

SHORT REPORT

Fast, simultaneous, and sensitive detection of staphylococci

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J Clin Pathol 2003;**56**:782–785

Aims: Bacterial infections are common and are involved in many forms of disease, ranging from arthritis to food poisoning. Of much concern are nosocomial infections, especially the increasing resistance of bacteria to methicillin. A prerequisite for the successful treatment of bacterial infections is a specific and sensitive method of detecting microorganisms.

Methods: Some methods to detect bacteria are time consuming, whereas others are faster but lack specificity and/or sensitivity. This article describes an optimised polymerase chain reaction (PCR) method that enables the simultaneous detection of different bacteria. A prerequisite for sensitive PCR is a method to isolate and recover extremely small amounts of bacterial DNA. This study used a new method to isolate DNA and compared the results to an established method.

Results: The method could detect fewer than 10 *Staphylococcus aureus*, methicillin resistant *S aureus*, *Staphylococcus epidermitis*, and other bacteria and it took less than two hours to perform.

Conclusion: The rapid DNA isolation method used in conjunction with the optimised PCR makes it possible to confirm the presence or absence of extremely small numbers of bacteria. Using real time PCR would shorten the procedure even further. This method might therefore contribute to more timely and specific interventions.

Staphylococcal infections are of serious concern for health care professionals. In some cases, more than 94% of *Staphylococcus aureus* have been reported to be resistant to treatment with penicillin.¹ Approximately 56% of the *S aureus* strains in the USA exhibit methicillin resistance. Another bacterium that plays an important role in nosocomial infections is *Staphylococcus epidermitis*, which is responsible for more than 75% of infections caused by catheters. Furthermore, *S epidermitis* is thought to be a major cause of chronic inflammation in patients with artificial joints, pacemakers, and/or artificial heart valves. In addition to their role in nosocomial infections, staphylococci are responsible for many other diseases, such as food poisoning, pneumonia, inflammation of heart and skeletal muscle, and encephalitis, and they are also thought to be responsible for 75% of cases of septic arthritis.²

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For these reasons a fast, sensitive, and specific test system is essential for the detection of staphylococci to enable early and specific intervention. Although there are several methods for the detection of staphylococci, most are not very useful because they are time consuming and insensitive. Here, we describe a test system based on multiplex PCR that allows the

specific detection of small numbers (less than 10) of *S aureus*, *S epidermitis*, and methicillin resistant staphylococci (MRSA) in less than two hours.

MATERIALS AND METHODS

Bacterial culture

Small scale broth culture, single colony isolation, streak plates, and dilution series were done using standard protocols.³ Dr M Manafi (University of Vienna, Austria), and Dr G Tuceck (Kaiser Franz Josef Spital, Vienna, Austria) kindly provided samples and strains of bacteria (*S aureus*, *S epidermitis*, and MRSA, as defined by conventional microbiological methods). In each case, using the streak plate method, a subculture was established to isolate a single colony. Uniformity in size and appearance of colonies indicated pure cultures. Dilution series (six times 1/10) of overnight broth bacteria cultures were made to define the number of bacteria used in subsequent experiments. Each of the resulting cultures was vortexed and aliquoted (0.1 ml). One aliquot each was spread on to agar plates and incubated overnight, after which the resulting colonies were counted. The remaining aliquots with the desired cell numbers were used in PCR experiments.

DNA isolation

First, samples were diluted 1/2 to 1/3 in saline or H₂O (highly viscous solutions such as synovial fluid were diluted 1/4) and centrifuged at 16 000 ×g for 10 minutes at 25°C. The pellet (sometimes invisible) was washed (H₂O) and centrifuged again at 16 000 ×g for five minutes, after which a solution containing 5 µl proteinase K (1 µg/µl), 5 µl lysostaphin (0.2 units/µl), and 90 µl Bactozyme dilution buffer (MRC Inc, Cincinnati, Ohio, USA) was added. Tubes were incubated at 45°C for 90 minutes at 700 revolutions/minute (rpm) on an Eppendorf Thermomixer. Subsequently, 10 µl of 10× Bactozyme solution (MRC Inc) was added and the solution was incubated for an hour at 45°C/700 rpm. After this, 400 µl of DNazol (MRC Inc) and 5 µl of polyacryl carrier were added, and the solution was mixed and incubated for 15 minutes at 50°C. The resulting DNA was precipitated, washed, and resuspended in Tris/EDTA buffer. The duration of this procedure was three hours.

Another method consisted of resuspending the bacterial pellets in 90 µl lysis buffer (50 mM Tris/HCl, pH 7.2, 50mM EDTA, 3% sodium dodecyl sulphate (SDS), and 1% β mercaptoethanol) and heating the samples in the centre of a microwave oven (600 Watts) for between 30 seconds and five minutes. Because evaporation becomes a problem when samples are exposed to microwaves for longer periods of time, mineral oil was added, and the heating procedure was interrupted after each minute for a few seconds. Although not

Abbreviations: MRSA, methicillin resistant staphylococci; PCR, polymerase chain reaction; rpm, revolutions/minute; SDS, sodium dodecyl sulfate

tested, submersion in a boiling waterbath might be equally effective, without running the risk of evaporation. It is important to test microwave ovens beforehand because overheating samples can result in violent evaporation. After microwaving, 400 μ l DNAzol was added and the solution was heated to 60°C for 15 minutes. The DNA was recovered as above. The duration of this procedure was 30 minutes.

PCR and primer sets

A Techne cyclor (Techgene, Duxford, Cambridge, UK) was used under the following standard conditions: initial denaturation for five minutes at 94°C, followed by 45 cycles of annealing for one minute at 52°C or 62°C, amplification for one minute at 72°C, and denaturation for one minute at 94°C, with a final extension at 72°C for 10 minutes. The duration of this procedure was two hours and 59 minutes. The short programme for *S aureus*/*S epidermitis* on the Techne instrument was initial denaturation for one minute, followed by 45 cycles of annealing for one second at 60°C, amplification for one second at 72°C, denaturation for one second at 94°C, with no final extension. The duration of this programme was 28 minutes.

The following primers were from MWG Biotech AG Ebersberg, Germany and were dissolved at a concentration of 100 pmol/ μ l in Tris/EDTA buffer. *Staphylococcus aureus* (SA): forward primer, 5'-GCGATTGATGGTGATACGGTT-3'; reverse primer, 5'-AGCCAAGCCTTGACGAATAAAGC-3'. *Staphylococcus epidermitis* (SE): forward primer, 5'-ATCAAAAAGTTGGCGAACCTTTTCA-3'; reverse primer, 5'-CAAAAGAGCGTGGAGAAAAGTATCA-3'. rRNA: forward primer, 5'-GGAGGAAGGTGGGGATGACG-3'; reverse primer, 5'-ATGGTGTGACGGGCGGTGTG-3'. MRSA: forward primer, 5'-GCGATTGATGGTGATACGGTT-3'; reverse primer, 5'-CGGACG TTCAGTCATTCTAC-3'. PCR was carried out in 0.5 ml tubes containing a total of 50 μ l of the following reagents: 5 μ l of 10 \times buffer, 1 μ l dNTP, 2 μ l each (forward and reverse) primer, 1 μ l Taq polymerase (5 units final concentration), DNA, and double distilled H₂O. The lengths of amplified fragments are: *S aureus*, 280 bp; *S epidermitis*, 124 bp; MRSA, 161 bp; and rRNA, 241 bp. The PCR products were separated on agarose gels (1% agarose, 0.5 μ g/ml ethidium bromide, 0.5 \times Tris/borate/EDTA buffer), scanned, and analysed on a Fluorimager 595 (Molecular Dynamics, Amersham Biosciences, Freiburg, Germany).

RESULTS

Specificity and sensitivity of PCR

The specificity of the PCR was shown by a match of the calculated and actual size of the amplified fragments. Sequencing of the amplified fragments confirmed specificity further (data not shown). Figure 1 shows the data from a representative experiment where DNA was isolated from five, 50, 500, 5000, and 50 000 bacteria (*S aureus*) and amplified as described. As demonstrated, fewer than 10 bacteria can be detected.

Recovery experiments

The recovery of bacteria from viscous solutions such as synovial fluid can be difficult. In addition, DNA isolated from blood samples can contain polymerase inhibitors. Therefore, we performed recovery experiments by spiking different solutions with equal amounts of bacteria. Aliquots of *S aureus*, calculated to contain approximately 100 bacteria, were used in this experiment. Figure 2 shows an experiment demonstrating that a sufficiently strong signal can be detected in all cases, although the recovery of synovial fluid bacteria is significantly weaker.

Detection of MRSA and multiplex PCR for the simultaneous detection of bacteria

Early detection of MRSA is becoming increasingly necessary for efficient patient care. Figure 3A shows an experiment where four serum samples were screened for the presence of

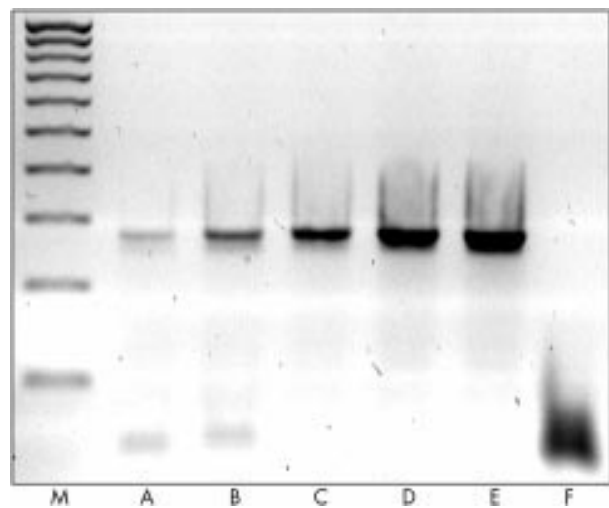


Figure 1 Polymerase chain reaction (PCR) sensitivity. Known numbers of bacteria (*S aureus*) were used for the isolation of DNA using sodium dodecyl sulfate and microwaving. Aliquots containing 5 (lane A), 50 (lane B), 500 (lane C), 5000 (lane D), and 50 000 bacteria (lane E) were amplified by PCR using a fast cycling programme, as described in the materials and methods. Lane F, no bacterial DNA (negative control); lane M, molecular size marker. These results also indicate that the quantification of cell numbers might be possible using a Fluorimager.

MRSA. Whereas large numbers of MRSA can be found in sample number 1, the faint band seen in sample number 3 might be the result of contamination with MRSA DNA or it might indicate the presence of very few methicillin resistant bacteria in this probe. Because of time saving considerations, it is desirable to screen several samples simultaneously for the presence of a series of bacteria. Possibilities of primer dimer formation make it necessary to test whether sets of primers are suitable for multiplex PCR. Figure 3B shows that the primers chosen are suited for the simultaneous detection of bacteria in general (universal rRNA primers), in addition to *S aureus* and/or *S epidermitis* through specific primers.

Isolation of DNA using SDS and microwaves

A suitable means to isolate and recover DNA is the single most important prerequisite for a sensitive PCR method. Staphylococci are known to be resistant to cell lysis.⁴ Special enzymes and methods have been developed to tackle this problem.⁴ Figure 4 shows a comparison between a published method

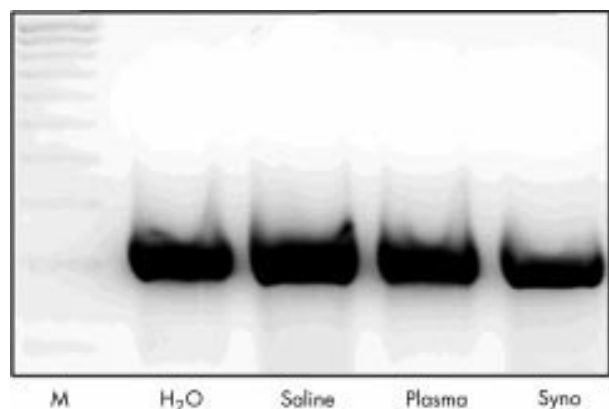


Figure 2 Recovery experiments. Equal amounts of bacteria were distributed into H₂O, saline solution, plasma, and synovial fluid (syno). The results show that the methods used can recover and amplify bacteria sufficiently well, even from viscous solutions such as synovial fluid. Lane M, molecular size marker.

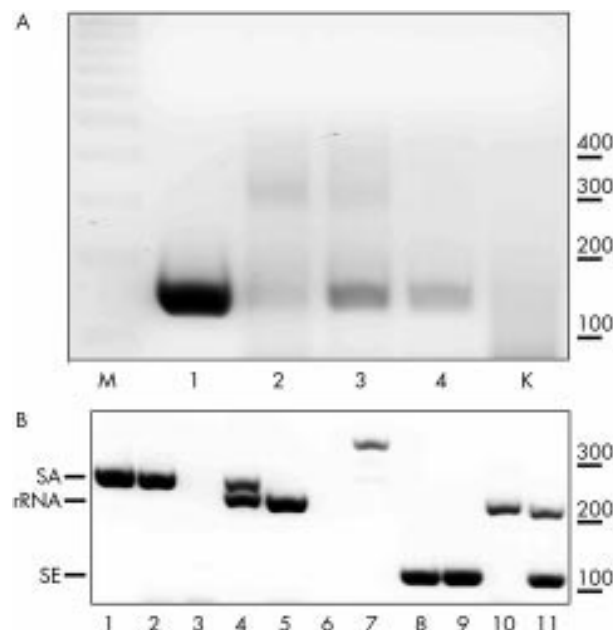


Figure 3 Methicillin resistance and multiplex polymerase chain reaction (PCR). (A) Several samples were screened for the presence of methicillin resistant staphylococci (MRSA). Samples 2 and 4 were negative for MRSA, whereas sample number 1 was highly positive. The faint band in sample 3 might indicate the presence of very few methicillin resistant bacteria. Lane M, molecular size marker lane; lane K, no bacterial DNA (negative control). (B) Lanes 1–5, *Staphylococcus aureus* (SA) DNA amplified with the following primers: lane 1, SA; lane 2, SA + SE; lane 3, SE; lane 4, SA + rRNA; lane 5, SE + rRNA. Lane 6, no bacterial DNA (negative control). Lanes 7–11, *Staphylococcus epidermitis* DNA amplified with the following primer sets: lane 7, SA; lane 8, SA + SE; lane 9, SE; lane 10, SA + rRNA; lane 11, SE + rRNA. These results show that it is possible to test for different bacteria simultaneously.

developed specifically for staphylococci and the method used in our laboratory to isolate DNA from bacteria. As shown, the published method⁴ is equally sensitive but takes considerably more time than our method.

Optimising PCR conditions can save considerable time

We tested whether standard PCR conditions can be shortened without sacrificing sensitivity. Figure 5 shows an experiment where equal amounts of *S aureus* DNA were amplified under identical conditions, with the exception that the times allocated for denaturation, annealing, and amplification were shortened. These data show that PCR programmes can be optimised/shortened to result in run times of 28 minutes

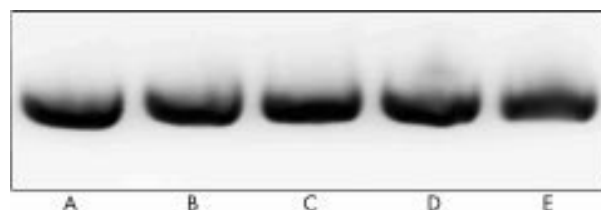


Figure 5 Frozen aliquots of complete polymerase chain reaction mixtures containing *Staphylococcus aureus* DNA were amplified under standard conditions and under conditions where the times allocated for denaturation, annealing, and amplification were shortened from one minute to one second. Lane A, four minutes of initial denaturation, followed by 45 cycles of denaturation for 30 seconds, annealing for 30 seconds, and amplification for 30 seconds, with a 10 minute final extension time. Lane B, same as lane A without a final extension. Lane C, four minutes of initial denaturation, followed by 45 cycles of denaturation for 30 seconds, annealing for 30 seconds, and amplification for 30 seconds. Lane D, four minutes of initial denaturation, followed by 45 cycles of denaturation for 10 seconds, annealing for 10 seconds, and amplification for 10 seconds. Lane E, four minutes of initial denaturation, followed by 45 cycles of denaturation for one second, annealing for one second, and amplification for one second.

compared with about three hours under standard conditions, without loss in sensitivity.

DISCUSSION

Bacteria are the cause of many diseases, ranging from arthritis to life threatening nosocomial infections. Early and reliable detection of such infections is essential. There are many methods for detecting bacteria; most are labour intensive and time consuming, whereas others are faster but lack specificity and sensitivity.^{5,6} PCR seems to be the method of choice because it unites speed with sensitivity and specificity. Here, we demonstrate the combination of an optimised PCR protocol with a method of isolating/recovering extremely small amounts of DNA, which enable the rapid and sensitive detection of bacteria in a variety of samples, including synovial fluid.

Several procedures are available for the isolation of genomic DNA from Gram positive bacteria, but most are time consuming and are not suitable for testing large numbers of samples.⁷ Microwaves have been used to isolate DNA before: they have been used successfully for the isolation of plasmid DNA in mini and maxipreps,^{8,9} and for the isolation of total genomic DNA from fungi, plants, and protists.¹⁰ However, a drawback of these methods is the large number of organisms required to obtain a sufficient amount of DNA. In most cases, the limited recovery of DNA presents no problem and is outweighed by the speed and the cost savings of such a procedure. We tested different lysis buffer systems and describe a

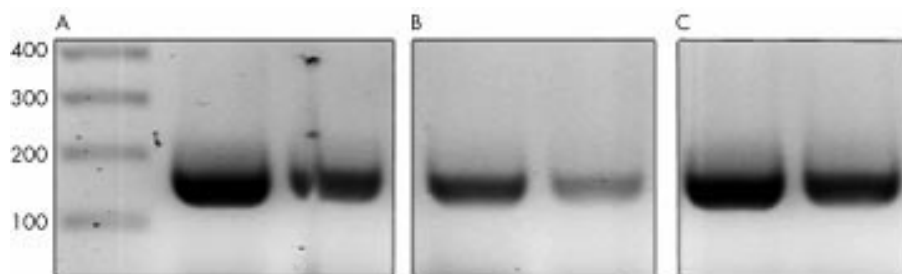


Figure 4 Comparison of DNA isolation methods. (A) DNA was isolated according to a published method using lysostaphin, proteinase K, and Bactozyme. (B, C) DNA was isolated from the same number of bacteria as in (A), but using sodium dodecyl sulfate and microwaving. In (B) the DNA was isolated immediately after microwaving, without further incubation in DNAzol at a high temperature, whereas in (C) the DNA was heated to 60°C for 15 minutes in DNAzol. These results indicate that a short period of microwaving plus a short exposure of the lysate to a high temperature in DNAzol is equally well suited for isolating DNA as the established sensitive but more time consuming method. Ten times more bacteria were used in the left hand lane of each panel than in the right hand lane.

new method that is suited to the recovery of extremely small amounts of DNA from Gram positive bacteria. DNA from staphylococci is notoriously difficult to isolate because of the membrane structure of these organisms.⁴ We have shown that lysis in an SDS/β mercaptoethanol buffer and exposure to microwaves is as effective as a longer, more elaborate, and more expensive method using specific enzymes for the detection of less than 10 microorganisms in each sample. It is the combination of the buffer system used together with the exposure to microwaves that makes the DNA isolation procedure fast and sensitive. We also showed that the chosen primer pairs were suited for multiplex PCR, the simultaneous testing of samples for the presence of *S aureus*, *S epidermitis*, and/or bacteria in general. In addition, we demonstrated that the assay conditions are suitable for detecting the presence of methicillin resistant forms of *S aureus*. The specificity of the PCR was confirmed by a comparison of the actual size with the calculated length of the fragment and by sequencing of the PCR product.

"It is the combination of the buffer system used together with the exposure to microwaves that makes the DNA isolation procedure fast and sensitive"

Our data also confirm that cycling parameters can be altered considerably without losing sensitivity. Because Taq polymerase incorporates about 50 nucleotides/second into a template, fragments of a few hundred base pairs will take only a few seconds to be elongated. In addition, Taq polymerase works acceptably well below and above the optimal temperature of 72°C, so that the one second allocated for elongation in the short PCR programme appears to be sufficient. Furthermore, a final elongation step does not improve PCR any further because incomplete fragments could be elongated to their full length through each following cycle.

Taken together, our method of rapid DNA isolation and the optimised PCR programme makes it possible to confirm the absence or presence of extremely small numbers of bacteria within two hours. Using real time PCR, which eliminates the need for running a gel, would shorten this procedure even further. The methods outlined above might therefore contribute to timely and specific intervention in the early phase of an illness.

ACKNOWLEDGEMENTS

This work was supported in part by the Austrian Ministry of Education, Science, and Culture, the Austrian Ministry of Social Security and Generations (GZ:236.065/7-VIII/A/6/00 and GZ:236.065/5-VIII/A/6/99), and the City of Vienna. Strains of bacteria (*S aureus*, *S epidermitis*, and MRSA) and samples containing bacteria were generously provided by Dr M Manafi, University of Vienna, Dr Tuceck, Kaiser Franz Josef Spital, Vienna, and Dr A Dunky, Willhelminenspital, Vienna.

Take home messages

- We describe a rapid DNA isolation method which can be used in conjunction with an optimised and shortened polymerase chain reaction (PCR) to confirm the presence or absence of extremely small numbers of bacteria
- This method can be used for the simultaneous testing of samples for the presence of *Staphylococcus aureus*, *Staphylococcus epidermitis*, methicillin resistant forms of *S aureus*, and/or bacteria in general.
- Using real time PCR would shorten the procedure even further
- This method might therefore contribute to more timely and specific interventions

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Accepted for publication 18 March 2003

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