

Short Report

Modified DNA Extraction for Rapid PCR Detection of Methicillin-Resistant Staphylococci

Aziz Japoni*, Abdolvahhab Alborzi, Manoochehr Rasouli and Bahman Pourabbas

Prof. Alborzi Clinical Microbiology Reserch Center, Shriaz University of Medical Sciences, Shiraz, Iran

Received 24 January 2003; revised 12 August 2003; accepted 24 August 2003

ABSTRACT

Nosocomial infection caused by methicillin-resistant staphylococci poses a serious problem in many countries. The aim of this study was to rapidly and reliably detect methicillin-resistant-staphylococci in order to suggest appropriate therapy. The presence or absence of the methicillin-resistance gene in 115 clinical isolates of *Staphylococcus aureus* and 50 isolates of Coagulase Negative Staphylococci (CNS) was examined by normal PCR. DNA extraction for PCR performance was then modified by omission of achromopeptadiase and proteinase K digestion, phenol/chloroform extraction and ethanol precipitation. All isolates with MIC>8 µg/ml showed positive PCR. No differences in PCR detection have been observed when normal and modified DNA extractions have been performed. Our modified DNA extraction can quickly detect methicillin-resistant staphylococci by PCR. The advantage of rapid DNA extraction extends to both reduction of time and cost of PCR performance. This modified DNA extraction is suitable for different PCR detection, when staphylococci are the subject of DNA analysis. *Iran. Biomed. J.* 8 (3): 161-165, 2004

Keywords: Methicillin-resistant staphylococci, PCR, DNA extraction

INTRODUCTION

Methicillin resistant strains of *Staphylococcus aureus* (MRSA) were identified shortly upon the introduction of methicillin into the clinical practice [1, 2]. In 1980, the first community acquired MRSA infection was reported in the United States [3]. There has been a steady increase in the prevalence of the methicillin-resistant strains of *S. aureus* isolated from hospital in the United States over the years that in 1997 approximately 25% of nosocomial isolates of *S. aureus* were methicillin-resistant [4]. Previous study in our center in Shiraz University affiliated hospitals showed MRSA had risen up to 33% [5].

Standardized methods of the susceptibility test have been used for the detection of resistant strain [6, 7]. However, phenotypic expression of methicillin-resistance is usually heterogeneous [8]. In addition, methicillin-resistance is influenced by culture conditions such as temperature, medium, pH

and NaCl content in medium [9]. These factors complicate the detection of methicillin-resistance, especially for strains with low level resistance.

Several PCR assays based on the DNA sequence information have been used for detection of MRSA strains [10-12]. The PCR methods have high sensitivity and specificity and were independent of the physical and chemical conditions of culture. To carry out normal PCR, 24 hours time is needed. This time is required for DNA preparation, PCR performance and detection of PCR products into the gel. We modified DNA extraction for PCR to reduce the test period and the cost.

MATERIALS AND METHODS

Bacterial strains. One hundred and fifteen *S. aureus* isolates and fifty Coagulate Negative Staphylococci (CNS) were obtained from different wards in Nemazi Hospital, Shiraz, Iran. The isolates were identified as a staphylococci based on

*Corresponding Author; Tel. (+98-711) 626 2225; Fax: (+98-711) 628 7071; E-mail: Japonia@hotmail.com

morphology, gram stain and catalase test. The *S. aureus* and CNS were differentiated from each other by coagulase and DNase activity. American Typing Culture Collections (ATCC), 25923 and 51153, were used as a *mecA* negative and positive, respectively. Methicillin Minimum Inhibitory Concentration (MIC) were determined by agar dilution plates as recommended by National Committee for Clinical Laboratory Standards (NCCLS) [13].

DNA extraction. Pure DNA was prepared by growing bacteria in 5 ml Trypticase Soy Broth (TSB) for 18 h (mid log). The grown bacteria were centrifuged at 800 ×g for 10 minutes and supernatant was discarded. The sediments were resuspended in 5 ml PBS, shaken for 30 minutes and then recentrifuged at 800 ×g. The washed bacterial cells were transferred into 1.5-ml microtubes and 10 µl achromopeptidase (10,000 u/ml) was added and incubated at 55°C for 30 min. Next, 10% SDS plus 5 µl (10 mg/ml) proteinase K were added and incubated in water bath at 37°C for 1 hour. The resulting suspension was extracted twice with phenol/chloroform and once with chloroform. Each step of the extraction was performed using a sterile microtube and centrifuged at 12,500 ×g. The bacterial DNA was recovered from supernatant by precipitated DNA in pure ethanol over night at -20°C freezer. Precipitated DNA was washed with 70% ethanol to separate any protein contamination and then resuspended in 40 µl TE buffer. A volume of 2.5 µl of this DNA was used as a DNA template.

PCR conditions. PCR mixture consists of 0.25 µM of each primer (5' AAA TCA GAT GGT AAA GGT TGG C3') and (5' AGT TCT GCA GTA CCG GAT TTG C3') (TLB Mol Bio Synthese Labor), 200 µM dNTP, 2.5 mM Mg₂Cl, 1.25 unit *Taq* polymearse (Cinna gen, Iran) and 2.5 µl of DNA template. DNA amplification was carried out for 40 cycles in 50 µl of reaction mixture as follows: denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 minutes with a final extension at 72°C for 5 minutes. PCR products (10 ml) were analyzed in 1.5% agarose gel. The gel containing amplified DNA was stained by immersion into the ethidium bromide suspension (10 mg/ml) for 10 min and destained with distilled water for 10 minutes. The PCR products were observed by visualized 533 bp band under automatic UV transiluminator (Uvtec, Sigma,

Germany). The image was stored in the computer for further analysis.

Modified DNA extraction method. For rapid PCR performance, the bacterial suspension was swabbed on Trypticase Soy Agar (TSA) while the surface of the medium was covered with standard vancomycin discs (3 discs for each plate) and incubated over night. The bacterial colonies from edges of the zone of inhibition were then resuspended in sterile distilled water and matched to 0.5 MacFarland standard (approximately 10⁸ cfu/ml). The bacterial suspension was heated at 95°C for 15 min and cooled at room temperature. Cured lysate mixture (2.5 µl) was used as a DNA template when modified DNA extraction for PCR performance was carried out.

RESULTS

Based on MIC values, all *S. aureus* strains were classified into three categories: Methicillin Sensitive *S. aureus* (MSSA, MIC = 0.25-1 Mg/ml), Borderline Methicillin-resistant *S. aureus* (BL, MIC 2-8 Mg/ml) and Methicillin-Resistance *S. aureus* (MRSA, MIC>8 µg/ml). MIC values for CNS showed up to 56% (28 out of 50) of these strains are methicillin resistant. These results are shown in Table 1.

Normal DNA extraction for PCR detection was optimized and modified for all isolates. No difference in PCR results have been observed when our rapid and normal DNA extraction have been performed (Fig. 1). All staphylococci with MIC>8 µg/ml, coagulase positive and negative strains, showed positive PCR results (Table 1). The time for rapid DNA extraction and PCR performance was 6 h instead of 24 hours.

DISCUSSION

Methicillin-resistant *S. aureus* produces a low-affinity penicillin-binding protein (PBP2/ or PBP 2a) in addition to usual PBP [14, 15]. Available data show that structural genes of this PBP (*mecA*) are present in resistant strains but not in susceptible ones [4]. The *mecA* genes and its associated elements are located in chromosome [16]. It has been proposed that extensive β-lactam antibiotic usage selected resistant strains. In our Hospital, there has been a 10% increase in the incidence of MRSA during the last four years (from 33% to 43%). These results are consistent with reports from

other countries [17-20]. If the strategy for antibiotic usage is not changed, we should expect

Table 1. Frequency of methicillin MIC for 115 *S.aureus* isolates and 50 Coagulase Negative Staphylococci (CNS) and their correlation to PCR results.

MIC µg/ml	0.25	0.5	1	2	4	8	16	32	64	128	256	>256
No/CNS(%)	4/8	5/10	2/4	3/6	2/4	6/12	2/4	1/2	4/8	8/16	5/10	8/16
PCR result	-	-	-	-	-	-	+	+	+	+	+	+
MIC range	0.25-1 µg/ml (MSCNS)			2-8 µg/ml (BLCNS)			MIC>8 µg/ml (MRCNS)					
Total No/CNS%	11/22			11/22			28/56					
No/CPS %	4/3.5	8/7	15/13	14/12.2	11/9.6	14/12.2	6/5.2	5/4.3	3/2.6	6/5.2	7/6.1	22/19
PCR result	-	-	-	-	-	-	+	+	+	+	+	+
MIC range	0.25-1 µg/ml (MSSA)			2-8 µg/ml (BLCPS)			MIC>8 µg/ml (MRSA)					
Total No/CPS%	27/23.5			39/34.1			49/42.4					

No, number; CPS, coagulase positive staphylococci; MSCNS, methicillin sensitive coagulase negative staphylococci; BLCNS, borderline resistant of coagulate negative staphylococci; MRCNS, methicillin resistant coagulase negative staphylococci; MSSA, methicillin Sensitive *S.aureus*; BLCPS, borderline resistant of coagulase positive staphylococci; MRSA, methicillin-resistant *S. aureus*; (-), negative PCR result; (+), positive PCR result.

more than 60% of staphylococci isolated from patients be methicillin-resistant in the near future. In the cases of CNS, 56% of the strains isolated from patients in the Hospital have already emerged methicillin- resistance. Therefore, it would be wise to change the patterns of antibiotic usage to reduce

selective pressure upon sensitive strains. However, we need reliable methods such as PCR for differentiation of sensitive and resistant strains that are independent of physical and chemical conditions of bacterial culture. This would be a guide for the clinician to use appropriate antibiotics. It has been

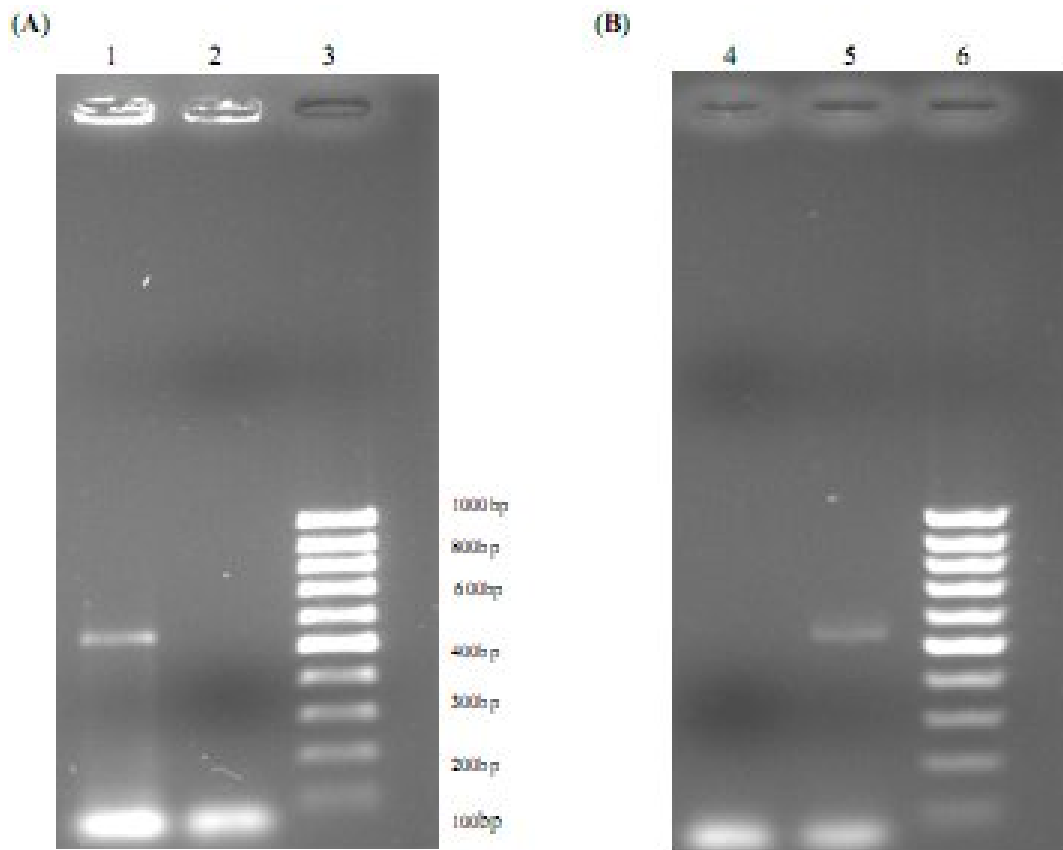


Fig. 1. Tracks 1 and 2 A, *mecA* positive (533 bp) and negative respectively when modified DNA extraction was carried out (precipitated DNA and proteins into the wells number1 & 2 are clearly observable); tracks 3 A and B, size markers; tracks 1 and 2 B, *mec A* negative and positive (533 bp) respectively when normal DNA extraction was carried out. bp., base pair.

suggested to use vancomycin for patients infected with *mecA* positive strains, whereas penicillinase resistant penicillins are the drug of choice for patients infected with *mecA* negative strains [21].

PCR is a sensitive, specific and rapid method for the detection of *mecA* positive strains. Nevertheless, staphylococci cell walls are surrounded by the thick peptidoglycan [22]. To weaken staphylococcal cell wall in normal DNA extraction, usually achromopeptidase or lysostaphin is recommended [23]. Whereas, in the case of Gram-negative bacteria like *E. coli*, with (1-3 nm diameters), thin peptidoglycan EDTA or lysozyme can be used to open cross-linked in peptidoglycan. Gram-negative bacteria treatment in this way can be simply lysed using SDS or boiling. On the other hand, for staphylococci with thick peptidoglycan (20-80 nm diameters), it would be difficult to penetrate into the cell wall except by specific enzymes (achromopeptidase or lysostaphin) adding to the lysis buffer [23]. We used vancomycin discs (cell wall inhibitor antibiotic) instead of expensive enzymes, because almost all staphylococci are sensitive to this antibiotic [24], and can weaken a thick bacterial cell wall. As a consequence, boiling of suspension containing several colonies of bacteria from the edge of inhibition zone will readily open the disintegrated cell wall.

Our method of DNA extraction is suitable for all PCR approaches where staphylococci are subject of the DNA analysis. Nevertheless, for some sensitive molecular methods such as restriction fragments length polymorphisms (RFLP) or DNA sequencing, at least one step phenol/chloroform extraction plus ethanol perception is needed to get rid of DNase and contaminated proteins [25].

REFERENCES

1. Barber, M. (1961) Methicillin Resistant Staphylococci. *J. Clin. Pathol.* 14: 385-393.
2. Stewart, G.T. and Holt, R.J. (1961) Evolution of natural resistance to the newer penicillin. *Br. Med. J.* 1: 308-311.
3. Center for Disease Control. Community-acquired methicillin-resistant *Staphylococcus aureus* infections-Michigan (1981) MMWR. *Morb. Mortal. Wkly. Rep.* 30: 185-187.
4. Chambers, H.F. (1997) Methicillin resistance in staphylococci. Molecular and biochemical basis and clinical implications. *Clin. Microbiol. Rev.* 10: 781-791.
5. Alborzi, A., Pourabbas, B.A., Salehi, H., Pourabbas, B.H., Obiidi, B. and Panjehshahin, M.R. (2000) Prevalence and pattern of antibiotic sensitivity of methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* in Shiraz. *Irn. J. Med. Sci.* 25: 1-8.
6. Barry, A.L. and Jones, R.N. (1987) Reliability of high-content discs and modified broth dilution test for detecting staphylococcal resistance to penicillin. *J. Clin. Microbiol.* 75: 1897-1901.
7. Thornsberry, C. and McDougal, L.K. (1983) Successful use of broth microdilution in susceptibility tests for methicillin resistant (heteroresistant) staphylococci. *J. Clin. Microbiol.* 18:1084-1091.
8. Hartman, B.J. and Tomasz, A. (1986) Expression of methicillin resistance in heterogeneous strains of *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 29: 85-92.
9. Sabath, L.D. (1982) Mechanisms of resistance to beta lactam antibiotics in strains of *Staphylococcus aureus*. *Ann. Intern. Med.* 97: 339-344.
10. Murakani, K.W., Minamide, K., Wada, W., Nakamura, E., Teraoka, H. and Watanbe, S. (1991) Identification of methicillin resistant strains of staphylococci by polymerase chain reaction. *J. Clin. Microbiol.* 29: 2240-2244.
11. Tokue, Y., Shoji, S., Satoh, K. and Motomyia, M. (1992) Comparison of a polymerase chain reaction assay and a conventional microbiologic method for detection of methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 36: 6-9.
12. Bartzavali-Louki, C., Dimitacopoulos, G. and Spiliopoulou, I. (2003) Polymerase chain reaction fingerprints of methicillin-resistant *Staphylococcus aureus* clinical isolates in Greece are related to certain antibiotypes. *J. Microbiol. Methods* 53: 417-422.
13. National Committee for Clinical Laboratory Standards (1985) Approved standard M7. A method for dilution of anti-microbial susceptibility tests for bacteria that grow aerobically. National Committee for Clinical Standards. Villanova, Pa.
14. Hartman, B.J. and Tomasz, A. (1984) Low affinity penicillin binding protein associated with β -lactam resistance in *Staphylococcus aureus*. *J. Bacteriol.* 156: 513-516.
15. Utsui, Y. and Yokota, T. (1985) Role of an altered penicillin binding protein in methicillin and cephem-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 28: 397-403.
16. Matthews, P.R., Reed, K.C. and Stewart, P.R. (1987) The cloning of chromosomal DNA associated with methicillin and other resistances in *Staphylococcus aureus*. *J. Gen. Microbiol.* 133: 1919-1929.
17. Lin, C.H., Hsu, R.B., Chang, S.C., Lin, F.Y. and Chu, S.H. (2003) Poststernomy mediastinitis due to methicillin-resistant *Staphylococcus aureus* epidemic in hospital. *Clin. Infect. Dis.* 37: 679-84.
18. Usukura, Y. and Igarashi, T. (2003) Examination of Severe hospital acquired infection affecting

- Extremely Low Birth Weight (ELBW) infants. *Pediatr. Int.* 45: 230-20.
19. Gastmeier, P., Geffers, C., Sohr, D., Schwab, F., Behnke, M. and Ruden, H. (2003) Surveillance of nosocomial infection in intensive care units. Current data and interpretations. *Wien. Klin. Wochenschr.* 115: 99-103.
 20. Senna, J.P., Pinto, C.A., Mateus, S., Quintana, A. and Santos, D.S. (2003) Spread of dominant methicillin-resistant *Staphylococcus aureus* (MRSA) clone between Uruguayan and South of Brazil hospitals. *J. Hosp. Infect.* 53:156-157.
 21. Murray, P.R. and Baron, E.J. (1999) Genetic methods for detection of antibacterial and antiviral resistance genes. In: *Manual of Clinical Microbiology*. 7th ed, Washington DC. American Society of Clinical Microbiology. pp. 1578-1592.
 22. Prescott, M.L., Harley, J.P. and Klein, D.A. (1996) Prokaryotic cell structure and function in Microbiology. WCB (Wm. C. Brown Publishers), Chicago. Pp. 33-72.
 23. Van Belkum, A., Bax, R., Beerbooms, P., Goessens, W.H.F., Van Leeuwen, N. and Quint, W.G. (1993) Comparison of phage typing and DNA finger printing by polymerase chain reaction for discrimination of methicillin resistant *Staphylococcus aureus* strains. *J. Clin. Microbiol.* 31: 798-803.
 24. Suzuki, K., Nishimura, T. and Baba, S. (2003) Current status of bacterial resistance in the otolaryngology field: result from the second nationwide survey in Japan. *J. Infect. Chemother.* 9: 46-52.
 25. Sambrook, J. and Russell, D.W. (2001) Molecular cloning. 3th ed., Cold Spring Harbor Laboratory Press, New York.