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# Development and Assessment of a Single Tube Internally Controlled Multiplex PCR Assay to Detect Different Pathogenic Bacteria Involved in Blood Stream Infections

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## ABSTRACT

***Background:*** Bloodstream infections are associated with high morbidity and mortality. Delayed etiological diagnosis and inadequate antimicrobial therapy are associated with treatment failures.
***Objectives:*** This study describes the development and assessment of a new multiplex PCR that includes an Internal Control (IC) for the assurance of the whole workflow from the extraction of the DNA until the revelation of the amplicons.
***Materials and Methods:*** A unique sequence was chosen for each pathogen and used for primer design. Primers for amplification of Enterobacteriaceae, Enterococcus spp, Staphylococcus spp, Acinetobacterbaumanii and IC were designed and tested for sensitivity and specificity on the basis of their standard strains.
***Results:*** The multiplex PCR showed a sensitivity ranging from 1 to 100 target copies per reaction or 50 to 100 colony forming unit (CFU) per ml to the whole blood depending on the bacterial species. The specificity of this method was elevated and no false positive amplification was identified for 17 different species other than the target microorganisms. Moreover, the detection of the IC was observed in the concentration as low as 1 copy per reaction. The correct co-amplification of IC for each single bacterial species showed a correct whole workflow procedure starting from the extraction step.
***Conclusion:*** This new assay permits a rapid and accurate detection of some pathogenic microorganisms, that are among the most commonly detected ones in blood stream infections in Iran, with a simple and cost-effective method which includes the use of an internal control to validate the whole procedure thus avoiding false negative results.

*Keywords:* Internal Control; Single Tube Multiplex PCR; Bacteremia



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Implication for health policy/practice/research/medical education:
This new assay permits a rapid and accurate detection of some pathogenic microorganisms, that are among the most commonly detected ones in blood stream infections in Iran, with a simple and cost- effective method which includes the use of an internal control for the validation of the whole procedure thus avoiding false negative results.

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## 1. Background

Bacteremias are recognized globally as a major cause of morbidity and mortality in hospitalized patients (1). Bloodstream infections account for 30-40% of all cases of severe sepsis and septic shock (2). Because of its ability to specifically amplify minute quantities of nucleic acid, PCR has been applied with great success in clinical diagnostics of bacterial infections (3-5). In particular, numerous broad range PCR assays targeting the 16SrDNA were developed to detect and identify the presence of cultivable and uncultivable bacteria from different specimens, and in particular in whole blood in a faster time than standard blood culture (6, 7). Relatively simple procedures to extract nucleic acid from clinical specimens provide samples with a reasonable purity without requiring hazardous chemicals and extensive manipulation (8). Nevertheless, extracted clinical specimens may contain small impurities that inhibit enzyme based nucleic acid amplification processes as it has been reported in previous studies (9-12). A positive IC result, ensures that negative results are truly negative. Inhibitory specimens can be identified by monitoring amplification of a second target nucleic acid, which serves as an internal control (IC). Obtaining a positive signal from the IC target demonstrates successful amplification, thereby validating a negative result for the microbial target. An internal control sequence (internal quality marker) in each individual sample allows truly internally-controlled DNA extraction and PCR reaction, in which experimental conditions for target and control templates are the same. The use of an internal control to exclude false negative results is highly desirable in order to keep the whole workflow under strict control to ensure the quality of the diagnostic process.

## 2. Objectives

A common cellular gene sequence, which is expected to be present in all specimens, can be used as an IC (13). This approach has the advantage of monitoring the integrity of the nucleic acid target, since in improperly collected, stored, or processed specimens, the endogenous target will be absent (or degraded) and failed to yield a positive amplicon. A disadvantage is that endogenous sequences may not accurately reflect amplification of the primary target due to differences in the primer sequence, size of the amplified product, and the relative amount of the two targets. Based on these considerations, the current study described the properties of the designed IC and explained how to use and interpret the results during routine clinical testing.

## 3. Materials and Methods

### 3.1. Design of Primer

The design of primers was carried out using Mega 4, Allel ID 6 software, Oligo 6 and Oligo analyzer to check out alignment, primer design, annealing temperature and multiplex condition respectively. An amplified 684 base-pair fragment in the Drosophila melanogaster chromosome was selected as the IC (GenBank accession number NC_004354.3). A 550 base pair (bp), 370bp, 118bp, 247 bp fragment for *Enterobacteriaceae* (GenBank accession number NC_000913.2), *Enterococcus* spp (GenBank accession number NC_017960.1), *Staphylococcus* spp (GenBank accession number NC_002745.2) and *Acinetobacter baumanii* (GenBank accession number NC_011595.1) were respectivelychosen as target sequences for the selected bacteria. The primer sets and genes target sequences with accession numbers are listed in *Table 1*.

**Table 1.** List of Oligonucleotide Primers Used for Conventional Multiplex PCR Amplification.

| Organism | Gene name | Forward primer | Reverse primer | Product size (bp) |
|---|---|---|---|---|
| **Drosophila melanogaster** | X chromosome | AGCATTCAAATCCTTCATACTG | ATGTTGGTGTAATCTGACTCG | 684 bp |
| ***Staphylococcus* spp.** | *rpoB* | CAGGAGAAGTTAAAGAACAAGAAG | GTGAACGAACTAATTGAGATACG | 118 bp |
| ***Enterococcus* spp.** | *rpoB* | AGAGAGTAAGGTCCGATTGAAC | GGTTGTTTCCCGTATTATGC | 370 bp |
| ***Acinetobacterbaumannii*** | *gyrA* | CACCAATCACACGCAATG | GTATTCCAACCGATATTCACC | 246 bp |
| ***Enterobacteriaceae*** | *rpoB* | CAGGTCGTCACGGTAACAAG | GTGGTTCAGTTTCAGCATGTAC | 512 bp |

### 3.2. Construction and Preparation of Standard Internal Control

The PTZ75 plasmid was used as the internal control DNA standard. This plasmid was constructed by ligating a PCR amplified fragment according to the instruction of the T/A cloning kit (Fermentas, Lithuania). The cloned fragment comprised a 684bp sequence from the D.melanogaster chromosome: in detail the selected sequence encompassed a region corresponding to the chromosome X (Genbank accession number NC_004354.3).

A series of 10 fold dilutions of the IC containing plasmid, ranging from 1 to 108copies/reaction were prepared and stored at - 80° C until used.

### 3.3. Bacterial Strains and DNA Extraction

Four different bacterial strains were selected as prototype of Gram positive (*Enterococcusfaecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213) and Gram negative

(*Acinetobacterbaumannii* ATCC 19606, *Escherichia coli* ATCC 25922) microorganisms, respectively. These bacteria were grown using the method of standard laboratory conditions (14). Each target sequence was cloned by using the T/A cloning kit (Fermentas, Lithuania) as reported below and enumerated by A260 absorbance. In addition, a panel of bacterial species, including gram positive and gram negative obtained both from the American Type Culture Collection (ATCC) and from clinical isolates collection (University of Isfahan) were tested.To evaluate the efficiencyof different techniques for the extraction of bacterial DNA, each singlebacterial suspension was treated with 4 diverse commercially available methods (QIAamp DNA mini blood kit (Qiagen, Germany), High Pure PCR Template Preparation Kit (Roche applied bioscience, Germany), Genomic DNA Purification Kit (Fermentas, Lithuania) and DNA extraction kit (Cinagen, Iran).) as follows: 200 µl of bacterial suspension (0.5 McFarland) were subjected to individual extraction protocols as indicated by each manufacturer. 1000 copies number of the DNA sequence, used as IC, were added to each sample before performing the extraction procedure. The final volume of the eluted DNA was adjusted to 100 µl.

### 3.4. Multiplex PCR

PCR conditions were optimized according to the manual of the emerald Amp MATHS PCR Master Mix (2X premix Bio Inc, Takara, Japan). Details of the procedure are given as follows: 0.2 mM of each primer and 1.25 U of Taq polymerase; optimal condition for annealing temperature was 60° C. As a template, 5 µl extracted DNA was added thus resulting in a total reaction volume of 25 µl. Amplification started with a cycle of 4 min at 94° C, followed by 35 cycles of denaturation at 94° C for 30 second, annealing at 60° C for 30 second and extension at 72° C for 1 min, subsequently final extension at 72° C for 10 min. The amplicons obtained for each set of species were distinguished by visualizing the PCR products by electrophoresis in 1% agarose gel and stained with ethidiume bromide (0.5 µg/ml). Stained gels were observed with UV light and images digitalized with (UVI doc HD2 LCD, UVITEC, UK).

Sensitivity was tested with serial dilutions of different plasmids containing the target sequences of the above indicated 4 prototype strains ranging from 1 to 108 copies number/reaction or serial dilutions of DNA extracted from human blood samples in vitro spiked by the 4 prototype strains. Specificity was tested with DNA extracted from the 17 bacterial species, including gram positive and gram negative obtained both from the American Type Culture Collection (ATCC) and from clinical isolates collection.

## 4. Results

### 4.1. Evaluation of Internal Control

The sensitivity of the IC DNA was tested by amplification of serial dilutions of quantified IC sequence containing plasmid. Specific amplification was obtained from all plasmid samples with concentration ranging from 1 to 108 copies per reactions, showing a high efficiency of amplification for the IC sequence selected (*Figure 1*).

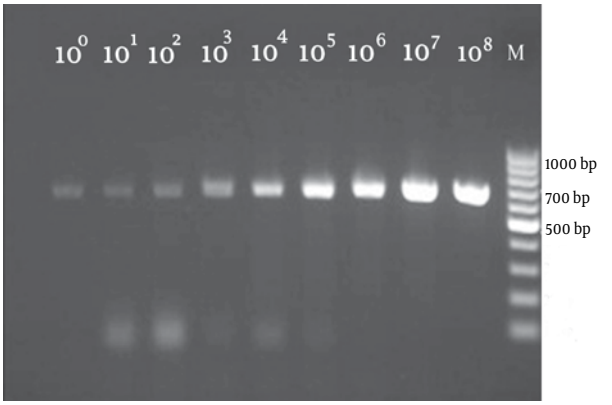


**Figure 1.** PCR amplification of the Internal Control (IC). The analytical sensitivity of the PCR was determined with serial dilutions of a plasmid containing the IC sequence ranging from 1to 108 copies. The lower detection limit for IC was 1 copy/reaction. The positions of the molecular weight markers (1 kbp) are indicated on the right.

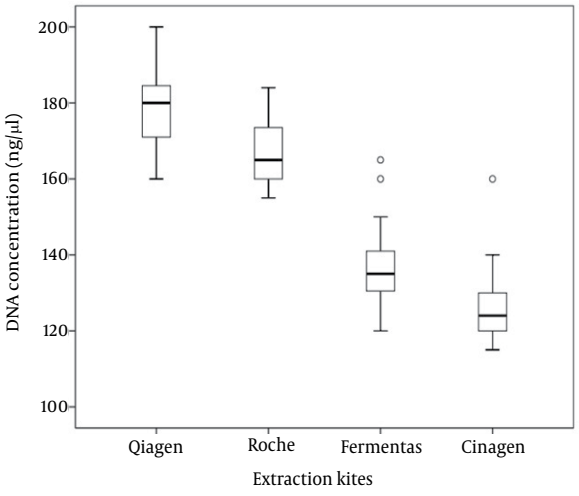


**Figure 2.** The box plot shows the total DNA concentration (ng/µl) obtained with the 4 different extraction kits. The Qiagen kit yeilded the highest DNA concentration. The boxplot shows that the highest purity with small variations was obtained by the Qiagen kit.

### 4.2. Qualitative Analysis of Different Extraction Methods

The quality of extracted bacterial DNA with the four kits was compared for each one of the four different prototype bacteria species, by using 5 replicate samples per germ. The mean OD value obtained with, Qiagen, Roche, Ferments and Cinagen kits were 1.93, 1.84, 1.56 and 1.32, respectively. The concentration of bacterial DNA (ng/µl)

obtained with Qiagen, Roche, Fermentas and Cinagen kits are reported in figure 2. There were statistically significant differences ($P < 0.05$) among them and the best quality was obtained with QIAamp DNA mini blood kit (Qiagen, Germany).

### 4.3. Sensitivity and Specificity of the Multiplex PCR Assay

In order to evaluate the sensitivity of the single tube multiplex PCR presented in the current study, the detection limits for all 4 prototype microorganisms were investigated, as reported above. The multiplex PCR assay identified all the 4 prototype bacterial strains successfully (*Figure 3*); moreover, no amplification products were detected for the 17 strains investigated for specificity of the method (data not shown). The lower detection limit for Gram positive were 102 copies per reactions or 100 CFU/ml for both *E.faecalis* (*Figure 3* B) and *S.aureus* (*Figure 3* D), whereas the lower detection limit for *A.baumannii* (*Figure 3* C) and *E.coli* (*Figure 3* A) were 10 copiesor 50 CFU/ml, respectively. The correct amplification of the IC showed that the whole workflow, starting from the extraction step, was performed in a reliable way.

## 5. Discussion

Rapid detection of bacteraemia is one of the most important tools in the clinical diagnosis of blood stream infections. Molecular techniques have been developed to achieve the diagnosis of bacterial infection by detecting bacterial DNA in blood faster (5, 15). The purity of the nucleic acid used in PCR is a critical point to achieve the highest sensitivity and to ensure reproducible results over time. Standard commercially available methods and kits are generally suitable for producing clean nucleic acid samples with sufficient concentration (16). The majority of the molecular assays are designed specifically to detect only one single organism, thus providing a high level of sensitivity and specificity (17-20). At the same time, a major limitation of this diagnostic approach is the capability to identify a unique bacterial species. Recently, a variety of a broad range PCR or multiplex PCR were applied in order to screen and detect the presence of multiple organismsin clinical samples (4, 21, 22). The major limitations of these methods are their cost to efficacy ratio and the fact that most of these techniques are based on time-consuming procedure for the final identification of the microorganism, such as: sequencing, restriction fragment length polymorphism or hybridization with germ specific probes (23-25).

The current study successfully combined the simultaneous detection of four different prototype bacteria with primers designed for the identification of *A.baumanii* and the whole genus to which these microbes actually belong, such as *Staphylococcus sppandEnterococcus* spp., and the most prominent pathogenic members of the family *Enterobacteriaceae* with a multiplex PCR assay. The current study results showed a detection limit variable between 10 to 100 copies of the target sequencesor 50 to 100 CFU/ml.Similar detection limits were observed for different molecular techniques when applied to different samples (26-28).

A previous study conducted by Bonilla et al.showed that the 37,5% of the culture positive samples were negative by PCR (22) and this fact is likely due to inhibition caused by a variety of factors, of which the most prominent is likely the ongoingantibiotic treatment. Moreover, a PCR inhibition was observed in whole blood samples when high level of leukocytosis was present (29). In particular, it was hypothesized that the PCR inhibition was probably due to the low performance of the extraction methods utilized or by the competition between the relatively low level of target DNA and the high level of human genomic DNA (29).

Based on these findings, the use of an internal control sequence can be suggested when whole blood samples or patient with special conditions, that are likely to generate PCR inhibition, must be investigated. Several compounds in blood have been suggested to possibly inhibit PCR, namely: heme, leukocyte derived host DNA (30), and anticoagulants (31, 32). Inhibition has been shown to affect the sensitivity of LCR-based amplification tests (33), TMA tests (34) and NASBA assays (35). An additional cause of false negative results, other than the presence of inhibiting factors, could be a number of target sequences below the assay detection limit or the presence of sequence variability (36). To monitor the process in genome amplification based tests, an internal control can be used (37, 38). The result of these studies demonstrates that incorporation of an internal control into PCR-based tests increase sensitivity by enabling the user to identify and retest samples that were scored as negative just for the presence of inhibition phenomena. Furthermore, a positive IC result indicates that the final amplification occurred and thus provides assurance that negative results are truly negative. Testing a dilution series of IC DNA revealed that the assay is able to detect concentrations as low as one copy of this sequence and this value is well comparable with the limits detected for the bacterial genes: this fact ensures that the IC could be properly detected in all the samples. The panel of the evaluated pathogenic bacteria encompasses several of the most prominent epidemiological causes of invasive infections in selected patient population in the Middle East area (39-42). It is indeed easy to perform the test reported in this manuscript which has an elevated cost effectiveness ratio due to its simplicity. It can consequently be proposed in the routine workflow of microbiology laboratories located in Middle East countries.

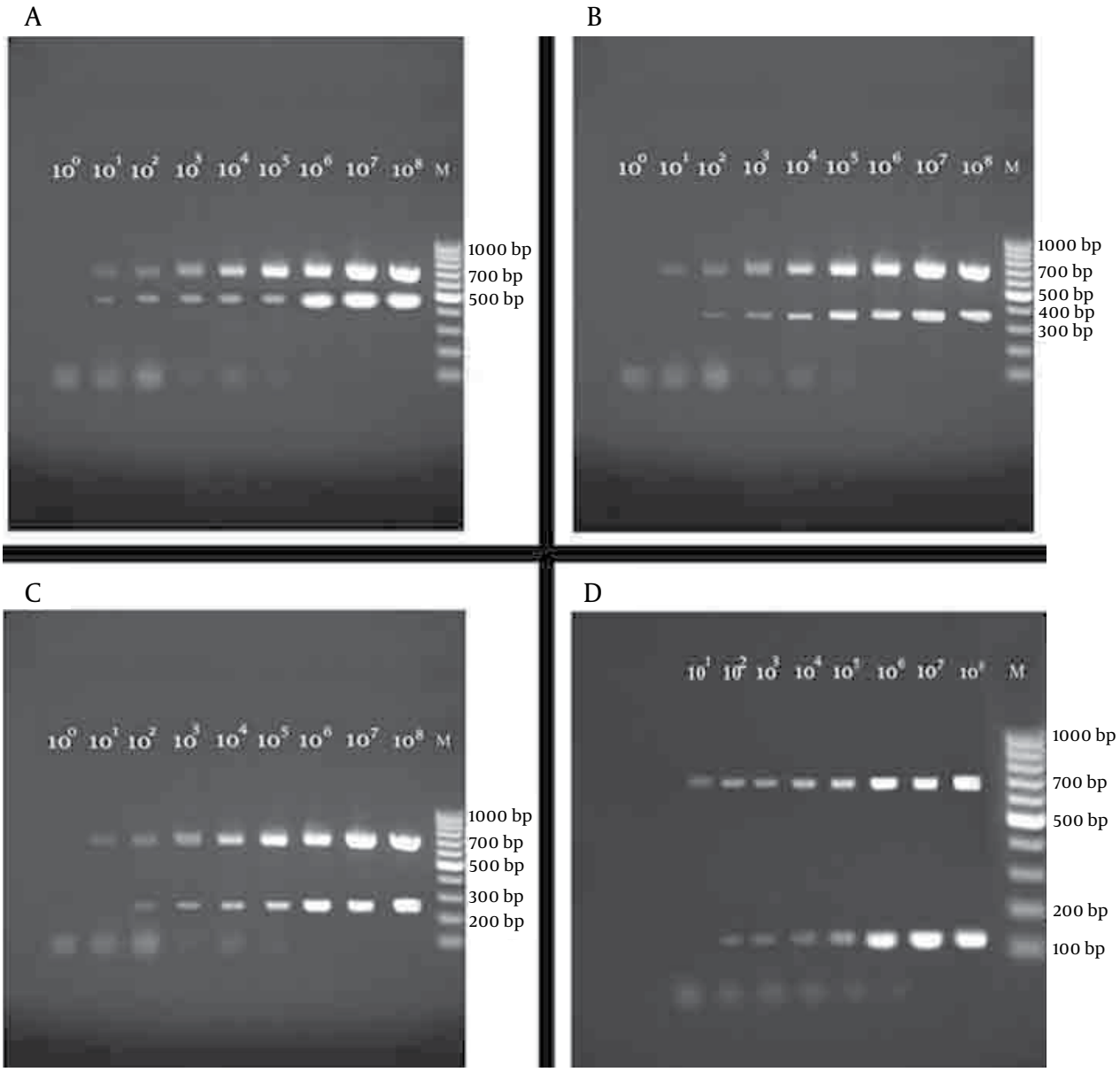


**Figure 3.** Amplification pattern of the 4 prototype bacterial strains. Detection limit of multiplex PCRs are shown in each panel. Each lane contains serial dilutions of plasmid DNA target sequences starting from 1 to 108 copies derived from: (Panel A) *E. coli* and IC (Panel B) *Enterococcus faecalis* and IC (Panel C) *Acinetobacter baumaniiand* IC (Panel D) *Staphylococcus aureus* and IC. The assays were able to detect as low as10 copies/reaction for *E.coli* and *A. baumanii* while the detection limit was 100 copies/reaction for *S.aureus* and *E.faecalis*.

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## Authors' Contribution

All authors had same contribution.

## Financial Disclosure

There is no conflict of Interest.

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