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Article

Detection of Bacteraemia in Patients with Fever and Neutropenia Using 16S rRNA Gene Amplification by Polymerase Chain Reaction

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Abstract Episodes of fever and neutropenia are common complications of treatment for cancer. The use of prophylactic and early empirical antibiotics has reduced mortality but decreases the sensitivity of diagnostic tests based on culture. The aim of this study was to determine the potential of a broad diagnostic approach (eubacterial) based on 16S rRNA gene amplification and sequencing to augment cultural methods of diagnosis of bacteraemia in patients with fever and neutropenia in a regional paediatric oncology centre. One hundred eleven patient-episodes of fever and neutropenia were evaluated during the study period, 17 of which were associated with positive blood cultures, as follows: *Staphylococcus epidermidis* (n=6 episodes), *Enterococcus faecium* (n=2), *Streptococcus sanguis* (n=3), *Streptococcus mitis* (n=3), *Staphylococcus aureus* (n=1), *Micrococcus* spp. (n=1), and *Stenotrophomonas maltophilia* (n=1). Eubacterial polymerase chain reaction (PCR) detected bacterial DNA in nine of 11 blood culture-positive episodes for which a sample was available for PCR; the species identified by sequence analysis were identical to those derived from the conventional identification of the cultured isolates. Bacterial DNA was detected in 20 episodes (21 bacterial sequences) associated with negative blood cultures, 18 of which occurred in patients who were receiving antibiotics at the time of sample collection. The species presumptively identified by partial 16S rRNA gene sequencing were as follows: *Pseudomonas* spp. (n=6 episodes), *Acinetobacter* spp. (n=5), *Escherichia* spp. (n=3); *Moraxella* spp. (n=3); *Staphylococcus* spp. (n=2); *Neisseria* spp. (n=1); and *Bacillus* spp. (n=1). The results of this study suggest that molecular techniques can augment cultural methods in the diagnosis of bacteraemia in patients who have been treated with antibiotics.

Introduction

Modern treatment of cancer is associated with significant risk of infection [1–3]. The single most important risk factor for infection is the degree of neutropenia [4], although other risk factors include disruption of physical defence barriers and alteration in microflora [2]. Virtually any organism can cause serious infection in the immunocompromised host, including commensal bacteria and organisms of low virulence that do not cause infection in the immunocompetent host [5]. Therapeutic decisions should ideally be based on microbial isolation and determination of antibiotic sensitivity [6]; however, early empirical antibiotic therapy may compromise the sensitivity of tests based on microbial culture [1, 7]. Currently, no pathogen is identified in 35 to 60% of episodes of fever and neutropenia [2, 8, 9]. Riikonen et al. [9] investigated blood culture-negative febrile neutropenic episodes and found serological evidence of bacterial infection in 35%.

Nucleic acid technology has recently found application in the diagnosis of infections caused by fastidious organisms [10, 11] and in life-threatening situations where

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antibiotics are given early [12]. Eubacterial 16S rRNA gene amplification with the polymerase chain reaction (PCR) uses primers that are complementary with sequences conserved in all bacteria and allows the detection of bacteria, even if nonculturable [13]. The application of eubacterial PCR offers a novel approach to the investigation of patients with fever and neutropenia. The aim of this study was to determine the potential of a broad diagnostic approach based on 16S rRNA gene amplification and sequencing to augment cultural methods of diagnosis of bacteraemia in patients with fever and neutropenia in a regional paediatric oncology centre.

Patients and Methods

Patients. The patient population included in the study were those undergoing either standard chemotherapy for malignancy or bone marrow transplantation (BMT) within the Royal Hospital for Sick Children, Bristol, UK. All patients who developed fever whilst neutropenic during the 7-month period from May to November 1995 were entered into the study. During the period of this study, BMT patients received prophylaxis with ciprofloxacin from day 3 following transplantation until the neutrophil count recovered to 1×10^9/l.

Definition of Episode of Fever and Neutropenia. An episode of fever and neutropenia was defined by a temperature of >38 °C on two occasions within 1 h or >38.5 °C on a single occasion in the presence of an absolute neutrophil count of $<1 \times 10^9$/l. No patients were excluded because of prior antibiotic therapy.

Patient Evaluation and Laboratory Information. At the start of a febrile episode, paired blood samples were collected for PCR and blood culture. If antibiotic therapy was altered for continuing fever, repeat paired samples were taken at 48 h and 96 h and intermittently during the course of prolonged fever. Up to 20 ml of blood for culture was collected from each lumen of the central venous catheter or from a peripheral vein in the absence of a central venous catheter and inoculated into aerobic and anaerobic broth dilution culture bottles (BacT/Alert; Organon Teknika, USA). The volume of blood cultured per episode was recorded using the scale on the side of each bottle. Blood cultures were considered contaminated (negative blood cultures) when coagulase-negative staphylococci [14] or environmental species were isolated from one of the two blood culture bottles only. Samples of 1 ml venous blood for PCR were placed in sterile DNA-free vacutainers containing EDTA (Becton Dickinson, France) and then stored at –70 °C until processed. These samples were coded so that the PCR operator was blind to clinical information.

Clinical data were retrieved by review of the case notes. Fever characteristics, treatment given, any complications of treatment, antibiotic changes, and information pertaining to potential causes of fever, including results of bacteriological, virological and mycological tests were noted.

Episode Analysis. Each patient-episode of fever and neutropenia was evaluated retrospectively using all available laboratory and clinical data and allocated to one of four categories. Multifactorial cases were allocated to the category considered most important: A, episode with bacterial isolation from blood culture; B, local or systemic infection with negative blood cultures; C, noninfective causes of fever [15–17]; D, episode of fever with no attributable cause (not in categories A, B, or C).

Statistical Methods. Duration of fever was compared using the Mann-Whitney U test. The chi-square test was used to compare the proportions of PCR-positive episodes in each of the four fever groups.

Methods for Polymerase Chain Reaction Analysis. The amplification, denaturing gradient gel electrophoresis, and sequencing methods have been described previously [18, 19]. Bacterial DNA in EDTA anticoagulated blood was detected using PCR amplification with primers B5 (5′-TCAGATTGAACGCTGGCGGC-3′) and B4 (5′-TATTACCGCGGCTGCTGGCA-3′). These primers are complementary to 16S rRNA gene sequences, at base-pair positions 20 and 513, respectively, which are highly conserved amongst bacteria. The amplification products were then visualised on 2% agarose gel, and the products from any sample with a positive signal were then reamplified using internal primers P2 (5′-ATTACCGCGGCTGCTGG-3′) and P3 (5′-CGCCCGCCGCGCGCGGGCGGGGGCACGGGGGGCCT ACGGGAGGCAGCAG-3'; 40 bp GC clamp attached at the 5' end) [20] (nested PCR) to produce a fragment that spans the variable V6 region of the 16 S rRNA gene. Different base-pair sequences were then separated by denaturing gradient gel electrophoresis (DGGE) [21]. Separation with DGGE was necessary to ensure that mixed amplification products were detected such as might be found in samples from patients with polymicrobial sepsis.

Control samples containing each of the reaction constituents except genomic DNA were run along with the samples under investigation (PCR negative). Additional control samples included blood samples from healthy volunteers (blood negative control) and blood samples spiked with *Escherichia coli* 10^4/ml (blood positive control) and extracted bacterial DNA (PCR positive). In each test run the sequence of samples was as follows: PCR negative, PCR positive; blood negative control, blood positive control; and then eight test samples. DNA extraction, preparation of the PCR reaction mixture, and post-PCR analysis were carried out in separate rooms using equipment designated for each area. DNA extraction and preparation of the PCR reaction mixture were carried out in separate class II laminar flow cabinets using filter-protected pipette tips (Aerogard; Alpha, UK). Samples were considered to have given a positive PCR product only when all of the control samples gave appropriate results. A sample was defined as PCR positive only if repeat extractions from the original blood sample gave a positive result in two separate experiments carried out on different days. Negative control blood samples gave positive results on less than 5% of test runs.

DNA Preparation. A modification of Talmud's method [22] using 300 µl of anticoagulated whole blood was used to extract DNA from blood samples. Following extraction, the samples were stored at –70 °C prior to PCR amplification.

Polymerase Chain Reaction Amplification. Reaction 1: The PCR reactions were performed in a total volume of 100 µl. The reaction mixture contained 0.5 units of Super TAQ Polymerase (HT Biotechnology, UK), buffer [10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% (v/v) Triton X-100, and 0.01% (w/v) gelatin], 20 µm of each dNTP (Pharmacia Biotech, Sweden), and 0.1 µM of each of the primers B4 and B5. Ten microlitres of the DNA extracted from the blood samples was added to the PCR mixture at 75 °C within a laminar flow cabinet, and the reaction mixture was overlaid with two drops of paraffin oil. The reaction mixtures were incubated for 3 min at 94 °C. A total of 35 cycles of PCR were performed using a thermal cycler (Omnigene; Hybaid, UK) consisting of a denaturation step for 30 sec at 94 °C, an annealing step for 1 min at 65 °C, and an extension step for 1 min at 72 °C. After the final cycle there was a step of 7 min at 72 °C.

The PCR products generated were visualised by ethidium bromide staining after electrophoresis in a gel containing 2% agarose. Any product giving a visible band was reamplified using internal primers (reaction 2).

Reaction 2: The PCR reactions were performed in a total volume of 50 µl. The reaction mixture contained the same constituents

already mentioned, with the addition of 0.1 μM of each of the primers P1 [21] and P2. One microlitre of PCR product from reaction 1 was added to this mixture at 75 °C and overlaid with two drops of paraffin oil. A total of 25 thermal cycles were performed. A Bio-Rad Protean II system (Bio-Rad Laboratories, UK) was used to perform DGGE as described previously [19].

Sequencing. Bands on the DGGE gel derived from blood samples were cut from the gel using a sterile scalpel blade and placed in 50 μl of water and left at 4 °C for 24 h. This DNA was then prepared for sequencing [19] and sequenced directly using the P1 primer on the ABI 377 automated fluorescent DNA sequencer using dye terminator chemistry. The sequences were analysed using the FastA program from the Wisconsin Genetics Computer Group suite of programs, Central Laboratory of the Research Councils, Daresbury Laboratory [23], to give sequence alignment and percentage identity.

Sensitivity of Polymerase Chain Reaction Methods. The sensitivity of the PCR methods, including the efficiency of DNA extraction, was tested using blood samples spiked with known concentrations of *Escherichia coli* determined using a modification of the Miles and Misra technique [24]. The PCR methods detected 4.3×10^3 colony-forming units (cfu) of *Escherichia coli* per millilitre (65 cfu/reaction) of spiked whole blood.

Results

All 111 patient-episodes of fever and neutropenia occurring during the study period are included in the analysis. In the standard chemotherapy group, there were 58 patient-episodes of fever and neutropenia in 36 children (median age 8 years, range 1–17 years). Twenty-one patients had haematological malignancies; the remainder had solid tumours. Nine patients had two episodes of fever and neutropenia, three had three episodes, one had four episodes, one had five episodes, and 22 had a single episode. In the BMT group there were 53 episodes of fever and neutropenia in 40 patients (median age 11 years, range 1–48 years). Thirty-seven patients had haematological malignancies, and three had genetic diseases. Nine patients had two episodes of fever and neutropenia, two had three episodes, and 29 had a single episode. Within this group, there were four patients who died during the febrile episode; one death was attributed directly to sepsis. Overall, there were 90 patient-episodes for which samples were available for analysis by PCR: 39 of 58 (67%) in the standard treatment group and 51 of 53 (96%) in the BMT group (Table 1).

Overall (Standard Chemotherapy and Bone Marrow Transplantation Patients) Episode Analysis, Group A. Bacteria were isolated from 17 blood culture-positive episodes in group A, as follows: *Staphylococcus epidermidis*, six episodes; *Enterococcus faecium*, two episodes; *Streptococcus sanguis*, three episodes; *Streptococcus mitis*, three episodes; *Staphylococcus aureus*, one episode; *Micrococcus* spp., one episode; and *Stenotrophomonas maltophilia*, one episode. Samples from 11 of these episodes were available for PCR analysis; nine (82%) were positive. The bacteria not detected by PCR were *Enterococcus faecium* and *Streptococcus mitis*. The sequences from the first eight samples positive in culture gave a result on the European Molecular Biology Laboratory, Cambridge, UK (EMBL) database identical to that derived from cultured bacteria. The ninth sequence was from a BMT patient with a line-associated *Stenotrophomonas maltophilia* infection, but there is no 16S rRNA gene sequence for this organism on the EMBL database.

Overall Episode Analysis, Group B. Diagnoses in group B included localised infection with *Staphylococcus aureus*, cellulitis, enterocolitis, urinary tract infection, atypical pneumonia, dental abscess, localised fungal lesion, disseminated viral infection (adenovirus, parainfluenza, cytomegalovirus, Epstein-Barr, coxsackie B, herpes simplex), otitis media, and disseminated candidiasis.

Overall Episode Analysis, Group C. Noninfective causes of fever (group C) included leukaemic presentation, drug reaction, haemolytic/uraemic syndrome, and graft-versus-host disease.

Overall, there were 20 episodes in which bacterial DNA was detected by PCR alone, and 21 bacterial

Table 1 Results of the polymerase chain reaction within assigned aetiological categories

Fever category*	Patient-episodes								
	Standard treatment group			BMT group			Standard group + BMT group		
	Total (n=58)	Analysed by PCR (n=39)	Positive by PCR (n=9)	Total (n=53)	Analysed by PCR (n=51)	Positive by PCR (n=20)	Total (n=111)	Analysed by PCR (n=90)	Positive by PCR (n=29)
A	5	0	0	12	11	9	17	11	9
B	18	14	2	12	12	2	30	26	4
C	9	5	1	8	7	0	17	12	1
D	26	20	6	21	21	9	47	41	15

* A, bacterial isolation from blood culture; B, local or systemic infection with negative blood culture; C, noninfective causes of fever; D, fever not in category A, B, or C

BMT, bone marrow transplantation; PCR, polymerase chain reaction

DNA sequences. There was a significant difference in the proportion of episodes with bacterial DNA detected by PCR in group A (patients with bacteraemia) compared with either group B (fever attributed to a nonbacteraemic infection) (9/11 vs. 4/26; chi-square with Yates correction = 12.2) or group C (fever attributed to a non-infective cause) (9/11 vs. 1/12: chi-square with Yates correction = 9.8). Bacterial DNA was detected by PCR in 15 of 41 episodes without an attributed cause of fever (group D). Fever duration (excluding patients who died during episodes of fever) was not significantly different in the blood culture-negative episodes in which bacterial DNA was detected by PCR (median 7 days; range 2–40 days) than in the blood culture-positive episodes (median 10 days; range 2–26 days). Fever duration was significantly longer in episodes that were PCR positive ($U = 64$; $P < 0.01$) or blood culture positive ($U = 75$; $P < 0.01$) than in episodes with no attributable cause of fever (after excluding those PCR-positive episodes) (median 2 days; range 1–19 days).

There were nine episodes in the standard treatment group that were blood culture negative and had bacterial DNA detected by PCR. There were 11 episodes in the BMT cohort that were blood culture negative and had bacterial DNA detected by PCR. Tables 2 and 3 show the presumptive identification, antibiotic treatment, and duration of fever associated with each of these patient-episodes. All of the identifications derived from sequence analysis showed >90% presumptive sequence identity using the EMBL database.

Review of clinical and laboratory records in both the BMT and the standard chemotherapy groups showed that two of the three episodes associated with detection of *Pseudomonas putida* DNA were associated with inflamed central venous catheter exit sites; two of the three episodes with *Moraxella phenylpyruvia* were associated with mucositis; one of the two episodes with *Escherichia coli* occurred in a patient with a previous episode of *Escherichia coli* bacteraemia diagnosed by blood culture; the episode with *Staphylococcus aureus* occurred in a patient from whom *Staphylococcus aureus* was cultured from sputum in large numbers; and the episode with *Staphylococcus epidermidis* was associated with the isolation of large numbers of *Staphylococcus epidermidis* from the tip of a central venous catheter removed at the end of the episode of fever and neutropenia. The significance of changes in antibiotic therapy are difficult to assess because of the unpredictable susceptibility profiles of many of the species presumptively identified by PCR and because of the difficulty in taking into account other factors such as removal of central lines and recovery of neutrophil counts.

Table 2 Clinical characteristics of episodes in the standard treatment group in which bacterial 16S rDNA sequences were identified by polymerase chain reaction alone

Presumptive identification	Relevant clinical information[a]	Antibiotic treatment at time of positive sample	Duration of fever (days)	Antibiotic treatment at time of clinical response
Pseudomonas medocina		none	2	cefuroxime, gentamicin
Pseudomonas aeruginosa		cefixime	3	ceftazidime, teicoplanin
Escherichia coli	previous blood culture positive for *E. coli*	cefixime	3	ceftazidime, teicoplanin
Acinetobacter johnsonii		cefuroxime, gentamicin	8	ceftazidime, teicoplanin
Acinetobacter johnsonii		ciprofloxacin	4	ceftazidime, teicoplanin
Acinetobacter lwoffii	enterocolitis	ceftazidime, teicoplanin	17	imipenem, vancomycin, amphotericin
Moraxella phenylpyruvica		none	4	ceftazidime, teicoplanin
Moraxella phenylpyruvica	mucositis	cefuroxime, gentamicin, flucloxacillin	fever continued until death	NA (death due to other complications)
Staphylococcus aureus[b]	*S. aureus* cultured from throat swab & sputum	cefuroxime, gentamicin, flucloxacillin		ongoing fever
Escherichia hermannii[b]	diarrhoea, vomiting	ceftazidime, teicoplanin	25	imipenem, vancomycin

[a] All patients had central venous catheters
[b] These samples were obtained at different times within the same patient episode.
NA, not applicable

Table 3 Clinical characteristics of episodes in the bone marrow transplantation group in which bacterial 16S rDNA sequences were identified by polymerase chain reaction alone

Presumptive identification	Relevant clinical information[a]	Antibiotic treatment at time of positive sample	Duration of fever (days)	Antibiotic treatment at time of clinical response
Pseudomonas putida	inflamed long line site	ciprofloxacin, flucloxacillin	5	imipenem, vancomycin
Pseudomonas putida	inflamed long line site	ceftazidime, teicoplanin	7	imipenem, vancomycin
Pseudomonas putida		ceftazidime, teicoplanin	11	imipenem, vancomycin
Pseudomonas pickettii	inflamed long line site	cefuroxime, gentamicin, flucloxacillin	13	ceftazidime, teicoplanin
Acinetobacter johnsonii		ciprofloxacin	12	imipenem, vancomycin
Acinetobacter calcoaceticus		imipenem, vancomycin	40	imipenem, vancomycin
Moraxella phenylpyruvica	mucositis	ciprofloxacin	6	ceftazidime, teicoplanin
Escherichia coli	diarrhoea	ceftazidime, teicoplanin	14	imipenem, vancomycin
Neisseria pharyngis		ciprofloxacin	6	teicoplanin
Bacillus spp.		ceftazidime, teicoplanin	fever continued until death	NA (death due to other complications)
Staphylococcus epidermidis	significant CVC tip colonisation: *S. epidermidis*	cefuroxime, gentamicin, flucloxacillin	5	ceftazidime, teicoplanin

[a] All patients had central venous catheters
CVC, central venous catheter; NA, not applicable

Discussion

Pathogens causing a large proportion of episodes of fever associated with neutropenia currently remain undetected when standard culture techniques are used [2, 8, 9]. There are many potential benefits associated with improving the sensitivity of tests for bacteraemia in patients with fever and neutropenia, such as a reduction in the number of other investigations, improved understanding of the incidence and epidemiology of bacteraemia, and better targeting of therapeutic strategies.

This study was designed to assess the extent to which the use of PCR with eubacterial primers could improve diagnostic yield rather than to quantify the benefits of better diagnosis. There are no absolute criteria to confirm the validity of positive PCR results associated with negative blood cultures. Overall, PCR detected bacterial DNA in significantly more episodes associated with positive blood cultures (group A) than in episodes with nonbacteraemic causes of fever (groups B and C). Most of the organisms identified by PCR have previously been associated with infections in immunocompromised patients [25, 26]. There were other positive cultures supporting the PCR identification in three patient episodes. Fever duration in the PCR-positive group was similar to that in the blood culture-positive group. The sensitivity of the methods reported here could be improved by the use of more efficient DNA extraction methods [27] and by the use of haemogloblin to reducing the inhibition of PCR by haemoglobin [28].

Both children and adults with fever and neutropenia have higher concentrations of bacteria in their blood than are found in immunocompetent adults with bacteraemia [29, 30]; this probably accounts for the good yield despite the poor sensitivity of our methods. Improved sensitivity may have led to the detection of small numbers of contaminating bacteria or PCR products [31–33]. In this study the contrast between the identifications of bacteria recovered by culture (predominantly gram-positive) and those detected by PCR alone (predominantly gram-negative) may be explained by the incomplete homology of the B5 primer for some gram-positive species and the relatively poor extraction of gram-positive DNA using our method. However, other recent studies have also reported an increasing frequency of gram-negative infections in immunocompromised patients [34, 35] The poor sensitivity of the methods used in this study and the diversity of medically-important species identified supports the contention that the majority of episodes in which bacterial DNA was detected were associated with bacteraemia rather than sample contamination. Even so, we cannot ex-

clude the possibility that a proportion of the PCR-positive samples was contaminated.

Since the organisms identified by PCR are culturable, we sought reasons to explain why the blood cultures remained negative. The mean blood volumes were less than would be considered ideal to maximise the rate of positive blood cultures obtained from adults, but the volume reflects the predominance of children in the study population. In the majority of episodes, patients were on antibiotics at the time an organism was identified by PCR [1, 7]. The *Acinetobacter* spp., *Pseudomonas* spp., and *Moraxella* spp. identified by PCR are nonfermentative, and the BacT/Alert system detects organisms by the colorimetric detection of CO_2 produced by growing organisms [36, 37]. The wards are located at a site separate from the laboratory and there is frequent delay in samples reaching the laboratory; as early processing is known to be important, this delay may have affected the detection rate using this blood culture system.

The factor contributing most to the poor relative performance of blood cultures was probably the empirical use of antibiotics. The bacterial DNA detected by PCR may have been derived from viable uncultured bacteria or from bacteria killed by empirical antibiotic therapy. Because we did not collect extra blood samples over and above those collected for culture, we cannot postulate on the half-life of circulating DNA in these patient-episodes.

The methods described in this report are labour intensive, time-consuming, and expensive. As automated systems become available, it will be possible to use this technology to inform clinical practice and to develop a more stringent protocol to determine the value of this approach in the management of patients with fever and neutropenia.

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