Technical report

Sensitive and universal method for microbial DNA extraction from blood products

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Abstract

A new method of extracting bacterial and yeast DNA from blood products dependent on guanidinium thiocyanate acid extraction and proteinase K treatment is described. In spiked samples the sensitivity per 0.1 ml of serum and blood, respectively, was 26 and 150 colony forming units (cfu) for *Escherichia coli*, 80 and 120 cfu for *Staphylococcus aureus* and 20 and 26 cfu for *Candida albicans*. This compared well with existing methodologies, worked on limited clinical samples and was not pathogen specific.

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The rational treatment of pyrexial infected patients with negative blood cultures would be greatly helped by the development of tests for detecting circulating microbial DNA. This would allow for the initial selection of specific therapy which would eliminate unnecessary side effects as a result of use inappropriate antibiotics and reduce costs. Recent work has seen the application of PCR with universal bacterial primers to the diagnosis of meningitis.[1] This work could be extended to blood provided that a sensitive method for preparing DNA was developed. An added complication is that yeasts account for 8% of all hospital acquired blood stream infections and are the fourth most common cause of septicaemia.[2] Blood cultures in patients with leukaemia have been reported as positive in between 25 and 82% of patients subsequently shown to have disseminated candidiasis.[3]

Empiric antibiotic treatment can be subdivided into Gram positive, Gram negative, fungal, and viral regimens. DNA based diagnostic tests capable of distinguishing between these rather than identifying an individual pathogen would thus be an appropriate tool for controlling antibiotic usage. The description of both universal bacterial and candidal primers based on the conserved ribosomal sequences where subsequent specificity is produced by probing is a method of doing this.[4 5] However, it is dependent on the development of a universal method of DNA isolation which will work on blood products from both bacteria and yeasts.

Previous techniques have looked at bacterial DNA separation from buffy coat smears,[6-8] which was time consuming and involves the isolation of white cells by density gradient centrifugation. In the case of 41 patients wtih a final clinical diagnosis of tuberculosis, 39 had a positive PCR using buffy coat smears. Twenty six of these were sputum smear negative.[8]

Methods for isolating human DNA, such as proteinase K digestion and salt-chloroform extraction,[9] glass powder extraction[10] and serum separator tubes,[11] have not been developed as a universal method for microbial DNA extraction. The trend has been to use microbe specific protocols involving either predigestion with enzymes, such as lysostaphin in the case of staphylococci[12] or zymolase for *Candida albicans*,[5 13 14] or a monoclonal antibody purification step prior to DNA extraction.[15]

Espy *et al*[16] compared three methods based on *IsoQuick*, phenol-chloroform and lysis for the extraction of viral nucleic acids from blood and the detection of cytomegalovirus and Epstein-Barr virus. They indicated that the most sensitive method was lysis, which is not sufficiently vigorous to break open a yeast such as *C albicans*.

Casas *et al*[17] reported a method for the extraction of viral RNA and DNA from cerebrospinal fluid dependent on guanidinium thiocyanate acid (GuSCN) extraction. This did not work on blood or serum as residual blood pigment inhibited the PCR reaction.

This paper reports the combination of guanidinium thiocyanate acid extraction with proteinase K treatment to produce a sensitive and universal method for microbial DNA extraction. This method has been assessed on blood and serum spiked with micro-organisms and on a limited amount of clinical material.

Methods

BLOOD SPECIMENS

Whole blood or serum (0.054 ml 0.34 M potassium EDTA/4.5 ml blood or 0.5 ml 0.105 M sodium citrate/4.5 ml blood) from a healthy human was used in the seeding experiments with clinical isolates of *Escherichia coli*, *Staphylococcus aureus* and *C albicans*. The colony

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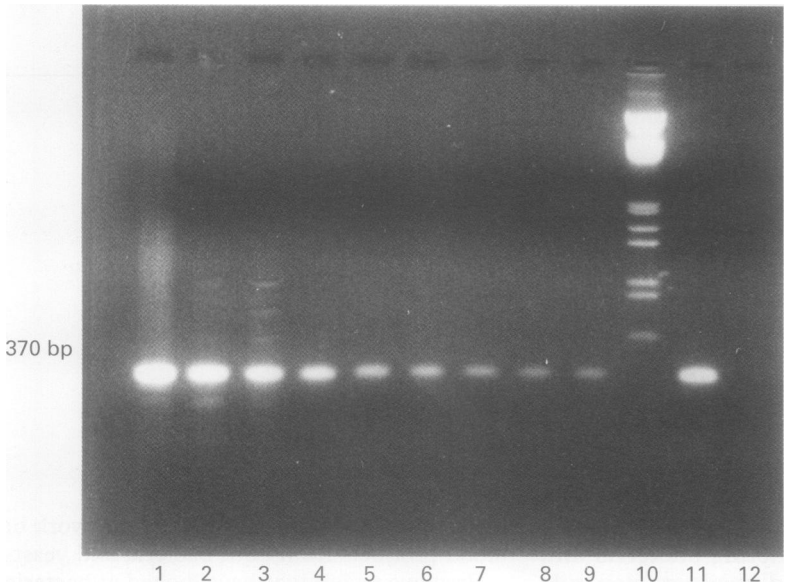

Figure 1 Lanes 1–9, titration from 2.6 × 10⁹/0.1 ml to 2.6 × 10¹ cfu/0.1 ml of E coli *after extraction and PCR with the universal bacterial primers. Product size 370 base pairs. Lane 10, molecular weight marker (EcoR1/HindIII digested DNA). Lane 11, positive control of 2.6 × 10⁷ cfu/0.1 ml of* E coli. *Lane 12, negative control lacking* E coli *DNA.*

counts were by a Miles and Misra method.[18] Each assay was performed at least twice and the average value presented. Serum samples were also obtained from patients with blood culture positive infection caused by *E coli, S aureus* or *C albicans*.

DNA EXTRACTION

From whole blood, 50 or 100 µl was added to a 1.5 ml Eppendorf tube containing 1 ml sterile double distilled water. After five minutes at room temperature, tubes were centrifuged at 14000 × *g* for 10 minutes. Nine hundred microlitres of the supernatant were removed and mixed with 400 µl lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 0.5% SDS) and 6 µl of proteinase K (20 mg/ml) (Sigma, Poole, Dorset, UK). In the case of serum 50–200 µl was added to a 1.5 ml Eppendorf tube with 300 µl lysis buffer and 6 µl proteinase K. The tubes were incubated for 30 minutes at 56°C, for four minutes at 100°C, and then cooled on ice for five minutes. Two hundred microlitres of 4 M GuSCN (Sigma) was added to each tube and the samples were mixed gently and incubated for 10 minutes at room temperature. After centrifugation (14 000 × *g* for 10 minutes at 4°C) the supernatant was transferred to a new Eppendorf tube and recentrifuged if still cloudy. To this was added a 1 in 10 volume of 3 M $NaOCH_3$ and an equal volume of cold isopropanol. After five minutes' incubation at room temperature, the DNA was precipitated by centrifugation (14 000 × *g* for 10 minutes). The isopropanol was removed and the pellet washed by the addition of 70% ethanol. After repeat centrifugation (14 000 × *g* for 10 minutes at 4°C), the ethanol was removed and the dried pellet dissolved in 50 µl sterile double distilled water.

DNA AMPLIFICATION

The sequences of the universal bacterial primers were 5′-AAC TGG AGG GTG GGG AT-3′ (RWO1) and 5′-AGG AGG TGA TCC AAC CGC A-3' (G974).[4] For *Candida*, these were 5′-GCA TCG ATG AAG AAC GCA GC-3′ (ITS3) and 5′-TCC TCC GCT TAT TGA TAT GC-3′ (ITS4).[5] DNA was amplified in a 50 µl reaction mixture consisting of 5 µl 10× PCR buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl (pH 8.3) (Boehringer Mannheim)), 4 µl 4 mM total deoxynucleosides (Promega), 2.5 µl 0.1 µg/µl of each primer, 1 unit of Taq DNA polymerase (Boehringer Mannheim), 19.6 µl double distilled water (BDH), and 16 µl of the DNA sample. The mixture was placed in a Perkin-Elmer DNA thermal cycler (Roche-9600) and incubated at 94°C for five minutes. PCR conditions were as follows: one minute at 94°C, one minute at 55°C and one minute at 72°C for 40 cycles and then incubated for seven minutes at 72°C. After amplification, 10 µl of the amplified product was run on a 1% agarose gel in the presence of ethidium bromide (0.5 µg/ml) for one to two hours at 55 volts and visualised by ultraviolet illumination.

IDENTIFICATION OF PRODUCTS

The sequences of specific probes were 5'-AT TGC TTG CGG CGG TAA CGT CC-3' for *C albicans*,[5] 5'-GCC GGT GGA GTA ACC TTT TAG GAG C-3' (RDR 327) for *S aureus*[4] and 5'-GGC GCT TAC CAC TTT GTG ATT CAT G-3' (RDR 140) for *E coli*.[4]

The PCR product was denatured by heating at 95°C for 10 minutes and transferred onto a nylon membrane (Hybond-N; Amersham). It was bound by ultraviolet crosslinking with a transilluminator (Stratalinker 2400) at 254 nm for three minutes. Probes were 3'-end labelled with digoxigenin (DIG)-ddUTP by the enzyme terminal transferase according to the manufacturer's directions (DIG-oligonucleotide 3'-end labelling kit; Boehringer Mannheim Biochemica). The DIG labelled probes were used to detect the membrane bound DNA in conjunction with an antibody conjugate (anti-digoxigenin alkaline phosphatase conjugate). A subsequent enzyme catalysed colour reaction with 5-bromo-4-chloro-3-indolylphosphate (X-phosphate) and nitro blue tetrazolium salt (NBT) produced an insoluble blue precipitate, which visualised the hybrid molecules.

Results

SENSITIVITY OF DNA EXTRACTION METHOD

The sensitivity for *E coli* detection for whole blood was 150 and for serum 26 colony forming units (cfu) per 0.1 ml. A titration of *E coli* from 2.6 × 10^9 cfu/0.1 ml to 26 cfu/0.1 ml is illustrated in fig 1. The equivalent figures for *S aureus* were 120 cfu/0.1 ml (whole blood) and 80 cfu/0.1 ml (serum) and for *C albicans* 26 cfu/0.1 ml (whole blood) and 20 cfu/0.1 ml (serum). The endpoint was visualised on an ethidium bromide stained gel and the identity confirmed by probing with DIG labelled oligonucleotides.

The technique was positive for the serum samples from individual patients with a septicaemia caused by *C albicans, S aureus* or *E coli*. The candidal product was absent from a

subsequent sample taken from the same patient 10 days later, which correlated with a clinical response to amphotericin B and negative blood cultures.

Discussion

This paper has reported a universal method of purifying microbial DNA. The sensitivity for *E coli* with serum was 26 cfu/0.1 ml. This can be compared favourably with the method of Hoon Song *et al*[6] for *Salmonella typhi*, in which mononuclear cells were obtained from whole blood after density centrifugation with Ficoll-Hypaque, incubated with lysozyme and proteinase K and the DNA was extracted with phenol-chloroform-isoamylalcohol. The sensitivity when purified *S typhi* DNA was mixed with mononuclear cell DNA was 4 ng, equivalent to 10^6 organisms.

The limit of detection for *S aureus* with serum was 80 cfu/0.1 ml. This is as good as the results reported with other Gram positive bacteria. Ubukata *et al*[12] performed PCR for the staphylococcal methicillin gene. DNA extraction was based on lysostaphin lysis which is specific to staphylococci and achieved a sensitivity of 5×10^2 cfu/tube for *S aureus* after probing. In the case of *Streptococcus pneumoniae*, whole blood digested by proteinase K followed by phenol-chloroform-isoamylalcohol extraction was positive at 4×10^2 cfu/0.1 ml.[7] A lysis method reported subsequently achieved a sensitivity of only 80% on clinical material despite being positive with as little as 4 cfu/ml in spiked specimens.[19]

The technique reported for candidal DNA extraction achieved a sensitivity comparable with other studies. Burgener-Kairuz *et al*,[13] applying PCR for detecting the cytochrome P-450 lanosterol-α-demethylase gene of *C albicans* after zymolase treatment, achieved a sensitivity of 10 to 20 cells/0.1 ml of seeded blood but only 71% in 80 clinical specimens. This was improved by the development of a nested PCR where five of seven patients with candidiasis were positive.[14] Lyticase extraction failed unless inhibitors were removed by glass adhesion[20] or the sensitivity was low at 150 to 200 cells/0.1 ml.[21] If non-yeast DNA was removed by pretreatment this increased to 10 to 15 cells/0.1 ml.[22] A combination of zymolase extraction, lysis, proteinase K treatment, and phenol chloroform extraction was positive for two *C albicans* cells/0.2 ml blood, but this was only after probing.[5]

Prior purification with candidal specific antibody resulted in an assay capable of detecting three cells of *C albicans*/0.1 ml.[15] A mixture of mechanical disruption with glass beads, protein digestion and DNA extraction produced a test with a detection rate of only 79% in cases of systemic candidiasis.[23]

In summary, this paper reports a universal method for extracting bacterial or fungal DNA from blood products. The sensitivity, as judged by seeded samples, was similar or better than that reported previously. This is likely to be increased when the technique is combined with probing. It was also evaluated successfully in a limited number of clinical samples. Thus, it could form the basis of a stategy for improving diagnosis. Microbial DNA extracted by this method would be amplified with universal primers. The pattern could be used to demonstrate whether the infection was Gram positive, Gram negative or yeast. This would lead to a more rational use of antibiotics. The precise microbe could then be identified by probing the product with a battery of specific probes.

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