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Evaluation of the Punch-it™ NA-Sample kit for detecting microbial DNA in blood culture bottles using PCR-reverse blot hybridization assay

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ABSTRACT

DNA extraction efficiency affects the success of PCR-based method applications. The Punch-it™ NA-Sample kit for extracting DNA by using paper chromatography is technically easy to use and requires just two reagents and only 10 min to complete. The Punch-it™ NA-Sample kit could be offered as a rapid, accurate, and convenient method for extracting bacterial and fungal DNA from blood culture bottles. We compared the efficiencies of the commercial kit (Punch-it™ NA-Sample kit) and an in-house conventional boiling method with Chelex-100 resin for DNA extraction from blood culture bottles. The efficiency of the two DNA extraction methods was assessed by PCR-reverse blot hybridization assay (PCR-REBA, REBA Sepsis-ID) for detecting Gram positive (GP) bacteria, Gram negative (GN) bacteria, and *Candida* species with 196 positive and 200 negative blood culture bottles. The detection limits of the two DNA extraction methods were 10^3 CFU/mL for GP bacteria, 10^3 CFU/mL for GN bacteria, and 10^4 CFU/mL for *Candida*. The sensitivity and specificity of the Punch-it™ NA-Sample kit by REBA Sepsis-ID were 95.4% (187/196) and 100% (200/200), respectively. The overall agreement of the two DNA extraction methods was 98.9% (392/396). Three of four samples showing discrepant results between the two extraction methods were more accurately matched up with the Punch-it™ NA-Sample kit based on conventional culture methods. The results indicated that the Punch-it™ NA-Sample kit extracted bacterial and fungal DNA in blood culture bottles and allowed extracted DNA to be used in molecular assay.

1. Introduction

Sepsis is a deadly medical condition that remains an important cause of death at intensive care units (Cohen, 2002). The mortality rate associated with sepsis ranges from 20% to 70% (Angus et al., 2001; Barnato et al., 2008; Dombrovskiy et al., 2007; Martin et al., 2003). The incidence of severe sepsis is approximately 90.4 cases per 100,000 population in European Union and 3.0 cases per 1000 population in the United States (Angus et al., 2001; Davies et al., 2001). The causative agents of sepsis are Gram-positive (GP) bacteria, Gram-negative (GN) bacteria, anaerobic bacteria, and fungi (Cohen, 2002; Jean-Baptiste, 2007). Blood culture systems are the current gold standard for the diagnosis of bloodstream infection (BSI). However, blood culture systems generally require 6 to 48 h for definitive identification of the infectious agent and the determination of its susceptibility to antibiotics (Beekmann et al., 2003; Peters et al., 2004).

Recently, molecular methods, such as PCR, real-time PCR, and microarray, have provided fast, accurate detection and identification of pathogenic bacteria and fungi (Millar et al., 2007). However, the ability of the assays to detect BSI pathogens is dependent on the bacterial load, the quantity and quality of the extracted bacterial DNA, and the elimination of PCR inhibitors (Al-Soud and Radstrom, 2001). The cetyl trimethyl ammonium bromide (CTAB) method and its modification have been widely used for the extraction of DNA (Jara et al., 2008; Kathiravan et al., 2015; Rajagopal et al., 2014). Although CTAB-based DNA extraction methods generally produce consistent results for DNA quantity and quality, most of them take a long time and involve the use of toxic and hazardous chemical solvents (Capote et al., 2012). Another approach depends on using Chelex-100, a chelating resin with a high affinity for multivalent metal ions. It is frequently used to release DNA from low numbers of cells by a boiling treatment, and it protects the DNA from the effects of boiling with resin beads (Walsh et al., 1991). Chelex has been used previously for DNA amplification from cultures or clinical samples for microbiology testing (De Lamballerie et al., 1992). There are also many commercial DNA extraction kits that are designed to extract

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high quality DNA and eliminate PCR inhibitors from blood samples or blood culture bottles. Nevertheless, most commercial DNA extraction kits are expensive and involve complicated procedures.

The Punch-it™ NA-Sample kit (NanoHelix Co., Yuseong, Republic of Korea) is designed for easy isolation of nucleic acid from a small amount of samples of various kinds, including stool, blood, urine, and tissue (NanoHelix Co., Ltd., 2009). The Punch-it™ NA-Sample kit uses the paper chromatographic method to efficiently remove PCR inhibitors from the samples. The purified nucleic acids bound on the membrane can be used directly in PCR or real-time PCR reactions as templates. This study is the first to evaluate the efficacy of the Punch-it™ NA-Sample kit using blood culture bottles collected from patients with presumed sepsis. In a prior study, we used a boiling method for DNA extraction from blood culture bottles. Although this method is inexpensive and simple, additional step to digest bacterial or fungal cell walls such as the freeze-thaw step is needed in order to successfully extract microbial DNA from blood culture bottles.

The objective of this study was to evaluate the Punch-it™ NA-Sample kit for its ability to isolate bacterial and fungal DNA from blood culture bottles for use with downstream molecular identification and to compare the results to those of our previously described boiling method from blood culture bottles (Park et al., 2014).

2. Materials and methods

2.1. Blood culture and collection of blood culture bottles

One-hundred ninety-six positive blood culture (PBC) and two hundred negative blood culture (NBC) bottles from patients with a delta neutrophil index greater than 2.7% were collected at Wonju Severance Christian Hospital from April to June 2015. To avoid redundancy of the enrolled samples, only one blood culture bottle per patient was allowed. This study was approved by the Institutional Ethics Committee of Yonsei University Wonju College of Medicine (approval no. CR312055-002).

The PBC bottles were eligible for enrollment if they had been flagged as positive by BACTEC FX (Becton Dickinson, Sparks, MD, USA) or BacT/ALERT 3D (bioMérieux, Durham, NC, USA) with a positive Gram stain. When bacterial growth was noted, the culture bottle was inoculated onto both blood agar and Mac-Conkey agar plates, which were then cultured overnight at 35 °C in a 5% CO_2 incubator. The isolates were presumptively identified based on colony morphology, Gram staining, and biochemical test results. Isolates were further verified with a Vitek 2 (bioMérieux), MicroScan (Siemens Healthcare Diagnostics, Sacramento, CA, USA) or Vitek 2 yeast identification card (bioMérieux) system. Antimicrobial susceptibility tests were performed with the Vitek 2 (bioMérieux) system or the MicroScan (Siemens) system. The NBC bottles were eligible for enrollment if they had not been flagged as positive by continuous monitoring blood culture system during 5 days of incubation after inoculation. Each 100-μL aliquot from the 196 PBC and 200 NBC bottles was aseptically aspirated and used for two DNA extraction tests as blood culture results were obtained.

2.2. Boiling method

DNA was extracted by using the following procedure. A 20-μL aliquot of the blood culture bottle was mixed with 1 mL of erythrocyte lysis buffer (ELB) (Sigma, St. Louis, MO, USA) at room temperature for 10 min to disrupt the erythrocytes. After centrifugation at 13,000 *g* for 5 min, the supernatant was removed. The pellet was washed with 1 mL of ELB to completely remove erythrocytes and then centrifuged under the same conditions. One hundred microliters of ELB were added to the pellet, which was then frozen and thawed twice. Next, 100 μL of DNA extraction solution (Optipharm, Osong, Republic of Korea) were added to the mixture, and the mixture was boiled for 15 min in the heat block (FINEPCR, Gunpo, Republic of Korea). After centrifugation at 13,000 *g* for 10 min, the supernatant was used as a DNA template for PCR.

2.3. Punch-it™ NA-Sample kit

In this study, the blood culture samples were processed with the Punch-it™ NA-Sample kit (NanoHelix Co., Yuseong, Republic of Korea) according to the manufacturer's protocol (Fig. 1). In brief, 20 μL of the blood culture bottle were mixed with 20 μL of lysis buffer. The lysate was transferred into the sample well of the Punch-it™ NA-Sample kit. After the sample solution was completely absorbed into the membrane, 200 μL of washing buffer were added to the washing well and incubated for 3 min. A puncher was used to extract a piece of the membrane (approximately 1 mm in size) from the bottom of the sample well. This membrane was transferred into 50 μL of 1× Tris-EDTA buffer (TE buffer; 10 mM Tris-HCl pH 8.0 containing 1 mM EDTA) and used as a DNA template for PCR. We used REBA Sepsis-ID to compare the performance of the Punch-it™ NA-Sample kit with the boiling method using the 196 PBC and 200 NBC bottles (Fig. 2).

2.4. Spiking experiment to determine the detection limit of the Punch-it™ NA-Sample kit

To determine the detection limit of the Punch-it™ NA-Sample kit, the cultured *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), and *Candida albicans* (ATCC 36802) were suspended separately in 1 × phosphate buffered saline, and the density of each organism was adjusted to 0.5 McFarland unit. A series of 5-fold dilutions, which ranged from 10^5 to 10^1 CFU/mL, were prepared from the bacterial or fungal suspensions, and 100 μL of each dilution were spread on a blood agar plate. Blood agar plates were incubated at 37 °C for 16 h. Each dilution was spiked into a negative blood culture sample, and each spiked sample was used for genomic DNA extraction with the Punch-it™ NA-Sample kit and boiling method.

2.5. PCR amplification

PCR was performed in a volume of 20 μL containing 10 μL of 2× PCR master mix (GeNet Bio, Daejeon, Republic of Korea), 5 μL of 1× primer mix (biotinylated primers for GP bacteria, GN bacteria, *Candida* species, and resistance genes for the *mecA* gene, the *van*A, and *van*B gene), and 5 μL of sample DNA. For the REBA Sepsis-ID (Optipharm), the first 10 PCR cycles involved initial denaturation at 95 °C for 30 s, followed by annealing and extension at 60 °C for 30 s. These 10 cycles were followed by 40 cycles of denaturation at 95 °C for 30 s, followed by annealing and extension at 54 °C for 30 s. After the final cycle, samples were maintained at 72 °C for 10 min to complete the synthesis of all strands. In every PCR assay, sterile distilled water was added to the PCR mixture in place of DNA extract as a negative control.

2.6. Reverse blot hybridization assay (REBA Sepsis-ID)

The REBA Sepsis-ID was performed according to the standard protocol provided by the manufacturer. In brief, biotinylated PCR products were denatured at 25 °C for 5 min in a denaturation solution and diluted in 970 μL of hybridization solution on the REBA membrane strip, in the provided blotting tray. Denatured, single-stranded PCR products were used to hybridize with the probes on the strip at 55 °C for 30 min. The strips were then washed twice in 1 mL of washing solution at 55 °C for 10 min with gentle shaking, incubated at 25 °C for 30 min with streptavidin-alkaline phosphatase conjugate (Roche Diagnostics, Mannheim, Germany) diluted to 1:2000 in conjugate diluent solution (CDS), and washed twice with 1 mL of CDS at room temperature for 1 min. Colorimetric hybridization signals were visualized with the addition of an alkaline phosphatase-mediated staining solution, i.e., nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate (NBT-