

Original Research Article

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Evaluation of a Multiplex PCR Assay for Direct Detection of Microbial DNA from Whole Blood of Suspected Cases of Late Onset Neonatal Sepsis in a Tertiary Care Hospital of Western Maharashtra, India

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ABSTRACT

Neonatal sepsis is one of the leading causes of neonatal mortality in India. There has been a reduction in early-onset sepsis due to advances in obstetric care while incidence of late-onset has increased especially in very low birth weight infants. Diagnosis of neonatal sepsis remains difficult due to non-specific signs and symptoms in neonates and delay in diagnosis leads to complications. Although blood culture has been the gold standard in detection of bloodstream pathogens it lacks rapidity and sensitivity which are overcome by molecular techniques. In the present prospective study conducted from April 2017 to March 2018, a multiplex PCR assay was evaluated for detection of pathogen DNA directly from whole blood of neonates with late-onset sepsis in a tertiary care hospital. Blood culture was positive in 71/210 (33.81%) neonates with Coagulase negative *Staphylococcus* species being the most common isolate (28.38%). PCR added a statistically significant advantage in sensitivity (40.71% vs 37.14%) and negative predictive value over blood culture and also added a diagnostic yield by detecting additional 17 isolates that were missed by conventional blood culture. PCR and blood culture can identify a partially overlapping set of bloodstream infections making PCR a useful adjunct to blood culture.

Keywords

Late-onset neonatal sepsis, Blood culture, Multiplex PCR

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Introduction

Neonatal sepsis is defined as systemic inflammatory response syndrome in the presence of or as a result of suspected or proven infection with or without

accompanying bacteraemia, documented by a positive blood culture in the first 28 days of life (Goldstein *et al.*, 2005). It encompasses various systemic infections of the new born such as septicaemia, meningitis, pneumonia, arthritis, osteomyelitis and urinary tract

infections. Sepsis is the third leading cause of neonatal mortality in developing countries after prematurity and intrapartum-related complications (or birth asphyxia). It is responsible for 13% of all neonatal deaths and 42% of deaths in the first week of life (Zea-Vera *et al.*, 2015). The burden of neonatal sepsis is huge in India. Hospital based studies suggest an incidence of 30 per 1000 live births while community based studies indicate an incidence of 2.7-17% of all live births. One-fifth of neonates with sepsis die in the hospital. The figure rises to up to 50% for those with culture proven sepsis (Sankar *et al.*, 2016).

Neonatal sepsis has been classified as either early-onset or late-onset depending on the time and mode of infection. Clinical manifestations of early-onset infections usually appear within 7 days of age (within 72 hours of life in case of preterm or very low birth weight neonates) and are most commonly caused by *Streptococcus agalactiae* and *Escherichia coli* acquired before or during delivery and usually represent vertical mother-to-infant transmission. Late-onset infections present after delivery, or beyond 3 to 7 days of age, and are attributed to organisms acquired from interaction with the hospital environment or the community most commonly Coagulase negative *Staphylococcus* (CoNS) and *Klebsiella pneumoniae* (Cortese *et al.*, 2016). Epidemiological studies have observed a general reduction in early-onset sepsis due to advances in obstetric care and the use of prophylactic intrapartum antibiotics. Meanwhile, the incidence of late-onset sepsis (LOS) has increased in parallel with the improved survival of premature infants, especially in those with very low birth weight (VLBW), indicating the role of hospitalisation and life-sustaining medical devices in the pathogenesis of neonatal LOS (Dong *et al.*, 2015).

Diagnosis of neonatal sepsis is difficult as the signs and symptoms of infection in neonates are subtle and non-specific and there is no one specific test to diagnose sepsis, and a number of different screening tools and biomarkers have been used. Manifestations are very difficult to differentiate from many other conditions and include: hypothermia or fever, lethargy, poor cry, refusal to suck, poor perfusion, prolonged capillary refill time, hypotonia, absent neonatal reflexes, bulging fontanel, brady/tachycardia, respiratory distress, apnea and gasping respiration, hypo/hyperglycaemia, and metabolic acidosis.

Delay in identifying affected infants may lead to prolonged and unnecessary therapy, emergence of resistant microorganisms, increase in health care spending, and a higher risk of complications such as cerebral palsy or intraventricular haemorrhage (Cortese *et al.*, 2016). Early diagnosis and the provision of appropriate antimicrobial therapy correlate with positive clinical outcomes.

Blood culture is the current “gold standard” for the detection of bloodstream microbial pathogens. Although it allows microbes to be identified and their susceptibility profiles to be tested, it presents several limitations. Lack of rapidity is a major problem. Detection of bacterial growth requires 12 to 48 hours or more in the case of fastidious bacterial or invasive fungal infection (MacVane *et al.*, 2016). Another limitation of blood culture is its low sensitivity for previous antibiotic treatment and/or low bacterial concentrations, due to the smaller amount of blood sampled from paediatric patients than from adults. Molecular amplification techniques like PCR help to overcome most of these limitations. PCR has the potential to rapidly identify bloodstream infection and is based on the direct detection of the microbe without relying on its growth curve or without suffering the bacteriostatic effect of antimicrobial therapy.

By using multiplex PCR simultaneous amplification of more than one gene loci using more than one primer pair can be used to differentiate various etiologic agents based on molecular weights of fragments amplified.

In the present study, a multiplex PCR assay is evaluated for the detection of bacterial and candidal DNA directly from whole blood of clinically suspected cases of late onset neonatal sepsis. Results were compared with those obtained from conventional blood cultures and other cultures from focus of infection, considering both clinical and laboratory data.

Materials and Methods

This study was conducted in the Department of Microbiology in a tertiary care hospital over a period of 12 months from April 2017 to March 2018. This prospective study enrolled a total of 210 neonates (infants <28 days of age) admitted in wards and intensive care units of the Department of Paediatrics with risk factors or clinical suspicion of late onset sepsis. Term neonates who had completed 7 days of life and preterm neonates (gestational age <32 weeks) and VLBW neonates (<1500 gm birth weight) who had completed 72 hours of life were included.

Ethical clearance was obtained from the Institutional Ethics Committee and written informed consent was obtained from the parents or guardians of neonates.

Sample collection and processing

Upon clinical suspicion of a bloodstream infection, before initiation of antibiotic treatment, a whole blood sample was collected under aseptic precautions from suitable peripheral vein for blood culture in BacT/Alert® PF Bottle (BioMerieux inc.). Due to limited amount of blood available only

the aerobic paediatric blood culture bottle was inoculated (0.5 to 1 mL). Bottles were incubated in BacT/Alert® 3D Microbial Detection System (BioMerieux inc.) for 7 days before giving negative result. This is an automated system based on the colorimetric detection of CO₂ produced by growing microorganisms (Thorpe *et al.*, 1990). When positive signal for growth was obtained, Gram staining was performed and plating was done on routine culture media. Bacterial isolates were identified to species level by using standard microbiological methods. Antibiotic susceptibility testing was performed by Kirby Bauer Disc Diffusion Method using CLSI (2017) guidelines.

At least 200 µL of additional whole blood sample for PCR analysis was obtained from the same peripheral vein immediately after the blood was drawn for blood culture. The samples were placed in sterile EDTA tubes and transferred to the Microbiology Laboratory within 2 hours. In the Microbiology Laboratory, the samples were maintained at 2-8 °C. DNA extraction was initiated within two days using HiPurA™ Blood Genomic DNA Miniprep Purification Kit (HiMedia® Labs). To avoid contamination, all molecular procedures were performed according to the manufacturer's instructions. Extracted DNA samples were maintained at -20 °C until amplification.

Extracted DNA samples were amplified in Prima-96 Plus™ Thermal Cycler (HiMedia® Labs) using selected primers enlisted below along with suitable positive and negative control (Reaction mixture control). Post amplification detection of DNA target was done by gel electrophoresis using HiPer™ Agarose Gel Electrophoresis Kit (HiMedia® Labs).

For detection of DNA, 4 sets of species-specific primers were combined in one

multiplex PCR mastermix tube to identify *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus* and *Staphylococcus epidermidis*. A second multiplex PCR mastermix tube contained 3 species-specific primers to identify *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Salmonella enterica* and also a generic primer for *Candida* species (Figure 1). These 8 primer sets were selected based on recent literature review of common organisms isolated from blood of late onset neonatal sepsis patients in tertiary care institutes in developing countries including India (Mohsen *et al.*, 2017; Agarwal *et al.*, 2016; Mehar *et al.*, 2013; Apparao *et al.*, 2017)

The test was validated by analysing the bands obtained on gel electrophoresis by amplifying DNA extracted from fresh culture growths of standard American Type Culture Collection (ATCC) strains for the respective organisms (Figure 1). Each primer pair was designed to produce a different sized PCR product. This difference is very important to judge the results of a multiplex PCR assay. We compared the multiplex PCR products with these size markers and judged whether samples were positive or negative (Figure 2 and 3).

Clinical data and case definitions

Data was extracted from the patient's medical records and bedside history was taken. A questionnaire was filled with demographic, clinical, and laboratory information of the patients which included: core temperature, heart and respiratory rates, leukocyte count (including differential count and immature: total neutrophil ratio), systolic blood pressure, significant maternal history, risk factors for bloodstream infections, underlying disease/cause of hospitalization, suspected or proven focus of infection, and concentration of serum C-reactive protein.

Individual patient cases were allocated to one of the following study groups (Hall *et al.*, 2006; Stoll *et al.*, 2002):

Group 1: Culture-proven sepsis was defined as having atleast one positive blood culture with a microorganism not considered contaminant (eg: *Bacillus* spp., *Micrococcus* spp., *Corynebacterium* spp., Viridans group *Streptococci* are common contaminants) Positive laboratory parameters (CRP >1.2mg/dL and Immature: Total neutrophil ratio >0.2) in case of isolated Coagulase negative *Staphylococci* (CoNS)

Antibiotic treatment continued for atleast 5 days or until removal of vascular access.

Group 2: Clinical sepsis- was defined as above but with negative blood culture(s).

Group 3: No infection/ No sepsis was defined as having negative cultures, negative laboratory parameters and in whom treatment was discontinued after 48-72 hours.

Those cases having more than one microorganism detected in blood by either PCR or blood culture were denoted as polymicrobial sepsis (Mancini *et al.*, 2009).

Results and Discussion

Demographic characteristics of total 210 neonates with risk factors or clinically suspected LOS were obtained. Male:Female ratio was 1.44:1 with mean age at presentation being 13 weeks and 19 (9.05%) neonates were born preterm (Table 1).

Blood culture was positive in 71/210 neonates (33.81%) excluding contaminants. CoNS was the most common isolate 21/74 (28.38%) followed by *Klebsiella pneumoniae* 12/74 (16.22%) and *Candida* species 8/74 (10.81%). 3 cases out of 71 had polymicrobial sepsis

yielding 2 isolates each by blood culture leading to a total of 74 isolates. These polymicrobial isolates were *Enterococcus faecalis* and *Enterobacter aerogenes* in one case, *Acinetobacter lwoffii* and CoNS in second case and *Klebsiella pneumoniae* and CoNS in third case (Table 2).

In 2 patients with isolated CoNS, the laboratory parameters (CRP, I/T ratio) were normal and antibiotics were discontinued after 48-72 hours, hence they were considered false positive by blood culture and the patients belonged to Group 3 with No Sepsis. Thus, a total of 69/210 (32.86%) neonates were culture proven cases of late onset sepsis (i.e., belonged to Group 1). 88/210 (41.90%) belonged to Group 2 (culture negative clinical sepsis) and 53/210 (25.24%) were in Group 3 (no sepsis).

Multiplex PCR was positive in 63/210 (30%) neonates of which 4 were polymicrobial with 2 isolates each, leading to a total of 67 isolates. No *Pseudomonas aeruginosa* isolates were identified by both PCR and blood culture (Table 3).

PCR could not identify 19 isolates in 17 cases which were detected by blood culture as they were not in panel, which included six *Enterobacter* species, three *Enterococcus faecalis*, two *Acinetobacter lwoffii*, one *Citrobacter koseri*, one *Burkholderia cephalia* complex, one *Serratia marcescens* and five CoNS other than *S. epidermidis*.

PCR and blood culture results were concordant positive in 52/210 (24.76%) cases, concordant negative in 128/210 (60.95%) cases and discordant in 30/210 (14.29%) cases. With blood culture as the gold standard, Sensitivity, specificity, positive predictive value and negative predictive value of PCR was found to be 73.24%, 92.09%, 82.54% and 87.07% respectively (Table 4). In 47 cases,

both PCR and blood culture identified the same organisms. In 5 cases although both PCR and blood culture were positive, they showed discordance with the organisms detected. Considering the organisms that were in panel, PCR could not detect one *S. aureus*, two *A. baumannii* and two *K. pneumoniae* isolates which were detected by blood culture.

PCR detected an additional 17 isolates which blood culture could not detect. Of these four *S. epidermidis* and one *Candida* species were detected in cases belonging to group 3 (no sepsis) and were ruled out as PCR contaminants. However, two *K. pneumoniae*, one *A. baumannii*, one *S. aureus*, two *E. coli*, two *Candida* species and four *S. epidermidis* (Total 12 isolates in 10 cases) were considered significant as the chart review proved the patients to be clinically ill and having other risk factors for nosocomial sepsis and showing similar isolates from culture from other clinical sites.

Comparing diagnostic accuracy of blood culture and PCR

Diagnostic accuracy was compared in 193 cases, since in 17 cases (out of 210) the organisms detected by Blood Culture were not in PCR panel (Table 5).

Defining a clinically ill patient as reference standard (Culture proven sepsis and culture negative clinical sepsis, i.e., Group 1 and Group 2 together) 140 cases were clinically ill while 53 had no sepsis. Sensitivity, specificity, Positive predictive value and negative predictive value of blood culture was found to be 37.14%, 96.23%, 96.29%, 34.23% respectively (Table 6). Sensitivity, specificity, Positive predictive value and negative predictive value of PCR was found to be 40.71%, 90.57%, 91.94%, 38.64% respectively (Table 7). In these clinically ill 140 cases, combined total of 62 isolates were

detected by PCR and BC. 48 (77.42%) isolates were detected by both methods, 5 (8.06%) exclusively by BC and 11 (17.74%) exclusively by PCR.

Time to result

Median time to positivity (TTP) of blood culture bottles while in the BacT/Alert Detection System was 14 hours (varying from 11-19 hours) and it required an additional 18-24 hours for identification of isolate, while Multiplex PCR results were available in the same day in less than 6 hours.

Positive clinical outcome in neonatal sepsis is by the prompt administration of appropriate antibiotics. Currently, the initial choice of antibiotics is empirical and broad and covers the most likely etiologic agents. The major limitation to a more targeted antibiotic strategy is the lack of an available diagnostic method for the rapid and reliable identification of the etiologic agent. Blood culture is the most widely utilized technique for the diagnosis of bloodstream infections. However, despite its high degree of specificity, its sensitivity remains low and the mean time to positivity is high (Lehmann *et al.*, 2008).

PCR added a statistically significant advantage in sensitivity (40.71% vs 37.14%) and Negative predictive value (38.64% vs 34.23%) over Blood culture ($p < 0.05$). PCR also added diagnostic yield, with PCR detecting an additional 17 isolates that were missed by blood culture. The clinical relevance of such PCR-positive but blood culture-negative situations is an important question (Westh *et al.*, 2009). Our results suggest that 12 organisms identified by PCR alone were clinically relevant on the basis of

chart review and confirmation of the result by other microbiological diagnostic methods. While comparing diagnostic accuracy in clinically ill cases, PCR added a significant 11 (17.74%) isolates ($p < 0.05$) and missed 5 (8.06%) exclusive isolates by Blood culture.

There were 5 episodes with false positive PCR results. This circumstance can be attributed to the high likelihood of DNA-contamination during blood sampling in neonates and sample processing, mainly with skin bacteria, a common problem of PCR techniques due to high PCR sensitivity and likewise found in other PCR studies (Tsalik *et al.*, 2010).

Potential reasons for the discordant blood culture-negative and PCR-positive results are numerous. An adequate volume of blood for culture is not available in neonates. Furthermore, the ability of PCR to detect DNA after antibiotic administration, could also have yielded these discordant results (Lucignano *et al.*, 2011).

Discordant blood culture-positive and PCR-negative results could be due to the intrinsic variability of PCR (ethanol introduced during the extraction process), inhibiting factors present in the patient's blood (eg- heme) or other unidentified factors, possibly due to genetic variability or mutations of the target site, inappropriate sample preparation, prolonged transport times causing DNA degradation (Troger *et al.*, 2016).

When there is concordance between PCR and blood culture results, the primary advantage to a PCR-based assay is the timeliness with which those results are available compared to the time to the availability of conventional blood culture results (Table 8).

Table.1 Demographic characteristics of total 210 neonates

Sex	Males	124 (59.05%)
	Females	86 (40.95%)
Mean age at presentation		13 days +/- 3 days
Mode of delivery	Intramural	65 (30.95%)
	Extramural	145 (69.05%)
Mean Gestational age		37.5 weeks +/- 3.5 weeks
	Preterm	19 (9.05%)
Mean Birth weight		2130 gm +/- 516 gm
	VLBW	14 (6.66%)
Admission	NICU/PICU	176 (83.81%)
	Ward	34 (16.19%)

Table.2 Isolates obtained by conventional blood culture

ORGANISM	NUMBER	PERCENTAGE
<i>Coagulase negative Staphylococcus species</i>	21 (<i>S. epidermidis</i> = 16)	28.38
<i>Klebsiella pneumoniae</i>	12	16.22
<i>Acinetobacter baumannii</i>	7	9.46
<i>Staphylococcus aureus</i>	6	8.11
<i>Escherichia coli</i>	5	6.76
<i>Enterobacter species</i>	6	8.11
<i>Enterococcus faecalis</i>	3	4.05
<i>Acinetobacter lwoffii</i>	2	2.70
<i>Salmonella enteric var Typhi</i>	1	1.35
<i>Citrobacter koseri</i>	1	1.35
<i>Burkholderia cephalia complex</i>	1	1.35
<i>Serratia marcescens</i>	1	1.35
<i>Candida species</i>	8	10.81
TOTAL	74	

Table.3 Isolates obtained by multiplex PCR

ORGANISM	NUMBER OF ISOLATES
<i>Staphylococcus epidermidis</i>	24
<i>Klebsiella pneumoniae</i>	12
<i>Acinetobacter baumannii</i>	6
<i>Staphylococcus aureus</i>	6
<i>Escherichia coli</i>	7
<i>Salmonella enterica</i>	1
<i>Pseudomonas aeruginosa</i>	0
<i>Candida species</i>	11
TOTAL	67

Table.4 Comparison of multiplex PCR with gold standard blood culture (BC)

	BC positive	BC negative	TOTAL
PCR positive	52	11	63
PCR negative	19	128	147
TOTAL	71	139	210
Sensitivity= 73.24%, Specificity= 92.09%, PPV= 82.54%, NPV= 87.07%			

Table.5 Comparing diagnostic accuracy of BC and PCR considering 193 cases with organisms in PCR panel

	BC positive	BC negative	TOTAL
PCR positive	51	11	62
PCR negative	3	128	131
TOTAL	54	139	193
McNemar's Chi square= 4.5714, p value= 0.03251 (<0.05, significant)			

Table.6 Diagnostic accuracy of Blood culture in clinically ill cases

	Clinically ill	No sepsis	TOTAL
BC positive	52	2	54
BC negative	88	51	149
TOTAL	140	53	193
Sensitivity= 37.14%, Specificity= 96.23%, PPV= 96.29%, NPV= 34.23%			

Table.7 Diagnostic accuracy of multiplex PCR in clinically ill cases

	Clinically ill	No sepsis	TOTAL
PCR positive	57	5	62
PCR negative	83	48	131
TOTAL	140	53	193
Sensitivity= 40.71%, Specificity= 90.57%, PPV= 91.94%, NPV=38.64%			

Table.8

Mastermix tube 1		Mastermix tube 2	
Organism	Target gene (Amplicon size)	Organism	Target gene (Amplicon size)
<i>E. coli</i> (ATCC 25922)	uspA (884 bp)	<i>P. aeruginosa</i> (ATCC 27853)	16S rRNA (961 bp)
<i>S. aureus</i> (ATCC 25923)	femA (450 bp)	<i>Salmonella enterica</i> var Typhi (ATCC 6539)	iroB (606 bp)
<i>K. pneumoniae</i> (ATCC 13883)	phoE (324 bp)	<i>A. baumannii</i> (ATCC 17978)	gyrB (290 bp)
<i>S. epidermidis</i> (ATCC 12228)	gseA (219 bp)	<i>C. albicans</i> (ATCC 10231)	28S rRNA (175 bp)

Fig.1 Standard PCR amplification image using ATCC controls

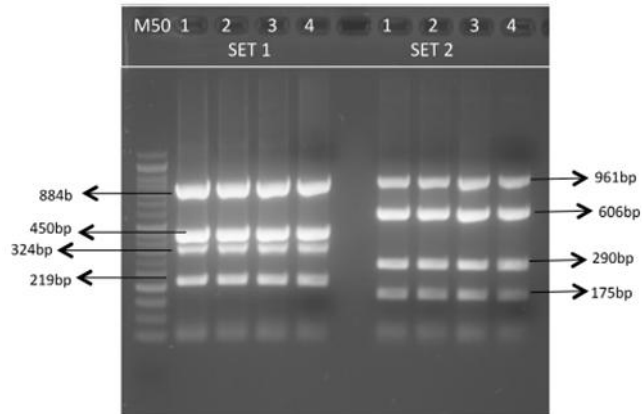
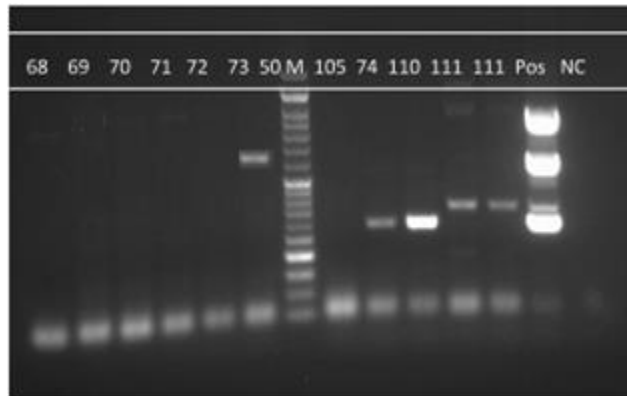
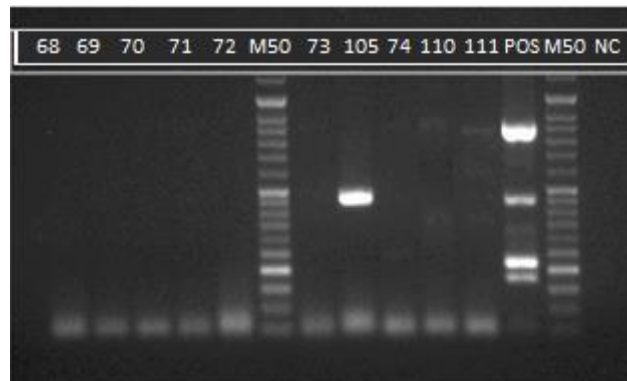


Fig.2 Results of multiplex PCR (mastermix tube 1)



Sample no. 73 is positive for *S. aureus*, Sample no. 74 and 110 are positive for *S. epidermidis*, Sample no. 111 (run twice) is positive for *K. pneumoniae*. 50M represents the 50 bp DNA ladder for comparison. POS represents positive control, NC represents negative control.

Fig.3 Results of multiplex PCR (mastermix tube 2)



Sample no. 105 shows positive band for *Salmonella*. M50 represents the 50 bp DNA ladder for comparison. POS represents positive control, NC represents negative control.

This may allow the treating physician to narrow antibiotic coverage early in the course of treatment, potentially avoiding the toxicity and costs associated with the use of broad and empirical antimicrobial therapy. PCR can also identify an infection that was not being adequately treated. For example, empirical antibiotic choices typically do not treat fungal infections. However, the identification of fungemia by PCR can allow the physician to appropriately expand the antimicrobial regimen early in the course of disease (Wallet *et al.*, 2010).

All polymicrobial infection episodes showed discordant results between isolates detected. This finding demonstrates the difficulty when diagnosing polymicrobial sepsis, such as the fact that blood culture regularly detects only the most rapidly growing microorganism (Mancini *et al.*, 2009).

PCR and blood culture can identify a partially overlapping set of bloodstream infections, making PCR a useful adjunct to blood culture. Prospective clinical trials for the assessment of treatment and patient outcomes as well as cost benefit analysis will be important to the further advancement of a multiplex PCR approach.

Multiplex PCR is a promising tool for the rapid identification of bloodstream infections as an adjunct to blood culture.

The benefit of rapid pathogen detection has to be balanced against the considerable risk of contamination, loss of information on antibiotic sensitivity pattern and increased costs.

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