Published in final edited form as:

J Microbiol Methods. 2014 April ; 99: 1–7. doi:10.1016/j.mimet.2014.01.013.

Evaluation of MolYsis™ Complete5 DNA Extraction Method for Detecting *Staphylococcus aureus* DNA from Whole Blood in a Sepsis Model Using PCR/Pyrosequencing

Chase D. McCann^a and Jeanne A. Jordan^a

Chase D. McCann: sphcdm@gwu.edu; Jeanne A. Jordan: jajordan@gwu.edu

^aThe George Washington University, School of Public Health and Health Services, Department of Epidemiology and Biostatistics; 2300 I Street NW, Washington, DC 20037 USA

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 *S. 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.

Keywords

DNA Extraction; Whole Blood; Molecular Diagnostics; Sepsis; Bacterial Bloodstream Infection

© 2014 Elsevier B.V. All rights reserved.

Corresponding Author: Jeanne A. Jordan, PhD, jajordan@gwu.edu, 202-994-7062, 2300 I Street NW, Ross 231, Washington, DC 20037 USA.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

In the US, bacterial bloodstream infections (BSI) are the 11th leading cause of death amongst adults and 7th amongst infants (Hoyert & Xu, 2012). *S. 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, Sherman, Ward, Fraser, & Kollef, 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 36–72 hours for Gram positive bacteria, and 48–96 hours for Gram negative bacteria (Jordan J. A., 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, Roque, Falcão, Figueiras, & Herdeiro, 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, Durso, Butchko, Jones, & Brozanski, 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, Jones, Krohn, & Jordan, 2006). Molecular methods are also capable of detecting bacterial DNA from non-viable, fastidious, or non-culturable microorganisms (Huttunen & 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 & 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 & 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, Hamilton, Scholes, & Bartlett, 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, Furtado, Brevnov, & Jordan, 2012).

The MoYsisTM 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, Bruggeman, & Wolffs, 2009). Bacteria-spiked blood samples, however, do not accurately represent the cellular environment and immunological response present in 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 MoYsis™ 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 MoYsis™ 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, Durso, Butchko, Jones, & Brozanski, 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 year 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 hours (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 consult 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, were 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 were 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% CO₂ 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 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 μ l) 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 MgCl₂, 0.1% Triton-X100) and centrifuged at $6,000 \times g$ for 5 minutes (min) at 4°C. Supernatant was removed and the pellet resuspended by vortexing in 100 μ l 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 H₂O and 800 μ l 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 $5,000 \times g$ 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 3M sodium acetate (NaOAc) (Sigma Chemical, St. Louis, MO), equal volume 99% isopropyl alcohol (Acros Organics), and 1 μ l 20 mg/ml glycogen (Cat# 10901393001; Roche Diagnostics, Indianapolis, IN). The sample was briefly vortexed and centrifuged at $13,000 \times g$ for 15 min at 4°C. Supernatant was carefully removed and the DNA pellet washed with 100 μ l 70% ethanol (Acros Organics) and centrifuged one final time at $13,000 \times g$ for 5 min at 4°C. Supernatant and all excess moisture in the tube was removed and the pellet was allowed to air dry for approximately 15 min at RT before being resuspended in 50 μ l of 1x 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 & 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, Scheer, Huenger, Vienna, & Conrads, 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 the recommended manufacturer's protocol. All reagents needed for this extraction were included in the kit. Chaotropic buffer CM (250 μ l) was added to 1 mL blood, briefly vortexed, and incubated for 5 min at RT to lyse blood cells. Buffer DB1 (250 μ l) and MolDNase B (10 μ l) were added to the lysate, briefly vortexed, and incubated for 15 min at RT to degrade nucleic acids released by the blood cells. Bacterial cells were pelleted by centrifugation at $12,000 \times g$ for 10 min and the resulting supernatant was removed. Buffer RS (1 ml) was added, mixed by vortex, and centrifuged at $12,000 \times g$ for 5 min where the resulting supernatant was discarded to remove any residual chaotrope or DNase activity. Buffer RL (80 μ l) was added and vortexed to resuspend the pellet followed by 20 μ l of Buglysis reagent vortexed for 10 seconds (sec) and incubated in a thermomixer (Cat# 535024744; Eppendorf AG, Hamburg, Germany) at 37°C and 1000 rpm for 30 min to degrade bacterial cell walls. Buffer RP (150 μ l) and Proteinase K (20 μ l) were added and incubated in a thermomixer at 56°C and 1000 rpm for 10 min after which 250 μ l of buffer CS and 250 μ l of buffer AB were added and vortexed for 10 sec to lyse remaining cells and denature proteins. DNA was isolated by centrifuging lysate through the provided spin column at $12,000 \times g$ for 30 sec, and washed with 400 μ l buffer WB centrifuged at $12,000 \times g$ for 30 sec followed by 400 μ l 70% DNA-free ethanol centrifuged at $12,000 \times g$ for 3 min. DNA was eluted in 100 μ l 70°C DNA-free deionized water via centrifugation at $12,000 \times g$ for 1 min and stored at -20°C prior to testing.

2.5. Concentration and Purity of DNA Extracts

Nucleic acid concentration from each extract was determined using a 1 µl volume analyzed via spectrophotometry (Nanodrop 2000, ThermoFisher Scientific, Waltham, MA). DNA purity was assessed using the ratio of light absorbance at 260 nm and 280 nm (A₂₆₀/A₂₈₀) using the Nanodrop 2000 Software v1.0. Average absorbance ratios were calculated for each of the two extraction methods and compared against a standard absorbance ratio of 1.8 representing pure, double-stranded DNA.

2.6 PCR Amplification and Melt Curve Analysis for Detection of an Endogenous Canine *p16* Gene Target

Previously described canine endogenous *p16* gene-specific PCR primers (Table 1) amplify a 143 bp target (Chaubert, et al., 2010). Five microliters of each DNA extract (40 extracts from 8 control dogs and 118 extracts from 36 infected dogs) in a total PCR reaction volume of 25 µl were analyzed for this target. The negative control consisted of 5 µl of sterile distilled water added to 20 µl of PCR master mix. PCR was carried out on a SmartCycler (Cepheid, Sunnyvale, CA) under cycling conditions of 45 cycles of 94°C for 30 sec, 55°C for 45 sec, and 73°C for 45 sec, followed by melt curve analysis (T_M) in which temperature conditions increased from 60°C to 95°C at a rate of 0.2°C/sec. This analysis was completed to assess presence of significant inhibitors within the DNA generated from the organic, bead-based method as well as to evaluate the effectiveness of the MoYsis™ method to remove genomic DNA from canine whole blood samples.

2.6. PCR Amplification of Bacterial *rRNA* Gene Targets

Both sets of DNA extracts were amplified via real-time PCR using the primers listed in Table 1. The previously described *Staphylococcus spp.*-specific PCR primers amplify a 243 base pair (bp) target within the *16S rRNA* gene whose internal sequence differentiates *S. aureus* from coagulase-negative *Staphylococcus spp.* (Jordan, Durso, Butchko, Jones, & Brozanski, 2006). Five microliters of purified DNA was combined with 20 µl of master mix containing 0.5 µl each of 10 uM concentrations of a biotinylated forward and unlabeled reverse primers (IDT, Coralville, IA) (Table 1), 12.5 µl of 2x SYBR PreMix Ex Taq polymerase master mix (Cat# RR041A; TaKaRa Biotechnologies Co., Dalian, Liaoning Province, Japan) and molecular grade water. PCR was carried out on a SmartCycler under cycling conditions of 95°C for 1 min, followed by 40 cycles of 95°C for 20 sec, 60°C for 1 min, and 72°C for 15 sec. A positive control, consisting of *S. aureus* DNA extracted using the bead-based organic extraction method and two negative controls consisting of master mix and sterile distilled water were included in each PCR run.

The previously described enteric gram-negative rod-specific PCR primers (Table 1) amplify a 280 bp target within a conserved region within the *23S rRNA* gene (Jordan, Jones-Laughner, & Durso, 2009). This PCR assay was performed selectively on DNA extracts with corresponding positive quantitative blood culture results as previously described (Moore, McCann, & Jordan, 2013). Five microliters of purified DNA was combined with 20 µl of PCR master mix containing 0.5 µl each of 10 uM concentrations of the 2 unlabeled forward primers and 1 biotinylated reverse primer (IDT, Coralville, IA) (Table 1), 12.5 µl of 2x SYBR PreMix Ex Taq polymerase master mix (Cat# RR041A; TaKaRa Biotechnologies Co.) and molecular grade water. PCR was carried out on a SmartCycler under cycling conditions of 95°C for 1 min, followed by 40 cycles of 95°C for 20 sec, 60°C for 1 min, and 72°C for 15 sec. A positive control, consisting of *E. coli* DNA extracted using the bead-based organic extraction method and two negative controls consisting of master mix and sterile distilled water were included in each PCR run.

2.7. Pyrosequencing Reaction

Pyrosequencing analysis was completed on a PyroMark ID Pyrosequencer (Qiagen) using the PyroMark Gold Q96 reagent kit (Cat# 972804; Qiagen) and PyroMark ID v1.0 software provided with the instrument. The *S. aureus*-specific pyrosequencing reaction uses an 11(ACTG) dNTP dispensation program as previously described along with one *Staphylococcus*-specific unlabeled sequencing primer (Table 1) (Moore, McCann, & Jordan, 2013). The presence of *S. aureus* was indicated by the following pyrogram-generated sequence: 5'-ACA TCA GAG AAG CAA GCT TCT CG-3' (Jordan, Durso, Butchko, Jones, & Brozanski, 2006). The enteric Gram-negative rod-specific pyrosequencing reaction uses an AGC, 12(CTGA) dNTP dispensation program as previously described along with two unlabeled sequencing primers (Table 1) (Moore, McCann, & Jordan, 2013). The sequences generated by pyrosequencing differentiate between a large number of medically-relevant enteric Gram-negative rods as previously described (Jordan, Jones-Laughner, & Durso, 2009).

2.8. Statistical Analysis

Mean values and standard deviations of DNA concentrations were calculated and compared using two-tailed Student's *t*-tests. A calculated *P*-value of < 0.05 was considered a significant difference between mean values. All graphs and statistical analyses were completed using Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA).

3. Results

3.1. Resulting purities of the two sets of DNA extracts

Purity of the resulting 118 DNA extracts (infected dogs) from each extraction method was evaluated by calculating the ratio of absorbance at 260 nm and 280 nm. Both the MoLYsis™ and organic bead-based extraction methods yielded relatively pure DNA with median absorbance ratios of 1.71 (IQR = 1.55–1.87) and 1.97 (IQR = 1.9–2.15), respectively (Figure 1).

3.2. Resulting concentrations of the two sets of DNA extracts

Spectrophotometric analysis of the 118 DNA samples (infected dogs) from each extraction method revealed that the organic, bead-based extraction method yielded significantly higher average DNA concentrations ($P < 0.05$) at each time point throughout the 96 h infection period compared to the MoLYsis™ extraction method (Figure 2; left axis). DNA concentrations resulting from the organic bead-based method were roughly 5 times those yielded by the MoLYsis™ method.

Changes in DNA concentrations yielded by the organic bead-based extraction method closely resembled the changes in the canine WBC counts observed over the 96 h period (Figure 2; right axis). Conversely, DNA concentrations yielded by the MoLYsis™ extraction method mirrored the changes in CFU/ml reflecting the non-*S. aureus* bacteria isolated from the quantitative blood cultures (Figure 3).

3.3. PCR and pyrosequencing results of the two sets of extracts screened for two bacterial DNA targets and an endogenous canine gene target

3.3.1 Molecular detection of endogenous p16 gene target—PCR and T_M analysis of the 158 DNA extracts (40 controls and 118 infected dogs) for the *p16* gene target revealed stark differences between the two extraction methods; 98.7% (156/158) of the bead-based extract PCR products compared to only 1.9% (3/158) of the MoLYsis™ extract PCR products were found to be positive for *p16*. Presence of the *p16*-specific 143 bp

amplicon correlated with a T_M peak of approximately 88.5°C. Samples lacking the *p16*-specific 143 bp amplicon also lacked a melt peak with that specific T_M .

3.3.2 Molecular detection of *S. aureus* 16S rRNA gene target—*Staphylococcus*-specific PCR/pyrosequencing analysis of the two sets of DNA extracts resulted in more MoYsis™-generated DNA extracts being positive for *S. aureus* DNA (58/82; 70.7%) than with the organic bead-based extracts (49/82; 59.8%) (Table 2). Because we were evaluating the extracts for *S. aureus* DNA, the 36 blood samples drawn at 0 h from the infected cohort were subtracted from the total 118 samples analyzed to generate a denominator of 82 for comparing the two extraction methods to detect this target. The highest rate of *S. aureus*-positive results was seen at 48 h for both the MoYsis™ generated extracts (87.0%) and the organic bead-based extracts (82.6%) (Table 2). No *S. aureus* DNA was detected in the 0 h time point (prior to the bacterial challenge) from either set of extracts, in the blood samples analyzed from the 36 *S. aureus* challenged dogs (Table 2). Neither was *S. aureus* DNA detected in any of the resulting 40 extracts of the blood samples drawn from the 8 mock-infected control dogs (data not shown). The peak heights from the resulting pyrograms generated after *S. aureus*-specific 16S rRNA gene PCR assay were compared between extraction methods. The DNA extracts from both the MoYsis™ and the organic bead-based protocols generated robust pyrograms that, if present, produced the *S. aureus*-specific DNA sequence associated with the 16S rRNA gene target. Overall, there was a trend towards the MoYsis™ extraction method producing pyrograms with higher peak intensity compared to the organic bead-based method (Figure 4).

3.3.3 Molecular detection of enteric gram-negative rods 23S rRNA gene target—For both subsets of DNA extracts with corresponding positive quantitative blood cultures, there was 100% agreement between the PCR/pyrosequencing identification and the blood culture results for the non-*S. aureus* bacteria isolated (data not shown). On average MoYsis™ extracts generated PCR Ct values that were 1.13 cycles lower than those generated using the organic bead-based extracts (data not shown).

3.4. Quantitative Blood Culture Results

Because the dogs in this study received oxacillin therapy beginning at 4 h post intrabronchial inoculation and continuing up through 72 h, *S. aureus* was not isolated from any of the quantitative blood cultures from either infected dogs or control dogs (data not shown). In contrast, 23.4% (37/158) of the quantitative blood cultures from the infected dogs were positive for oxacillin-resistant non-*S. aureus* bacteria and consisted mainly of enteric Gram-negative rods (see Figure 3 footnote; data not shown). These positive cultures occurred mainly in bloods drawn at 48h, 72h and/or 96h. Figure 3 illustrates the increases seen in CFU/mL of these bacteria over time from those quantitative cultures which mirrored the increase in DNA concentrations seen over time in the MoYsis™ DNA extracts.

4. Discussion

Molecular testing of specimens for diagnosing infectious diseases is becoming more commonplace in hospitals replacing the slower, culture-based methods (Emmadi, et al., 2011). In blood culture applications, this approach provides a number of important advantages, the most significant of which, is a reduced time to result (Moore, McCann, & Jordan, 2013). Having blood culture results sooner would enable timelier tailoring of antibiotic therapy for improved patient survival and outcome (Gaieski, et al., 2010). The crucial step in any molecular diagnostic test, however, is the initial nucleic acid extraction from biologic samples.

Previous evaluations of the MoLYsis™ Complete5 DNA extraction kit show how effectively non-bacterial target DNA is removed from whole blood resulting in increased sensitivity of downstream molecular analysis using bacteria-spiked blood samples (Hansen, Bruggeman, & Wolffs, 2009; Loonen, Jansz, Kreeftenberg, Bruggeman, Wolffs, & van den Brule, 2010; Horz, Scheer, Huenger, Vienna, & Conrads, 2008). In this study, we evaluated the efficacy of the MoLYsis™ extraction kit for removal of host cell DNA and effect on downstream molecular analysis compared to our laboratory-developed organic, bead-based extraction method using whole blood samples from a *S. aureus*-infected canine model of sepsis. MoLYsis™ generated extracts had significantly lower overall DNA concentrations compared to the organic, bead-based method, effectively removing nearly all genomic DNA with only 1.9% of DNA extracts having amplifiable endogenous *p16* gene target. In contrast, 98.7% of DNA extracts generated using the organic bead-based method were positive for the *p16* gene target by PCR; a method in which the resulting DNA represented to a great extent, mammalian genomic DNA released from lysed WBC, rather than bacterial DNA, if present. WBC counts and quantitative blood culture results supported this idea as changes in DNA concentrations yielded over time by the organic bead-based extraction method closely mimicked the change in total WBC counts over time (Figure 2), while changes in DNA concentrations over time from MoLYsis™-generated DNA extracts closely simulated changes in CFU/ml of the non-*S. aureus* bacteria isolated from positive quantitative blood cultures (Figure 3). We believe that the organisms isolated from quantitative blood cultures may represent environmental contamination associated with blood sampling from the established cephalic vein IV line that was used consistently over the 4-day experiment to withdraw blood samples and provide various drugs.

Using the MoLYsis™ method, where DNA extracts were first depleted of canine genomic DNA prior to enriching for intact bacteria, resulted in higher rates of detection of *S. aureus* DNA compared to the organic bead-based methodology (Table 2). The MoLYsis™ extracts also resulted in pyrograms with slightly higher peak heights on average, than those generated using organic bead-based extracts (Figure 4). However, there were no instances in this investigation where lower peak heights resulted in ambiguous interpretation of the DNA sequence.

The greatest percentage of DNA extracts from whole blood found to be positive for *S. aureus* by PCR/pyrosequencing occurred at 48 h for both the MoLYsis™ (87.0%) and organic bead-based (82.6%) extraction methods. After that time, the percentages of extracts positive for *S. aureus* DNA decreased but were still measurable in smaller percentages of samples even up to 96 h post-infection. Molecular detection of bacterial DNA in culture-negative samples is an important observation when considering the implementation of a molecular approach for diagnosing BSI. While standard of care dictates that blood drawn from patients being evaluated for BSI should be collected before antibiotic(s) are administered, this is not always the case in practice. Consequently, knowing how long non-replicating, yet intact organisms remain in the bloodstream and are able to be detected by a molecular approach after initiating antibiotic therapy is important information to begin to collect for the purposes of interpreting molecular-based results for diagnosing bloodstream infections.

These data illustrate the effectiveness of the MoLYsis™ extraction kit in removing genomic DNA from WBC contained in whole blood samples, which if present could otherwise reduce the sensitivity of the molecular assay by interfering or competing with primers binding to their specific DNA targets during PCR, while still successfully capturing DNA from intact bacterial cells. This observation about genomic DNA interfering with bacterial DNA amplification was described in a previous study where we saw the negative impact that elevated WBC counts (>35,000/μl) has on the sensitivity of a real-time PCR when

attempting to detect bacterial DNA directly from blood of neonates being evaluated for sepsis using the organic bead-based extraction method (Jordan & Durso, 2005). This current study was performed using a well-characterized canine model, which simulated the sepsis syndrome encountered in humans, and provided a tightly controlled, reproducible clinical environment for *in vivo* testing of the efficacy of molecular diagnosis and comparison of whole blood DNA extraction methods. It was important to utilize such a physiologically relevant model of infection as opposed to the more traditional spiked blood model because it allowed for both the presence of an infection as well as a genuine immune response to the organism.

The study was limited by the fact that the dogs received antibiotic therapy during this study beginning at 4 h post *S. aureus* inoculation, with the last dose being given at 72 h. Our study comparing DNA extraction methods in a canine sepsis model was not the primary aim of these experiments, so we did not have the liberty to modify or eliminate the use of antibiotics for these purposes. Drawing blood for culture after antibiotics are administered is counter to recommended clinical practice, however, in this case antibiotic therapy was essential to the goals of the overall study for which this model was originally intended. So although we could compare the two extraction methods using a molecular approach, we were unable to compare the molecular results with quantitative blood cultures for *S. aureus*. It is important to note that while no *S. aureus* grew out in culture from either the control or infected dogs, *S. aureus* DNA was detected by PCR and pyrosequencing in a percentage of dogs even out to 96 h post-infection. These results reinforce the fact that bacterial viability is not a requirement for molecular-based detection. However, based on the MolYsis™ test principle, the DNA extracted represented intact bacteria, and not free DNA from previously lysed cells circulating in blood samples, as free DNA from lysed cells would have been degraded with the addition of the first chaotropic buffer in the extraction procedure.

This study supports previous findings that the DNA extraction method utilized is critical, playing a major role in the quality of further molecular analysis (Regan, Furtado, Brevnov, & Jordan, 2012). In the case of molecular diagnosis of bacterial BSI using PCR and pyrosequencing, removal of mammalian genomic DNA from WBCs, which is present at much higher levels than bacterial DNA in whole blood, improved our ability to detect *S. aureus* DNA target in those samples. The MolYsis™ Complete5 DNA extraction kit proved to be an effective method for isolating and purifying bacterial DNA from whole blood samples in this canine sepsis model. Currently, the MolYsis™ extraction method is being evaluated for use with whole blood clinical samples collected from human subjects suspected of BSI using this same PCR/pyrosequencing method of molecular identification.

Acknowledgments

We would like to thank Jing Feng, Al Hilton and Melinda Fernandez for their technical expertise and support of the canine sepsis model. Dr. Jordan received funding for this research from the National Institutes of Health under grant 1-R01-AI073342.

References

- Al-Soud WA, Radstrom P. Purification and characterization of PCR-inhibitory components in blood cells. *J Clin Microbiol.* 2001; 39:485–493. [PubMed: 11158094]
- Blot SI, Depuydt P, Annemans L, Benoit D, Hoste E, DeWaele JJ, et al. Clinical and Economic Outcomes in Critically Ill Patients with Nosocomial Catheter-Related Bloodstream Infections. *Clinical Infectious Diseases.* 2005; 41(11):1591–1598. [PubMed: 16267731]
- Brozanski BS, Jones JG, Krohn MJ, Jordan JA. Use of polymerase chain reaction as a diagnostic tool for neonatal sepsis can result in a decrease in use of antibiotics and total neonatal intensive care unit length of stay. *Journal of Perinatology.* 2006; 26:688–692. [PubMed: 17024143]

- Chan KY, Lam HS, Cheung HM, Chan AC, Li K, Fok TF, et al. Rapid Identification and Differentiation of Gram-Negative and Gram-Positive Bacterial Infections by Quantitative Polymerase Chain Reaction in Preterm Infants. *Critical Care Medicine*. 2009; 37(8):2441–2447. [PubMed: 19531943]
- Chaubert P, Baur Chaubert AS, Sattler U, Forster U, Bornand V, Suter M, et al. Improved polymerase chain reaction-based method to detect early-stage epitheliotropic T-cell lymphoma (mycosis fungoides) in formalin-fixed, paraffin-embedded skin biopsy specimens of the dog. *J Vet Diag Invest*. 2010; 2(1):20–29.
- Edmond MB, Wallace SE, McClish DK, Pfaller MA, Jones RN, Wenzel RP. Nosocomial Bloodstream Infections in United States Hospitals: A Three-Year Analysis. *Clinical Infectious Diseases*. 1999; 29(2):239–244. [PubMed: 10476719]
- Emmadi R, Boonyaratanakornkit JB, Selvarangan R, Shyamala V, Zimmer BL, Williams L, et al. Molecular methods and platforms for infectious diseases testing a review of FDA-approved and cleared assays. *J Mol Diagn*. 2011; 13(6):583–604. [PubMed: 21871973]
- Fredricks D, Relman D. Improved Amplification of Microbial DNA from Blood Cultures by Removal of the PCR Inhibitor Sodium Polyanetholesulfonate. *Journal of Clinical Microbiology*. 1998:2810–2816. [PubMed: 9738025]
- Gaieski DF, Mikkelsen ME, Band RA, Pines JM, Massone R, Furia FF, et al. Impact of Time to Antibiotics on Survival in Patients with Severe Sepsis or Septic Shock in Whom Early Goal-Directed Therapy was Initiated in the Emergency Department. *Critical Care Medicine*. 2010; 38(4):1045–1053. [PubMed: 20048677]
- Garnacho-Montero J, Ortiz-Leyba C, Herrera-Melero I, Aldabó-Pallás T, Cayuela-Dominguez A, Marquez-Vacaro JA, et al. Mortality and morbidity attributable to inadequate empirical antimicrobial therapy in patients admitted to the ICU with sepsis: a matched cohort study. *J Antimicrob Chemother*. 2008; 61(2):436–441. [PubMed: 18056733]
- Hansen WL, Bruggeman CA, Wolffs PF. Evaluation of new preanalysis sample treatment tools and DNA isolation protocols to improve bacterial pathogen detection in whole blood. *J Clin Microbiol*. 2009; 47:2629–2631. [PubMed: 19535529]
- Horz H, Scheer S, Huenger F, Vienna ME, Conrads G. Selective Isolation of Bacterial DNA from Human Clinical Specimens. *Journal of Microbiological Methods*. 2008; 72(1):98–102. [PubMed: 18053601]
- Hoyert DL, Xu J. Deaths: Preliminary Data for 2011. *National Vital Statistics Report*. 2012; 61(6)
- Huttunen R, Aittoniemi J. New Concepts in the Pathogenesis, Diagnosis and Treatment of Bacteremia and Sepsis. *Journal of Infection*. 2011; 63(6):407–419. [PubMed: 21840338]
- Ibrahim EH, Sherman G, Ward S, Fraser VJ, Kollef MH. The influence of inadequate antimicrobial treatment of bloodstream infections on patient outcomes in the ICU setting. *Chest*. 2000; 118(1):146–155. [PubMed: 10893372]
- Jordan JA. Molecular Diagnosis of Neonatal Sepsis. *Clinics in Perinatology*. 2010; 37(2):411–419. [PubMed: 20569815]
- Jordan JA, Durso MB. Real-time Polymerase Chain Reaction for Detecting Bacterial DNA Directly from Blood of Neonates Being Evaluated for Sepsis. *The Journal of Molecular Diagnostics*. 2005; 7(5):575–581. [PubMed: 16258155]
- Jordan JA, Durso MB, Butchko A, Jones JG, Brozanski BS. Evaluating the Near-Term Infant for Early Onset Sepsis: Progress and Challenges to Consider 16S rDNA Polymerase Chain Reaction Testing. *Journal of Molecular Diagnostics*. 2006; 8(3):357–363. [PubMed: 16825509]
- Jordan JA, Jones-Laughner J, Durso M. Utility of Pyrosequencing in Identifying Bacteria Directly from Positive Blood Culture Bottles. *J Clin Microbiol*. 2009; 47(2):368–72. [PubMed: 19091813]
- Kollef MH, Zilberberg MD, Shorr AF, Vo L, Schein J, Micek ST, et al. Epidemiology, microbiology and outcomes of healthcare-associated and community-acquired bacteremia: A multicenter cohort study. *J Infect*. 2011; 62:130–5. [PubMed: 21195110]
- Kumar A, Roberts D, Wood KE, Light B, Parillo JE, Sharma S, et al. Duration of Hypotension Before Initiation of Effective Antimicrobial Therapy is the Critical Determinant of Survival in Human Septic Shock. *Critical Care Medicine*. 2006; 34(6):1589–1596. [PubMed: 16625125]

- Loonen AJ, Jansz AR, Kreeftenberg H, Bruggeman CA, Wolffs PF, van den Brule AJ. Acceleration of the direct identification of *Staphylococcus aureus* versus coagulase-negative staphylococci from blood culture material: a comparison of six bacterial DNA extraction methods. *Eur J Clin Microbiol Infect Dis*. 2010; 30:337–342. [PubMed: 20972809]
- Martin GS, Minnino DM, Eaton S, Moss M. The Epidemiology of Sepsis in the United States from 1979 through 2002. *New England Journal of Medicine*. 2003; 384(16):1546–1554. [PubMed: 12700374]
- McLaughlin JC, Hamilton P, Scholes JV, Bartlett RC. Clinical laboratory comparison of lysis-centrifugation and BACTEC radiometric blood culture techniques. *Journal of Clinical Microbiology*. 1983; 18(5):1027–1031. [PubMed: 6358246]
- Minnecci PC, Deans KJ, Hansen B, Parent C, Romines C, Gonzales DA, et al. A Canine Model of Septic Shock: Balancing Animal Welfare and Scientific Relevance. *American Journal of Physiology Heart and Circulatory Physiology*. 2007; 293:H2487–H2500. [PubMed: 17644570]
- Moore MS, McCann CD, Jordan JA. Molecular-based Detection of Culture-confirmed Bacterial Bloodstream Infections Using Limited Enrichment Time. *J Clin Microbiol*. 2013; 51(11):3720–5. [PubMed: 23985915]
- Murray PR, Masur H. Current approaches to the diagnosis of bacterial and fungal bloodstream infections in the intensive care unit. *Crit Care Med*. 2012; 40(12):3277–3282. [PubMed: 23034460]
- Pittet D, Li N, Woolson RF, Wenzel RP. Microbiological Factors Influencing the Outcome of Nosocomial Bloodstream Infections: 6-Year Validated, Population-Based Model. *Clinical Infectious Diseases*. 1997; 24(6):1068–1078. [PubMed: 9195059]
- Regan JF, Furtado MR, Brevnov MG, Jordan JA. A Sample Extraction Method for Faster, More Sensitive PCR-Based Detection of Pathogens in Blood Culture. *The Journal of Molecular Diagnostics*. 2012; 14(2):120–129. [PubMed: 22245225]
- Rodrigues T, Roque F, Falcão A, Figueiras A, Herdeiro M. Understanding physician antibiotic prescribing behaviour: a systematic review of qualitative studies. *International Journal of Antimicrobial Agents*. 2012
- Srinivasan L, Harris MC. New Technologies for the Rapid Diagnosis of Neonatal Sepsis. *Current Opinion in Pediatrics*. 2012; 24(2):165–171. 279–280. [PubMed: 22273634]
- van de Sande-Bruinsma N, Grundmann H, Verloo D, Tiemersma E, Monen J, Goossens H, et al. Antimicrobial Drug Use and Resistance in Europe. *Emerging Infectious Diseases*. 2008; 14(11):1722–1730. [PubMed: 18976555]
- Wilson IG. Inhibition and facilitation of nucleic acid amplification. *Appl Environ Microbiol*. 1997; 63:3741–3751. [PubMed: 9327537]

Highlights

- Selective isolation of bacteria from whole blood resulted in relatively pure DNA
- Selective isolation of bacteria resulted in a higher rate of *S. aureus* detection
- [DNA] values after selective isolation mirrored quantitative blood culture results

Figure 1.

Box plot comparing the 260/280 nm absorbance ratios of the resulting DNA extracts generated from the 118 samples drawn using the two extraction methods as determined by spectrophotometry. Each plot reveals the median (midline), inter-quartile range (IQR; box), 10th and 90th percentiles (error bars), and minimum/maximum outliers (Δ = min, + = max). Outliers were defined as any values greater or less than 1.5 times the IQR. An absorbance ratio of 1.8 was used as the standard for pure DNA (dashed line).

Figure 2.

Comparison of average DNA concentrations of the DNA extracts generated from the 118 samples using the two extraction methods measured at five time point intervals along with WBC counts. The line graph represents average white blood cell (WBC) counts x 1000 per μl of blood measured over the same 96 h period. Error bars represent standard error.

Significant differences in DNA concentrations were observed at all time points between extraction methods ($P < 0.05$). Differences in sample size (n) throughout the study period reflect death of animals due to pulmonary complications caused by the bacterial infection.

Figure 3.

Comparison of average DNA concentrations of the DNA extracts generated from the 118 samples using the two extraction methods measured at five time points against the number of colony-forming units of non-*S. aureus* bacteria per milliliter (CFU/ml) of whole blood as determined by the quantitative blood culture method. Non-*S. aureus* organisms isolated by blood culture included: *E. coli*, *K. pneumonia*, *P. fluorescens*, *P. aeruginosa*, *E. cloacae*, *S. maltophilia*, *C. xerosis*, and *A. baumannii*.

Figure 4.

Example of pyrograms generated from *Staphylococcus spp.*-specific *16S rRNA* gene target amplicons comparing the two manual DNA extraction methods from the same blood sample. The resulting 23 base pair sequence predictive of *S. aureus* is: 5'-ACA TCA GAG AAG CAA GCT TCT CG-3' (Jordan, Durso, Butchko, Jones, & Brozanski, 2006).

Table 1

PCR and Pyrosequencing primer sequences

Target	Gene	PCR Primers (5'-3')	Pyrosequencing Primers (5'-3')
<i>Staphylococcus</i>	16S rRNA	F _B , TGC CTA ATA CAT GCA AGT CGA GCG R, GTT GCC TTG GTA AGC CGT TAC CTT	S, GTG TTA CTC ACC CGT CCG CCG CTA
Enteric GNR	23S rRNA	F ₁ , CTA AGG CGA GGC CGA AAG F ₂ , CTA AGG CGA GGC TGA AAA G R _B , CTA CCT GAC CAC CTG TGT CG	S ₁ , GGT TGT CCC GGT TTA S ₂ , GGT CGT CCC GGT TCA
dcpl6-ex2	<i>p16</i>	F, CAG GTC ATG ATG ATG GGC AGC G R, AGC ACC ACC AGC GTG TCC AGG	N/A

GNR: Gram-negative rods, F_x: Forward, R_x: Reverse, S_x: Sequence, X_B: Biotinylated, N/A: Not applicable.

16S rRNA gene, base pair position 46–288 in reference strain *S. aureus* accession number X70648 (Jordan et al, 2006)

23S rRNA gene, base pair position 1346–1625 in reference strain *E. coli* accession number AJ278710 (Jordan et al., 2009)

Table 2

Detection of *S. aureus* DNA by PCR/pyrosequencing from two DNA extraction methods

Extraction Method	0 h		24 h		48 h		72 h		96 h		Total Detected	
	n/total	%	n/total	%	n/total	%	n/total	%	n/total	%	n/total*	%
MolYsis™	0/36	0.0	25/31	80.6	20/23	87.0	9/14	64.3	4/14	28.6	58/82	70.7
Bead	0/36	0.0	20/31	64.5	19/23	82.6	6/14	42.9	4/14	28.6	49/82	59.8

* The n/total fraction represents the sum of the 24 h, 48 h, 72 h and 96 h data, and excludes the data from samples drawn at 0 h.