COMPARISON OF RAPID METHOD OF DNA EXTRACTION USING MICROWAVE IRRADIATION WITH CONVENTIONAL PHENOL CHLOROFORM TECHNIQUE FOR USE IN MULTIPLEX PCR FOR *mec A* AND *fem B* GENES TO IDENTIFY GENOTYPES OF MRSA FROM CULTURES

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Abstract

Methicillin Resistant *Staphylococcus aureus* (MRSA) infection is a major cause of morbidity and mortality in hospitalised patients and requires vancomycin for effective therapy. Rapid identification of MRSA is vital to control MRSA outbreaks in hospitals. Identification of MRSA is a time consuming process requiring more than 48 hours and is labour intensive involving culture, biochemical tests and antimicrobial susceptibility testing. In this study we have used microwave irradiation of the bacterium obtained from cultures which was then directly subjected to a multiplex PCR technique to accurately and rapidly identify the presence of *mec A* and *fem B* genes which characterise MRSA. This has been compared with the standard method of lysing the bacterium and DNA extraction using phenol chloroform method followed by multiplex PCR. The microwave lysis method followed by direct PCR has been found to be less time consuming, 5 hours, as compared to 9 hours by conventional technique. Use of this strategy would enable early identification and early implementation of control measures.

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KEY WORDS: Microwaves; MRSA; Multiplex PCR; Rapid diagnosis.

Introduction

Methicillin-resistant strains of *S aureus* (MRSA) cause life-threatening infections. MRSA are highly resistant to antibiotics and are only sensitive to vancomycin or teicoplanin. Incorrect or missed identification of MRSA results in inappropriate or ineffective treatment of infections. MRSA with reduced susceptibility to vancomycin have also emerged [1]. On the basis of evidence from countries where MRSA is not a problem, it has been suggested that early detection, effective infection control measures, and rational antibiotic use will limit the transmission of these organisms; however, spread is still increasing in many countries [2]. Multiplex PCR for rapid identification, of the *mec A* gene (conferring methicillin resistance) and *fem B* or *fem A* genes (constitutive gene of coagulase positive *Staphylococcus*) have been described [3,4,5]. They involve a time consuming DNA extraction step. In this study we have standardised a protocol involving lysing the bacteria by microwave irradiation and directly carrying out multiplex PCR without DNA extraction, thus saving both time and labour.

Material and Methods

20 cluster forming Gram positive cocci were obtained in pure cultures from routine clinical samples on blood agar. The colonies

were examined by conventional tests for slide coagulase, tube coagulase and antimicrobial susceptibility (ABST) by the comparative Stokes method. ABST to methicillin was done using a disc diffusion technique. Known control strains of methicillin sensitive *S aureus* (MSSA) (NCTC 11561) and known MRSA were plated in two quadrants of a Mueller Hinton agar and 2 test strains were plated on the other two quadrants. A 5µg disc of methicillin was placed in the centre and incubation at 30°C was carried out for 24 hours. Results were read against known controls.

In the conventional method for DNA extraction about 10 colonies on each plate were picked up and dissolved in 500µL of lysis buffer containing 50µg of proteinase K, in NaCl (1.168%), Tris (0.02 M), EDTA (0.05 M) and SDS (1%) Buffer. After incubation at 65°C for 2 hours the solution was vortexed with an equal volume of phenol chloroform isoamyl alcohol (50:49:1), centrifuged at 5000 rpm for 15 minutes. The supernatant was washed with an equal volume of chloroform, and DNA precipitated with 0.7 volumes of isopropanol. The dried pellet was redissolved in 15µL TE buffer and 1µL used for PCR was added to 24µL of premixed multiplex PCR solution.

For the Microwave lysis method 10 colonies were lightly touched with a straight wire loop and emulsified in 2µL of TE buffer deposited in a microcentrifuge tube. Bacteria were then lysed by irradiation in a BPL microwave oven using seven pulses of 60 seconds each with a 60 second interval in between. No further DNA extraction was carried out. After irradiation 24µL of premixed multiplex PCR solution was added.

Each 24µL reaction mix contained : 10xPCR Buffer 2.5µL, GATC Mix 1.5µL, distilled water 19.5µL, Primer mix (2.5pM each) 1µL, Taq Polymerase 0.25µL (The PCR core Kit, Bangalore Genie) Primers sequences used were as previously described [3].

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Mec A 1-5':GTA GAA ATG ACT GAA CGT CCG ATA A,

Mec A 2-5':CCA ATT CCA CAT TGT TTC GGT CTA A,

Fem B 1-5':TTA CAG AGT TAA CTG TTA CC

Fem B 2-5':ATA CAA ATC CAG CAC GCT CT.

PCR was carried out in a thermal cycler (Hybaid Omni E). Cycling parameters were : a hot start of 94°C for 4 min was followed by 30 cycles of melting at 94°C for 45 sec, annealing at 50°C for 45 sec and extension at 72°C for 60 sec. Electrophoresis was carried out using 10µL of product in a 2% agarose gel containing ethidium bromide at 50V for 2 hours. A 100 bp ladder was simultaneously loaded as molecular weight marker. The results were read in a UV transilluminator and photographed using a Wratten No 9 filter.

Results

Of the organisms selected for the present study, 6 organisms were defined as MRSA (coagulase positive and methicillin resistant *Staphylococci*), 8 were MSSA (coagulase positive and methicillin sensitive *Staphylococci*), 5 were MRCNS (coagulase negative and methicillin resistant *Staphylococci*) and 1 was MSCNS (both coagulase negative and methicillin sensitive *Staphylococci*). They gave unequivocal results on conventional biochemical testing.

In the conventional technique DNA extraction took about 6 hours, the PCR took 3 hours, and gel electrophoresis took 90 minutes (Total time approximately 9.5 hours).

The microwave lysis of the organism took about 20 minutes, the PCR took 3 hours, and gel electrophoresis took 90 minutes (Total time 5 hours) (Table 1).

TABLE 1

Time required for various steps of PCR

Test	DNA extraction	PCR	Electrophoresis	Total time
Conventional DNA extraction with Multiplex PCR	6 hours	3 hours	1.5 hours	9.5 hours
Modified microwave lysis with Multiplex PCR	20 mins	3 hours	1.5 hours	5 hours

The result of gel electrophoresis of representative strains is shown in Fig 1. The *mec A* gene gave a 310 bp fragment while the *fem B* gene gave 651 bp fragment on multiplex PCR. MRSA showed the presence of both the *mec A* and *fem B* bands (lane 4), MSSA showed presence of a single band denoting presence of the *fem B* gene (lane 3), MRCNS gave a single *mec A* band (lane 2) while MSCNS did not give any band (lane 5). In our present study the results of both the conventional DNA extraction method as well as by microwavelysis method followed by multiplex PCR were identical and concordant.

Discussion

Identification of a cluster forming Gram positive coccus following isolation of pure colony from culture plate requires coagulase testing and a methicillin susceptibility testing. This requires between 24-48 hours after the sample has been received for a provisional report as 'possible MRSA' to be given using routine

protocols [6]. Moreover routine testing does not reveal the presence of borderline MRSA.

Conventional PCR for *S.aureus* requires the lysis of the bacterium using proteinase K or lysostaphin, followed by a phenol chloroform extraction and isopropanolol precipitation of DNA. This procedure takes at least 6 hours. This is followed by PCR, which usually takes up around 3 hours and an electrophoretic gel run, which takes 1.5 hours. Our study showed that using a multiplex PCR with direct microwave lysis of the organism, a definitive result could be obtained within 5 hours of obtaining growth on the primary culture plate.

Identification of MRSA by drug-susceptibility tests alone poses a serious problem, because a considerable number of clinical *S.aureus* isolates are borderline resistant to methicillin. Hence a quick and sensitive method of PCR based amplification for the detection of the *mec A* gene is necessary. The *mec A* gene codes for the drug resistant penicillin-binding protein 2a(PBP2a) or 2(PBP2'), and mediates the clinically relevant resistance to all beta-lactam antibiotics [4]. For appropriate treatment of MRSA infection, rapid detection of *mec A* is extremely important. However, identical *mec A* genes have been found in both coagulase-positive and coagulase-negative methicillin resistant *Staphylococcus*. A second gene related to the expression of methicillin-resistance has been called *fem A*. The *fem* group of genes are involved in pentaglycine side chain formation, inter peptide bridging as

Fig. 1: The photograph shows an ethidium bromide gel after electrophoresis of PCR products of representative organisms from each group. Lane 1 has a 100 bp ladder, Lane 2 shows MRCNS, Lane 3 shows MSSA, Lane 4 shows MRSA and Lane 5 shows MSCNS. The *mec A* gene produces a 315 bp band while *fem B* produces a 651 bp band

well as expression of methicillin resistance. Oshima et al [5] amplified both *mec A* and *fem A* genes by PCR and found the *mec A* gene was positive in all MRSA strains and 6% of MSSA strains (this is probably due to absence of phenotypic expression in a strain genotypically capable of clinical resistance). The *fem A* gene was positive in all MRSA and MSSA strains. On the other hand, the *fem A* gene was absent from coagulase-negative *Staphylococcus* strains with the methicillin-resistant phenotype [6]. Towner et al [3] used the *mec A* and *fem B* gene to detect MRSA. Cotter et al [7] used a one-tube triplex PCR wherein three genes, the methicillin-resistance gene *mec A*, *fem A* and the extracellular thermonuclease gene, *nuc*, were simultaneously amplified. MSSA and coagulase-negative *Staphylococci* were also tested and the assay was found to be MRSA specific.

Our study showed that from a pure growth of organism *mec A* and *fem B* genes in MRSA could be quickly and easily demonstrated within 6 hours using the modified protocol. Lysis of the bacterial cell wall using a microwave oven was followed by a direct PCR without an intervening DNA extraction protocol. It was seen that when minimal amounts of bacteria were taken, the PCR assay worked satisfactorily. However use of large amounts of bacterial isolate resulted in inhibition of the reaction. When carefully carried out, the modified multiplex PCR was seen to be a sensitive and reliable procedure for the rapid diagnosis of MRSA infection by detecting the presence of the *mec A* and *fem B* genes. It could also easily differentiate MRSA from MRCNS and MSSA. The main advantage was however the speed of diagnosis and the accuracy of the reaction as compared to conventional testing which is cumbersome and often inaccurate. Thus

the method could accurately demonstrate the presence of *mec A* and *fem B* genes within 5 hours of bacterial isolation and is a useful tool for rapid and unequivocal identification of MRSA. This would be of great significance in rapid diagnosis of MRSA and would help control and prevent spread of hospital infections due to MRSA.

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