

# Real-time broad-range PCR versus blood culture. A prospective pilot study in pediatric cancer patients with fever and neutropenia

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## Abstract

**Materials and methods** In a pilot study, results of real-time broad-range (16S rRNA) polymerase chain reaction (PCR) performed on 45 blood samples of pediatric cancer patients with fever and neutropenia were compared with blood culture results.

**Results** The PCR assay used, having proven a high sensitivity in artificially spiked blood samples, was positive

in only three of ten blood culture-positive samples, and it was positive in 10 of 35 (29%) culture-negative samples.

**Conclusion** This broad-range PCR assay, which may identify not-grown bacteria potentially contributing to fever, needs improvement in sensitivity, and different reasons for positive PCR in negative blood culture samples need to be assessed before clinical application.

**Keywords** Bacteremia · Broad-range · Fever · Neutropenia · Polymerase chain reaction

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## Introduction

Blood cultures are the gold standard to diagnose bacteremia since about a century. Their potential for further methodological improvement is negligible despite limitations in speed of detection and sensitivity for slow-growing or fastidious organisms or after start of antibacterial therapy [6]. Broad-range polymerase chain reaction (PCR) might offer a promising auxiliary diagnostic tool. It bases on amplifying DNA regions coding for 16S or 23S rRNA which is highly conserved in all bacteria [6, 7]. Subsequent sequencing of amplicons allows for identification of bacterial species [1, 6, 7].

In fever and neutropenia (FN) after chemotherapy, bacteremia is potentially life threatening [8]. The sensitivity and speed limitations of blood cultures are thus especially important. The aim of this two-center pilot study was to assess the applicability and potential problems of broad-range PCR in the daily routine setting by comparing its results with those of routine blood cultures in blood from pediatric cancer patients with FN.

## Materials and methods

### Patients and samples

Cancer patients aged 1 to 17 years presenting with fever (axillary temperature greater than 38.0°C for more than 2 h, or greater than 38.5°C once) and severe neutropenia (absolute neutrophil count less than  $0.5 \times 10^9/l$ ) induced by nonmyeloablative chemotherapy were eligible. Besides oral antiseptic rinsing, antimicrobial prophylaxis was restricted to *Pneumocystis jiroveci* pneumonia prophylaxis by oral cotrimoxazole or inhaled pentamidine. Multiple FN episodes per patient were allowed. Based on an estimated proportion of 20% blood culture-positive samples [8], the study aimed at ten blood culture positive episodes to determine the proportion of blood culture-negative but PCR-positive samples with a 95% confidence interval less than 33% wide. Patients or their legal guardians gave written informed consent. The respective cantonal ethics committees approved the study.

Before starting empirical intravenous antimicrobial therapy, blood was drawn into a syringe, from which two blood culture bottles and a 5-ml K3E Vacutainer™ glass tube (Becton Dickinson, Basel, Switzerland) for PCR analysis were filled. Blood cultures were processed in routine automated systems (BACTEC, Becton Dickinson, or BacT/ALERT, bioMérieux, Geneva, Switzerland). The Vacutainer™ tubes were transported without preprocessing to the laboratory performing broad-range PCR.

### Erythrocyte lysis, DNA extraction and real-time broad-range PCR

Lysis of erythrocytes was performed upon receipt of the sample in the laboratory as described [10], and the pellet

was stored at  $-80^\circ\text{C}$  until further processing. The Wizard SV genomic DNA purification system (Promega, Madison, WI) augmented with lysozyme, lysostaphin, and proteinase K, was used for DNA extraction as described [10], the final elution volume being 200  $\mu\text{l}$ .

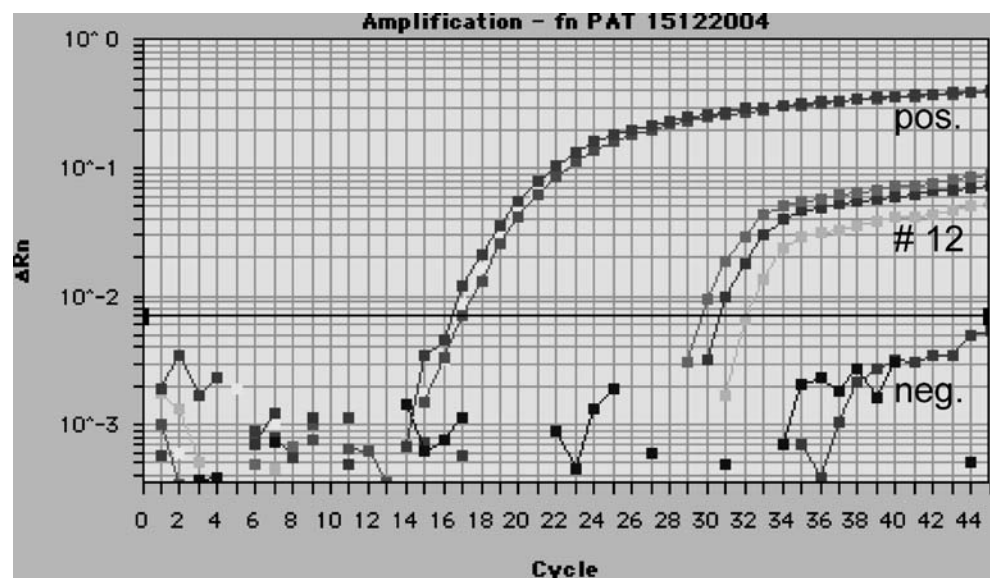
Real-time broad-range PCR was performed on 2  $\mu\text{l}$  DNA elution to amplify a conserved DNA sequence encoding for the 16S rRNA gene using forward primer Bak11W 5'-AGTTTGATC(A/C)TGGCTCAG-3', reverse primer Bak2 5'-GGACTAC(C/T/A)AGGGTATCTAAT-3' [5], and probe Tap 5'-CGTATTACCGCGGCTGCTGGCAC-3' [9] with TaqMan® Universal PCR Master Mix and No AmpErase® UNG (Applied Biosystems, Cheshire, UK) on an ABI PRISM 7700 Sequence Detector (Applied Biosystems) as described [10]. Each PCR was performed in triplicate. When at least two threshold cycle values were below 38 or one was below 34 [10], results were considered positive if sequencing of the amplicon allowed for bacterial identification and considered indeterminate if sequencing did not allow for identification. Negative controls (blood from healthy volunteer) and positive controls (blood spiked with  $10^6$  cfu *Escherichia coli* per PCR) were determined in each run (Fig. 1).

The PCR assay using this specific combination of DNA extraction and primer pair had proven to be the most sensitive of nine assays tested. It detected 18 of 22 bacterial species tested by spiking physiological saline at concentrations of  $10^2$  cfu per PCR or below [10]. Applying the probe used together with this primer pair, the sensitivity to detect *E. coli* was identical when spiking blood instead of physiological saline [10].

### Sequencing and bacterial identification

Amplicons were sequenced using primer Bak2 and the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied

**Fig. 1** Real-time quantitative broad-range PCR (TaqMan®) for sample no. 12. The fluorescence ( $\Delta Rn$ ), measured in each PCR cycle for each sample, is plotted on the  $y$ -axis against cycle number on the  $x$ -axis for the sample (# 12) in triplicate and the positive (*pos.*;  $10^6$  cfu *E. coli* per PCR) and negative controls (*neg.*; blood from healthy volunteer) each in duplicate. The threshold cycle ( $C_T$ ) value corresponds to the cycle number where fluorescence exceeds the  $\Delta Rn$  threshold set here at  $7 \times 10^{-3}$  (horizontal line)



**Table 1** Samples with positive blood cultures and/or positive or indeterminate broad-range PCR results<sup>a</sup>

Sample		Blood culture	Broad-range PCR, sequencing, and identification		
Number	Age (years)	Result	Sensitivity <sup>b</sup>	Result <sup>c</sup>	Identification (score in bits, similarity)
1	8.8	Viridans streptococcus	10	Negative	–
6	1.4	<i>Escherichia coli</i>	1	Negative	–
10	8.9	<i>Enterobacter cloacae</i>	10,000	Negative	–
		Viridans streptococcus	10		
12	2.7	CoNS	1	Positive	<i>Staphylococcus epidermidis</i> (444, 96%)
15	13.5	CoNS	1	Negative	–
		<i>Bacillus fragilis</i>	100		
16	13.5	<i>B. fragilis</i>	100	Indeterminate	–
26	2.8	CoNS	1	Indeterminate	–
27	15.6	CoNS <sup>c</sup>	1	Negative	–
38	13.2	<i>Klebsiella pneumoniae</i> <sup>d</sup>	10,000	Positive	<i>Klebsiella pneumoniae</i> (488, 92%)
41	3.0	<i>K. pneumoniae</i>	10,000	Positive	<i>Stenotrophomonas maltophilia</i> (438, 91%)
		<i>Pseudomonas aeruginosa</i>	100		
2	1.7	Negative	NA	Positive	<i>Pseudomonas aeruginosa</i> (82, 88%)
3	4.5	Negative	NA	Positive	<i>S. maltophilia</i> (174, 84%)
9	6.7	Negative	NA	Positive	<i>Pseudomonas</i> spp. (466, 92%) <i>S. maltophilia</i> (462, 92%) <i>Acinetobacter calcinoaceticus</i> (462, 92%)
18	2.8	Negative	NA	Indeterminate	–
19	9.5	Negative	NA	Positive	<i>S. maltophilia</i> (527, 94%) <i>A. calcinoaceticus</i> (527, 94%)
20	13.0	Negative	NA	Indeterminate	–
21	13.8	Negative	NA	Positive	<i>S. maltophilia</i> (492, 93%)
23	4.1	Negative	NA	Positive	<i>S. maltophilia</i> (206, 85%)
25	4.7	Negative	NA	Positive	<i>S. maltophilia</i> (357, 89%)
30	3.1	Negative	NA	Positive	<i>S. maltophilia</i> (258, 87%)
31	2.9	Negative	NA	Positive	<i>S. maltophilia</i> (76, 87%)
40	3.8	Negative	NA	Positive	<i>S. maltophilia</i> (145, 84%)
43	5.0	Negative	NA	Indeterminate	–
44	4.9	Negative	NA	Indeterminate	–

<sup>a</sup>In 21 episodes of fever and neutropenia, results of both methods were negative (median age at sampling, 11.2 years)

<sup>b</sup>Analytical sensitivity (cfu per PCR) of this combination of DNA extraction and PCR for species detected in water [10]

<sup>c</sup>Positive, if at least two threshold cycle ( $C_T$ ) values below 38 or at least one  $C_T$  value below 34 and bacterial identification possible by sequencing the amplicon; indeterminate if at least two threshold cycle ( $C_T$ ) values below 38 or at least one  $C_T$  value below 34 but no bacterial identification by sequencing; negative in the remaining samples

<sup>d</sup>Two different strains isolated

CoNS indicates coagulase-negative staphylococcus; NA not applicable, as blood cultures were negative

Biosystems). The samples were analyzed on an ABI PRISM 310 Genetic Analyzer (PerkinElmer, Boston, MA) as described [10]. Bacterial identification was done in a two-step procedure as described [1, 10]. First, the FASTA algorithm of the GCG Wisconsin software package (Accelrys) with visual inspection of the electropherogram, and sequence correction where necessary was performed. It was followed by a second search using the basic local alignment search tool (<http://www.ncbi.nlm.nih.gov/blast>). Criteria for identification to the genus or species level were used as described [1]. For each sequence, only the highest-scoring and, if applicable, the next highest-scoring species were recorded.

## Statistics

StatXact 6.3.0 (Cytel, Cambridge, MA) was used for Zelen's exact test of homogeneity of odds ratios, and for the exact Mann–Whitney test, where applicable.

## Results

Blood cultures detected bacteremia in 10 (22%) of 45 FN episodes in 31 patients with FN recruited consecutively in two centers during a 6-month period in 2003. Patient's median age at first FN was 8.8 years (range, 1.4–16.5).

Diagnoses included acute lymphoblastic leukemia in 14, acute myeloid leukemia in two, lymphoma in five, and solid tumors in ten patients.

Broad-range PCR, performed after optimization of the method [10] in 2005, was positive in three and indeterminate in two of ten blood culture-positive samples and positive in 10 and indeterminate in 4 of 35 blood culture-negative samples (Table 1). There were no significant associations of these ratios with center, diagnostic group, fever rule, and peripheral versus central venous access (Zelen's test; data not shown). Among patients with blood culture-negative samples, a positive versus negative PCR result was significantly associated with younger age (median, 4.3 versus 11.2 years; Mann–Whitney statistic 394,  $p=0.013$ ).

In the three samples with both positive blood culture and PCR results, bacterial identification confirmed the species detected in blood cultures in two samples (*Staphylococcus epidermidis* and *Klebsiella pneumoniae*), whereas in the third sample, growing *K. pneumoniae* and *Pseudomonas aeruginosa* in blood cultures, *Stenotrophomonas maltophilia* was identified.

In nine of the ten samples with negative blood culture but positive PCR result, *S. maltophilia* with or without other species (*Acinetobacter calcoaceticus* and *Pseudomonas* spp.) was identified. The sequences of these nine samples all differed from each other (data not shown). *Pseudomonas aerigompsa* was identified in one sample.

## Discussion

In contrast to the results of the study that had established the specific assay of broad-range PCR used [10], the sensitivity of real-time broad-spectrum PCR was not high in this pilot study. Two main reasons might explain this. First, the analytical sensitivity of the applied combination of DNA extraction and PCR protocol ranges, depending on the bacterial species, between 1 cfu and 10,000 per PCR in physiological saline [10]. However, there was no evident association between sensitivity and result of broad-range PCR (Table 1). In contrast, the blood culture method may, at least theoretically, detect bacteremia with only 1 cfu per blood culture bottle for each species growing in this system. Second, bacterial blood loads are expected to be lower after nonmyeloablative than after myeloablative chemotherapy, where sensitivity of broad-range PCR reported is higher [3, 6]. In the three PCR- and blood culture-positive samples, species identification was correct in two samples, whereas the apparent discrepancy in the third sample likely resulted from the high agreement of 16S sequences of *P. aeruginosa* grown in culture and *S. maltophilia* (formerly classified as *Pseudomonas maltophilia*) identified by PCR.

Broad-range PCR was positive in 29% of the samples with negative blood culture, which is comparable to published results (up to 42%) [4, 6]. As broad-range PCR aims at detection of additional cases of bacteremia compared to blood cultures as the nonperfect gold standard, this proportion cannot be converted into a specificity. In nine of ten blood culture-negative but PCR-positive samples, *S. maltophilia* was identified, twice together with *A. calcoaceticus* and once with *Pseudomonas* spp, whereas *P. aerigompsa* was identified once. These ubiquitous gram-negative aerobe rods that can cause nosocomial infections are known to grow well in routine blood culture systems. Thus, it remains unclear to which entity the positive PCR result in samples with negative blood culture correspond to. First, they might represent contamination, an inherent drawback in PCR-based methods [2, 6]. The fact that the sequences of the nine samples with *S. maltophilia* identified all differed from each other strongly argues against laboratory contamination. Second, they might correspond to clinically not relevant background bacterial DNA, i.e., residues of dead or degraded bacteria, reported in the blood of healthy subjects [4, 6]. Background DNA of *S. maltophilia* and *Pseudomonas* spp. has been described [4]. Third, they might represent the clinically relevant additional detection of bacterial infection, e.g., of bacterial compounds spilled over from a localized infection into the blood stream, not detected by blood culture. Another reason for the failure to detect *S. maltophilia* in blood cultures might have been the routine prophylactic application of oral trimethoprim–sulfamethoxazole to prevent *P. jiroveci* pneumonia, as this antimicrobial shows activity against most strains of *S. maltophilia*. In a different setting, namely, in patients receiving intravenous antibacterial therapy, the detection of *Pseudomonas* spp. and *Acinetobacter* spp. by PCR in samples with negative blood cultures has been described [3].

The statistically significant younger patient age in blood culture-negative samples with positive versus negative PCR might result from greater bacteremia in younger children, or it might reflect younger children showing less containment of local bacterial infections than older children. Thus, the identification of bacteria in blood samples showing no growth in cultures by broad-range PCR could help identify neutropenic patients developing fever because of a bacterial infection. Such information could assist in the devise of empiric antimicrobial treatment in FN.

In conclusion, this pilot study demonstrates that real-time broad-range PCR may be worth further studying in FN because of its potential advantages over blood cultures including faster detection and identification within one to two working days [10], quantitative results allowing longitudinal assessment and better sensitivity for slow-growing or fastidious organisms, or after antimicrobial

therapy [6]. Large prospective studies are needed to optimize its performance and then to assess its final clinical value. Less than optimal sensitivity, the inherent potential for contamination, and the difficulty to distinguish background DNA from real bacteremia seem to be the main drawbacks at this moment, with blood culture remaining as the reference test. Possible optimizations are preincubation in blood culture media to enhance sensitivity, nested PCR to reduce the risk of contamination and to allow for sequencing in infection with multiple germs [3], and the simultaneous detection of DNA coding for 16S rRNA and the corresponding rRNA itself as a measure of viability [6].

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