ORIGINAL ARTICLE

Evaluation of Nested broad-range PCR for Pathogen Detection in Negative Blood Cultures

Sunny Saurabh[1], Zinnu Rain[2], Pradyot Prakash[1,2], Deepak Kumar[1,*]

[1]Department of Microbiology, IMS, BHU, Varanasi, Uttar Pradesh, INDIA.
[2]Bacterial Bio-film and Drug Resistance Research Laboratory, Department of Microbiology, IMS, BHU, Varanasi, Uttar Pradesh, INDIA.

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ABSTRACT

Background: Bloodstream infections are a major cause of morbidity and mortality among all age groups. In the laboratory, it is routinely diagnosed by performing a blood culture which is considered an imperfect gold standard. The diagnostic limitations and uncertainties of blood cultures are related to low sensitivity, particularly in antibiotic-exposed cases, and extended time for pathogen detection that increases turn-around-time of the results. An alternative and recently introduced nucleic acid amplification method for 16S rDNA, a conserved DNA sequence common to all bacteria, has gained significant. It has high sensitivity and an ability to detect organisms that are non-cultivable or nonviable owing to prior antibiotic treatment and where the organism fails to grow on a culture medium. **Objectives:** To determine the utility of broad-range PCR in the detection of pathogens from negative blood culture was the aim of our study. **Materials and Methods:** A total of 50 negative blood cultures after 5 days of incubation on BacT/Alert continuously monitored blood culture system were included in this study. From each blood broth sample, DNA was isolated which was subjected to a broad range 16S rDNA nested PCR. The PCR products obtained were visualized on agarose gel electrophoresis and the results were interpreted. **Results:** Broad range bacterial 16S rDNA nested PCR detected the desired amplicon in 4/50 (8%) of the blood culture-negative samples. **Conclusion:** The broad range bacterial nested PCR method if used in combination with routine culture techniques in clinical microbiology laboratories will increase the detection of Bloodstream pathogens.

Keywords: Bloodstream infection (BSI), NAAT, Blood culture, broad-range PCR.

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Correspondence

Dr. Deepak Kumar
Associate Professor, Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi-221005, Uttar Pradesh, INDIA.
Email id: deepak.mln30@gmail.com

INTRODUCTION

Bloodstream infections (BSIs) are the leading cause of morbidity and mortality in people of all ages.[1] Globally, BSI affects about 30 million people leading to 6 million deaths annually.[2] BSI is diagnosed by performing a blood culture which is considered the "gold standard" for confirming the clinical suspicion of sepsis till date.[3] Despite the technical improvements of continuous monitoring of blood culture systems, the value of blood cultures for confirming the clinical suspicion of sepsis has been shown to be sub-optimal. The overall positivity may be as low as 30 to 40% in most of the setups despite the proper implementation of standard procedures, adequate blood volume collection, and substantial clinical suspicion of BSI.[4] Nevertheless, the median time to positivity of blood cultures is 15 hr (range, 2.6 to 127 hr) which is too high and thus affects the final reporting time. False-negative results usually due to difficulty to cultivate pathogens constitute significant errors, which can radically affect clinical outcomes in bacteremic patients. Even the continuous monitoring automated blood culture systems are known to report falsely negative in around 3% of the cases.[5,6] In fact, none of the available blood culture systems is appropriate for the detection of all potential bloodstream pathogens. Some

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micro-organisms like *Coxiella burnetii*, *Bartonella* spp. *Chlamydia* spp. *Tropheryma whippelii* and organisms such as the HACEK [*Haemophilus aphrophilus, H. paraphrophilus, H. parainfluenzae, Actinobacillus actinomycetemcomitans, Cardiobacterium hominis, Eikenella corrodens, Kingella* spp.] group, *Legionella* spp. grow poorly, or not at all, in blood culture and systems.[7]

To overcome the limitations of blood culture systems, Nucleic acid amplification techniques (NAAT) have been introduced in the last few decades as an alternative for blood culture in the diagnosis of BSI.[8] NAAT works by recognizing the conserved DNA sequence that is common to all bacteria (e.g. 16S rDNA).[9] The most common approach involves universal broad-range assays targeting specific sequences in the bacterial genome (e.g. pan bacterial 16S) in blood directly or blood-broth. NAAT-based testing holds significant promise because of the potential for high sensitivity and the ability to detect organisms that are non-cultivable or nonviable owing to prior antibiotic treatment and where the organism fails to grow on a culture medium. With an objective to detect the potential pathogens which are missed during the blood culture, we have tried to evaluate the clinical utility of broad-range PCR to detect the pathogen from blood culture-negative cases in our setup.

MATERIALS AND METHODS

Ethical consideration

The study plan was approved by the Institute Ethics Committee of IMS BHU Varanasi, U.P., India, and informed consent was obtained from each of the participants/guardians.

Study design

The present study is observational, carried out in the Department of Microbiology, IMS, BHU, Varanasi. The total duration of the study was one year: from July 2020 to June 2021. Blood cultures from patients suspected of systemic infection were processed routinely in IMS, BHU Microbiology Laboratory. Samples collected in Biomerieux - BACT/ALERT® culture bottles (bioMérieux USA) were incubated using the BacT/Alert continuously monitored blood culture system and reported as negative after 5 days. In the study, a total of 50 single negative blood cultures were selected from discarded culture bottles for further testing.

Processing of the sample

Blood culture method

The blood culture bottles declared negative after 5 days of incubation using the BacT/Alert blood culture system were subjected to Gram stain, as well as a terminal subculture on 5 % blood agar, chocolate agar, and MacConkey agar and incubated for 48 hr at 37°C under aerobic conditions. The culture and Gram stain results were accounted for. Around 1ml of blood-broth was drawn from each blood culture bottle using a sterile syringe for PCR study and was stored at -20°C until further use. The bottles were kept at -20°C until the DNA extraction was performed.

PCR method

Extraction of DNA

DNA was extracted using 1 ml of blood broth obtained after overnight incubation of the blood culture bottle using the QIAamp 96 DNA Blood Kit (Qiagen, Hilden, Germany). A volume of 200 µl of blood broth was used for extraction, and DNA was eluted in 100 µl of elution buffer. The entire procedure was performed according to the manufacturer's instructions.

Buffers

Taq polymerase enzymes and customized primers were procured from SBS Genetech Co., Ltd., China.

PCR primers

Bacterial rDNA consists of highly conserved nucleotide sequences that are shared by all bacterial species, interspersed with variable regions that are genus- or species-specific. Primers based on the conserved sequences of the 16S rDNA gene, ubiquitous in bacteria, were used to detect the presence of bacterial DNA in blood broth samples (Table 1).[10]

Amplification of DNA

The PCR reaction was carried out in 25 µl volume with the reaction mix containing 10 x reaction buffer (5 µl/sample), dNTPs (2 µl/sample), forward and reverse primers, and Taq polymerase enzyme (0.66 pmol). Amplification was carried out on BIO-RAD system with a heated lid. The hot start method was employed by heating at 94°C for 5 min initially. Thereafter, amplification was carried out for 35 cycles at 90°C for 1min (denaturation), 65°C for 1 min (annealing), and 72°C for 1 min (extension). An extra extension was carried out at 72°C for 7 min. The amplification products of primary PCR was again amplified with nested primers following the same protocol. The final amplification product (1000 bp) was separated on 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining. The 100 bp DNA ladder was used as a DNA molecular weight standard. Gel electrophoresis with 1.2% agarose gels was conducted with 1X TBE buffer (0.1 M Tris, 0.09 M boric acid, 1 mM EDTA).

Table 1: Primers and amplification conditions targeting Broad range 16S rDNA gene.

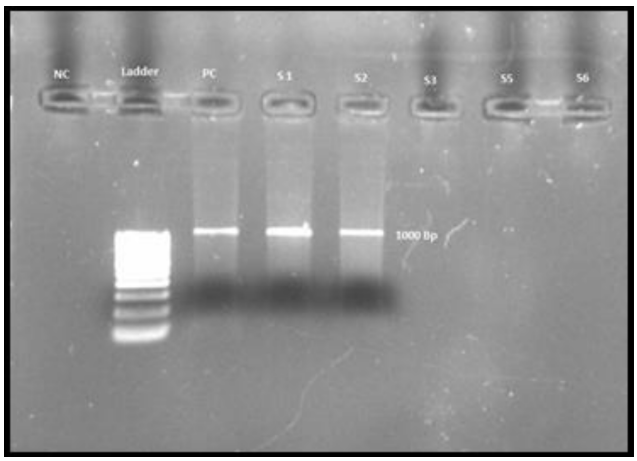
Target gene	Cycle	Primer names and sequences	Amplicon size	Annealing temperature and number of cycles
16S rDNA (Broad range primers)	Primary cycle	16S F 5'-TTG GAG AGT TTG ATC CTG GCT C-3'	1194 bp	56°C for 30 s for 30 cycles
		16S R 5'-ACG TCA TCC CCA CCT TCC TC-3'		
	Nested cycles	NF 5'-GGC GGC AKG CCT AAY ACA TGC AAG T-3'	1025 bp	56°C for 30 s for 30 cycles
		NR 5'-GAC GAC AGC CAT GCA SCA CCT GT-3'		

T: thymine; A: adenine; G: guanine; C: cytosine

Staphylococcus aureus clinical isolate as the positive control; Double distilled water as negative control were run with each batch of samples analyzed. Documentation of gel was done by Gel doc system BIO-RAD made in the USA.

RESULTS

Over the study period, blood culture was sent from 68 patients admitted to SS Hospital ICU BHU Varanasi suspected of having bloodstream infection. Of these, blood cultures were reported negative in 50 patients (73.6%) after an incubation period of 5 days on BacT/Alert continuously monitored blood culture system. The terminal Gram stain performed from the blood broth failed to demonstrate the presence of any pathogen, while the terminal subculture from the blood broth did not grow any micro-organism on culture plates after 48 hr of incubation. Broad range bacterial 16S rDNA nested PCR detected the desired amplicon in 4/50 (8%) of the blood culture-negative samples (Figure 1).



NC Negative control; L Ladder; PC Positive control; S1-S6 samples

Figure 1: Gel image showing 1000 bp size target amplicon after nested PCR using broad range 16S rDNA primers.

DISCUSSION

Through this study, we tried to demonstrate the suitability of broad-range nested PCR for determining bloodstream pathogens. The methodology was designed to explore whether it can provide add-on information to what is provided by blood culture. The present work has shown that the broad-range nested PCR could detect the bacterial genome in blood broth which was declared as negative after 5 days of incubation. The broad range of nested PCR could detect more cases of bacteremia than blood culture in this study. This can be explained as NAAT having the ability to detect the DNA of nonviable and non-cultivable micro-organisms. Thus NAAT could be beneficial to diagnose difficult cases which may be either due to prior antibiotic exposure or due to the presence of fastidious or non-cultivable pathogens. Studies in the past have evaluated several different protocols and systems targeting broad-range PCR and found it to be extremely beneficial for testing in situations like blood culture-negative endocarditis.[11] Yet another study conducted on CSF showed similar benefits to resolve difficult-to-diagnose cases, including those where patients were receiving antibiotic therapy at the time of CSF collection.[12]

To implement any novel diagnostic method into the routine, algorithms must be designed that would benefit the patient the most. It should be able to detect the true pathogens earliest that are missed by the culture technique. The timing of performing the NAAT in BSI is of utmost importance as an early diagnosis is life-saving. In the present study, we performed this test on blood culture declared negative after 5days of incubation to standardize our protocol. However in the interest to detect an early bacteremia blood broth can be used for NAAT after an incubation of 24 hr as suggested by Paul *et al*.[13] Thus, the PCR analysis can be performed within a working day, and information on the infectious state of patients can be provided earlier than the BC. This is extremely crucial in managing the BSI in critically ill patients.

NAAT does have limitations of its own which must be considered while interpreting any results. Although the molecular technique can be expected to have a lower limit of detection than BC, they may still struggle to match BC performance, in part because of the significant differences in the sample volume tested. It is worth mentioning here that the molecular test is performed on 0.5 ml-1 ml of blood broth while blood culture is performed with a larger volume. Blood volume is the single most important factor affecting the performance in terms of the positivity of blood culture.[14]

NAAT also faces challenges concerning specificity when compared to blood culture. There are risks of false-positive and false-negative PCR results which often arise due to the detection of contaminating bacterial DNA and interference from an excessive amount of human DNA, respectively.[15] Transient bacteremia may also occur even in healthy individuals, and NAAT testing may detect the presence of pathogen DNA circulating even after successful treatment hence the result may be interpreted in reference to the clinical presentation of patients.[16] Studies examining healthy donors have shown the presence of bacterial genetic material in the circulation of a significant proportion (31%) of donors tested.[17] In addition, contamination can be introduced in blood culture at any step from the environment during the specimen collection or testing process. We in our study have explored utility of single blood cultures rather than paired which is usually associated with a lower sensitivity to detect bacteremia, as well as difficulty in differentiating contaminants from clinically significant pathogens. Hence, the PCR algorithm must be designed taking every necessary precaution to minimize bacterial contaminations and to selectively detect DNA from only living micro-organisms, while human and extracellular bacterial DNA must be excluded. Detection using broad-range PCR and subsequent correct identification to the genus and/or species level is possible by nucleotide sequence analysis of the amplified 16S rDNA fragment. Hence further study must be planned to incorporate a large number of samples.

The cost of NAAT-based testing is yet another challenge in routine use in clinical microbiology laboratories. The laboratories that implement such assays should therefore need to identify patients that would benefit from testing most. Whether culture-based systems will remain the diagnostic methods of choice into the next century or be replaced by molecular techniques remains to be determined.

CONCLUSION

Broad Range 16S rDNA PCR, if used in combination with routine blood culture technique in clinical microbiology laboratories will increase the detection of blood stream pathogens.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

1. Laupland KB, Church DL. Population-based epidemiology and microbiology of community-onset bloodstream infections. Clin Microbiol Rev. 2014;27(4):647-64. doi: 10.1128/CMR.00002-14, PMID 25278570, PMCID PMC4187633.
2. Abram TJ, Cherukury H, Ou CY, Vu T, Toledano M, Li Y, *et al*. Rapid bacterial detection and antibiotic susceptibility testing in whole blood using one-step, high throughput blood digital PCR. Lab Chip. 2020;20(3):477-89. doi: 10.1039/c9lc01212e. PMID 31872202, PMCID PMC7250044.
3. Timsit JF, Ruppé E, Barbier F, Tabah A, Bassetti M. Bloodstream infections in critically ill patients: An expert statement. Intensive Care Med. 2020;46(2):266-84. doi: 10.1007/s00134-020-05950-6. PMID 32047941, PMCID PMC7223992.
4. Afshari A, Schrenzel J, Ieven M, Harbarth S. Bench-to-bedside review: Rapid molecular diagnostics for bloodstream infection - a new frontier? Crit Care. 2012;16(3):222. doi: 10.1186/cc11202, PMID 22647543.
5. Klaerner HG, Eschenbach U, Kamereck K, Lehn N, Wagner H, Miethke T. Failure of an automated blood culture system to detect non-fermentative gram-negative bacteria. J Clin Microbiol. 2000;38(3):1036-41. doi: 10.1128/JCM.38.3.1036-1041.2000, PMID 10698992, PMCID PMC86332.
6. Meessen NE, De Vries-Hospers HG. Failure of an automated blood culture system to detect non-fermentative gram-negative bacteria. J Clin Microbiol. 2000;38(7):2803-4. doi: 10.1128/JCM.38.7.2803-2804.2000, PMID 10979755, PMCID PMC87044.
7. Brouqui P, Raoult D. Endocarditis due to rare and fastidious bacteria. Clin Microbiol Rev. 2001;14(1):177-207. doi: 10.1128/CMR.14.1.177-207.2001, PMID 11148009, PMCID PMC88969.
8. Peker N, Couto N, Sinha B, Rossen JW. Diagnosis of bloodstream infections from positive blood cultures and directly from blood samples: Recent developments in molecular approaches. Clin Microbiol Infect. 2018;24(9):944-55. doi: 10.1016/j.cmi.2018.05.007. PMID 29787889.
9. Prakash V, Kumar D, Singh S, Nath G. Bacterial profiling of healthy bone marrow using polymerase chain reaction technique. Iraqi J Hematol. 2017;6(2):69. doi: 10.4103/ijh.ijh_21_17.
10. Fournier PE, Thuny F, Richet H, Lepidi H, Casalta JP, Arzouni JP, *et al*. Comprehensive diagnostic strategy for blood culture-negative endocarditis: A prospective study of 819 new cases. Clin Infect Dis. 2010;51(2):131-40. doi: 10.1086/653675, PMID 20540619.
11. Tkadlec J, Peckova M, Sramkova L, Rohn V, Jahoda D, Raszka D, *et al*. The use of broad-range bacterial PCR in the diagnosis of infectious diseases: A prospective cohort study. Clin Microbiol Infect. 2019;25(6):747-52. doi: 10.1016/j.cmi.2018.10.001, PMID 30321604.
12. Paul P, Pandey N, Gupta V, Tilak V, Kumar D, Tilak R. Semi-nested polymerase chain reaction over blood culture in detection of bloodstream

fungal infection in leukemic children with febrile neutropenia. J Appl Hematol. 2020;11(4):174. doi: 10.4103/joah.joah_41_20.

13. Huber S, Hetzer B, Crazzolara R, Orth-Höller D. The correct blood volume for paediatric blood cultures: A conundrum? Clin Microbiol Infect. 2020;26(2):168-73. doi: 10.1016/j.cmi.2019.10.006, PMID 31654793.
14. Grahn N, Olofsson M, Ellnebo-Svedlund K, Monstein HJ, Jonasson J. Identification of mixed bacterial DNA contamination in broad-range PCR amplification of 16S rDNA V1 and V3 variable regions by Pyrosequencing of cloned amplicons. FEMS Microbiol Lett. 2003;219(1):87-91. doi: 10.1016/S0378-1097(02)01190-4, PMID 12594028.
15. Samuel L. Direct detection of pathogens in bloodstream during sepsis: Are we there yet? J Appl Lab Med. 2019;3(4):631-42. doi: 10.1373/jalm.2018.028274, PMID 31639731.
16. Perez PF, Doré J, Leclerc M, Levenez F, Benyacoub J, Serrant P, *et al*. Bacterial imprinting of the neonatal immune system: Lessons from maternal cells? Pediatrics. 2007;119(3):e724-32. doi: 10.1542/peds.2006-1649, PMID 17332189.

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