Journal of Microbiological Methods 99 (2014) 1–7

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Evaluation of MolYsis™ Complete5 DNA extraction method for detecting *Staphylococcus aureus* DNA from whole blood in a sepsis model using PCR/pyrosequencing

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ARTICLE INFO

Article history:
Received 19 December 2013
Received in revised form 21 January 2014
Accepted 22 January 2014
Available online 3 February 2014

Keywords:
DNA extraction
Whole blood
Molecular diagnostics
Sepsis
Bacterial bloodstream infection

ABSTRACT

Bacterial bloodstream infections (BSI) and ensuing sepsis are important causes of morbidity and mortality. Early diagnosis and rapid treatment with appropriate antibiotics are vital for improving outcome. Nucleic acid amplification of bacteria directly from whole blood has the potential of providing a faster means of diagnosing BSI than automated blood culture. However, effective DNA extraction of commonly low levels of bacterial target from whole blood is critical for this approach to be successful. This study compared the Molzyme MolYsis™ Complete5 DNA extraction method to a previously described organic bead-based method for use with whole blood. A well-characterized *Staphylococcus aureus*-induced pneumonia model of sepsis in canines was used to provide clinically relevant whole blood samples. DNA extracts were assessed for purity and concentration and analyzed for bacterial *rRNA* gene targets using PCR and sequence-based identification. Both extraction methods yielded relatively pure DNA with median A260/280 absorbance ratios of 1.71 (MolYsis™) and 1.97 (bead-based). The organic bead-based extraction method yielded significantly higher average DNA concentrations ($P < 0.05$) at each time point throughout the experiment, closely correlating with changes observed in white blood cell (WBC) concentrations during this same time period, while DNA concentrations of the MolYsis™ extracts closely mirrored quantitative blood culture results. Overall, *S. aureus* DNA was detected from whole blood samples in 70.7% (58/82) of MolYsis™ DNA extracts, and in 59.8% (49/82) of organic bead-based extracts, with peak detection rates seen at 48 h for both MolYsis™ (87.0%) and organic bead-based (82.6%) methods. In summary, the MolYsis™ Complete5 DNA extraction kit proved to be the more effective method for isolating bacterial DNA directly from extracts made from whole blood.

1. Introduction

In the US, bacterial bloodstream infections (BSI) are the 11th leading cause of death among adults and 7th among infants (Hoyert and Xu, 2012). *Staphylococcus aureus* is the most common cause of healthcare-associated BSI and the second most common cause of community-acquired BSI, accounting for 35.6% and 29.4% of all culture-confirmed BSI, respectively (Kollef et al., 2011). Early detection and rapid treatment with appropriate antibiotics are vital for improving outcome in patients with suspected BSI. For every hour of delay in antimicrobial administration a 7.6% average decrease in survival can be seen (Kumar et al., 2006). Administration of inadequate or ineffective antimicrobial treatment was found to be an independent determinant of hospital mortality (Ibrahim et al., 2000).

The current diagnostic gold standard for BSI requires growth of the organism in culture followed by Gram staining and phenotypic identification of the purified isolate, with result reporting time ranging from 36 to 72 h for Gram positive bacteria, and 48–96 h for Gram negative bacteria (Jordan, 2010). Physicians begin treating patients for suspected BSI with a regimen of broad-spectrum antibiotic(s) immediately after drawing blood for culturing using a continuously monitoring automated instrument. However, overuse of empiric therapy can result in negative consequences for both the individual—through potential adverse drug reactions or destruction of protective gut microbiota—and the greater community—by increasing the opportunity for emergence of antibiotic resistant bacteria as well as the increased costs of treatment (Rodrigues et al., 2012; van de Sande-Bruinsma et al., 2008).

There is an urgent need for more rapid, yet accurate diagnostics to reduce the number of doses of ineffective or unnecessary broad-spectrum antibiotics received by uninfected patients to allow for the more timely administration of a more tailored and effective antibiotic therapy to those who do have a BSI. Recent studies have shown that molecular-based identification of highly conserved bacterial 16S and 23S ribosomal DNA targets is a viable option for BSI diagnosis (Jordan et al., 2006; Chan et al., 2009). This approach does not require lengthy incubation periods, and could detect or rule out BSI sooner and thereby reduce the number of doses of unnecessary or ineffective antibiotics administered to patients (Brozanski et al., 2006). Molecular methods are

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http://dx.doi.org/10.1016/j.mimet.2014.01.013

also capable of detecting bacterial DNA from non-viable, fastidious, or non-culturable microorganisms (Huttunen and Aittoniemi, 2011). This is especially important for cases in which patients are already receiving antibiotics when blood is drawn for culture—a clinical decision that greatly reduces the diagnostic yield of standard blood culture (Srinivasan and Harris, 2012).

Despite these many potential advantages, the performance characteristics of a molecular-based method for diagnosing BSI is completely dependent upon the quality and quantity of DNA template generated. Blood contains a number of PCR inhibitors (Al-Soud and Radstrom, 2001; Wilson, 1997), and low colony counts (~1–100 colony-forming units per milliliter of blood) are the rule rather than the exception in most individuals with culture-confirmed sepsis (McLaughlin et al., 1983). With so few organisms and significant inhibitors present, it is critical to select an effective DNA extraction method for use with whole blood that is compatible with molecular diagnostics (Regan et al., 2012).

The MolYsis™ Complete5 DNA extraction protocol was developed to selectively isolate and purify bacterial DNA from whole blood (Horz et al., 2008). Previous studies show success with this protocol using spiked blood samples, removing virtually all human non-target DNA and improving the limit of detection of molecular assays when compared to other DNA extraction methods (Hansen et al., 2009). Bacteria-spiked blood samples, however, do not accurately represent the cellular environment and immunological response present in the blood of an individual during systemic infection. This study sought to utilize a well-characterized *S. aureus*-induced pneumonia model of sepsis in dogs to provide a more clinically relevant set of blood samples to extract using this method (Minneci et al., 2007). To our knowledge, this study was the first of its kind to evaluate the efficacy of the MolYsis™ Complete5 DNA extraction kit using whole blood samples collected from a *S. aureus*-induced pneumonia model of sepsis in canines. The objective was to evaluate the MolYsis™ method for its ability to isolate and purify bacterial DNA directly from whole blood of *S. aureus*-infected or mock infected dogs for use with downstream molecular identification compared to our previously described organic bead-based extraction method (Jordan et al., 2006) from the same whole blood samples.

2. Materials and methods

2.1. S. aureus-induced pneumonia model of sepsis in canines

In total, 44 healthy purpose-bred Beagles (male, 1–2 years old, 10–14 kg) were studied, ~4 dogs at any one time. Following intravenous (IV) sedation and analgesia maintained throughout the study, animals were randomized to receive either intrabronchial inoculation of 1.5×10^9 CFU/kg of *S. aureus* (infected dogs, n = 36) or an equivalent volume of saline (control dogs, n = 8), time recorded as 0 h (h). The oxacillin-sensitive *S. aureus* isolate used in this study originated from an ICU patient with culture-confirmed BSI, which was cryopreserved for future use. The inoculums used in these studies were prepared from fresh overnight cultures of the organism. The dose of *S. aureus* given to the dogs was chosen to result in ~70% mortality based on previously completed dose response studies (unpublished results) for the purpose of developing a canine model for studying outcomes during septic shock. The bacterial concentration in the intrabronchial inoculum given to the dogs was determined spectrophotometrically (DU730, Beckman Coulter, Indianapolis, IN) and confirmed turbidimetrically using a 0.5 McFarland standard. At 4 h post inoculation, the recommended dose of oxacillin (20 mg/kg, q4, IV) was initiated with continuance through 72 h. Standard care was initiated for the dogs providing hemodynamic, oxygenation and ventilatory support through 96 h. Vital signs were monitored throughout the duration of this study with a supervisor or attending investigator available for consultation at all times. Use of these dogs in this study was approved by NIH IACUC # CCM10-02 and The George Washington University IACUC# A226.

2.2. Sample collection, WBC count and quantitative blood culture

Several blood samples were collected consecutively from an established cephalic vein IV line from the dogs in this study at 0, 24, 48, 72 and 96 h post inoculation. These blood samples were kept at room temperature until processed as follows: Seven milliliters of whole blood, used for the two DNA extraction methods, was collected into K2 EDTA tubes (BD Vacutainer, Franklin Lakes, NJ) and processed within 2 h of draw time; 1 ml of whole blood was collected and analyzed immediately for total white blood cell count (Hemavet 950, Drew Scientific, Dallas, TX). A subset of the dogs in this study had blood drawn from the cephalic vein for quantitative culture; 1.5 ml of drawn blood was aseptically inoculated into Wampole Isolator™ tubes (Cat# 50C5; Inverness Medical, Princeton, NJ) and processed within the allotted time recommended by the manufacturer with 100 μl each of inoculated sample being plated onto both MacConkey agar and 5% sheep blood agar (Cat# 215197, 297759; Becton Dickinson, Sparks, MD). These inoculated agar plates were incubated at 37 °C with 5% CO_2 for 4 days and examined daily for bacterial growth and enumeration of colonies present. The number of CFU/ml was calculated using the equation found in the manufacturer's packet insert, and the bacteria were identified using manual, routine biochemical and enzymatic tests that assessed their phenotypic characteristics.

2.3. Organic bead-based extraction method (bead)

This method does not eliminate cellular DNA, but extracts total DNA. Blood (500 μl) from a K2 EDTA tube was added to 10 ml RBC lysis buffer (0.32 M sucrose, 10 mM Tris HCl [pH 7.5], 5 mM $MgCl_2$, 0.1% Triton-X100) and centrifuged at 6000 ×*g* for 5 minutes (min) at 4 °C. Supernatant was removed and the pellet resuspended by vortexing in 100 μl of a lysis buffer containing 5 M Guanidine-HCl in 0.1 M Tris HCl (pH 8.0). The resulting lysate was added to a 1.5 ml tube containing 0.24 g of 0.1 mm zirconium silica beads (Biospec, Bartlesville, OK) and vortexed, using a horizontal adapter (Cat#13000-V1; Mo Bio Laboratories Inc., Carlsbad, CA), for 5 min. Four hundred microliters of distilled H_2O and 800 μl 99% benzyl alcohol (Acros Organics, New Jersey) were added to the lysate/bead mixture and vortexed. After settling for 5 min at room temperature (RT), the lysate was centrifuged at 5000 ×*g* for 5 min. The resulting aqueous layer was removed and transferred to another 1.5 ml tube where the organic extraction step was repeated a second time. DNA was precipitated from the aqueous layer by adding a 1/10th volume of 3 M sodium acetate (NaOAC) (Sigma Chemical, St. Louis, MO), equal volume 99% isopropyl alcohol (Acros Organics), and 1 μl 20 mg/ml glycogen (Cat# 10901393001; Roche Diagnostics, Indianapolis, IN). The sample was briefly vortexed and centrifuged at 13,000 ×*g* for 15 min at 4 °C. The supernatant was carefully removed and the DNA pellet washed with 100 μl 70% ethanol (Acros Organics) and centrifuged one final time at 13,000 ×*g* for 5 min at 4 °C. The supernatant and all excess moisture in the tube were removed and the pellet was allowed to air dry for approximately 15 min at RT before being resuspended in 50 μl of 1× TE buffer consisting of 10 mM Tris HCl (pH 8.0), 1 mM EDTA (pH 8.0), and stored at −20 °C prior to testing. This extraction protocol was adapted from a previous publication (Fredricks and Relman, 1998).

2.4. Molzyme MolYsis™ Complete5 (MolYsis™)

The proprietary MolYsis™ technology utilizes selective isolation of bacterial DNA from intact organisms in whole blood (Horz et al., 2008). In this study, blood (1 ml) from a K2 EDTA tube (BD Vacutainer) was processed using the MolYsis™ Complete5 DNA extraction kit (Cat# D-321-100; Molzym GmbH & Co. KG, Bremen, Germany) according to