DETECTION OF BACTERIAL, METHICILLIN RESISTANCE, AND β-LACTAMASE GENES FOUND IN WOUND SWABS BY MULTIPLEX POLYMERASE CHAIN REACTION

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Abstract- Coagulase-positive and coagulase negative, methicillin-resistant staphylococci are major causes of serious nosocomial infections and it is very important to have a reliable test to detect these bacteria. A multiplex polymerase chain reaction (mPCR) was used on 100 clinical samples for simultaneous amplification of the universal bacterial, mec-A encoding the penicillin binding protein 2a, which is associated with staphylococcal methicillin resistance and TEM-1 encoding the β-lactamase, which accounts for the majority of all cases of the plasmid β-lactamase resistance worldwide. Out of 100 wound swabs tested, 99% with universal primers, 26% with TEM-1 primers and 6% with mec-A primers were positive. Dot blot Digoxigenin hybridization on the 30 samples was carried out to confirm identified bacteria with specific bacterial probes. Out of 100 wound swabs, 38% were positive with *Staphylococcus aureus* probe, 23% were positive with enteric bacteria probe, 7% were positive with *Streptococcus agalactia* probe and 1% were positive with *Haemophilus influenza* probe. The mPCR method used in this study, was designed to be incorporated into the workflow of the clinical microbiology laboratory and allows for the identification of intrinsic resistance in a timely and reliable manner.

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Key words: Staphylococcus aureus, methicillin resistance, β-lactamase, universal primer, multiplex polymerase chain reaction (mPCR)

INTRODUCTION

Methicillin resistance in staphylococci is due to acquisition of the mec-A gene encoding a new penicillin-binding protein (PBP2′ or PBP2a) that has a lower affinity to methicillin than the endogenous PBPs. PBP2′ is involved in the assembly of the cell wall peptidoglycan in the presence of high concentrations of β-lactams that otherwise inhibit the endogenous PBPs. The production of PBP2′ is under dual control by its own mecR1-mecI- and the penicillinase b1aR1-b1aI-encoded regulatory elements (1). Resistance to high levels of methicillin depends, in addition to PBP2′, on chromosomally encoded factors that are involved in the synthesis and degradation of the peptidoglycan. Any mutations that reduce peptidoglycan precursor formation or change the chemical composition of the muropeptide precursor result in lowered resistance (1,2). The expression of PBP2a, a 78 KDa cell membrane protein (3, 4) encoded by the mec-A gene, may be influenced by some other genes (5). The mec-A gene has been detected by both nucleic acid hybridization (6-9) and polymerase chain reaction (PCR) (10-13), which has directly been applied on the bacteria isolated from blood cultures (13).

Some investigators have suggested that the disclosure of methicillin resistance in staphylococci may be based on mec-A detection instead of, or as a supplement to, the standard *in vitro* susceptibility tests (6, 8, 10-13). It should be taken into consideration that the susceptibility tests may not be reliable for

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heterogenous strains with a low frequency of cells expressing the resistance (10,11). β-lactam antibiotics, including penicillins, cephalosporins, carbapenems, and monobactams act by inhibiting bacterial cell wall synthesis and cell division. The most common form of resistance to these agents is destruction of drugs by β-lactamase, which can be encoded for in both chromosomes and plasmids (14,15). Coagulase-positive and coagulase negative, methicillin-resistant staphylococci are major causes of serious nosocomial infections and, on the other hand, they frequently show resistance to a variety of the antibiotics (16). It is, therefore, very important to have a reliable test to detect these bacteria. The PCR offers reliable detection of mec-A and the potential of the same day diagnosis of *Staphylococcus aureus* in cultures of blood or other clinical specimens (13). In the multiplex PCR (mPCR), primer sets targeting different genetic determinants are combined in a same reaction mixture.

The aim of this study was to introduce a PCR method using combined primers targeting the universal staphylococcal mec-A and TEM-1 sequences in a same reaction mixture. Using this method, detection of the infective microorganisms and of bacterial β-lactamase and mec-A genes, differentiation between *S. aureus* and non- *S. aureus* and also between methicillin-resistant and susceptible isolates in just one single step would be possible.

MATERIALS AND METHODS

Briefly, a batch of 100 wound swab samples was divided in two groups; the first group included 70 single result samples and the second one contained 30 multiple result samples. Both groups were subjected to the mPCR while only in the latter group the reactions were further confirmed by Digoxigenin-labeled oligonucleotide hybridization.

Primers and the probes

a) Nucleotide sequence 5′ to 3′ RW01 (AAC TGG AGG AAG GTG GGG AT) and DG74 (AGG AGG TGA TCC ACC CGC A) (Roche Molecular System). These universal bacterial primers amplified a 370 bp segment (17).

b) H-1 (5′ CGG CAT TTT GCC TTC CTG TTT TTG C 3′) H-2 (5′ GGC CCC AGT GCT GCA ATG ATA CCG 3′). The expected length of the amplified product of the target sequence with these primers (TEM-1 and TEM-2) was 702 bp (18).

c) MRS-1 (5′ GAA ATG ACT GAA CGT CCG AT) MRS-2 (5′ GCG ATC AAT GTT ACC GTA GT). This set of primers amplified a 150 bp long segment of the mec-A gene (13).

The haemophilus species probe RDR 125 was prepared using sequence 5′ GGA GTG GGT TGT CCA GAA GTA GAT 3′, corresponding to the nucleotides from positions 1416-1440. The *Streptococcus pneumoniae* probe RDR 462 was prepared using the sequence 5′ AAC TGA GAC TGG CTT TAA GAG ATT A 3′, corresponding to the nucleotides from 1278-1302. The *Escherichia coli* and enteric bacteria probe RDR 140 was prepared using the sequence 5′ GGC GCT TAC CAC TTT GTG ATT CAT G 3′, corresponding to the nucleotides from 1458-1482. The *Streptococcus agalctia* probe KG 0001 was prepared using the sequence 5′ TAA TCT CTT AAA GCC AAT CTC AGT T 3′, corresponding to the nucleotides from 1278-1302. The *Staphylococcus aureus* probe RDR 327 was constructed using the sequence 5′ GCC GGT GGA GTA ACC TTT AGG AGC 3′, corresponding to the nucleotides from 1435-1458 (17).

DNA extraction from clinical samples

Wound swab was immersed in 200 µl of lysis buffer, containing 0.5 M GuSCN, 0.05% NLS, 1 mM DTT, 0.2 mM sodium citrate, in a 1 ml Eppendorf tube and stirred to release bacteria. Then 250 µl of cold (-20 ºC) isopropyl alcohol was added to precipitate nucleic acid. After 5 mins, the Eppendorf tube was centrifuged at 14000 g for 10 mins at 4 ºC. The isopropyl alcohol was removed and the pellet was washed with 500 µl of 70% ethanol. After further centrifugation (10 mins at 4 ºC at 14000 g), the ethanol was removed and the dried pellet dissolved in 10 µl of sterile double distilled water (19).

Multiplex PCR for detection of bacterial TEM-1 and mec-A genes

Amplification reactions were done in a 25µl (6 primers) reaction volume. Reaction tubes contained primers (RW01, DG74, H-1, H-2, MRS-1, and MRS-2, 1.5 µl of each), dNTPs 4 µl, X10 PCR buffer 2.5 µl, 25mM $MgCl_2$ 3 µl, PCR H_2O 5 µl, template DNA 1 µl, taq polymerase 0.5 µl, and mineral oil 60 µl. Control setup of this study included Oxford *Staphylococcus aureus* NCTC5671 as a methicillin-sensitive bacterium, *Staphylococcus aureus* MUCOB 393 (Manchester University Collection of Bacteria) as a methicillin-resistant bacterium, β-lactamase positive, β-lactamase negative, no DNA,

Acanthamoeba sp., Candida sp., and φx174 DNA/Hae III (Promega, UK) as a marker. Thirty cycles of amplification were performed in a Perkin Elmer Cetus DNA thermal cycler. A 5 minute step was carried out at 95 ºC, 30 seconds for annealing at 55 ºC, and a 30 second extension step at 72 ºC, then extension step at 72 ºC for 7 minutes (13,20).

Digoxigenin (Dig) oligonucleotide 3′- end labeling

To detect Dig-labeled nucleic acids, the manufacturer instructions of DIG Nucleic Acid Detection Kit (Boehringer Mannheim Biochemica) were followed. To a 1.5 ml microfuge tube on ice, the following reagents were added in order: 5X tailing buffer 4 µl, 25 mM $CoCl_2$ solution 4 µl, oligonucleotide 100 ρM, Dig-11- ddutp 1 µl, terminal transferase 1 µl, and H_2O up to the final volume of 20 µl. The reaction tube was incubated for 25 mins at 37 ºC, and then placed on ice and 1 µl glycogen solution and 1 µl EDTA was added to it. The labeled oligonuceotide was precipitated with 2.5 µl of 4 M LiCl and 75 µl of pre-chilled absolute ethanol. The reagents were mixed and then the tube was incubated at –70 ºC for 30 minutes. The tube was then centrifuged at 13000 *g* for 15 minutes at 4 ºC. The ethanol was decanted and after washing the pellet with 50 µl of 70% cold ethanol, it was centrifuged again at 13000 *g* for 5 mins at 4 ºC. The ethanol was decanted again and the pellet was dried and resuspended in 20 µl of sterile redistilled water.

Hybridization reaction with Dig-labeled oligonucleotides

The DNA was denatured at 95 ºC for 10 minutes. After placing the reaction mixture on ice for 10 minutes, 1 µl of each DNAs was spotted onto the nitrocellulose membrane, which was then left to dry. The DNA was fixed on moistened membrane (by 5 × SSC) by UV. In pre-hybridization step, the membrane was folded inside a nylon bag (moistened by 5 × SCC) and placed inside the roller tube. After adding of bout 30 ml of hybridization solution, it was placed in a hybridization oven at 64 ºC for 60 minutes. Then 24 µl of Dig-labeled oligonucleotide was added to a 6 ml of hybridization solution. In hybridization step, the hybridization solution was discarded and then replaced for by a hybridization solution containing Dig-labeled probes and then left at 64 ºC for 6 hrs. In the post-hybridization step, the membrane was washed twice (5 minutes / wash) in the 2 × SSC solution at room temperature and again washed twice (15 minutes / was) in 0.3 × SSC solution at 64 ºC so that unbound probe, which otherwise could lead to high background, was removed. The washing temperature corresponded to the hybridization temperature. The membrane was taken out from roll tube and placed in the buffers 1 and 2 for 1 min and 3 minutes, respectively. The membrane was incubated in anti-Dig alkaline phosphatase pre-diluted in buffer 2 to 1:5000 for 30 minutes at room temperature. Then anti-Dig alkaline phosphatase was discarded and washed twice with buffer 2 (15 minutes / wash). The membrane equilibrated in 20 ml of buffer 3 for 2 minutes. To make the substrate, 45µl NBT solution and 35 µl of X phosphate solution were mixed to 10 ml of buffer 3. The buffer was decanted and the substrate was added. The color reaction allowed developing in the dark and it was usually completed within 16 hours.

RESULTS

In the present study a multiplex PCR with the universal bacterial primers, i.e., mec-A and TEM-1, was performed on 70 clinical samples, out of which 61 (87%) showed corresponding band on gel electrophoresis (Fig. 1). With TEM-1 (penicillin) primers, in 16 out of 70 clinical samples, and with mec-A (methicillin) primers, in 4 out of 70 clinical samples the corresponding bands on gel electrophoresis were detected. In mPCR using universal bacterial primers, where there was only one species of bacteria isolated on culture, 9 false positive results out of 70 samples (12.8%) were found. In mPCR with the 30 clinical samples with the universal bacterial primers, where there was more than one bacterial species isolated, 29 cases (96.7%) had a band on gel electrophoresis. Sensitivity of universal bacterial primers according to the bacterial group was between 75% and 100%. The sensitivity of universal bacterial primers for the bacteria isolated from 70 clinical samples was 87%. The sensitivity and specificity for TEM-1 probe were 19% and 100% and for mec-A probe were 100% and 96%, respectively. In PCR with universal bacterial primers only 2 clinical samples had no corresponding band on gel electrophoresis. Those two samples were of a same patient. In PCR with TEM-1 and mec-A primers, 5 out of 30 (16.6%) and 3 out of 30 (10%) samples had a band on gel electrophoresis, respectively. In PCR with TEM-1, there were 42 false negative out of 70 clinical samples (60%). This means that the detection of TEM-1 as a part of mPCR was very insensitive.

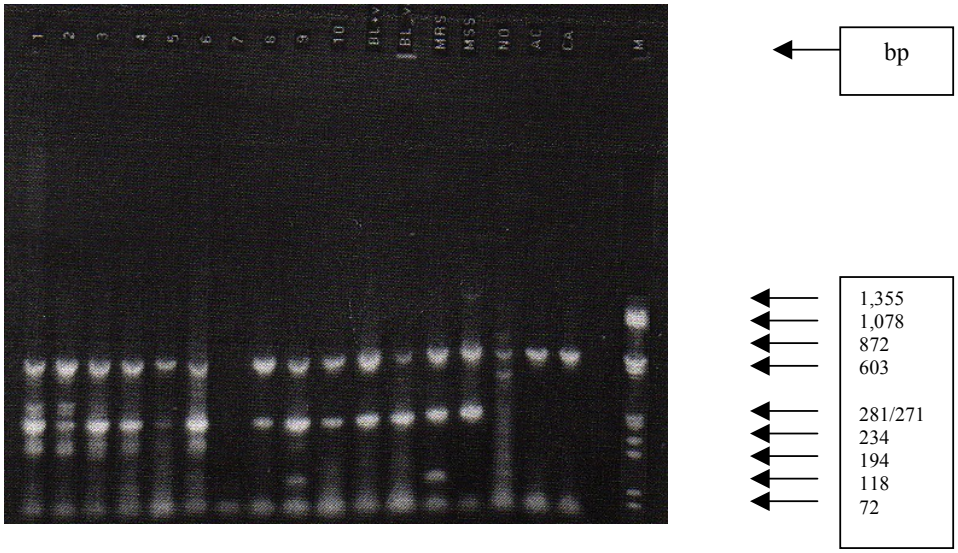

Fig. 1. A pattern of gel electrophoresis from multiplex PCR products. Lanes 1 to 6, 8, and 10 had universal and TEM-1 bands. Lane 9 had universal, TEM-1 and mec-A. Lane 7 had no band. Lane 11 was β-lactamase negative, as a negative control. Lane 13 was a methicillin-resistant *Staphylococcus aureus* (MRSA), as a positive control. Lane 14 was a methicillin-sensitive *Staphylococcus aureus* (MSSA), as a negative control. Lane 15 was sterile distilled water, as a no DNA control. Lanes 16 and 17 were candida sp. and *Acanthamoeba sp.*, as eukaryotic (no universal band have shown) and lane 18 was φx174 DNA/Hae III (Promega, UK), as a marker.

In the mPCR with 30 clinical samples, where there were more than one bacterium isolated looking at the TEM-1 primer, 5 cases (16.6%) were detected with a corresponding band on gel electrophoresis. These 5 samples were β- hemolytic streptococci + coliform (2 samples), *Staphylococcus aureus* + coliform, β-hemolytic streptococci + hemolytic streptococci group G, and β- hemolytic streptococci + anaerobes.This further emphasized the lack of sensitivity of the TEM-1 assay. The PCR products of the 30 clinical samples were confirmed by Digoxigenin labeling with specific probe (Fig. 2). The probes used in this study were of *Streptococcus pneumonia*, *Staphylococcus aureus*, *Hemophilus influenza*, Eenterobacteriaceae, and *Streptococcus agalctia*. The time needed to perform the Digoxigenin assay was two days.

DISCUSSION

The introduction and increasing use of antibiotics for antibacterial therapy has initiated a rapid development and expansion of antibiotic resistance in microorganisms, particularly in human pathogens. Additionally, a shift to an increase in number and severity of Gram-positive infections has been observed in the last decades. Common to these pathogens is their tendency to accumulate multiple resistances under antibiotic pressure and selection. Methicillin-resistant *Staphylococcus aureus* (MRSA), that have acquired multiresistance to all classes of antibiotics, have become a serious nosocomial problem. Recently, the emergence of the first MRSA with reduced vancomycin susceptibility evoked the specter of a totally resistant *S. aureus*. Problems with multiresistance expand also to penicillin-resistant *Streptococcus pneumoniae* that are partially or totally resistant to multiple antibiotics, and to vancomycin-resistant *Enterococcus spp.*, completely resistant to all commonly used antibiotics.

The rapid development of resistance is due to mutational events and/or gene transfer and acquisition of resistance determinants, allowing strains to survive antibiotic treatment (21). Methicillin-resistant staphylococci depend on efficient PBP2′ production and are modulated by chromosomal factors. Depending on the genetic background of the strain that acquired mec-A, resistance levels range from phenotypically susceptible to highly resistant. A common characteristic of most methicillin-resistant staphylococci is the heterogenous expression of resistance, which is due to the segregation of a more highly resistant subpopulation upon challenge with methicillin. Maximal expression of resistance by PBP2′ requires the efficient and correct synthesis of the peptidoglycan precursor. Genes involved in cell-wall precursor formation and turnover, regulation, transport, and signal transduction may determine the level of resistance that is expressed.

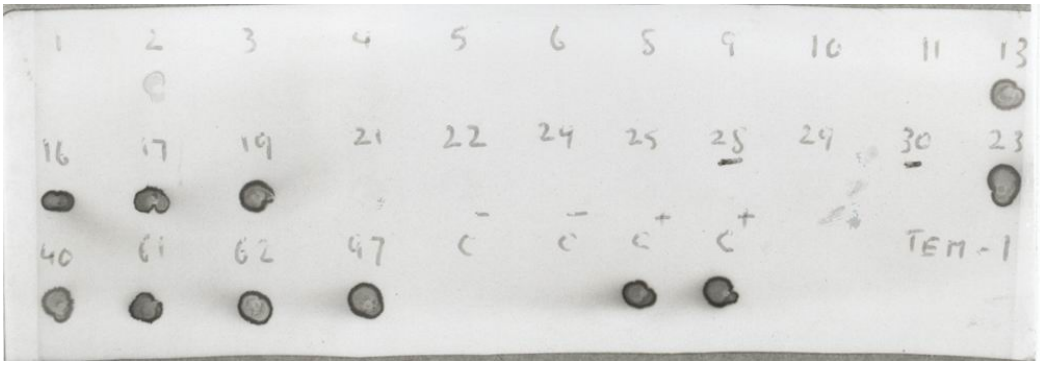

Fig. 2. Digoxigenin (Dig) oligonucleotide 3′- end labeling test of TEM-1 positive samples with specific TEM-1 probe. Ten out of 26 TEM-1 positive samples were positive with specific TEM-1 probe.Key: C^-, negative control; C^+, positive control.

At this stage, however, there is no information available on the functionality or efficacy of such factors in clinical isolates in relation to methicillin resistance levels (22). β-lactamase confers resistance to penicillins, cephalosporins, and related antibiotics by their ability to bind and hydrolyze these drugs before they reach their target sites. In Gram-negative bacteria these targets, known as penicillin-binding proteins, are located on the outer surface of the inner membrane of the cell envelope. β- lactamases are usually located in the periplasm, ready to intercept incoming β-lactam molecules. The most prevalent and successful β-lactamase genes are carried by plasmids, which unlike their chromosomally-encoded counterparts, are usually produced constitutively so that any changes in their ability to confer resistance stem from alterations in the structure of the protein rather than from deregulation of gene expression. The most successful plasmid-encoded β-lactamases, in terms of clinical significance, are the members of the TEM-1. Expression of the TEM-1 β-lactamase is the commonest mechanism of the resistance to the β-lactam group of drugs in gram-negative bacteria (23-25). This resistance gene has regularly been identified in Enterobacteriaceae but is not confirmed to this group of bacteria. The gene is usually located on the promiscuous transposon Tn3, although it and its derivatives may be located on the Tn2 (26, 27). A series of transposition and rearrangement events have allowed the TEM-1 gene migration into the many bacterial species, including *Haemophilus*, *Neisseria*, and *Vibrio* species (28, 29).

Detection of antibiotic resistance genes in clinical isolates with a reliable method is therefore of great importance in clinical scene. In this study, primers RW01, DG74 correspond to the regions of the 16S rRNA gene, which are highly conserved among divergent groups of bacteria and therefore would be expected to amplify DNA from most pathogenic bacteria. The primer locations were chosen to be relatively specific for eubacterial genes, i.e., at the 3′ ends. There are numerous mismatches with the small subunit rRNA (nuclear and mitochondrial) found in eukaryotes such as humans and fungi. The RW01 and DG74 primers were initially tested against DNA from a panel of 102 bacterial species. The panel included Gram negative and Gram-positive bacteria as well as spirochetes and mycoplasmas (17). The primers described here were designed to detect bacteria present in clinical specimens such as wounds, blood, spinal fluid, urine, etc.

It has been shown that the latex agglutination test is highly sensitive to detect MRSA if performed after induction by cefoxitin. However, inconclusive results must then be rapidly confirmed on the same day by real-time PCR used to detect mec-A and femA genes (30). In one study, low-level methicillin resistance of staphylococci was correlated with the presence of the mec-A gene and overproduction of β-lactamase (31). However, methicillin-resistant strains were recently proposed to be classified as β-lactamase hyperproducers (32). Another technically important problem is the stability of mec genes. It has been reported that methicillin-resistant *Staphylococcus epidermidis* strains appeared relatively stable, with 57.9% of isolates containing the whole regulatory region. Alterations within the mec-A gene were detected more often in other coagulase-negative staphylococci, which also had a higher percentage with deletions of regulatory genes. On the other hand, among MRSA a genetically heterogenous population was identified, with several alterations and deletions of mec genes (33). It has been said that the detection of the mec-A gene by the PCR technique is a rapid and accurate way to identify methicillin resistance (34) and it was found to be valuable when a more

definitive determination of intrinsic methicillin-resistance was desired (35). However, a combination of conventional methods alone or together with a molecular method should be used every time *S. aureus* is tested for detection of methicillin resistance (36). The mPCR method used in this study, was designed to be incorporated into the workflow of the clinical microbiology laboratory and allows for the identification of intrinsic resistance in a timely and reliable manner.

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