *et al.*, (72.5%), Jyothi *et al.*, [7] (71.42%)

In our study, all the MRSA isolates showed 100% sensitivity to vancomycin and linezolid. Which is close to the study done by Gupta V *et al.*, [6], MRSA also showed 90.9% sensitivity to doxycycline and clindamycin (90.90%).

Among four coagulase negative staphylococci isolated only one CONS isolate is methicillin resistant, which showed 100% sensitivity to vancomycin and linezolid.

44.44% of the isolates were methicillin resistant Staphylococcus aureus. This percentage of MRSA is very much close to the studies of Arora S *et al.*, [8] (46%) Sangeetha Joshi *et al.*, [9] (42%), Anila A Mathews *et al.*, [10] (34.09%) Priest D H *et al.*, (32.5%)

Results of amplification based detection of four target genes of Staphylococcus by Multiplex PCR

All the Staphylococcus aureus and CONS were included in genotyping.

Presence of 16SrRNA gene confirmed all the isolated samples as the genus Staphylococcus.

Presence of femA gene confirmed 23(100%) isolated Staphylococci as Staphylococcus aureus. Absence of femA gene in one isolated Staphylococcus confirmed it as CONS.

Presence of mecA gene in 20 Staphylococcus aureus and four CONS confirmed them as methicillin resistant. Absence of mecA gene in rest of the three Staphylococcus aureus confirmed them as methicillin sensitive.

Presence of lukPVL gene confirmed 8(40%) MRSA samples as community acquired MRSA. Absence of lukPVL gene confirmed rest of the 12 MRSA samples as hospital acquired MRSA.

CORRELATION OF CEFOXITIN DISC DIFFUSION TEST WITH GENOTYPIC mecA DETECTION

In the present study the results of Cefoxitin disc diffusion test and multiplex PCR are correlating with the studies done by Kunsang O bhutia *et al.*, [11], Mathew A A *et al.*, [10], Anand K B [12], Bosgelmez-Tmaz G *et al.*, (2006), Pascal Vannuffel *et al.*, [13], Gabriella Septiani Nasution *et al.*, and Sajith Khan AK *et al.*

In the present study it is interesting to note that 20 out of 23 Staphylococcus aureus were MRSA by cefoxitin disc diffusion method .all 20 showed presence of mecA gene.

Phenotypic expression of mecA gene may be altered depending on the growth conditions for *S. aureus*, such as temperature or osmolarity of the medium, and this may affect the accuracy of the methods used to detect methicillin resistance.

Though conventional culture technique for MRSA diagnosis is common and inexpensive, such techniques are time consuming, taking 48-72 hrs and standardization is difficult.

Results of multiplex PCR

In the present study presence of femA and mecA corelated 100% with the studies done by Pascal vannuffel *et al.*, [13], Hassanain Al-Talib *et al.*, [14], Moussa *et al.*, [15], Perez-Roth *et al.*, [16], Charles Emeka Okolie *et al.*, [17] .

In the present study 40%were community acquired strains which corelated with the studies of Bocher *et al.*, (47%) and Moussa *et al.*, [15] (37.6%).

CONCLUSION

MRSA is the major organism isolated in the present study. As there is heterogenous expression of MR, and alteration in phenotypic expression of *mecA* depending on growth conditions, multiplex PCR being gold standard has overcome all these disadvantages. Simultaneous detection of *femA* and *mecA* genes in the same PCR tube has the added advantage of identifying both species and its phenotypic resistance. PCR has an advantage of differentiating CA-MRSA (*lukPVL* gene) from HA-MRSA. Hence multiplex PCR is a rapid, accurate, reliable and sensitive method for detection of MRSA and used for appropriate therapeutic management of patients in the hospital and community.

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