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Comparing the Accuracy Rate of Two Different Universal Primers in Enteric Pathogen Diagnosis From Blood

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Background: Detecting enteric bacteria in blood by culture is a slow assay with low accuracy rate. PCR might be a suitable alternative assay but as several species can cause bacteremia, it is necessary to use universal primers.
Objectives: In this study we evaluated and compared two pairs of universal primers in detecting four enteric bacteria in blood, which are common causes of bacteremia in human.
Materials and Methods: Standard strains of *E. faecalis*, *S. typhi*, *E. coli*, and *S. Aeruginosa*, were used in this study. A serially diluted bacterial suspension of all strains was made for inoculation to four sets of defibrinated sheep blood which were used to prepare blood specimens with different bacterial contents for performing routine assay and PCR. PCR was performed using two different universal primers designed from two ribosomal genes, 16sr RNA and 23sr RNA.
Results: PCR with 16sr RNA universal primer showed more accuracy rate than both blood culture and PCR with 23sr RNA universal primer. Mean time for performing PCR assay and blood culture was eight and 48 hours, respectively.
Conclusions: Both PCR with 16sr RNA and 23sr RNA universal primers have more accuracy rate than blood culture and are faster in detection of bacteremia. PCR with 16sr RNA universal primer is more accurate than both PCR with 16sr RNA universal primer and blood culture for diagnosis of bacteremia.

Keywords: Bacteremia; Prisma Universal Bond 3 Primer; Culture; Polymerase Chain Reaction

1. Background

Bacteremia is a serious and urgent problem (1) caused by several bacteria (2). For effective treatment of bacteremia, diagnosis of the causative bacteria and their susceptibility to antimicrobial agents is critical (1, 3). Blood culture is the most common assay (4) and the culture results for most bacteria is reported positive after 24-72 hours although, the essential time to achieve culture results might be longer for fastidious bacteria and in cases receiving antibiotics before taking sample for culture (5, 6). Moreover, additional time is needed for performing antimicrobial susceptibility test and minimum inhibitory concentration (MIC) (7-9). Some physicians prefer to do empiric treatment with several antibiotics or prefer using a wide spectrum drug (6). This might result in widespread resistance to antibiotics in bacteria (6, 9). In recent years, several automatic culture systems are introduced that are more rapid than traditional procedure (10, 11). In addition, some molecular diagnostic assays are investigated in different studies (12-15). Of many advantages of PCR one is its ability to detect anaerobic bacteria (14) and another is more applicability for diagnosis of bacteremia with polymicrobial origin. Every bacteria will present a single band in PCR by using universal primer but they definitely show different types of colonies in culture, similar to contaminated cases (7, 8). As several bacteria cause bacteremia, for rapid diagnosis of these cases it would be necessary to use a multiplex-PCR assay with several specific primers or to use a pair of universal primers. It is too difficult to establish a multiplex-PCR with several sets of primers for multiplex-PCR assay, because their annealing temperature

Implication for health policy/practice/research/medical education:
Detecting bacteria in blood by culture is a slow assay with low accuracy rate. PCR might be a suitable alternative assay; however, several species can cause bacteremia and it is necessary to use the universal primers. In this study, two pairs of universal primers for detecting seven bacteria in blood were evaluated and compared, which are common causes of human bacteremia. We found that both PCR with 16sr RNA and 23sr RNA universal primers have high accuracy rate and are faster than blood culture in detection of bacteremia. PCR with16Sr RNA universal primer is more accurate in diagnosis of bacteremia than both PCR with 16sr RNA universal primer and culture.

would be different. A wide spectrum universal primer which is designed from consensus regions of bacterial genome especially from ribosomal genes 16sr RNA and 23sr RNA, are more applicable to detect most bacteria in any specimen (16-18). Consensus parts of these two genes are useful for designing universal primers, taxonomic and classification aims (19, 20), and for bacteria typing using RFLP (21).

2. Objectives

This study was designed to evaluate and compare accuracy rate of two universal primers: 16sr RNA and 23sr RNA, for detection of four enteric pathogenic bacteria strains, which are common causes of bacteremia.

3. Materials and Methods

Standard strains of four enteric bacteria, which are most common causes of bacteremia, were used in this study. Enterococcus faecalis PTCC 1447, Pseudomonas aeruginosa ATCC 25873, *Esherichia coli* PTCC 1553, and Salmonella typhi PTCC 1609 were the bacteria. They were prepared from bacteria and fungi collection center of Iranian Institute of Industrial and Scientific Researches. Well-grown colonies of each strain on Tryticase Soy Agar (TSA) were used for preparing bacterial suspensions with certain crowd (10^8 cfu/mL) by mixing them in normal saline. Bacterial crowd was adjusted using spectrophotometer in 680 nm comparing to 0.5 McFarland solution. By using serially diluting procedure, four bacterial suspensions with 10^6 cfu/mL, 10^4 cfu/mL, and 10^2 cfu/mL crowd were made for each strain. By adding certain amount of any bacterial suspension to several sets of defibrinated sheep blood with t same volumes, blood specimens with certain crowd of bacteria were made. Actually, we made ten sets of six serially diluted blood samples with different bacterial content for each strain including 10^4 cfu/mL, 10^3 cfu/mL, 10^2 cfu/mL, 10^1 cfu/mL, 5 cfu/mL, and 0 cfu/mL. These were used as specimens for performing routine diagnosis and PCR assays. Each assay was repeated ten times for each strain. In routine diagnostic method, 100µL of each specimen was inoculated in 1 mL TSB, incubated at 37°C for 24 hours, transferred to TSA, and incubated for an additional 24 hours.

DNA extraction for each specimen was performed using a sophisticated process as follows: adding 100 µL of sample to 400 µL sterile distilled water in a 2 mL tube, incubation in 4 °C for 60 minutes, adding 500 µL red cell lyses buffer ($NAHCO_3$ 10Mm, NH_4CL 0,155 M, pH = 7), additional incubation in 37°C for one hour, centrifuging in 104 rpm for 15 minutes, adding 200 µL bacterial lyses buffer (Tris 10 mM, sucrose 0.3 M, $MgCL_2$ 5 mM) and 10 µL lysozym (0.1 mg/mL, Sinagen, Lot: MR7735) to the pellet, incubation in 37°C for one more hour, adding 4 µL proteinase K (900 u/mL, Fermentaze Lot: 00022411) and incubation in 65°C for one hour, extraction of DNA by phenol-chloroform method, precipitation of DNA by cold isopropanol, washing DNA two times with 70% cold ethanol, and storing the extracted DNA in freezer.

We used a pair of 16sr RNA universal primers designed by Kariyama (18) with the following sequences:F-5-GGATTAGATACCCTGGTAGTCC-3 and R-5- TCGTTGCGGGACTTAACCCAAC-3 that make 320bp fragments, and also a pair of 23sr RNA universal primers designed by Anthony (22) with the following sequences: F-5-GCGATTTCTGAATGGGGAAACCC-3 and R-5-TTCGCCTTTCCCTCACGGTACT-3 that make 349 bp fragments. PCR mix was made as follows: PCR Buffer (10X) 3 µL, $MgCL_2$ (25 mM) 2 µL, dNTP (10 mM) 0.5 µL, forward primer 400pM, reverse primer 400 pM, DNA pol (5u) 0.2 µL, DNA 2 µL, and ddH_2O until 25 µL. PCR program was adjusted as follows: initial denaturation at 94°C for 7 min, 35 cycles of denaturation at 94°C for 40 s, annealing at 60°C for 40s, extension at 72°C for 50s, and final extension with 72°C for 10 minutes. Electrophoresis of PCR product was performed on 1.5% agarose gel.

4. Results

Results of culture and PCR assays for all bacteria are summarized in Table 1. PCR with both primers showed positive results indicating their potency for diagnosis of all studied strains in blood. PCR with 16sr RNA universal primer showed more accuracy rate than both culture and other PCR assay for *S. aeruginosa* (5cfu/mL, 10^3 cfu/mL, and 10^2 cfu/mL, respectively) (Figure 1). The assay presented the same accuracy rate as blood culture and PCR with 23sr RNA universal for *E. faecalis* (5 cfu/mL for all) (Figure 2) but showed more accuracy rate than blood culture and other PCR assays for *S. typhi* (10^2 cfu/mL, 10^3 cfu/mL, and 10^3 cfu/mL, respectively). Finally the assay presented the same accuracy rate for blood culture (both 10^2 cfu/mL) but showed more accuracy rate than PCR with 23sr RNA universal primer for *E. coli* (10^2 cfu/mL versus 10^3 cfu/mL).

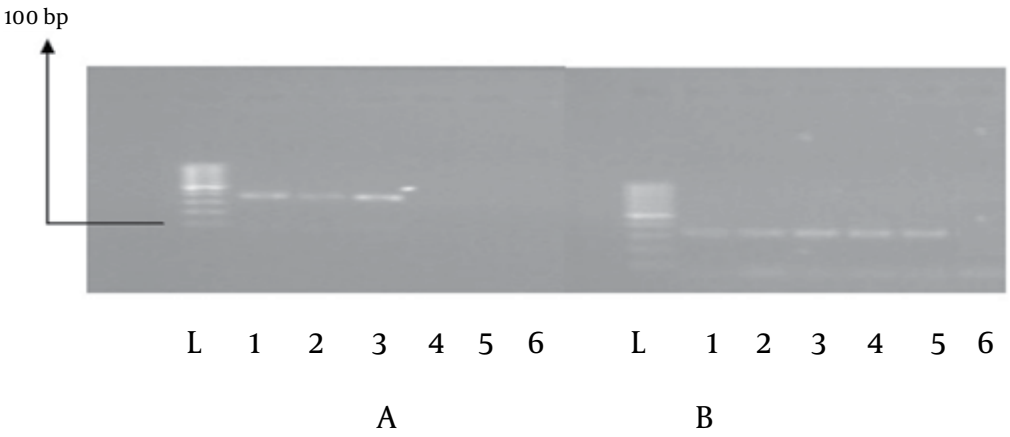
Generally, PCR with 16sr RNA universal primer showed more accuracy rate than both blood culture and the PCR with 23sr RNA universal primer for all the study bacteria species. PCR with 23sr RNA universal primer had the same accuracy rate as blood culture for *E. faecalis* and S. typhi, and showed more accuracy rate for *E. coli*. However, it presented less accuracy rate for *S. aeruginosa*. Mean time for PCR assay was eight hours while it was more than 48 hours for blood culture. Both PCR assays and culture did not have any not false positive results, hence, their specificities were the same (100 %.).

Table 1. Results of Cultures and Two PCR Assays of All Specimens

Bacterial Content in Blood Sample, (cfu/mL)	104, I [a], II [a], III [a]	103, I, II, III	102, I, II, III	101, I,II, III	5, I, II, III	0, I, II, III
Test Strain						
E. faecalis	+, +,+	+, +,+	+, +, +	+, +, +	+, +, +	-, -, -
S. typhi	+, +,+	+, +,+	+, -, -	-, -, -	-, -, -	
E. coli	+, +,+	+, -, +	+, -, +	-, -, -	-, -, -	-, -, -
E. coli	+, +,+	+, +,+	+, -, +	+, -, -	+, -, -	-, -, -

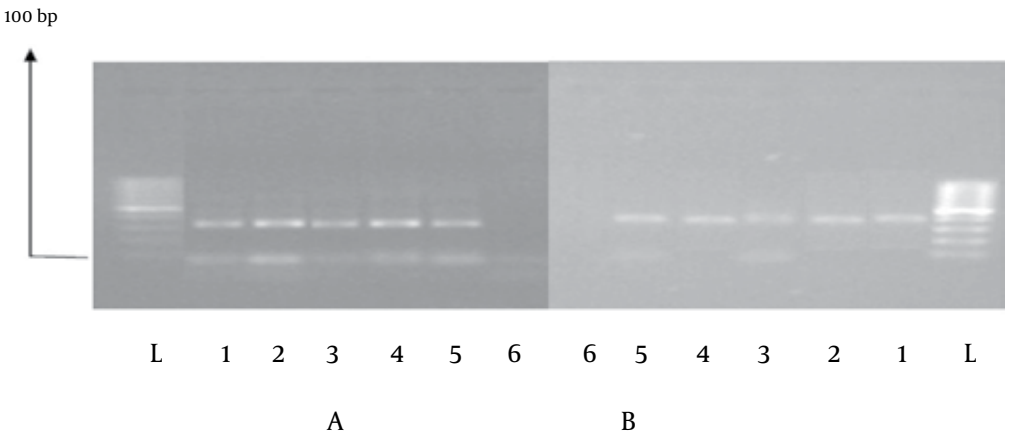
[a] I, Culture; II, PCR with 16sr RNA primer; III, PCR with 23sr RNA primer.

Figure 1. Results of Blood Inoculated PCR With Different Amounts of *S. aeruginosa*



A. PCR with 23sr RNA primer (350bp); B. PCR with 16sr RNA primer (320bp). L, Ladder 100bp. 1, blood with 10^4 cfu/mL bacterial cells; 2, blood with 10^3 cfu/mL bacterial cells; 3, blood with 10^2 cfu/mL bacterial cells; 4, blood with 10^1 cfu/mL bacterial cells; 5, blood with 5 cfu/mL bacterial cells; 6, negative control (blood with no bacteria). As this figure shows, PCR with 16sr RNA primer for this bacterium, seems to be more sensitive than 23sr RNA primer PCR. (5 cfu/mL vs. 10^2 cfu/mL).

Figure 2. Result of PCR for blood inoculated with different amounts of *E. faecalis*



A. PCR with 16sr RNA primer (320 bp); B. PCR with 23sr RNA primer (350 bp); L. Ladder 100bp. 1, blood with 10^4 cfu/mL bacterial cells; 2, blood with 10^3 cfu/mL bacterial cells; 3, blood with 10^2 cfu/mL bacterial cells; 4, blood with 10^1 cfu/mL bacterial cells; 5, blood with 5 cfu/mL bacterial cells; 6, negative control (blood with no bacteria). As this figure shows, PCR with both 16sr RNA and 23sr RNA primers have the same sensitivity for this bacterium.

5. Discussion

Most important aspect of targeting consensus parts of 16sr RNA and 23sr RNA genes is the ability to detect most pathogenic bacteria; therefore, it might have higher sensitivity for bacteremia diagnosis (14). This assay has been tried by several researchers. Ono et al. used 16sr RNA universal primer for differential diagnosis of bacterial and nonbacterial sepsis (17). Klauseger et al. used a pair of 16sr RNA universal primers to successfully detect 62 pathogenic bacteria (23). The main problem of PCR for bacteremia diagnosis is false positive results due to sample contamination (17) which might be diminished significantly by purifying of extracted DNA (24) or by using a high quality commercial DNA extraction kits (25). Different 16sr RNA universal primers and different protocols were tried and showed variable sensitivity and specificities (20, 23-26). An in-house protocol tried by Wellinghausen et al. (27) showed equal rates of sensitivity and specificity (85%).

PCR is more applicable for diagnosis of bacteremia in patients who have already used antibiotic before the test (27). Another advantage of PCR is the speed of assay. It can be performed in one-day whereas routine assay usually needs 48 to 72 hours to be done. It must be noted that false positive rates of PCR might be more than routine assays because it also detects dead bacteria in blood (17). The second disadvantage of PCR is false negative results that might be related to low number of bacteria in the specimen. Therefore, using an internal control is recommended (22). The third disadvantage of PCR with universal primer is its inability to determine antimicrobial susceptibility pattern of the bacteria because it needs bacteria isolation The fourth disadvantage is impossibility of detecting bacterial identity in species level; however, it can be performed using a typing method such as RFLP on PCR product (21) or by using several specific probes (22). The first way is time consuming but it is more economic (28).

Bacterial DNA extraction with high quantity and quality from whole blood is a serious technical problem. Moreover, there are many PCR inhibitors in blood. Therefore, the extraction method has important role in the accuracy of PCR assay (17). We used RBC lysis buffer and proteinase K for decreasing inhibitors but it showed low efficacy. Zhang et al. used Quiagen kit and detect 5 cfu/mL *S. pneumonia* in blood (29). Newcombe et al. used Boom method (30). Klauseger et al. used DNazol buffer for lysis of bacteria and blood cells (23). of Red blood cell lysis with sterile distilled water and DNA extraction with boiling method was used by Anthony et al. however, it was not effective for *S. aureus* (22). Rothman et al. used initial enrichment of blood in TSB for four to six hours before extraction (24).

Accuracy rate and spectrum of universal primers are other problems with PCR although it is not the same for different primers. The 16sr RNA universal primer designed by Christensen et al. was not a wide spectrum one (31). Greisen et al. detected 90 bacteria by using one pair primer and detected 102 bacteria by using two pairs universal primers (28). The 16sr RNA universal primer designed by Lu et al. (21) showed 92.3% sensitivity for CSF bacteria detecting and presented high accuracy rate to detect 10 cfu/mL gram-negative and 250 cfu/mL gram-positive bacteria in blood. Primers designed by Klauseger et al. (23) also showed different sensitivities toward gram-positive and gram-negative bacteria. In the present study, both universal primers showed higher accuracy rate for gram-positive bacteria; PCR assay with 16sr RNA universal primer showed higher accuracy rate and had lower false negative rate with more efficiency than 23sr RNA primer for detecting most studied bacteria. It seems that the limitations of these studies might have diminished significantly by using initial enrichment as Rothman et al. did (24), by using a more efficient commercial kit as Zhang et al. used (29), and finally by optimization the PCR assay as Heininger et al. have performed (32). The present study demonstrated that detecting of bacteria in blood by PCR using 16sr RNA universal primer has more accuracy rate than PCR with 23sr RNA universal primer for all studied bacteria. In addition, it is more accurate and more rapid than blood culture for diagnosis of bacteremia, however, their specificity are the same.

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Authors' Contribution

All listed authors have contributed sufficiently to the project.

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