DETECTION OF MICROBES AND MICROBIAL INFECTION

Daniel Zweitzig

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by

Daniel Robert Zweitzig

Born on February 27th, 1978

In Abington, Pennsylvania, U.S.A.

This dissertation has been approved by:

Supervisor:

Prof. L.W.M.M. Terstappen, MD, PhD

Members of the committee:

Chairman/Secretary:

Prof. J.L. Herek, PhD

University of Twente

Supervisor:

Prof. L.W.M.M. Terstappen, MD, PhD

University of Twente

Members:

Prof. A. Kocer, PhD

University of Twente

Prof. P. Jonkheijm, PhD

University of Twente

Prof. L. Visser, MD, PhD

Leiden University

Prof. E. J. Kuijper, MD, PhD

Leiden University

Prof. M. Koopmans, DVM, PhD

Erasmus University

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Thesis motivation

Microorganisms are present in overwhelming numbers within the world around us, as well as on and inside our bodies. They play key roles within the environment, and our global ecosystem would fall apart without them. Similarly, commensal microorganisms are an important part of human physiology and are beneficial to us in a variety of ways. However, our microscopic co-inhabitants can also exhibit 'Jekyll and Hyde'-type behavior. For example, when a human host becomes immunocompromised, otherwise harmless microorganisms can quickly turn into deadly attackers. Other microorganisms are 'natural born killers' and are pathogenic even to a healthy host after being ingested, transferred from person to person, or introduced via contaminated pharmaceuticals and/or blood products. In fact, infectious disease is a leading cause of mortality worldwide, resulting millions of deaths annually and significant healthcare costs. One of the first lines of defense against microorganism-related mortality is a timely and accurate diagnosis for infected patients. Consequently, there is a constant need for new infectious disease-related diagnostic methods, as well as sensitive techniques for detecting contaminated biologics and/or pharmaceuticals. A goal of this thesis was to develop novel methodology for improved detection of microorganisms, then demonstrate its utility for various clinically relevant applications. Another goal was to demonstrate the feasibility of a microsieve-based immunoassay device for detecting pathogen-induced proteins from human blood products.

Thesis contents

Chapter one provides an overview of the various methodologies currently used to directly and indirectly detect infectious disease. The second chapter describes the development and characterization of novel methodology that links the conserved biochemical activity of microbial DNA polymerase to quantitative PCR. This methodology was termed enzymatic template generation and amplification (ETGA). In chapter three, a model system is presented in which the ETGA methodology is combined with a selective cell lysis procedure to enable detection of microbes from spiked blood culture samples (termed the ETGA Blood Culture Assay). These studies provided the basis for conducting a clinical study aimed at verifying the utility of this approach. Chapter four details a clinical study wherein the ETGA Blood Culture Assay was used to detect the presence of hematopathogens within blood culture samples derived from patients suspected of having a bloodstream infection. During this study, the time to detection of microorganisms was monitored for the ETGA approach relative to traditional blood culture instruments. In chapter five, the ETGA Blood Culture Assay was modified to enable sensitive detection of various clinically relevant bacteria from platelet concentrates, with the goal of rapid and universal contamination testing prior to transfusion. In addition to directly measuring the presence of a pathogenic agent, diagnosis of an infection can also be made by detecting microorganism-induced biomolecules from blood. Consequently, in chapter six the focus of the thesis shifts from ETGA-based detection of microbes, to demonstrating the feasibility of using microsieves as a simple and rapid immunoassay platform capable of detecting several different infectious disease-induced antibodies from human serum. In chapter seven, the microsieve-based immunoassay platform was adapted for detecting a sepsis-induced biomarker, termed sPLA2-IIA, from human plasma. Chapter eight describes experiments aimed at enabling microsieve-based detection of sPLA2-IIA from whole blood derived from a finger prick, towards generating proof of concept for adaptation onto a point-of-care device.

Chapter 1

Overview of Infectious Disease Detection Methods

Daniel Zweitzig and Leon Terstappen

1.1 Introduction

The human body is a complex microbiological ecosystem containing regions of highly concentrated bacteria and fungi, in close proximity to sterile liquids (1-4). For example, the human mouth is a rich source of bacteria, with millions of them per milliliter of saliva (5). The large intestine is also teeming with microbes, as there are an estimated 10^{11} bacteria per gram of stool (6). Skin, nostrils, and urogenital regions are also laden with microorganisms, and the diversity of species present at each anatomical region can vary significantly among individuals (1,7,8) (Figure 1).

Figure 1 – Diversity of the microorganisms present at various anatomical regions
Adapted from Lloyd-Price, et.al (1)

In stark contrast, our bodies are also home to several normally-sterile liquids; such as blood, cerebrospinal fluid, pleural fluid, and pericardial fluid (2-4). When microorganisms are properly segregated from sterile fluids, and maintained at the appropriate relative proportions within non-sterile sites, humans can co-exist with their prokaryotic counterparts in a healthy and even symbiotic manner (9,10). For example, the intestinal microbiome plays a crucial role in digestion, as well as other physiological processes such as immune system function (11-13) (Figure 2).

Figure 2 – The microbiome plays an important role in many normal physiological processes
Adapted from Lloyd-Price, et.al (1)

However, this is a fragile balance since pathophysiological conditions can arise when sterile bodily fluids are exposed to opportunistic endogenous microbes, or if the amount of certain microbial species becomes deregulated within non-sterile sites (14). For example, if a localized infection enters the bloodstream and metastasizes to other organs, a deadly pathophysiological condition termed sepsis can rapidly occur (15) (Figure 3).

Figure 3 – Schematic of how bacterial infections can induce sepsis
(<https://newsnetwork.mayoclinic.org/discussion/mayo-clinic-q-and-a-understanding-sepsis-and-septic-shock/>)

In addition to this inherent risk, our bodies can also be exposed to non-resident and potentially pathogenic microbial species via various mechanisms including, but not limited to; interaction with other humans, transfusion of blood products, injection of pharmaceuticals, and ingestion of contaminated food or water (17-20). Considering the above, it should not be surprising that detection of microbes from numerous biological (e.g. blood, wounds, cerebrospinal fluid, etc...) and non-biological sources (e.g. pharmaceuticals, water/food sources, etc...) is an important part of public health and safety (21-23).

Over the past few centuries, two main approaches have emerged for detecting infection of humans/animals, as well as contamination in biologics/pharmaceuticals, food/beverages, or environmental samples. The first approach depends upon direct detection or visualization of the target microorganism via various culture-based and culture-independent methodologies, as well as combinations thereof. The second and more indirect approach relies on measurement of biomolecules that are produced or activated by a host organism in response to an infectious agent. Below, an overview is provided for several methodologies currently used to directly detect the presence of microbes from clinical samples, and will also briefly review indirect detection of infections via serologic approaches.

1.2 Direct Detection of Microbes

1.2.1 Microscopy

The microscope has been a valuable scientific tool for hundreds of years and its early usage set the stage (no pun intended) for modern day clinical diagnostic microbiology (24-26). In fact, it can be argued that microscopy and diagnostic microbiology share a common origin, in the person of Antonie van Leeuwenhoek (24,27, 28). While working as a janitor in the Netherlands, van Leeuwenhoek's hobby of lens grinding evolved into the development of rudimentary microscopes that enabled the first visualization of protozoa in 1675, then bacteria in 1683 (24,27,28) (Figure 4).

Figure 4 – Image of Antonie van Leeuwenhoek and his early microscope
<https://scientistsinformation.blogspot.com/2015/05/antonie-van-leeuwenhoek-24th.html>

By the end of the nineteenth century, notable scientists such as Weigert, Koch, and Erlich expanded upon van Leeuwenhoek's work by developing microbial staining techniques, which enhanced the ability to visualize disease-causing agents under the light microscope (27). An important differential staining technique, termed Gram staining, was developed by the Danish physician Hans Christian Gram in the late 1800s, and is still widely utilized during the early phase of diagnostic algorithms within the clinical microbiology laboratory (27). The Gram staining technique enables differentiation of two major classes of bacteria, 'Gram-negative' and 'Gram positive', based upon how dyes interact with the respective bacterial cell wall components (Figure 5).

Figure 5 – Schematic overview of a generalized Gram staining procedure
<https://laboratoryinfo.com/gram-staining-principle-procedure-interpretation-and-animation/>

Over the past century, more sophisticated microscopy techniques and instrumentation have been developed for clinical diagnostic purposes (24-28). One such technique, termed fluorescent microscopy, can be used to directly detect microbes that are not easily stained and visualized under the light microscope (26). In this approach, samples are incubated with microorganism-specific antibodies that have been previously conjugated to a fluorophore (Figure 6A), then imaged using a fluorescent microscope (29, 30) (Figure 6B).

Figure 6A - Simplistic overview of a direct fluorescent antibody staining assay (29)

Figure 6B - Example images of *Legionella* bacteria obtained from fluorescent antibody staining and subsequent microscopic analysis (30)

Electron microscopy represents an even more specialized technology, that enables direct visualization of smaller infectious disease-causing agents that less powerful microscopes are unable to detect. The most common application of this approach within the clinical diagnostic setting is for direct imaging of viruses from specimens such as stool or urine (31).

Despite being considered the diagnostic gold standard for certain infections (e.g. malaria and giardiasis), microscopy is associated with some inherent drawbacks that limit its usage to mostly an adjunct diagnostic tool. Those drawbacks include labor intensive sample preparation procedures, requirement for highly skilled scientists to analyze the slides, and lack of sensitivity for detecting the presence of microbes from non-cultured samples. However, innovative solutions such as the QBC capillary tube-based system have been developed, which significantly reduce the sample processing time and complexity; thus, making microscopy-based detection of microorganisms (e.g. malaria) more user-friendly (32).

1.2.2 Culture

Microbiology pioneers such as van Leeuwenhoek, Pasteur, and Koch, were some of the first to demonstrate that small numbers of microbes can increase significantly if provided with the proper environmental conditions and nutritional requirements (27). One of the earliest microbial growth medias developed by Louis Pasteur was based upon a filtered extract of boiled yeast, sugar, and chalk to neutralize the pH (27). Over time, the development of specialized media formulations (e.g. selective, chromogenic, etc.) have enabled differentiation of a microorganism's genus/species via growth limitations and/or biochemical activity (32,33) (Figure 7).

Figure 7 – Example images of specialized culture media used to differentiate microbes (33)

The use of specialized media, in combination with additional biochemical testing, is still common within modern day clinical microbiology laboratories, and provide diagnoses ranging from the presumptive type to the gold standard type (32,33). However, culture-based diagnostic methodologies are often hindered by lengthy incubation requirements (34). Also, certain fastidious microorganisms are unable to propagate on solid medium and/or within liquid culture; potentially prohibiting their detection (34, 35). For example, *Treponema pallidum* (the spirochete that causes syphilis) has never been successfully cultivated *in vitro*; thus, requiring development of alternative detection approaches (32).

Blood culture is also a commonly utilized methodology within diagnostic algorithms for detecting patients at risk of developing sepsis. In this technique, blood is drawn into multiple bottles containing growth media optimized for propagation of either aerobic or anaerobic bacteria, as well as fungi (36-38) (Figure 8).

Figure 8 – Example of blood culture collection

<https://www.bd.com/en-us/offering/capabilities/microbiology-solutions/blood-culture/blood-culture-collection>

Despite its widespread adoption, the use of blood culture for this purpose suffers from several limitations. For example, some studies have demonstrated that blood culture is insensitive for detecting certain fastidious organisms, and those that are detected can require anywhere from 1 to 5 days of incubation (36-38). These lengthy time requirements can prove fatal for some patients, as bloodstream infections can progress to the deadly condition of septic shock over the course of hours, not days (36-38).

Given the limitations of culture-based methods, numerous culture-independent microbial detection methodologies have emerged over the past several decades (39); some of which are discussed below.

1.2.3 Immunocapture

Directly detecting the presence of microorganisms is also made possible by immunocapture methodologies. Immunocapture assays come in a variety of formats and offer a relatively rapid option for detecting pathogens within the clinical microbiology setting (40). While the exact assay formats may vary (e.g. ELISA, lateral flow, CLIA, etc.), immunocapture assays generally

utilize two different antibodies that are specific to biomolecules present on or within the targeted microbiological agent (41). Typically, a capture antibody is immobilized to a solid support, and the other antibody is coupled to a reporter molecule for detecting assay signal. One example of an immunocapture assay widely utilized in the clinical setting is the lateral flow-based group A *Streptococcal* antigen test. This approach is routinely used in doctor's offices, and a positive result obtained from a throat swab sample provides sufficient clinical information to prescribe proper antibiotics, without the need for culture. There are many other immunocapture assays, which include, but are not limited to, those utilized for rapid detection of *Cryptococcus species*, *Streptococcus pneumoniae*, Ebola virus, and *Legionella pneumophila* (40-42) (Figure 9).

Figure 9 – Schematic of *Cryptococcus* antigen capture assay (42)

Despite the relatively rapid turnaround time, and ability to obviate the need for culture, immunocapture methodologies generally exhibit poor sensitivity from non-cultured samples, which may cause negative results to be considered clinically unhelpful. Consequently, more sensitive methodologies for rapid and direct detection of infectious microorganisms have been developed.

1.2.4 Nucleic Acid Detection

Over the past few decades, the drawbacks associated with the above-mentioned microorganism detection approaches, combined with the expansion of nucleic acid sequence availability, have sparked the development of numerous rapid, sensitive, and specific molecular diagnostic (MDx) assays (43). In most cases, MDx assays rely upon detection of microorganism-specific RNA or DNA via enzymatic amplification approaches (e.g. PCR), hybridization of labeled nucleic acid probes to pre-cultured samples, and various combinations thereof.

Some of the earliest MDx approaches relied upon hybridization of radiolabeled DNA probes to pre-cultured clinical samples. In fact, the first such assay to gain FDA approval in 1986 utilized a radiolabeled DNA probe complementary to a specific *Legionella* rRNA sequence (44, 45). Since then, more practical non-radioactive hybridization approaches have been developed, which utilize nucleic acid probes conjugated to fluorophores for direct detection of microorganisms from pre-cultured clinical samples. For example, commercially available Peptide Nucleic Acid Fluorescence in Situ Hybridization (PNA FISH™) assays are useful for confirming the presence of hematopathogens such as Gram-positive and Gram-negative bacteria, as well as yeast from positive blood cultures (46-48) (Figure 10).

Figure 10 – Example of PNA FISH staining of different bacteria, as well as representative fluorescent microscopy images of each (48)

Although hybridization approaches are quite specific and can be readily designed to identify a wide variety of microorganisms from complex mixtures, they generally lack the analytical sensitivity required for use with non-cultured clinical samples.

In 1992, the first FDA-cleared diagnostic Polymerase Chain Reaction (PCR) assay for detection of *Chlamydia trachomatis*, termed the Amplicor CT™, marked the beginning of a revolution within the infectious disease diagnostic testing arena (44). By combining high levels of specificity associated with DNA primer hybridization, exquisite sensitivity of exponential amplification, and rapid turnaround time; PCR-based diagnostic assays often represent an ideal solution to the shortcomings associated with culture and immunocapture-based microbiological detection methodologies. Early PCR diagnostic tests, such as the Amplicor CT mentioned above, required detection of amplification products via a separate hybridization-based method such as ELISA, or gel analysis. However, the advent of quantitative PCR (qPCR) assays that monitor amplification in real time via fluorescent nucleic acid binding dyes or intra-amplicon hybridization probes, collectively represent a huge leap forward in diagnostic utility. Early generation qPCR-based MDx assays, commonly referred to as monoplex tests, effectively targeted nucleic acid sequences of individual bacterial, viral, or fungal pathogens. The GeneOhm™ test systems are an example of FDA-cleared monoplex MDx assays that were once utilized (44). In the procedures associated with the GeneOhm assays, bacteria such as *C. difficile* and *S. aureus* (including MRSA) are captured via swabbing, eluted into a buffer, and transferred into tubes containing glass beads. The captured bacteria are lysed via mechanical disruption, then transferred again into thermocycling tubes already containing microbe-specific PCR reagents (49,50). Since manual sample preparation procedures such as those used for the GeneOhm assays are becoming less desirable within modern clinical diagnostic laboratories, more user-friendly and automated MDx assays have been developed, such as the Xpert™ test system (44). In this approach, bacterial or viral pathogens can also be collected via swabbing, then inserted into a cassette where the subsequent elution, lysis, thermocycling, and detection steps are rapidly performed in an automated fashion (44,51) (Figure 11).

Figure 11 – Example images of the Cepheid Xpert detection system and assay cartridges (51)

Despite being highly effective for certain focused applications (e.g. tuberculosis detection, MRSA detection, Group B Streptococcus, etc.), MDx assays that are only able to detect a few targets are not ideal for diagnosing syndromic infections where multiple causative pathogens are suspected; such as the respiratory tract infections, meningitis, and bloodstream infections. This need has sparked the development of numerous MDx platforms that are able to detect numerous pathogens within a single test; collectively termed multiplex assays (39). Multiplex MDx assays come in a variety of formats and associated platforms. For example, the Seegene™ test system can screen for the presence of over 90 different sepsis-causing microorganisms, then subsequently identify 27 of them (52). This assay outcome is achieved by utilizing multiple primer sets during an initial PCR-based screen to determine if Gram positive bacteria, Gram negative bacteria, or fungi are present. Another multiplex test system, termed Septifast™, also utilizes multiple primer sets to detect the presence of the same three major groups of microorganisms, however subsequent organism identification is made via melting curve analyses after thermocycling (53). Despite being able to utilize whole blood as a sample matrix,

both the Seegene and Septifast assays require separate instrumentation to perform processes such as nucleic acid isolation, thermocycling, and organism identification. Furthermore, complex test systems such as these can be costly, require highly skilled personnel, exhibit reduced sensitivity in exchange for universality, and can take anywhere from 3-8 hours to obtain results; collectively limiting adoption by clinical laboratories. Consequently, more rapid, cost effective, and user-friendly multiplex MDx assays and platforms are being developed, such as the FilmArray[®] system. In the FilmArray approach, patient samples are loaded directly into a ready-to-use assay cartridge. Once the sample has been loaded, microbe lysis, nucleic acid purification, multiplex PCR amplification, and organism identification all occur rapidly in a fully automated fashion within the cassette (54, 55) (Figure 12).

Figure 12 – Example images of the BioFire Film Array detection system and assay cartridges (55)

Additional fully automated molecular diagnostic systems such as the Roche Cobas, Hologic Panther, and BD Max are also commercially available. In general, these systems offer additional benefits such as random-access capabilities, fully integrated sample processing modules,

and/or greater sample throughput (44, 56). Several of the assays/platforms listed above, as well as a summary of their key features, have been summarized Table 1.

System	Ease of Sample Preparation	Biomarkers per Assay	Throughput
BD GeneOhm	+	+	+
SeeGene	+	++++	+
Roche Septifast	+	++++	+
BioFire FilmArray	++	++++	+
Luminex Aries	++	++++	+
GenPOC Revogene	+++	+++	+
Cepheid Xpert	+++	++	++
BD MAX	+++	++	++
Hologic Panther	+++	+	+++
Roche Cobas	+++	++	++++

Table 1 – List of selected MDx platforms and their relative capabilities

Development of more sophisticated MDx platforms is ongoing, and currently include various PCR-based approaches that are tethered to microorganism detection/identification technologies such as mass spectrometry and nucleic acid sequencing (44, 57-59).

1.3 Indirect Detection of Infections

1.3.1 Antibody Detection

In addition to directly detecting microbes, infectious disease-related diagnoses can also be made by measurement of antibodies produced during a host’s immune response to an infectious agent; commonly referred to as serology testing. Serology testing for infectious disease has been utilized for many decades and is common practice within clinical diagnostic laboratories (60, 61). In many cases, pathogen-specific antibodies of the IgM and/or IgG isotypes are measured together or in separate assays, depending upon the purpose of the testing. For example, assays intended for detecting acute infection will normally be designed for detecting pathogen-specific antibodies of the IgM isotype, whereas assays intended to detect infections that have progressed further into the seroconversion process will detect antibodies of the IgG isotype. However, some infectious disease serology assays intended for

broader screening of exposure to a pathogen are designed to simultaneously detect both IgM and IgG antibodies. Regardless of the exact format, serology assays generally rely upon capture of serum-derived antibodies by pathogen-specific antigens in the form of entire microorganisms, purified native proteins, recombinant proteins, or peptides (41, 60, 61).

As mentioned previously, the spirochete *Treponema pallidum* is unable to be cultured *in vitro*, thus serology assays are routinely used as an aid in diagnosing syphilis. Fluorescent Treponemal Antibody (FTA) tests used for diagnosing syphilis are an example of an old, but still utilized, serology assay format (62). In the FTA approach, intact *Treponema pallidum* bacteria (usually purified from infected rabbits) are immobilized and fixed onto a microscope slide, then incubated with a patient's serum. If anti-Treponemal antibodies are present in the serum, they will bind to the immobilized microorganism. After non-specific serum components are washed away, the anti-Treponemal antibodies are detected using an anti-human IgG or IgM antibody that has been previously conjugated to a fluorophore. Once the assay procedure has been completed, slides are imaged via fluorescent microscopy during which a qualitative assay result is determined manually by a trained operator. While useful, serology assays based upon fluorescent microscopy are sometimes undesirable due to subjectivity of result interpretation, laborious procedures, and limited throughput. In an effort to overcome such limitations, widely utilized methodologies such as enzyme linked immunosorbent assays (ELISA) have been developed, with early versions dating back to as early as the 1960s (63,64). Like other methodologies, diagnostic ELISAs come in a variety of formats; however, microbe-specific antigens are typically coated onto a 96-well plastic plate (a.k.a 'antigen-down format'), then incubated with patient samples during which serum-derived antibodies are captured onto the plate. After washing away non-specific serum components, an anti-human antibody conjugate is added, then washed again and detected by an appropriate instrument (Figure 13).

Figure 13 – Schematic diagram of a typical ‘antigen down’ ELISA approach (64)

Once data collection is complete, qualitative or quantitative assay results can be determined objectively using data reduction algorithms pre-programmed into software, thus overcoming the subjectivity associated with most microscopy-based serology tests. Over time, instrumentation has been developed to carry out all critical steps associated with ELISA procedures in an automated fashion; such as dilution/addition of patient samples, reagent dispensing/aspiration, temperature-controlled incubation, signal detection, and data reduction. Another automatable serology assay format that has grown in popularity over the past decade relies upon antigen coupling to magnetic beads, which are loaded along with reagents into an instrument that allows patient samples to be analyzed in a random-access manner. This technology typically generates and measures chemiluminescent signal, which can provide improved sensitivity and linear dynamic measuring ranges relative to approaches that employ colorimetric detection such as traditional ELISA (61, 65). A summary of some serology assay approaches, and their respective features, is presented in Table 2.

Assay Type	Ease of Use	Biomarkers per Assay	Throughput	Sensitivity
*Fluorescent Microscopy	+	+	++	+
Lateral Flow	++++	++	+	++
ELISA	++	+	+++	+++
Luminex Lx200	++	++++	+++	+++
**CLIA	++++	+	+++	++++
* Refers to techniques such as Fluorescent Treponemal Antibody detection and Indirect Fluorescent Antibody detection using antigens fixed onto a glass microscope slide				
** Refers to automated chemiluminescent immunoassay platforms				

Table 2 - Overview of selected serology assay formats and their relative capabilities

1.3.2 Detection of other biomolecules (non-antibody)

Besides pathogen-specific antibodies, the host's physiological response to an infection can also cause an increase in the serum concentration of various inflammatory biomolecules (66, 67). Measurement of these biomolecules can be effectively used as an adjunct to other assays or clinical symptoms within diagnostic algorithms (Figure 14).

Figure 14 – Example of ‘non-antibody’ biomarkers upregulated in response to infection (67)

Measurement of serum procalcitonin (PCT) levels is an example of a ‘non-antibody’ detection approach utilized to add in the diagnosis of infectious disease. Despite being produced by numerous cell types under normal conditions, serum levels of PCT have been shown to increase significantly in response to bacterial infections and/or other pathophysiological conditions (68, 69). In fact, the BRAHMS PCT assay has recently been cleared by the FDA for use in diagnosing sepsis-related infections (70). In addition to PCT, numerous other serum biomarkers have been demonstrated to be elevated in serum during inflammatory conditions such as sepsis, and

many of them are currently being evaluated for potential diagnostic and or prognostic utility (71).

1.4 Conclusion

In the fragile balance between microbes and man, there is ongoing need for development and improvement of infectious disease-related detection methodologies. In the following thesis, novel methodologies will be presented for both direct detection of microbes via a novel molecular approach, as well as detection of infection-induced biomolecules via a new immunoassay approach.

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Chapter 2

Characterization of a Novel DNA Polymerase Activity Assay Enabling Sensitive, Quantitative and Universal Detection of Viable Microbes

Daniel R. Zweitzig, Nichol M. Riccardello, Bruce I. Sodowich and
S. Mark O'Hara

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Abstract

During the past fifty years, *in vitro* measurement of DNA polymerase activity has become an essential molecular biology tool. Traditional methods used to measure DNA polymerase activity *in vitro* are undesirable due to the usage of radionucleotides. Fluorescence-based DNA polymerase assays have been developed; however, they also suffer from various limitations. Herein we present a rapid, highly sensitive and quantitative assay capable of measuring DNA polymerase extension activity from purified enzymes or directly from microbial lysates. When tested with purified DNA polymerase, the assay detected as little as 2×10^{-11} U of enzyme (≈ 50 molecules), while demonstrating excellent linearity ($R^2 = 0.992$). The assay was also able to detect endogenous DNA polymerase extension activity down to at least 10 colony forming units of input Gram-positive or Gram-negative bacteria when coupled to bead mill lysis while maintaining an $R^2 = 0.999$. Furthermore, preliminary evidence presented here suggests that DNA polymerase extension activity is an indicator of bacterial viability, as demonstrated by the reproducibly strong concordance between assay signal and bacterial colony formation. Together, the innovative methodology described here represents a significant advancement toward sensitive detection of potentially any microorganism containing active DNA polymerase within a given sample matrix.

2.1 Introduction

DNA polymerase activity is indispensable for genome replication and organism propagation across all biological domains (1-3). Since its initial characterization (4), the ability to harness DNA polymerase activity *in vitro* has become a fundamental tool in the field of molecular biology research (5). Above and beyond its established importance in research, *in vitro* measurement of DNA polymerase activity potentially offers numerous useful applications within the pharmaceutical and clinical setting. For instance, since bacterial DNA polymerase is actively being targeted for development of novel antimicrobial agents (6, 7), a rapid and sensitive assay capable of measuring DNA polymerase activity is desirable. Also, loss or gain of DNA polymerase activity is intimately involved in human disease. For example, emerging links between DNA polymerase activity and genetic aberrations are designating the enzyme as a target for anti-cancer therapies (8,9). Deficiencies in DNA polymerase activity have also been linked to mitochondrial disorders (10). Furthermore, measurement of DNA polymerase activity has the potential to be used as a rapid and sensitive diagnostic tool, capable of detecting virtually any organism harboring active DNA polymerase within a given environmental or biological matrix where sterility is expected.

The most common method used to measure DNA polymerase activity *in vitro* depends upon incorporation of radiolabeled nucleotides (11). However, routine use of such DNA polymerase assays is undesirable due to the inherent risks and restrictions associated with radioisotopes. Consequently, over the past few decades numerous non-radioactive *in vitro* polymerase assays have been developed. Some rely upon the measurement of fluorescence generated by DNA polymerase-mediated release of single stranded binding protein (12) or binding of PicoGreen™ to double stranded DNA (13,14). Other methods rely on microplate coupling and detection of fluorescently-labeled nucleotides (15). More recently, molecular beacon-based (16) and electrochemical-based (17) DNA polymerase assays have been developed. Despite successfully averting the use of radioactivity, the above assays are limited by either poor sensitivity, a small linear dynamic range of measurement or the use of purified polymerase.

Our lab has an ongoing interest in methodology involving enzymatic template generation and amplification (ETGA). Herein we describe the initial characterization of novel ETGA

methodology based upon the measurement of DNA polymerase extension activity coupled to a quantitative PCR readout. For the remainder of the manuscript, we will refer to this assay as DNA polymerase extension coupled polymerase chain reaction (DPE-PCR). The DPE-PCR assay is used to measure low levels of purified enzyme and is capable of detecting endogenous DNA polymerase extension activity directly from microbial cell lysates.

2.2 Materials and Methods

2.2.1 DNA substrate design and preparation

The sequences of the DNA substrate were adapted from DNA oligos previously used to measure bacterial-derived ATP via T4 DNA ligase (18). Oligo 1 (5'- gccgatatcggacaacggccgaactgggaaggcgaga ctgaccgaccgataagctagaacagagagacaacaac -3') and Oligo 2 (5'- uaggcgucggugacaaacggccagcguuguugu cucu[dideoxyCytidine] -3') were synthesized by Integrated DNA Technologies (Coralville, Iowa). The "u" in Oligo 2 represents deoxyUridine. DideoxyCytidine (ddC) was included as the last base on the 3' end of Oligo 2 to block DNA polymerase-mediated extension (see Figure 1 schematic). First, lyophilized Oligo 1 and Oligo 2 were resuspended to a final concentration of 100 μ M in sterile Tris-EDTA (T.E.) pH 8.0 (Ambion). Routine pre-annealing of the substrate was performed as follows. To begin, 100 μ L of Oligo1 (100 μ M stock) and 100 μ L of Oligo 2 (100 μ M stock) were added to 800 μ L of annealing buffer (200 mM Tris, 100 mM Potassium chloride and 0.1 mM EDTA) pH 8.45 resulting in a 1 mL mixture of Oligo 1 and Oligo 2 each at 10 μ M. One hundred micro-liter aliquots of the 10 μ M oligo mixture was dispensed into thin-walled 0.2 mL PCR tubes, capped, placed into a GeneAmp® 9700 thermocycler (Applied Biosystems) and the following pre-annealing program was performed: 95° C for 2 minutes, ramp at default speed to 25° C and incubate for 5 minutes, ramp at default speed to 4° C. A substrate dilution buffer was prepared by diluting oligo annealing buffer (described above) 1:10 in sterile water (Ambion, cat#AM9932). The pre-annealed DNA substrate was subsequently diluted to a final concentration of 0.01 μ M (10X stock) in oligo dilution buffer, aliquoted and stored at -20° C.

2.2.2 DNA polymerase extension reaction conditions

DNA Pol I (NEB cat# M0209L), Klenow (NEB cat# M0210S) and Klenow exo(-) (NEB cat# M0212S) were diluted to the indicated U/ μ L stock in sterile T.E. pH 8.0. To begin, 2 μ L of DNA polymerase stock at each concentration were placed into a 50 μ L DNA polymerase extension reaction mixture containing the following components: 50 μ M dNTP, 20 mM Tris pH 8.0, 10 mM Ammonium sulfate, 10 mM Potassium chloride, 2 mM Magnesium sulfate, 1% BSA, 0.1% Triton X-100, 0.1% Tween 20, and 0.001 μ M pre-annealed DNA substrate (described above). Two micro-liters of T.E. (without DNA polymerase) was routinely added to an additional tube containing complete DNA polymerase extension reaction mixture and is referred to as a “No Input Control” (NIC). Reactions containing DNA polymerase (or No Input Controls) were vortexed briefly and placed at 37° C for 20 minutes. After 20 minutes, 3 μ L of each reactions containing purified DNA polymerase were immediately placed into a qPCR reaction (see below for qPCR conditions).

2.2.3 Heat treatment of DNA polymerase extension reaction components

Prior to usage, DNA polymerase extension reaction reagent stocks (minus DNA substrate) were heat treated as follows: 10X dNTP mixture [500 μ M dATP, dCTP, dGTP, dTTP] was heated at 90 ° C for 30 minutes. 10X core reaction mix [200 mM Tris pH 8.0, 100 mM Ammonium sulfate, 100 mM Potassium chloride, 20 mM Magnesium sulfate] was heated at 90 ° C for 30 minutes. 1.43X BSA/Detergent mix [1.43 % BSA, 0.143 % Triton X-100, 0.143 % Tween 20] was heated at 75 ° C for 45 minutes. Substrate annealing buffer (200 mM Tris, 100 mM Potassium chloride and 0.1 mM EDTA) pH 8.45 was heated at 90 ° C for 30 minutes. Bead mill tubes were heated at 95 ° C for 20 minutes.

2.2.4 Quantitative PCR primers, probes and competitive internal control design

The DPE-PCR primers described here were previously used to amplify a DNA substrate modified by T4 DNA ligase (18) and are as follows: Forward primer (5'- ggacaacggccgaactgggaaggcg -3'), Reverse primer (5'- taggcgtcggtgacaaacggccagc -3'). The detection probe used in this study was (5' FAM- actgaccgaccgataagctagaacagagag -IABk-FQ 3'). As a tool to monitor qPCR inhibition,

a competitive internal control was generated and contains the following sequence (5'-gccgatatcggacaacggccgaactgggaaggcgagatcagcaggccacacgttaaagacagagagacaacaacgtggccgtttgtcaccgacgccta-3'). The internal control sequence was synthesized and cloned as a "minigene" by Integrated DNA Technologies (Coralville, Iowa). Upon receipt, the internal control minigene plasmid was linearized using the restriction enzyme PvuI (New England Biolabs) and re-purified using a PCR cleanup column (Qiagen). The purified internal control was quantified using a Nanodrop spectrophotometer (Thermo Scientific, ND-1000), diluted to the desired concentration in T.E. and stored at -20° C. A probe, specific for the internal control DNA, was synthesized by Integrated DNA Technologies (5' TX615- atcagcaggccacacgtt aaagaca -IAbRQSp 3'). A detailed schematic containing the relative positioning of the primers/probes within the substrate/competitive Internal Control can also be found in Figure 1.

2.2.5 Quantitative PCR composition and thermocycling parameters

Each 30 µL qPCR reaction contained: 1X LightCycler 480 Master Mix (from 2X stock, Roche cat# 04707494001), 333 nM of forward and reverse primers, 166 nM detection probe (FAM), 166 nM internal control probe (TxRed), 1.2 U of Uracil DNA Glycosylase (abbreviated hereafter as UDG, Bioline cat# BIO-20744) and 40 copies of the competitive Internal Control DNA (described above). Three micro-liters of each DNA polymerase extension reaction (from purified DNA polymerase or microbial cell lysates) were added to 27 µL of qPCR master mix and a two-step thermocycling protocol was run on a SmartCycler (Cepheid, Sunnyvale CA) as follows: Initial incubation of 40° C for 10 minutes and 50° C for 10 minutes and at 95° C for 5 minutes (to activate Taq and complete UDG-mediated DNA backbone hydrolysis of Oligo 2), followed by 45 cycles of 5s denaturation at 95° C and 20s annealing/extension at 65° C. Cycle threshold (Ct) values were generated automatically by the SmartCycler software using 2nd derivative analysis of the emerging qPCR curves. An example of a typical DPE-PCR reaction containing all controls (including target and competitive internal control curves) is presented in Supplemental Figure 1.

2.2.6 Bead mill lysis tube composition

Bead mill lysis tubes are generated by pipetting 60 µL (wet volume) of 0.1mm glass beads (Scientific Industries cat# SI-G01) using a 100 µL size Eppendorf tip and 50 µL (wet volume) of

0.5mm glass beads (Scientific Industries cat# SI-BG05) using a modified 1000 μ L size Eppendorf tip (To enable more reproducible and accurate dispensing of the 0.5mm beads, the end of the 1000 μ L size Eppendorf tip was cut to a 1mm inner diameter using a sterile razor blade). Once a slurry of both size beads were dispensed into a 1.5 mL tube (with screw cap), the aqueous supernatant was subsequently aspirated using a sterile gel loading pipette tip attached to a vacuum source. After aspiration, tubes were capped and heat treated prior to use (see above heat treatment section).

2.2.7 Contamination prevention recommendations

A sufficient aseptic working environment can be readily achieved by routinely working in a positive air pressure bench top hood/box equipped with HEPA filter feed (Sentry Air Systems) or comparable HEPA filtered PCR workstation. Pipettes and work surfaces should also be frequently cleaned with a lab wipe that has been moistened with 10% bleach solution that has been prepared weekly.

2.2.8 Bacterial strains and media

Staphylococcus aureus (ATCC 25923) and *Escherichia coli* (ATCC 25922) were primarily used in this study. Cultures were grown in/on Brain-Heart Infusion liquid media/agar (Teknova). The ATCC reference numbers and growth media for the additional 17 microorganisms tested are listed in Supplemental Figure 9.

2.2.9 Detection of bacterial DNA polymerase extension activity following bead mill lysis

S. aureus and *E. coli* cultures were grown to an OD₆₀₀ of 1.0 ± 0.2 (approximately 1×10^9 cfu/mL.) For each organism, 1 mL of culture was pelleted and washed three times in T.E. Bacterial suspensions were serially diluted in T.E., and 5 μ L of each stock were added to bead mill lysis tubes containing 50 μ L DNA polymerase extension reaction mixture (see above for composition). A titration curve of 1×10^5 to 1×10^0 cfu/reaction was performed in triplicate for each organism, including triplicate reactions without bacterial suspension (No Input Control). After the addition of 5 μ L bacterial stock (or No Input Control), reaction tubes were bead milled for 6 min. at 2800 rpm using a digital Vortex Genie equipped with a disrupter head (Scientific Industries). Immediately after disruption, sample tubes were placed at 37° C for 20 minutes. After the 20

minute incubation, sample tubes were transferred to 95° C for 5 min. and removed to cool at room temperature. Sample tubes were then spun at 12k x g for 30 seconds and 3 µL of each reaction were placed into the qPCR portion of the DPE-PCR assay. Five micro-liters of each bacterial stock was plated to obtain more accurate *cfu* input levels. Gene-specific PCR was also performed on the same lysates used for DNA polymerase detection. Primer and probe sequences for *S. aureus* and *E. coli* gene specific PCR are listed in Supplemental Figure 15.

2.2.10 Dideoxy termination experiments

Termination of purified DNA polymerase extension activity with ddCTP:

DNA polymerase extension reactions were prepared as described above with a 50 µM [dATP, dGTP, dTTP] mixture supplemented with either 50 µM dCTP or 50 µM ddCTP (Affymetrix #77332.) 50 µL DNA polymerase extension reactions with a 50 µM [dATP, dGTP, dTTP] mixture, supplemented with either dCTP or ddCTP, were spiked with 2 µL of a 1×10^{-9} U/µL stock of DNA polymerase I (New England Biolabs # M0209). Triplicate reactions were incubated at 37° C for 20 minutes and 3 µL of each reaction were subsequently placed into qPCR.

Elimination of microbial detection via ddCTP:

S. aureus and *E. coli* cultures were grown, washed and diluted as described above. To demonstrate ddCTP-dependent termination of microbial DNA polymerase extension, 5 µL of bacterial stock were added to bead lysis tubes containing 50 µL of DNA polymerase extension reaction buffer with a 50 µM [dATP, dGTP, dTTP] mixture supplemented with either 50 µM dCTP or 50 µM ddCTP. Bead mill lysis, DNA polymerase extension reaction and qPCR were performed as described above. Five micro-liters of each bacterial stock were plated to determine more accurate *cfu* input levels. Gene specific PCR of genomic DNA was also performed on the same lysates used for DPE-PCR.

dCTP rescue of microbial detection:

S. aureus and *E. coli* cultures were grown, washed and diluted as described above. Five micro-liters of bacterial stock were added to bead lysis tubes containing 50 µL of DNA polymerase extension reaction buffer containing a 50 µM [dATP, dGTP, dTTP, **ddCTP**] mixture. Just prior to lysis, 1 µL of dCTP at [2.5 mM, 0.25 mM 0.025 mM and 0.0025 mM] was added to separate ddCTP-containing reactions. Reactions containing 50 µM [dATP, dGTP, dTTP, dCTP] alone and 50 µM

[dATP, dGTP, dTTP, **ddCTP**] alone were run in parallel as “non-terminated” and “terminated” comparators. Bead mill lysis, DNA polymerase extension reaction and qPCR were performed as described above. Five micro-liters of each bacterial stock were plated to determine more accurate *cfu* input levels. Gene-specific PCR was also performed on the same lysates used for DPE-PCR.

2.2.11 Viability Assessment Experiments

S. aureus and *E. coli* cultures were grown, washed and diluted as described above. Two hundred micro-liters of bacterial stocks at approximately 2000 *cfu*/μL (in T.E.) were incubated at 25° C, 45° C, 65° C, 85° C and 105° C for 20 minutes. After heating, samples were cooled to room temperature and 5 μL of each bacterial stock were added to bead mill lysis tubes containing 50 μL of DNA polymerase extension reaction buffer. Bead mill lysis, DNA polymerase extension reaction and qPCR were performed as described above. Five micro-liters of each bacterial stock (treated at various temperatures) were also added to 1 ml of T.E. and 50 μL were plated for colony count determination. Gene specific PCR was also performed on the same lysates used for DPE-PCR.

2.3 Results and Discussion

2.3.1 Overview of the DPE-PCR assay

The measurement of DNA polymerase extension activity could represent a useful tool with far reaching applications such as, but not limited to, screening candidate-polymerase inhibitors *in vitro*, or detecting the presence any microbe (harboring active DNA polymerases) within a diverse range of sample types. If intended for these purposes, routine use of traditional polymerase assays that incorporate radiolabeled nucleotides is unattractive. Consequently, numerous non-radioactive DNA polymerase extension assays have been developed in recent decades. Despite successfully averting the use of radioactivity, current fluorescence-based DNA polymerase assays also suffer from various deficiencies. For example, detection of DNA polymerase activity via several existing non-radioactive assays is dependent upon the binding of PicoGreen™ to newly-generated double stranded DNA (13,14). If intended to analyze DNA polymerase activity from freshly lysed organisms, PicoGreen™-based assays would likely be

hampered by background fluorescence via binding of PicoGreenTM to genomic DNA.

Microplate-based DNA polymerase assays have also been developed (15). Decreased sensitivity of microplate-based assays can be expected for numerous reasons, including dependence upon intermediate binding of either product or substrate to a microplate and/or inefficient incorporation of modified dNTPs by DNA polymerase. More recently, real-time measurement of DNA polymerase activity via molecular beacons has been described (16). Despite improved sensitivity, direct measurement of molecular beacon fluorescence could also potentially be hindered by exposure to crude cellular lysates.

We set out to develop a rapid, simple, highly sensitive and quantitative assay capable of measuring DNA polymerase extension activity derived from purified commercial sources or freshly lysed cells. Figure 1A contains a schematic overview of the mechanisms involved in coupling DNA polymerase extension activity to qPCR.

Figure 1. Basic overview of the DPE-PCR assay.

(A) DNA polymerase is incubated with a substrate consisting of pre-annealed Oligo-1 and Oligo-2. DNA polymerase extends only the 3' end of Oligo-1 during a 20 minute incubation at 37°C. Three micro-liters of the DNA polymerase extension reaction mixture is subsequently transferred into a hot start qPCR reaction containing uracil DNA glycosylase (UDG). Prior to and during activation of Taq, UDG degrades the deoxyuridine within Oligo-2, leaving only a single stranded product derived from DNA polymerase-mediated extension of Oligo-1. After activation of Taq, PCR-based amplification is initiated via primer binding to the Oligo-1 extension product. **(B)** The sequence of a competitive internal control DNA is presented. The competitive internal control is present at 40 copies within each PCR reaction.

Notably, Oligo 2 is eliminated by uracil DNA glycosylase (UDG) prior to and during Taq activation, thus preventing undesired Taq-dependent extension of the substrate just prior to PCR cycling. A microbial detection method linking T4 DNA ligase activity to PCR amplification has been previously reported (18), which contains similarities to our DPE-PCR assay and is another example of an ETGA methodology. However, in our hands a modified version of this method, aimed at detecting microbial-derived NAD-dependent DNA ligase activity, suffered from a lack of sensitive and universal microbial detection, leading us to the development of the improved novel DNA polymerase-based approach named DPE-PCR described herein.

2.3.2 Sensitive and linear detection of purified DNA polymerase extension activity

To begin, we set out to determine the approximate analytical sensitivity of the DPE-PCR assay using commercially available DNA polymerase I. In this experiment, DPE-PCR signals derived from decreasing amounts of DNA polymerase I were compared to parallel reactions without input DNA polymerase (Referred to hereafter as the “No Input Control” or NIC). As shown in Figure 2A, detection of DNA polymerase I extension activity was achieved over a wide range of input enzyme. In fact, DNA polymerase I extension activity was distinguishable from the NIC down to as little as 2×10^{-11} units (U) of enzyme (equivalent to approximately 50 molecules of polymerase). To our knowledge, detection of DNA polymerase extension activity at this level is unrivaled in existing DNA polymerase assays. In theory, this level of sensitivity could enable single microbe detection as *E. coli* has been reported to contain approximately 400 DNA polymerase I molecules per cell (11). Regression analysis also showed a strong positive linear correlation ($R^2 = 0.992$) between the DPE-PCR cycle threshold (Ct) values and Units of input commercial DNA polymerase I after graphing data from two independent limit of detection experiments (Figure 2B and Supplemental Figure 3A).

After sensitivity and linearity experiments were performed, it was important to determine if the DPE-PCR assay signal was independent of intrinsic exonuclease activity. To this end, we subsequently compared signals generated by 2×10^{-7} U of DNA polymerase I to those generated from DNA polymerase I lacking 5'→3' exonuclease activity (Klenow) and another version of the enzyme lacking all exonuclease activity (Klenow exo -). For additional specificity and background signal determination, *E. coli* DNA ligase at 2×10^{-7} U and a NIC were tested in parallel. As shown in Figure 2C, both Klenow and Klenow exo - were detected at similar levels when compared to wild type DNA polymerase I, providing evidence that the DPE-PCR assay signal is derived from DNA polymerase-dependent extension and not intrinsic exonuclease activity (Also see Supplemental Figure 3B).

In addition to using exonuclease free polymerases, we set out to further demonstrate that DPE-PCR assay signal is derived from DNA polymerase-dependent extension of the DNA substrate

prior to qPCR. Since incorporation of dideoxy nucleotides is a well-established method used for termination of DNA polymerase chain extension activities (19,20), we chose to substitute dCTP with dideoxyCTP (ddCTP) within our DNA polymerase extension reaction mix. The schematic shown in Figure 2D reveals the first possible position within the substrate that ddCTP can be incorporated by DNA polymerase. If ddCTP is incorporated into this position, the extension product of Oligo 1 would be insufficient in length for subsequent detection by qPCR primer 1 (See Figure 1 schematic). As shown in Figure 2D, substitution of dCTP with ddCTP eliminates signal generated by DNA polymerase I, thus demonstrating that the DPE-PCR assay signal is dependent upon DNA polymerase extension of the substrate prior to qPCR.

Figure 2. Sensitive detection of purified DNA polymerase using DPE-PCR

The presence of a low copy competitive internal amplification control confirms that qPCR was not inhibited by the presence of low amounts of ddCTP that are carried over from the DNA polymerase assay reagents (See supplemental Figure 4). In addition, we feel it is important to note that we sporadically observe a weak, but detectable signal in the absence of input-DNA polymerase (No Input Control). Due to the exquisite sensitivity of the DPE-PCR assay, we have demonstrated that weak background noise signals can be attributed to “contaminant” DNA polymerase activity present in the DNA polymerase extension stock reagents prior to reaction assembly. Consequently, pre-treatment of the DNA polymerase extension reagents (see materials and methods section) is routinely performed and is sufficient to eliminate the contaminant DNA polymerase signal observed (See supplemental Figure 2A for an example). Additionally, we have demonstrated that a major potential source of unwanted Taq-dependent signal could arise from the operator’s failure to add active UDG to the qPCR mastermix. For example, intentional omission of UDG from the qPCR mastermix results in a high background signal derived from Taq-dependent extension of the DNA substrate (see Supplemental Figure 2B), however we have never observed high background signals (resulting from UDG failure) when UDG is added as described in the methods section. Another hypothesized source of increased background signal could be derived from DNA polymerase introduced by the operator during experimental setup. It is therefore recommended that the operator exhibit good aseptic technique when preparing samples and reagents for the DNA polymerase extension and qPCR portions of the assay (see materials and methods section for contamination prevention recommendations). Considering the above, we feel it is very important that an NIC be run in parallel with each experiment to verify that the starting reagents are free of contamination and that UDG has been added to the qPCR mastermix.

2.3.3 Sensitive universal detection of microbes via measurement of endogenous DNA polymerase extension activity directly from cell lysates

In addition to detecting purified polymerase activity a simple, sensitive and universal method that measures microbial-derived DNA polymerase activity would be highly desirable. For

instance, measurement of DNA polymerase extension activity could be used to screen environmental or biological samples for the presence of any microorganism harboring active DNA polymerase. To this end, we developed a simple method that couples microbial lysis to our DPE-PCR assay. As shown in Figure 3, a liquid sample known to contain, or suspected of containing, microbes is added to a bead mill lysis tube, disrupted and immediately transitioned into the DPE-PCR assay. As shown in Figure 3, a liquid sample known to contain, or suspected of containing, microbes is added to a bead mill lysis tube, disrupted and immediately transitioned into the DPE-PCR assay.

Figure 3. Schematic overview of coupling bead lysis to DPE-PCR

We chose one Gram negative bacteria (*E. coli*) and one Gram positive bacteria (*S. aureus*) to demonstrate the ability of our assay to measure microbial-derived DNA polymerase extension activity in crude cellular lysates. As shown in Figure 4A, when linked with bead mill lysis, the DPE-PCR assay is capable of detecting a wide dynamic range of input *E. coli*, down to and below 10 colony forming units (*cfu*) per lysis tube. Linear regression analysis of *E. coli* detection was also performed down to 10 *cfu* of input bacteria and showed a strong positive linear correlation between input *cfu* and DNA polymerase extension activity signal as indicated by an R^2 value of 0.999 (Figure 4B). Colony count plating and *E. coli*-gene specific qPCR (gsPCR) were run in parallel, confirming both the input level of *cfu* per reaction and the ability to monitor intact genomic DNA from the exact same lysates. DNA polymerase extension activity from *S. aureus* lysates was detected to a similar input level (Figure 4C). *S. aureus* detection was plotted down to 10 *cfu* of input bacteria and also showed a strong linear correlation between input *cfu* and DNA polymerase extension activity signal ($R^2 = 0.999$, Figure 4D).

Figure 4. DPE-PCR enables sensitive and quantitative detection of Gram negative and Gram positive bacteria via measurement of DNA polymerase extension activity in crude lysates

(A) Decreasing amounts of *E. coli* cfu were spiked into bead lysis-coupled DPE-PCR. No Input Controls (NIC) were also included to monitor reagent background levels. All cfu spikes and NICs were performed in triplicate. A representative DPE-PCR curve is shown below for each level of bacterial input. Colony count plating and gsPCR were performed in an effort to obtain a better estimate of the actual cfu placed into each reaction and is presented in Supplemental Figure 5 **(B)** A plot of *E. coli* DNA polymerase activity and linear regression analysis is presented. Graphs were generated using the average Ct values obtained from triplicate reactions of bacterial spikes ranging from 1×10^5 - 1×10^1 input cfu. **(C and D)** cfu titration experiments were performed for *S. aureus* exactly as described above for *E. coli*. Colony count plating and gsPCR were performed in an effort to obtain a better estimate of the actual cfu placed into each reaction and is presented in Supplemental Figure 6.

Colony count plating and gsPCR were performed in parallel to confirm the amount of *S. aureus* present in each bead lysis tube, as well as the presence of directly analyzable genomic DNA. Complete tables of plating, gsPCR and DNA polymerase activity results for both *E. coli* and *S. aureus* can be found in Supplemental Figures 5 and 6. We subsequently tested the ability of the DPE-PCR assay to measure DNA polymerase activity from seventeen additional clinically relevant microorganisms. As shown in Table 1, we were able to detect DNA polymerase activity from all seventeen additional organisms including six Gram-negative bacteria, six Gram-positive bacteria and five *Candida* species. Detection of the seventeen additional microbes exhibited a

strong positive linear correlation to input *cfu* with impressive low limits of detection. The upper linear dynamic ranges have yet to be fully characterized.

Bacterial panel	Lower Limit <i>cfu</i> Detected by DPE-PCR	R ² (1e4-1e1 <i>cfu</i>)
<i>Klebsiella pneumoniae</i>	< 10	0.9957
<i>Pseudomonas aeruginosa</i>	< 10	0.9860
<i>Enterobacter cloacae</i>	< 10	0.9995
<i>Acinetobacter baumannii</i>	< 10	0.9980
<i>Haemophilus influenzae</i>	< 10	0.9996
<i>Serratia marcescens</i>	< 10	0.9956
<i>Enterococcus faecalis</i>	< 10	0.9963
<i>Enterococcus faecium</i>	< 10	0.9899
<i>Streptococcus pyogenes</i>	< 10	0.9945
<i>Streptococcus agalactiae</i>	< 10	0.9969
<i>Streptococcus pneumoniae</i>	< 10	0.9999
<i>Staphylococcus epidermidis</i>	< 10	0.9990

Candida panel	Lower Limit <i>cfu</i> Detected by DPE-PCR	R ² (1e5-1e3 <i>cfu</i>)
<i>Candida albicans</i>	≈ 20	0.9945
<i>Candida tropicalis</i>	≈ 20	0.9969
<i>Candida glabrata</i>	≈ 40	0.9111
<i>Candida parapsilosis</i>	≈ 20	0.9950
<i>Candida krusei</i>	≈ 15	0.9868

Table 1: Sensitive and linear detection of 17 additional clinically relevant microbial species.

More comprehensive results containing parallel plating data and DPE-PCR results for each of the 17 additional microbes are presented in Supplemental Figures 10-14. Together, these data support the notion that DPE-PCR has the potential to be useful as a universal “pan” test for the sensitive detection of any microbe in a normally sterile environment.

2.3.4 Elimination of DPE-PCR detection of microbes via ddCTP substitution

As previously shown in Figure 2D, substitution of dCTP with ddCTP in the DNA polymerase extension reaction mix represents a powerful tool for blocking extension of Oligo 1 within our

assay. To demonstrate that the signal derived from bacterial spikes was dependent upon their DNA polymerase extension activity, and not the other endogenous bacterial enzyme activities present in the lysates, we set up an experiment to compare DPE-PCR signals obtained from *E. coli* and *S. aureus* using a standard DNA polymerase reaction mix containing (dATP, dTTP, dGTP, dCTP) versus a reaction mix containing (dATP, dTTP, dGTP, ddCTP). As shown in Figure 5A, when compared to the standard reaction mix, substitution of ddCTP blocked the generation of signal derived from *E. coli cfu* spikes (Figure 5A). A dCTP rescue experiment was subsequently performed by comparing DNA polymerase extension activity from bacteria lysed in a DNA polymerase extension reaction mix containing 50 μ M (dATP,dTTP,dGTP, ddCTP only), to those containing increasing amounts of supplemented dCTP (see materials and methods for a detailed description of rescue experiments). Figure 5B demonstrates the rescue effect that increasing amounts of supplemented dCTP has on quantifiable DNA polymerase extension activity derived from *E. coli* lysates. In addition to measuring microbial DNA polymerase extension activity, gSPCR was run in parallel to verify that equivalent amounts of *E. coli* were present in each of the assayed lysates. A graphical comparison of DNA polymerase activity versus presence of genomic DNA is presented in Figure 5C. Signal termination (via ddCTP) and dCTP rescue experiments were subsequently repeated with *S. aureus* and similar results were obtained (Figure 5D-F).

Figure 5. Detection of bacteria by DPE-PCR is blocked by ddCTP and rescued with dCTP

(A) 5 μ L of *E. coli* suspension were added to bead lysis-coupled DNA polymerase assays comprised of a 50 μ M [dATP, dGTP, dTTP] mixture supplemented with either 50 μ M dCTP or 50 μ M ddCTP. DPE-PCR curves representing *E. coli*-derived DNA polymerase activity is presented. Approximate cfu input as determined by plating is presented in the upper left region of the qPCR graph (B) 5 μ L of *E. coli* suspension were added to bead lysis tubes containing 50 μ L reaction buffer comprised of a 50 μ M [dATP, dGTP, dTTP] mixture supplemented with either 50 μ M dCTP or 50 μ M ddCTP. Prior to lysis, 1 μ L of dCTP [2.5 mM, 0.25 mM, 0.025 mM, 0.0025 mM] was added to selected ddCTP-containing reactions. Reactions containing 50 μ M [dATP, dGTP, dTTP, dCTP] alone or 50 μ M [dATP, dGTP, dTTP, ddCTP] alone were run in parallel as “non-terminated” and “terminated” comparators. The resultant DPE-PCR curves representing *E. coli*-derived DNA polymerase activity is presented. Approximate cfu input as determined by plating is presented in the lower left region of the qPCR graph (C) *E. coli* gene specific PCR was also performed on the same lysates used for DNA polymerase detection presented in Figure 2B. Linear plots of dCTP-dependent rescue of bacterial DNA polymerase detection vs. gsPCR of genomic DNA are shown. Plots were generated using the average qPCR Ct values from triplicate reactions at the indicated conditions (D-F) ddCTP termination and dCTP rescue experiments were performed for *S. aureus* exactly as described above for *E. coli*.

Tables containing DPE-PCR and gsPCR data for both *E. coli* and *S. aureus* can be found in Supplemental Figures 7 and 8. qPCR competitive internal control values are provided to demonstrate that low levels of ddCTP carried over into qPCR are not inhibitory, and thus are

not responsible for the disappearance of DNA polymerase activity signal (Highlighted in Supplemental Figures 7A and 8A). Together, the data presented in Figure 5 strongly support the claim that the DPE-PCR assay is specifically detecting microbial DNA polymerase extension activity and signal is not derived from substrate modification via enzymatic activities other than DNA polymerase.

2.3.5 Measurement of DNA polymerase extension activity as an indicator of bacterial viability

Traditional methods for determining bacterial viability are dependent upon growth and visualization of a particular microbe on solid medium (21). Although bacterial growth and visualization is the current industry gold standard, the traditional *cfu* viability determination methods are undesirable due to the length of time required for *cfu* formation. Furthermore, the ability to grow on solid media or in liquid culture can vary dramatically from one microbe to another, thus potentially limiting the detection of certain fastidious organisms (22). Due to the aforementioned limitations of traditional methods, there is a growing need in a wide variety of pharmaceutical (23), environmental, food processing and clinical testing arenas for the rapid assessment of microbial viability. Consequently, numerous molecular methods have been developed in an effort to quickly assess microbial viability status within a given matrix (24). Despite being rapid and sensitive, molecular methods that detect the presence of nucleic acid often fall short of representing an accurate measurement of cell viability. For example, amplification of endogenous DNA or RNA is a poor indicator of bacterial viability, due to the persistence of nucleic acid after cell death (25, 26). We set out to determine the feasibility of using DNA polymerase extension activity as an indicator of bacterial viability. To this end, an experiment was designed to compare detection of DNA polymerase extension activity and PCR-mediated detection of genomic DNA as indicators of bacterial viability following various amounts of heat treatment. To begin, *E. coli* suspensions were treated at increasing temperatures for a fixed period of time. After heat treatment, bacteria were subsequently assayed for the presence of both DNA polymerase extension activity and genomic DNA. Heat treated and non-heat treated bacterial stocks were also plated in parallel to monitor bacterial viability via the presence of visible *cfu*. Figure 6A represents the levels of *E. coli* DNA

polymerase extension activity measured after the indicated amounts of heat treatment. Notably, a significant drop in *E. coli* DNA polymerase extension activity was observed after incubation of bacterial suspensions between 45° C and 65° C (Figure 6A). In contrast, gsPCR signal obtained from the same lysates remained relatively constant at all temperatures and is graphically compared to DNA polymerase activity in Figure 6B. Plating results presented below the graph further demonstrate that increasing levels of heat treatment are sufficient to prevent *cfu* formation and are paralleled by a dramatic loss of DNA polymerase activity; however, dead cells still contribute genomic DNA levels very close to their original input levels confirming that gsPCR is a poor indicator of the presence of viable cells (Figure 6B). In Figure 6C, the bar graphs further highlight the relative abilities of DPE-PCR and gsPCR to monitor the disappearance of *cfu* in response to lethal amounts of heat treatment.

Figure 6. DPE-PCR as an indicator of *E. coli* viability in response to heat treatment

(A) 200 μL aliquots of an *E. coli* suspension ($\sim 2000 \text{ cfu}/\mu\text{L}$) were incubated at 25°C, 45°C, 65°C, 85°C and 105°C for 20 minutes. After heating, each bacterial stock was cooled to room temperature and 5 μL were transferred to the bead lysis-coupled DPE-PCR assay. DPE-PCR curves representing *E. coli*-derived DNA polymerase activity following each of the indicated temperature treatments are presented. (B) Plots were generated from triplicate DPE-PCR reactions and gPCR of genomic DNA (from the same lysates) after the indicated temperature treatments of *E. coli* suspensions. Parallel plating was also performed in triplicate for each of the treated *E. coli* suspensions. Representative cfu monitoring plates are presented below the graph, revealing bacterial viability status after treatment at each temperature. (C) DPE-PCR is compared to gPCR of genomic DNA in response to the various temperature treatments. “Fold Reduction of qPCR Signal” was calculated using the indicated equation and the values obtained were used to generate comparative bar graphs.

Subsequently, we wanted to test whether measurement of DNA polymerase extension activity could be used to indicate the viability status of a Gram positive organism as well. The previous *E. coli* experiments were repeated with *S. aureus* under the same conditions. Figure 7A-C, show similar results obtained from heat treatment experiments repeated with *S. aureus*.

Figure 7. DPE-PCR as an indicator of *S. aureus* viability in response to heat treatment

(A) 200 μL aliquots of an *S. aureus* suspension ($\sim 2000 \text{ cfu}/\mu\text{L}$) were incubated at 25°C, 45°C, 65°C, 85°C and 105°C for 20 minutes. After heating, each bacterial stock was cooled to room temperature and 5 μL were transferred to the bead lysis-coupled DPE-PCR assay. DPE-PCR curves representing *S. aureus*-derived DNA polymerase activity following each of the indicated temperature treatments are presented. (B) Plots were generated from triplicate DPE-PCR reactions and gsPCR of genomic DNA (from the same lysates) after the indicated temperature treatments of *S. aureus* suspensions. Parallel plating was also performed in triplicate for each of the treated *S. aureus* suspensions. Representative *cfu* monitoring plates are presented below the graph, revealing bacterial viability status after treatment at each temperature. (C) DPE-PCR is compared to gsPCR of genomic DNA in response to the various temperature treatments. “Fold Reduction of qPCR Signal” was calculated using the indicated equation and the values obtained were used to generate comparative bar graphs.

Collectively, the strong concordance between the presence of *cfu* and DNA polymerase extension activity shown in Figures 4, 6, 7 and Table 1 demonstrates that DPE-PCR has potential to be used as a general indicator of cell viability. Additional experiments are underway to measure relative DNA polymerase extension activity from microbes exposed to other clinically or pharmaceutically relevant agents (bacteriostatic and bactericidal) aimed at reducing cell proliferation or viability.

2.4 Conclusions

In summary, we have developed a novel, highly sensitive, quantitative and rapid DPE-PCR assay. In addition to quantitative detection of extremely low levels of purified enzyme, we have demonstrated the ability of DPE-PCR to reproducibly measure DNA polymerase extension activity from less than 10 *cfu* of bacteria via coupling to bead lysis. We have also demonstrated the potential for DPE-PCR to universally detect microbes by testing a panel of microorganisms comprised of seven Gram-negative bacteria, seven Gram-positive bacteria and five *Candida* species. Furthermore, preliminary evidence that the DPE-PCR assay can be used to assess bacterial viability was provided via the reproducibly strong correlation between DNA polymerase extension activity and proliferation as indicated by the presence of *cfu*. Considering the data presented here, we strongly believe that ETGA methodology such as our DPE-PCR assay has the potential to become a useful tool for a wide range of testing applications within pharmaceutical, environmental, food and clinical settings.

2.5 Supplemental Data

Supplemental Figure 1 – Example of a typical DPE-PCR assay readout and brief description of routine controls

Supplemental Figure 2

- (A) Example of “contaminant” DNA polymerase activity detected in DNA polymerase extension stock reagents and its elimination/reduction after reagent pre-heat treatment.
- (B) UDG within the qPCR mastermix is essential for elimination of Taq-dependent background noise. A dilution of UDG within the qPCR mastermix is also presented.

Supplemental Figure 3

- (A) Raw data used to generate linearity graphs for DNA Pol I LOD depicted in Figure 2B
(B) Raw data from triplicate reactions of Figure 2C

A

Units of DNA pol I per reaction	Run 1a Target-Ct (FAM)	Run 1b Target-Ct (FAM)	Run 2a Target-Ct (FAM)	Run 2b Target-Ct (FAM)	Avg Target-Ct (FAM)	StdDev Target-Ct (FAM)
2e-5	16.2	16.4	17.4	17.5	16.875	0.67
2e-6	20	20.1	20.8	20.8	20.425	0.43
2e-7	23.5	23.9	24.8	24.9	24.275	0.68
2e-8	27.9	27.9	28.6	28.6	28.25	0.40
2e-9	31.8	31.1	32.2	32.8	31.975	0.71
2e-10	34.3	35.1	35.8	35.3	35.125	0.62
2e-11	36.6	36.7	37.8	36.7	36.95	0.57
NIC	38.1	ND	ND	37.5	NA	NA
PCR blank	ND	ND	ND	ND	NA	NA

B

Sample Assayed	Reaction 1-Target Ct (FAM)	Reaction 2-Target Ct (FAM)	Reaction 3-Target Ct (FAM)	Avg Ct	StdDev Ct
Pol I	25.5	25.1	25.8	25.47	0.35
Klenow	23.7	26.3	23.9	24.63	1.45
Klenow exo-	25.1	23.8	23.3	24.07	0.93
Ligase	ND	ND	ND	NA	NA
NIC	36.9	37.9	ND	37.40	NA

Supplemental Figure 4

- (A) Raw data from triplicate DPE-PCR reactions containing DNA pol I reactions with dCTP or ddCTP shown in Figure 2D. PCR blank controls are included to demonstrate that the minor amounts of ddCTP carried from the DNA polymerase extension reaction into the qPCR reaction are not inhibitory for Taq. See Supplemental Figure 1 for an example of a typical DPE-PCR readout.
(B) Corresponding Target (FAM) curves
(C) Corresponding competitive Internal Control (TxRed) curves

A

Sample ID	Target-Ct	Internal Control - Ct
Pol-dCTP	31.2	32.2
Pol-dCTP	31.7	32.8
Pol-dCTP	31.4	32.7
Pol-ddCTP	37	36.6
Pol-ddCTP	ND	35.6
Pol-ddCTP	ND	36.5
PCR blank	ND	36.6
PCR blank	ND	36.3
PCR blank	ND	36.7

40 copies of competitive Internal Control DNA is still detected in the presence of ddCTP carryover

B

C

"Flattened" competitive internal control curves derived from Pol-dCTP are given a Ct of ≈ 32.5 by SMART Cyclet software

Supplemental Figure 5

- (A) Raw data from triplicate reactions used to generate linearity graphs for *E. coli* in Figure 4B
(B) Results from triplicate *cfu* plating
(C) Raw data from triplicate reactions of *E. coli* gene specific PCR. Data was generated from the same lysates used to determine DNA polymerase activity

A

Polymerase Activity Detection - FAM Ct Value

Targeted <i>cfu</i> input	Reaction-1	Reaction-2	Reaction-3	Avg Ct	StdDev Ct
e5	20.3	19.9	19.7	19.97	0.31
e4	22.9	23.1	23.3	23.10	0.20
e3	26.1	26.3	27.7	26.70	0.87
e2	30.6	29.8	29.8	30.07	0.46
e1	34.8	33.2	34	34.00	0.80
e0	ND	36.8	ND	NA	NA
NIC	ND	37.2	37	37.10	0.14

B

Colony Count Plating - *cfu* observed

Targeted <i>cfu</i> input	Plate 1	Plate 2	Plate 3	Avg	StdDev
e5	TNTC	TNTC	TNTC	NA	NA
e4	TNTC	TNTC	TNTC	NA	NA
e3	TNTC	TNTC	TNTC	NA	NA
e2	95	92	152	113.00	33.81
e1	8	6	19	11.00	7.00
e0	1	1	0	1.00	NA

C

Gene Specific PCR - FAM Ct value

Targeted <i>cfu</i> input	Reaction-1	Reaction-2	Reaction-3	Avg Ct	StdDev Ct
e5	26.31	26.57	26.94	26.61	0.32
e4	29.87	30.04	30.25	30.05	0.19
e3	33.5	33.33	33.82	33.55	0.25
e2	36.48	37	37.95	37.14	0.75
e1	ND	38.59	ND	NA	NA
e0	ND	ND	ND	NA	NA
NIC	ND	ND	ND	NA	NA

ND = None Detected

NA = Not Applicable

TNTC = Too Numerous To Count

Supplemental Figure 6

- (A) Raw data from triplicate reactions used to generate linearity graphs for *S. aureus* in Figure 4D
(B) Results from triplicate *cfu* plating
(C) Raw data from triplicate reactions of *S. aureus* gene specific PCR. Data was generated from the same lysates used to determine DNA polymerase activity

A

Polymerase Activity Detection - FAM Ct Value

Targeted <i>cfu</i> input	Reaction-1	Reaction-2	Reaction-3	Avg Ct	StdDev Ct
e5	19.8	19.4	19.8	19.67	0.23
e4	22.1	23.8	23.7	23.20	0.95
e3	26	26	26.6	26.20	0.35
e2	29.3	29.6	29.5	29.47	0.15
e1	32.3	33	32.8	32.70	0.36
e0	34.6	35.5	35.6	35.23	0.55
NIC	ND	ND	ND	NA	NA

B

Colony Count Plating - *cfu* Observed

Targeted <i>cfu</i> input	Plate 1	Plate 2	Plate 3	Avg	StdDev
e5	TNTC	TNTC	TNTC	NA	NA
e4	TNTC	TNTC	TNTC	NA	NA
e3	TNTC	TNTC	TNTC	NA	NA
e2	304	374	348	342.00	35.38
e1	21	42	41	34.67	11.85
e0	4	2	10	5.33	4.16

C

Gene Specific PCR - FAM Ct Value

Targeted <i>cfu</i> input	Reaction-1	Reaction-2	Reaction-3	Avg Ct	StdDev Ct
e5	23.15	22.7	23.4	23.08	0.35
e4	27.67	26.52	27.91	27.37	0.74
e3	30.63	21.51	31.02	27.72	5.38
e2	33.92	35.36	34.16	34.48	0.77
e1	36.94	36.41	36.75	36.70	0.27
e0	ND	ND	ND	NA	NA
NIC	ND	ND	ND	NA	NA

ND = None Detected

NA = Not Applicable

TNTC = Too Numerous To Count

Supplemental Figure 7

- (A) Raw data from triplicate reactions used to generate dCTP rescue graphs for *E. coli* in Figure 5C
(B) Raw data from triplicate reactions of *E. coli* gene specific PCR. Data was generated from the same lysates used to determine DNA polymerase activity

A *E. coli* DPE-PCR data

Sample ID	Target-Ct (FAM)	Internal Control-Ct (TbRed)	Avg Target-Ct (FAM)	StdDev Target-Ct (FAM)
dCTP	25.1	ND	25.63	0.46
dCTP	25.9	ND	—	—
dCTP	25.9	ND	—	—
d6CTP	ND	36.8	NA *	NA
d6CTP	ND	37	—	—
d6CTP	ND	37.3	—	—
d6CTP +50uM dCTP	26.3	ND	26.03	0.29
d6CTP +50uM dCTP	25.7	ND	—	—
d6CTP +50uM dCTP	26.2	ND	—	—
d6CTP +5uM dCTP	27	ND	27.00	0.10
d6CTP +5uM dCTP	26.9	ND	—	—
d6CTP +5uM dCTP	27.1	ND	—	—
d6CTP +0.5uM dCTP	29.2	ND	29.90	0.26
d6CTP +0.5uM dCTP	29.6	ND	—	—
d6CTP +0.5uM dCTP	30	ND	—	—
d6CTP +0.05uM dCTP	35.7	35.7	35.73	0.35
d6CTP +0.05uM dCTP	35.4	35.8	—	—
d6CTP +0.05uM dCTP	36.1	36.4	—	—
NIC	ND	36.1	NA	NA
NIC	ND	36.6	—	—
NIC	37.5	36.3	—	—

B *E. coli*-gsPCR Data

Sample ID	Target-Ct (FAM)	Avg Target-Ct (FAM)	StdDev Target-Ct (FAM)
dCTP	34.3	34.00	0.89
dCTP	34.7	—	—
dCTP	33	—	—
d6CTP	33.6	34.43	0.72
d6CTP	34.8	—	—
d6CTP	34.9	—	—
d6CTP +50uM dCTP	34.3	34.80	0.44
d6CTP +50uM dCTP	35.1	—	—
d6CTP +50uM dCTP	35	—	—
d6CTP +5uM dCTP	33.8	34.30	0.50
d6CTP +5uM dCTP	34.8	—	—
d6CTP +5uM dCTP	34.3	—	—
d6CTP +0.5uM dCTP	35.3	34.90	0.36
d6CTP +0.5uM dCTP	34.6	—	—
d6CTP +0.5uM dCTP	34.8	—	—
d6CTP +0.05uM dCTP	35.2	34.37	0.72
d6CTP +0.05uM dCTP	33.9	—	—
d6CTP +0.05uM dCTP	34	—	—
NIC	ND	NA	NA
NIC	ND	—	—
NIC	ND	—	—

No inhibition of competitive qPCR internal control due to ddCTP carryover

* Depicted as 45 Ct in graph to provide baseline for dCTP rescue curves. No actual Ct was calculated by the SmartCycler

ND = None Detected

Supplemental Figure 8

- (A) Raw data from triplicate reactions used to generate dCTP rescue graphs for *S. aureus* in Figure 5F
(B) Raw data from triplicate reactions of *S. aureus* gene specific PCR. Data was generated from the same lysates used to determine DNA polymerase activity

A *S. aureus* DPE-PCR data

Sample ID	Target-Ct (FAM)	Internal Control-Ct (TbRed)	Avg Target-Ct (FAM)	StdDev Target-Ct (FAM)
dCTP	24.8	ND	24.83	0.15
dCTP	24.7	ND	—	—
dCTP	25	ND	—	—
d6CTP	ND	36.2	NA *	NA
d6CTP	ND	36.4	—	—
d6CTP	ND	36.6	—	—
d6CTP +50uM dCTP	24.7	ND	24.67	0.15
d6CTP +50uM dCTP	24.5	ND	—	—
d6CTP +50uM dCTP	24.8	ND	—	—
d6CTP +5uM dCTP	24.6	ND	26.00	2.69
d6CTP +5uM dCTP	29.1	ND	—	—
d6CTP +5uM dCTP	24.3	ND	—	—
d6CTP +0.5uM dCTP	25.9	ND	25.90	0.00
d6CTP +0.5uM dCTP	25.9	ND	—	—
d6CTP +0.5uM dCTP	25.9	ND	—	—
d6CTP +0.05uM dCTP	30	32	30.70	0.62
d6CTP +0.05uM dCTP	31.2	31.8	—	—
d6CTP +0.05uM dCTP	30.9	33.7	—	—
NIC-dCTP	ND	36.5	NA	NA
NIC-dCTP	ND	36.8	—	—
NIC-dCTP	37.8	36.3	—	—

B *S. aureus*-gsPCR Data

Sample ID	Target-Ct (FAM)	Avg Target-Ct (FAM)	StdDev Target-Ct (FAM)
dCTP	31.4	31.20	0.10
dCTP	31.3	—	—
dCTP	31.2	—	—
d6CTP	31.4	31.40	0.20
d6CTP	—	—	—
d6CTP	31.2	—	—
d6CTP	31.6	—	—
d6CTP +50uM dCTP	31.2	31.40	0.20
d6CTP +50uM dCTP	31.4	—	—
d6CTP +50uM dCTP	31.6	—	—
d6CTP +5uM dCTP	31.4	32.17	1.69
d6CTP +5uM dCTP	34.1	—	—
d6CTP +5uM dCTP	31	—	—
d6CTP +0.5uM dCTP	31.1	30.93	0.15
d6CTP +0.5uM dCTP	30.8	—	—
d6CTP +0.5uM dCTP	30.9	—	—
d6CTP +0.05uM dCTP	31.1	31.90	0.75
d6CTP +0.05uM dCTP	21.6	—	—
d6CTP +0.05uM dCTP	32	—	—
NIC	ND	NA	NA
NIC	ND	—	—
NIC	ND	—	—

No inhibition of competitive qPCR internal control due to ddCTP carryover

* Depicted as 45 Ct in graph to provide baseline for dCTP rescue curves. No actual Ct was calculated by the SmartCycler

ND = None Detected

Supplemental Figure 9

(A) Source and growth media for the 17 additional microorganisms tested by DPE-PCR

A

Microorganism	ATCC #	Liquid Growth Media	Solid Growth Media
<i>Klebsiella pneumoniae</i>	700721	Brain Heart Infusion	Brain Heart Infusion
<i>Pseudomonas aeruginosa</i>	10145	Brain Heart Infusion	Brain Heart Infusion
<i>Enterobacter cloacae</i>	13047	Brain Heart Infusion	Brain Heart Infusion
<i>Acinetobacter baumannii</i>	15308	Brain Heart Infusion	Brain Heart Infusion
<i>Haemophilus influenzae</i>	35039	Resuspended Colony	814GC
<i>Serratia marcescens</i>	13880	Brain Heart Infusion	Brain Heart Infusion
<i>Enterococcus faecalis</i>	51299	Brain Heart Infusion	Brain Heart Infusion
<i>Enterococcus faecium</i>	19434	Brain Heart Infusion	Brain Heart Infusion
<i>Streptococcus pyogenes</i>	19615	Brain Heart Infusion	Brain Heart Infusion
<i>Streptococcus agalactiae</i>	13813	Brain Heart Infusion	Brain Heart Infusion
<i>Streptococcus pneumoniae</i>	6303	Resuspended Colony	Blood Agar
<i>Staphylococcus epidermidis</i>	12228	Brain Heart Infusion	Brain Heart Infusion
<i>Candida albicans</i>	11006	YM	YM
<i>Candida tropicalis</i>	750	YM	YM
<i>Candida glabrata</i>	66032	YM	YM
<i>Candida parapsilosis</i>	22019	YM	YM
<i>Candida krusei</i>	14243	YM	YM

Supplemental Figure 10 - Parallel plating results for 17 additional microorganisms tested

Microorganism	Target cfu assayed by DPE-PCR					cfu counted after parallel plating of 100 target	cfu counted after parallel plating of 10 target (A)	cfu counted after parallel plating of 10 target (B)	cfu counted after parallel plating of 10 target (C)	cfu counted after parallel plating of 1 target (A)	cfu counted after parallel plating of 1 target (B)	cfu counted after parallel plating of 1 target (C)
<i>Klebsiella pneumoniae</i>	[10,000]	[1,000]	[100]	[10]	[1]	105	16	13	6	1	3	0
<i>Pseudomonas aeruginosa</i>	[10,000]	[1,000]	[100]	[10]	[1]	188	21	29	28	3	4	5
<i>Enterobacter cloacae</i>	[10,000]	[1,000]	[100]	[10]	[1]	290	39	49	41	8	4	0
<i>Acinetobacter baumannii</i>	[10,000]	[1,000]	[100]	[10]	[1]	111	17	16	14	2	1	1
<i>Haemophilus influenzae</i>	[10,000]	[1,000]	[100]	[10]	[1]	≈ 500	105	101	4	10	6	
<i>Serratia marcescens</i>	[10,000]	[1,000]	[100]	[10]	[1]	160	12	25	11	3	1	2
<i>Enterococcus faecalis</i>	[10,000]	[1,000]	[100]	[10]	[1]	170	23	17	16	6	3	3
<i>Enterococcus faecium</i>	[10,000]	[1,000]	[100]	[10]	[1]	53	3	5	8	0	0	0
<i>Streptococcus pyogenes</i>	[10,000]	[1,000]	[100]	[10]	[1]	85	13	15	9	0	7	0
<i>Streptococcus agalactiae</i>	[10,000]	[1,000]	[100]	[10]	[1]	235	41	36	25	9	1	2
<i>Streptococcus pneumoniae</i>	[10,000]	[1,000]	[100]	[10]	[1]	41	7	5	7	0	1	0
<i>Staphylococcus epidermidis</i>	[10,000]	[1,000]	[100]	[10]	[1]	195	25	24	27	4	4	5
<i>Candida albicans</i>	[100,000]	[10,000]	[1,000]	[100]	[10]	≈ 200	18					
<i>Candida tropicalis</i>	[100,000]	[10,000]	[1,000]	[100]	[10]	90	18					
<i>Candida glabrata</i>	[100,000]	[10,000]	[1,000]	[100]	[10]	≈ 300	36					
<i>Candida parapsilosis</i>	[100,000]	[10,000]	[1,000]	[100]	[10]	≈ 200	20					
<i>Candida krusei</i>	[100,000]	[10,000]	[1,000]	[100]	[10]	65	13					

Supplemental Figure 11 - DPE-PCR results for 17 additional microorganisms tested

Microorganism	DPE-PCR Result at 100,000 cfu target level	DPE-PCR Result at 10,000 cfu target level	DPE-PCR Result at 1,000 cfu target level	DPE-PCR Result at 100 cfu target level	DPE-PCR Result at 10 cfu target level	DPE-PCR Result at 1 cfu target level
<i>Klebsiella pneumoniae</i>		Positive	Positive	Positive	3 of 3	1 of 3
<i>Pseudomonas aeruginosa</i>		Positive	Positive	Positive	3 of 3	2 of 3
<i>Enterobacter cloacae</i>		Positive	Positive	Positive	3 of 3	2 of 3
<i>Acinetobacter baumannii</i>		Positive	Positive	Positive	3 of 3	3 of 3
<i>Haemophilus influenzae</i>		Positive	Positive	Positive	3 of 3	3 of 3
<i>Serratia marcescens</i>		Positive	Positive	Positive	3 of 3	3 of 3
<i>Enterococcus faecalis</i>		Positive	Positive	Positive	3 of 3	2 of 3
<i>Enterococcus faecium</i>		Positive	Positive	Positive	3 of 3	2 of 3
<i>Streptococcus pyogenes</i>		Positive	Positive	Positive	3 of 3	1 of 3
<i>Streptococcus agalactiae</i>		Positive	Positive	Positive	3 of 3	1 of 3
<i>Streptococcus pneumoniae</i>		Positive	Positive	Positive	3 of 3	1 of 3
<i>Staphylococcus epidermidis</i>		Positive	Positive	Positive	3 of 3	3 of 3
<i>Candida albicans</i>	Positive	Positive	Positive	2 of 2 *	2 of 2 *	
<i>Candida tropicalis</i>	Positive	Positive	Positive	2 of 2 *	2 of 2 *	
<i>Candida glabrata</i>	Positive	Positive	Positive	1 of 2 *	1 of 2 *	
<i>Candida parapsilosis</i>	Positive	Positive	Positive	2 of 2 *	1 of 2 *	
<i>Candida krusei</i>	Positive	Positive	Positive	2 of 2 *	2 of 2 *	

* Detection of *Candida* species becomes non-linear below 1000 cfu and positivity is dependent upon the weak background noise associated with each specific DPE-PCR run.

Supplemental Figure 12 - Selected DPE-PCR curves for 6 additional gram negative bacteria

Supplemental Figure 13 - Selected DPE-PCR curves for 6 additional gram positive bacteria

Supplemental Figure 14
- Selected DPE-PCR curves for 5 *Candida* species

Supplemental Figure 15

- (A) Primer and probe sequences used for gene specific PCR-mediated detection of *E. coli* genomic DNA
- (B) Primer and probe sequences used for gene specific PCR-mediated detection of *S. aureus* genomic DNA
- (C) Thermoprofile for both gene specific PCR assays

A

uidA gene of *E. coli*
Forward Primer: 5' caccgacatgtggaatgaag 3'
Reverse Primer: 5' cggtgaagatccctttctt 3'
Probe (5' FAM labeled): 5' ccgcgtctttgatcgcgtca 3'

B

nuc gene of *S. aureus*
Forward Primer: 5' ctgaagcaagtcattacgaa 3'
Reverse Primer: 5' agccaagccttgacgaactaa 3'
Probe (5' FAM labeled): 5' tatgctgatgaaaaatggtaaacaaagc 3'

C

<u>Pre PCR</u>	<u>45 cycles</u>	
95 ° C	95 ° C	
5 min.	5 sec.	60 ° C 1 min.

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Chapter 3

Feasibility of a Novel Approach for Rapid Detection of Simulated Bloodstream Infections via Enzymatic Template Generation and Amplification (ETGA)-mediated Measurement of Microbial DNA Polymerase Activity

Daniel R. Zweitzig, Bruce I. Sodowich, Nichol M. Riccardello and
Shawn M. O'Hara

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Abstract

Bloodstream infections (BSI) caused by bacteria and fungi are associated with significant morbidity and mortality. Currently, blood culture is the gold standard for confirming a suspected BSI, but is often criticized due to the lengthy Time-to-Detection (TTD) required for indicating the presence of microbes. Detection of conserved microbial nucleic acid sequences within blood culture samples via PCR has been demonstrated to offer potential for reduced TTD of BSI, however these approaches have been criticized due to various limitations. We report a novel approach toward rapid detection of microbes from simulated BSI via differential hematopoietic cell lysis followed by enzymatic template generation and amplification (ETGA)-mediated measurement of microbial DNA polymerase extension activity. The differential cell lysis procedure effectively reduced the level of detectable DNA polymerase extension activity associated with human-derived hematopoietic cells present in blood culture samples taken from healthy donors. After treatment with the differential cell lysis procedure, the ETGA assay detected a panel of clinically prevalent bacteria and *C. albicans* from spiked blood culture samples. The ETGA blood culture method also reduced by 3-fold the TTD required for simulated BSI when compared to a continuous-monitoring blood culture instrument. In summary, this study represents the feasibility of an innovative approach towards a rapid, sensitive, and universal screen for microbes within blood culture samples. Clinical application and automation are discussed.

3.1 Introduction

Bloodstream infections (BSI) are a global problem associated with serious health risks and hospital costs.¹⁻⁸ Once BSI is suspected, the clinical microbiologist plays a key role in managing patient care by (i) confirming the presence of a microorganism in a patient's blood, (ii) determining its gram status, (iii) and sub-culturing the causative microorganism for subsequent identification and antimicrobial susceptibility procedures.⁹⁻¹² Currently, blood culture is an essential tool for confirming a suspected BSI and is firmly established as the industry gold standard.¹³⁻¹⁷ However, even modern blood culture systems still require 1-2 days of incubation to detect the presence of most microbes,^{11,17,18} thus, they also represent a rate-limiting step by delaying the initiation of critical downstream testing such as organism identification and antimicrobial susceptibility procedures. Since delays in organism identification and antimicrobial susceptibility determination have been linked to decreased patient survival and increased healthcare costs,^{5,6,19,20} reducing Time-to-Detection (TTD) for BSI has potential to improve patient outcomes.²¹ Consequently, numerous nucleic acid and non-culture-based rapid microbial detection methods have been developed in an effort to reduce the TTD for BSI and subsequent administration of effective antimicrobial agents.²²⁻²⁴ Although, several drawbacks still exist that have limited the widespread adoption of such methodologies.²⁵ For example, 'universal' PCR assays have been criticized due to studies revealing that the sequences targeted by the primers are not actually universal.²⁶⁻²⁸

Therefore, there is still a pressing need for an assay that can rapidly and sensitively detect the presence of any microorganism present in a blood culture sample. If achieved, such an assay could improve patient outcomes by expediting the initiation of subsequent microbiological testing, thus decreasing the overall time until appropriate therapy is administered. Since 90% of all suspected BSI cultures are negative, another important advantage of reduced TTD for BSI would be to predict the true negative blood cultures earlier than the current gold standard, which could reduce the time and costs of unnecessary hospital stays and facilitate improved antimicrobial stewardship.

Our lab is actively developing assays based upon the enzymatic template generation and amplification (ETGA) methodology.²⁹ We recently reported the initial characterization of a

novel ETGA methodology termed DNA polymerase extension- polymerase chain reaction (DPE-PCR), which enabled sensitive, quantitative and universal detection of viable microbes.²⁹ In this study we set out to determine the feasibility of using ETGA to detect the presence of microorganisms within spiked blood culture samples via measurement of their endogenous DNA polymerase activity. Considering that blood culture samples contain vast numbers of DNA polymerase-harboring hematopoietic cells (HC), each sample theoretically has the potential to contribute significant background signal to the current version of the ETGA assay. Consequently, we set out to determine if a differential cell lysis procedure, that when combined with ETGA, could enable detection of microbes by selective measurement of their DNA polymerase activity within blood culture samples. To our knowledge the methodology and its application presented in this study represent an innovative approach towards improved TTD of BSI.

3.2 Materials and Methods

3.2.1 Blood collection from healthy donors

Eight to ten milliliters of blood were drawn from consented healthy donors into BD BACTEC™ Plus + Aerobic/F bottles (BD, cat# 442192). For the healthy donor screen used for establishment of the model system positivity threshold, five milliliters of blood were also drawn into a parallel EDTA tube (BD, cat# 1172785) and sent to LabCorp (Raritan NJ) for complete blood count (CBC) analysis.

3.2.2 Differential Cell Lysis Procedure

We adapted a differential cell lysis procedure using detergent and alkali based upon previous studies that demonstrated the ability of such reagents to effectively lyse HC, without negatively affecting microbes.³⁰⁻³² An additional schematic overview of the ETGA blood culture assay protocol is also presented (See Supplemental Figure S1). To begin, a 0.75 mL aliquot is removed from the blood culture bottle using a 21 gauge needle and transferred to a 1.5 mL tube already containing 250 uL of 1% Triton X-100. The sample tube is then capped and inverted four times to mix. The sample is incubated for 5 minutes at room temperature and then spun at 8000 x g

for 3 minutes. The supernatant is poured off into a waste container and the tube is then inverted onto a plastic-backed lab wipe to drain any residual liquid. Using an extended pipette tip, 1 ml of 5 mM NaOH is added and subsequently pipetted up and down ten times to resuspend blood debris. The sample tube is then capped, incubated for 5 minutes at room temperature and spun at 8000 x g for 3 minutes. The supernatant is poured off into a waste container and the tube is then inverted onto a plastic-backed lab wipe to drain any residual liquid. Next, 0.6 mL of wash buffer (20 mM Tris, 10 mM Ammonium sulfate, 10 mM Potassium chloride, 2 mM Magnesium sulfate) is added and pipetted up and down 5 times to resuspend the sample, and simultaneously transferred to pre-labeled bead mill lysis tube. At this stage, 0.6 mL of wash buffer is added to an additional beadmill tube that will serve as the No Input Control (NIC). Next, 0.6 mL of wash buffer is added to an additional beadmill tube that will become the DNA Polymerase Control (DPC). All beadmill lysis tubes are then spun at 8000 x g for 3 minutes. After spinning, supernatants are carefully removed using a 1 mL pipette. After aspirating the supernatant, the samples are now ready for the transitioning to the core ETGA assay (described below).

3.2.3 The core ETGA assay

Complete details regarding the composition of the core ETGA assay (including DNA substrate and primer/probe sequences) have been previously reported.²⁹ Briefly, 50 µL of DNA polymerase extension (DPE) reaction mixture [50 µM dNTP, 20 mM Tris pH 8.0, 10 mM Ammonium sulfate, 10 mM Potassium chloride, 2 mM Magnesium sulfate, 1% BSA, 0.1% Triton X-100, 0.1% Tween 20, and 0.001 µM pre-annealed DNA substrate] is added to bead mill tubes containing either a processed blood culture sample or NIC/DPC controls. Bead mill tubes containing DPE reaction mixture are placed into a disrupter adapter (Scientific Industries) and vortexed for 6 minutes at 2800 rpm. After milling, 5 µL of DNA polymerase (NEB, cat# M0209S pre-diluted to a concentration of 1×10^{-7} Units/µL) is added to the designated DPC tube and immediately transfer all tubes to 37° C for 20 minutes, then to 95° C for 5 minutes. Following the 95° C incubation, tubes are cooled to room temperature and spun at 12,000 x g for 30 seconds. Four microliters of each reaction are subsequently placed into a SMART Cyclor reaction tube containing 27.2 µL of a qPCR mastermix, resulting in a mixture comprised of [1X

LightCycler 480 Master Mix (from 2X stock, Roche cat# 04707494001), 333 nM of forward and reverse primers, 166 nM detection probe (FAM), 166 nM internal control probe (TxRed), 1.2 U of Uracil DNA Glycosylase (Bioline cat# BIO-20744) and 40 copies of a competitive Internal Control DNA. Thermocycling is performed using a SmartCycler (Cepheid, Sunnyvale CA) as follows: Initial incubation of 40° C for 10 minutes and 50° C for 10 minutes and at 95° C for 5 minutes, followed by 45 cycles of 5s denaturation at 95° C and 20s annealing/extension at 65° C. Cycle threshold (Ct) values are generated automatically by the SmartCycler software using 2nd derivative analysis of the emerging qPCR curves.

3.2.4 Detection of hematopoietic cell (HC)-derived DNA polymerase activity via ETGA

Since erythrocytes were not suspected to contain DNA polymerases and are an established source of DNA polymerase inhibitors,³³ we used SST fractionation to deplete erythrocytes from the HC prior to being subjected to ETGA assay analysis. To begin, three milliliters of whole blood from two healthy donors were each aseptically transferred from an EDTA tube to a Serum Separation tube (SST) (BD, cat# 367988). The SST's were centrifuged at 2,000 x g for 10 minutes. After spinning, the plasma supernatant was aspirated, and the erythrocyte-depleted HC were gently resuspended from the surface of the gel plug with 1mL of Phosphate Buffered Saline (PBS). Each HC suspension was transferred to a new tube, the volume was raised to 4mL with PBS, and centrifuged at 3000 x g for 3 minutes. The supernatant was removed and the HC pellets were resuspended again in 1mL of PBS, followed by raising the volume to 4mL. This procedure was repeated an additional two times for a total of three washes in a volume of 4mL. From each washed sample, 0.75mL was added to two bead-mill tubes. The bead-mill tubes were centrifuged at 8000 x g for 3 minutes and the supernatant was carefully aspirated down to the top of the bead bed. Samples were subsequently processed according to the core ETGA assay protocol procedure provided above. Since ddCTP has been previously demonstrated to block DNA polymerase-dependent extension of the ETGA assay substrate,²⁹ we decided to substitute ddCTP for dCTP in parallel DPE reactions containing HC lysates. Accordingly, the paired samples from each donor were subjected to DPE reactions with either a standard deoxynucleotide triphosphate mix (dATP, dCTP, dGTP, and dTTP), (New England Biolabs cat#

NO446S) or with a deoxynucleotide triphosphate mix substituted with ddCTP (dATP, dGTP, dTTP and ddCTP). The NIC and DPC were run in parallel and are described above. Following ETGA assay analysis, the samples were also analyzed for the presence of HC-derived genomic DNA (gDNA) using a BRAF qPCR assay (described below).

3.2.5 Reduction of HC-derived ETGA assay signal via the differential cell lysis procedure

Two aliquots of whole blood (3mL each from a single healthy donor) were each transferred from EDTA tubes into two SST's. From each SST, the HC suspensions were harvested, washed and pooled into a total of 8mL. Next, 0.75mL aliquots of the HC suspension were placed into six sterile 1.5 mL tubes containing 0.25 mL of 1% Triton X-100. Three of these tubes were immediately spiked with approximately 1,000 CFU of *S. aureus* and the other three were not spiked. These six samples were subsequently subjected to differential cell lysis and ETGA analysis as described above. Three additional pre-washed 0.75mL aliquots of the HC suspension were placed into bead-mill tubes and were processed in parallel using only the core ETGA assay in an effort to serve as 'non-lysed' comparators. The appropriate assay controls were included as described above. Following ETGA assay analysis, the lysates were also measured for the presence of *S. aureus* gDNA using a gene specific qPCR assay (described below.)

3.2.6 Description of gene specific qPCR Assays

A human gDNA assay was designed from the Homo sapiens v-raf murine sarcoma viral oncogene homolog B1 (BRAF). BRAF oligonucleotide sequences are as follows: Forward Primer 5'- TTGCCAGCTATCACATGTCC-3', Reverse Primer 5'- TAACCCAGGCTAACC GACTG-3', and hydrolysis probe /56-FAM/TG CCTGTGTTGCAGGTGAGAAGTTG /3IABkFQ/. A *C. albicans*-specific assay was designed against a yeast 18s rRNA sequence. *Candida albicans*-specific oligonucleotide sequences are as follows: Forward Primer 5'- TTTATCAACTGTACACCAG-3', Reverse Primer 5'- ATCCCGCCTTACCACTACCG -3', and hydrolysis probe /56-FAM/TGAACGCACATTGCGCCCTC /3IABkFQ/. All oligonucleotides were manufactured by Integrated DNA Technologies (Coralville, IA). The PCRs were prepared as such: 300nM forward and reverse

primers, 100nM hydrolysis probe, 12.5μL Light Cycler 480 Probes Master (Roche Applied Sciences cat# 04902343001), nuclease free water (Life Technologies, cat# AM9932) to a volume of 21μL, and 4μL of sample to be analyzed. The samples were analyzed on a Cepheid Smart Cycler II (Sunnyvale, CA) using the following protocol: 95°C for 5 minutes, and then 45 cycles of 95°C for 15 seconds followed by 60°C for 40 seconds where the optical data is collected. The gene specific qPCR assays targeted to the *nuc* gene of *S. aureus* and the *uidA* gene of *E. coli* were previously described.²⁹

3.2.7 Spike, recovery and detection of Gram positive and Gram negative bacterial panels from blood culture

The panel of Gram positive and Gram negative bacteria tested herein were chosen based upon their established prevalence among clinical blood culture isolates.²⁴ A complete list of all microbial strains (including source and growth conditions) used is provided (See Supplemental Table S1). For both Gram positive and Gram negative bacterial spikes, blood was collected into a BD BACTEC™ Plus + Aerobic/F bottle from a consented healthy donor as described above. After collection, the blood culture bottle was placed into a BACTEC 9050 incubator for approximately 1 hour until the dilution of the bacterial stocks were completed. Bacterial strains were prepared as follows. With the exception of *S. pneumoniae*, 2-3 freshly grown colonies of each strain were inoculated into 5 mL of BHI broth and were incubated at 37° C with shaking until reaching an OD₆₀₀ of 1.0 ± 0.2 (approximately 10⁹ CFU/mL). For *S. pneumoniae*, 1-2 freshly grown colonies were removed from a blood agar plate and resuspended in 1 mL of PBS (approximately 10⁶ CFU/mL). All strains were diluted in PBS to an approximate target concentration of 1000 CFU/ μL prior to spiking into blood culture. After bacterial strains were diluted to the target concentration of 1000 CFU/μL, 0.75 mL aliquots were removed from the blood culture bottle using a sterile syringe equipped with a 21 gauge needle and transferred to sterile 1.5 mL tubes. Ten microliters of each bacterial stock (at 1000 CFU/ μL) were immediately added to individual 0.75 mL blood culture aliquots. For each bacterial strain used, stocks were further diluted to 1 CFU/ μL and parallel plating of 100 μL were also performed in an effort to verify approximate bacterial input. Ten microliters of sterile PBS (used to dilute each bacterial strain) was added to an additional 0.75 mL blood culture aliquot to serve as a

“No spike” control (referred to hereafter as the NSC). After the addition of bacteria (and PBS for the NSC), 250 µL of 1% Triton X-100 were added to each sample. Tubes were subsequently capped, inverted and samples were subjected to the differential cell lysis and ETGA assay procedures as described above.

3.2.8 Model system threshold establishment via ETGA blood culture assay analysis of fifteen healthy donors

Blood culture bottles from a total of fifteen healthy donors were collected as four separate sample sets: Sample Set A = Donors 1-4, Sample Set B = Donors 5-8, Sample Set C = Donors 9-12, Sample Set D = Donors 13-15. Prior to aliquot removal, the BACTEC bottles were placed into a BACTEC 9050 incubator for 1 to 2 hours to enable collection of additional donors and to assay setup. After samples from all of the donors were collected, duplicate 0.75 mL aliquots of blood culture were removed from each BACTEC bottle using a sterile 1 mL syringe equipped with a 21 gauge needle and the differential cell lysis and ETGA assay procedure was performed immediately. After aliquot removal, the blood culture bottles were replaced into the BACTEC incubator for a routine 5 day period to confirm that the samples were negative. The raw ETGA assay data from fifteen healthy donors was exported from the SMART Cycler and re-assembled using Microsoft® Excel®. Samples that were assigned cycle threshold (Ct) values (derived from duplicate ETGA blood culture assay analysis of the fifteen healthy donors) were used to generate a model system threshold value with the following formula: **Threshold = [Average Ct - (3 x Standard Deviation Ct)]**.

3.2.9 Time course growth monitoring of *S. aureus*, *E. coli* and *C. albicans* in blood culture via ETGA blood culture protocol

For each strain tested, two blood culture bottles were collected from a consented healthy donor as described above. One bottle was designated for spiking of microbes and the other bottle served as the NSC. The presence of microbial growth versus time was subsequently assessed for spiked and donor-matched NSC blood culture bottles by BACTEC 9050 Time to Detection (TTD), ETGA blood culture assay, gene specific PCR (gsPCR) and traditional microbial plating analysis as follows. After collection, the blood culture bottles were placed into a

BACTEC incubator for approximately 1 hour during preparation of microbial stocks. For *C. albicans*, 2-3 freshly grown colonies were inoculated the previous day into YM broth and grown overnight at 30° C (Resulting in a stock at approximately 10⁸ CFU/mL). Bacterial stock cultures were grown as described above, and diluted in PBS to an approximate stock concentration of 1 CFU/μL prior to spiking into blood culture. For *C. albicans*, the culture was diluted in PBS to an approximate stock concentration of 20 CFU/μL prior to spiking into blood culture. For each strain, 100 μL of stock solution (at either 1 CFU/μL for bacteria or 20 CFU/μL for *C. albicans*) was added to a blood culture bottle using a sterile 1 mL syringe equipped with a 21 gauge needle. Microbial stocks were also plated to verify the approximate input levels. 100 μL of the appropriate sterile diluent was added to a parallel (donor matched) blood culture bottle to serve as the NSC. Spiked and non-spiked blood culture bottles were inverted to mix and duplicate 1 mL aliquots were immediately removed and labeled as “t = 0”. For time course analysis of bacterial spikes, the blood culture bottles were subsequently placed into the BACTEC incubator and duplicate 1 mL aliquots were removed at 2hr, 4hr, 6hr and 8 hr time points and stored at 4° C overnight in an effort to synchronize sample preparation. For time course analysis of *C. albicans*, 1 mL aliquots were removed at time zero and the blood culture bottles were returned to the BACTEC incubator. Duplicate 1 mL aliquots were subsequently removed at 6hr, 10hr, and 26hr time points. After overnight storage at 4° C (to synchronize sample preparation), a 0.75 mL portion of the time course aliquots were processed together according to the differential cell lysis and ETGA assay procedures. One hundred micro-liters of each unprocessed blood culture aliquot were also plated in parallel to monitor CFU during the time course incubation. Gene specific PCR was performed on 4 μL of the ETGA assay lysates derived from each of the time course samples as described above.

3.3 Results

3.3.1 Detection of DNA polymerase extension activity from erythrocyte-depleted HC

In this manuscript we propose a novel approach for rapid detection of microbes from blood culture samples via differential cell lysis followed by ETGA assay analysis, collectively referred to

hereafter as the ETGA blood culture assay (See Figure 1 diagram).

Figure 1 The novel approach for detecting microbes in blood culture samples via differential cell lysis followed by ETGA assay analysis is presented

Before acquiring and testing blood culture samples, we initially chose to assess the level of ETGA assay signal derived from DNA polymerase-harboring HC. As shown in Figure 2A, the erythrocyte-depleted HC fractions taken from two independent healthy donors yielded strong ETGA assay signals. Next, we wanted to confirm that the ETGA assay signal obtained from HC was dependent on DNA polymerase extension activity. To this end, ddCTP was substituted for dCTP within the DPE reaction mixture. The substitution of ddCTP for dCTP effectively blocked substrate extension and reduced the ETGA assay signal derived from HC to the level of assay baseline noise (Figure 2A). Quantitative PCR analysis of a human genomic DNA marker (BRAF) was performed on the same lysates used in Figure 2A and confirmed that equivalent amounts of nucleated HC were present in the ddCTP-containing DPE reactions (Figure 2B).

3.3.2 Selective reduction of HC-derived ETGA assay signal using the differential cell lysis procedure

By establishing that HC contribute ETGA assay signal in the absence of input microbes, we confirmed that our proposed approach for detection of microbes is reliant upon effective removal of HC-derived DNA polymerase extension activity. Consequently, the next objective was to determine if the proposed differential cell lysis procedure could selectively reduce the ETGA assay signal derived from erythrocyte-depleted HC while maintaining a microbe-derived signal. To this end, an additional blood sample was collected from a healthy donor and erythrocyte-depleted HC suspensions were prepared and subjected to ETGA assay analysis as described above. As shown in Figure 2C, 'non-lysed' HC were reproducibly detected by ETGA assay at a similar level to that shown previously in Figure 2A. However, after being subjected to the differential cell lysis procedure, ETGA assay signal derived from HC aliquots was reduced to assay baseline noise (Figure 2C). A graphical representation of ETGA data obtained from triplicate 0.75 mL aliquots of non-lysed and lysed HC is presented and is used to generate a 'Fold Reduction of ETGA assay Signal' value using the indicated equation (Figure 2D). In addition to effectively reducing ETGA assay signal from HC, our proposed differential cell lysis procedure must also enable detection of clinically relevant microbes. To this end, triplicate parallel 0.75 mL HC aliquots were spiked with approximately 10^3 CFU of *S. aureus* and subjected to the ETGA blood culture assay procedure. As shown in Figure 2C, after being processed by the ETGA blood culture assay, *S. aureus*-containing HC aliquots were easily distinguishable from the parallel non-spiked HC suspensions. Gene specific PCR was also performed on the ETGA lysates and confirmed the presence and absence of *S. aureus* in the spiked and non-spiked HC aliquots respectively (See Supplemental Figure S2).

Figure 2 (A) Erythrocyte-depleted HC taken from two independent blood donors were analyzed by the core ETGA assay. The resultant assay curves are presented and indicate the level of ETGA signal detected from these cells. Substitution of ddCTP for dCTP within parallel DPE reaction mixes block the generation of ETGA assay signal, thus confirming that the HC-dependent background signal is indeed derived from DPE activity (B) In an effort to verify that equivalent amounts of HC were present in the ddCTP-containing DPE reactions (shown in Figure 2A), qPCR was performed for the human genomic DNA marker BRAF. (C) Representative ETGA curves are presented and demonstrate that the proposed differential cell lysis procedure dramatically reduces the ETGA assay background signal derived from erythrocyte-depleted HC, while still enabling detection of *S. aureus*. (D) The average Ct values obtained from triplicate ETGA blood culture assay preparations (Figure 2C) were used to determine the ‘Fold Reduction of ETGA Signal’ that was achieved by the differential cell lysis procedure.

3.3.3 Establishment of an ETGA positivity threshold

Next, we set out to test the ability of the differential cell lysis procedure to prevent significant detection of DNA polymerase extension activity from negative blood culture samples. The primary goals of these experiments were to: 1.) verify that the differential cell lysis procedure is effective at removing ETGA signal from blood culture samples taken from healthy donors and 2.) establish a model system positivity threshold using the ETGA data obtained from the healthy donor screen. To begin, duplicate 0.75 mL blood culture aliquots (removed from blood culture

samples obtained from fifteen healthy donors) were separated into four sample sets and processed using the ETGA blood culture assay protocol. As shown in Figure 3A-D, a range of low-level ETGA blood culture assay background signals were detected from the fifteen donors analyzed. The raw data from all thirty ETGA blood culture assay preparations were extracted from the SMART Cyclyer software, combined and re-graphed using Microsoft® Excel® (Figure 3E). DNA polymerase positive controls were also included for each of the four separate ETGA blood culture assay runs in an effort to verify equivalent performance of the core ETGA assay reagents (Figure 3E). After collection and assembly, we subsequently performed statistical analysis of the ETGA blood culture assay data obtained from healthy donors and established a model system positivity Cycle Threshold (Ct) of 35.24 that could be used as an objective means for determining sample positivity in subsequent experiments presented herein (See formula and threshold value presented adjacent to Figure 3E).

Figure 3 (A-D) Duplicate blood culture aliquots derived from fifteen healthy donors were separated into four separate groups and were analyzed using the ETGA blood culture assay. ETGA assay curves are presented and indicate the level of background signal obtained from the various donors. (E) The data obtained from ETGA blood culture assay analysis of the fifteen healthy donors were re-assembled using Microsoft® Excel®. An ETGA blood culture assay model system positivity threshold value was subsequently generated using the indicated equation.

After aliquot removal, blood culture bottles from each of the fifteen healthy donors were returned to the BACTEC 9050 incubator for a routine 5 day period to verify they were not contaminated with microbes during the collection procedure and/or sample aliquoting. The corresponding complete blood cell counts (CBC) and BACTEC results obtained from the fifteen healthy donors were also obtained (See Supplemental Table S2).

3.3.4 The ETGA blood culture assay protocol enables detection of Gram positive and Gram negative bacteria spiked into blood culture samples

After determining that the ETGA blood culture assay was effective at limiting signal from numerous blood donors, we next wanted to verify that it enabled detection of a clinically prevalent panel of Gram positive and Gram negative bacteria. After performing ETGA blood culture assay analysis, all seven Gram positive bacteria were detected well-above the model system positivity threshold and were easily distinguishable from the NSC (Figure 4A). Bar graphs relating CFU input to ETGA blood culture assay signal are presented in Figure 4B. As shown in Figure 4C, signals obtained from ETGA blood culture assay analysis of all six Gram negative bacterial strains were also well above the model system positivity threshold, proportionate to CFU input and were easily distinguishable from the NSC. Bar graphs relating CFU input to ETGA blood culture assay signal are also presented for the Gram negative bacterial panel in Figure 4D.

Figure 4 (A) Seven Gram positive bacterial species were spiked into blood culture aliquots and were processed in parallel with an NSC using the ETGA blood culture assay protocol. The resultant ETGA curves are presented. (B) The ETGA assay data obtained from Figure 4A was used to generate a bar graph. In an effort to relate CFU input to ETGA Ct values, the approximate CFU input for each of the Gram positive bacterial species are present above each bar within the graph. (C) Six Gram negative bacterial species were spiked into blood culture

aliquots and were processed in parallel with an NSC using the ETGA blood culture assay protocol. The resultant ETGA curves are presented. (D) The ETGA assay data obtained from Figure 4C was used to generate a bar graph. In an effort to relate CFU input to ETGA Ct values, the approximate CFU input for each of the Gram negative bacterial species are present above each bar within the graph.

Independent experiments, aimed at ETGA blood culture assay-mediated detection of Gram positive and Gram negative bacteria in blood culture aliquots were repeated by a different operator and yielded very similar results. All blood culture bottles were replaced into the BACTEC incubator and no growth was detected after a routine 5 day period, confirming the samples were not contaminated during the manipulations associated with blood collection and aliquoting.

3.3.5 Monitoring of *S. aureus* time course growth in blood culture via the ETGA blood culture assay

We set out to assess the ability of the ETGA blood culture assay to detect simulated BSI by monitoring the time course growth of clinically prevalent microbes spiked into blood culture samples obtained from healthy donors. The first microorganism chosen for growth monitoring was *S. aureus*. To begin, growth of *S. aureus* (spiked at 100 CFU per bottle) was compared to an NSC bottle as described in the materials and methods section. The BACTEC 9050 instrument properly designated the bottle spiked with *S. aureus* as positive, with a TTD of 12.17 hours (Figure 5A). The BACTEC 9050 did not detect growth within the donor-matched NSC bottle after a routine 5 day incubation period (Figure 5B). Despite requiring 12.17 hours for detection by the BACTEC 9050 instrument, ETGA blood culture assay analysis of 0.75 mL blood culture aliquots detected the presence of *S. aureus* in aliquots removed after only 2 hours of incubation, and with decreasing Cycle Threshold (Ct) values (representing increasing amounts of *S. aureus*) at each of the subsequent time points (Figure 5A). ETGA blood culture assay analysis of aliquots taken from the donor-matched NSC bottle did not yield assay signal higher than the positivity threshold, and thus were in overall agreement with the BACTEC 9050 negativity result (Figure 5B). Parallel plating of the same blood culture time course aliquots confirmed the presence of viable *S. aureus*, correlated with the corresponding signals in Figure

5A & 5B and further verified that no cultivatable bacteria were present in the NSC bottle (Figure 5C and 5D). Although poorly visible within the presented image, 2 CFU of *S. aureus* were present on the perimeter of the 2 hour plate (See note under the 2 hour plate within Figure 5C). Gene specific PCR was also performed on the exact same lysates used to generate ETGA blood culture assay data and further confirmed the presence or absence of *S. aureus* growth in the spiked or non-spiked bottles respectively (Figure 5E and 5F).

Figure 5 (A) Time course-aliquots taken from a blood culture bottle spiked with *S. aureus* were analyzed by the ETGA blood culture assay. The CFU input, resultant ETGA curves, and BACTEC TTD are presented. (B) ETGA curves and BACTEC results are presented for the parallel time course analysis from a donor-matched NSC bottle. (C and D) Parallel plating was performed using 0.1 mL from each of the time course-blood culture aliquots and is presented directly below the respective ETGA curves. (E and F) *S. aureus*-specific qPCR analysis was also performed on the same lysates used to generate the time course-ETGA data presented in Figure 5 A and B. The resultant *S. aureus*-specific qPCR curves are presented.

3.3.6 Monitoring of *E. coli* time course growth in blood culture via ETGA blood culture assay

The next microorganism used to simulate a BSI was *E. coli*. Similar to *S. aureus*, 100 CFU of *E. coli* were spiked into a freshly obtained blood culture bottle and growth was compared to a donor-matched NSC via various methodologies. The BACTEC 9050 properly designated the bottle spiked with *E. coli* as positive, with a TTD of 12.5 hours (Figure 6A) and no growth was detected in the donor-matched NSC bottle after a routine 5 day incubation period (Figure 6B). ETGA blood culture assay analysis detected the presence of *E. coli* in aliquots removed as early as 2 hours after inoculation, and with decreasing Ct values at subsequent time points (Figure 6A). ETGA blood culture assay analysis of 0.75 mL aliquots taken from the donor-matched NSC bottle did not yield assay signal above the model system positivity threshold and were therefore in overall agreement with the BACTEC result (Figure 6B). Parallel time course plating analysis indicated presence of viable *E. coli*, correlated well with corresponding signals in Figure 6A & 6B and confirmed that the NSC was free of cultivatable bacteria (Figure 6C and 6D). Gene specific PCR analysis was performed on the exact same lysates used for ETGA blood culture assay analysis and further verified the presence of *E. coli* in the spiked samples, and its absence in aliquots derived from the NSC bottle (Figure 6E and 6F).

Figure 6 (A) Time course aliquots taken from a blood culture bottle spiked with *E. coli* were processed by the ETGA blood culture assay. The CFU input, resultant ETGA curves, and BACTEC TTD are presented. (B) ETGA curves and BACTEC results are presented for the parallel time course analysis from a donor-matched NSC bottle. (C and D) Parallel plating was performed using 0.1 mL from each of the time course-blood culture aliquots and is presented directly below the respective ETGA curves. (E and F) *E. coli*-specific qPCR analysis was also performed on the same lysates used to generate the time course-ETGA data presented in Figure 6 A and B. The resultant *E. coli*-specific qPCR curves are presented.

3.3.7 Monitoring of *C. albicans* time course growth in blood culture via ETGA blood culture assay

In addition to bacteria, we felt it was important to assess whether the ETGA blood culture assay could detect and monitor the growth of *C. albicans* spiked into a blood culture sample.

Previous reports have demonstrated that simulated Candidemia exhibits inconsistent blood culture positivity and increased TTD when spiked at as much as 1000 CFU per bottle.^{34, 35}

Therefore, to minimize nonlinear growth behavior for feasibility of growth monitoring, we targeted 1000 CFU which resulted in 2000 CFU of *C. albicans* that were spiked into a freshly

obtained blood culture bottle and growth was compared to a donor-matched NSC via various methodologies. The BACTEC 9050 properly designated the blood culture bottle spiked with 2000 CFU of *C. albicans* positive, with a TTD of 21 hours (Figure 7A). Again, no growth was detected by the BACTEC 9050 in the donor-matched NSC bottle after a routine 5 day incubation period (Figure 7B). Likely due to the inoculation level, the ETGA blood culture assay was able to detect the presence of *C. albicans* above the positivity threshold at time zero and effectively monitored its growth at subsequent time points (Figure 7A). Once again, ETGA blood culture assay analysis of 0.75 mL aliquots taken from the donor-matched NSC bottle did not yield assay signal above the model system positivity threshold and were therefore in overall agreement with the BACTEC result (Figure 7B). Parallel time course plating of blood culture aliquots (Figure 7C and 7D) demonstrated that the presence of viable *C. albicans* CFU correlated well with the corresponding ETGA assay signals in (Figure 7A & 7B) and confirmed the absence of *C. albicans* growth within the NSC bottle. Parallel gsPCR analysis of these exact same ETGA blood culture lysates also confirmed the presence of *C. albicans* genomic DNA in aliquots taken from the spiked bottle and its absence within the NSC bottle (Figure 7E and 7F).

Figure 7 (A) Time course-aliquots taken from a blood culture bottle spiked with *C. albicans* were processed by the ETGA blood culture assay. The CFU input, resultant ETGA curves, and BACTEC TTD are presented. (B) ETGA curves and BACTEC results are presented for the parallel time course analysis from a donor-matched NSC bottle. (C and D) Parallel plating was performed using 0.1 mL from each of the time course-blood culture aliquots and is presented directly below the respective ETGA curves. (E and F) *C. albicans*-specific qPCR analysis was also performed on the same lysates used to generate the time course-ETGA data presented in Figure 7 A and B. The resultant *C. albicans*-specific qPCR curves are presented.

3.3.8 Assembly of microbial time course growth data

ETGA blood culture assay and gsPCR data obtained from *S. aureus*, *E. coli* and *C. albicans* growth monitoring experiments (Figures 5-7) were subsequently assembled into table format. For ETGA blood culture assay analysis, a positive or negative call was assigned to each sample result using the model system positivity threshold as a guide. For gsPCR, a positive or negative call was assigned to each sample result via the presence or absence of a detectable amplification curve via the SMART Cycloer™ software. Complete time course results for each of the organisms tested are presented in Tables 1-3.

Sample ID	ETGA Ct	ETGA Result*	gsPCR Ct	gsPCR Result [†]
0 hr- No spike	36.3	Negative	ND	Negative
2 hr- No spike	37.9	Negative	ND	Negative
4 hr- No spike	37.4	Negative	ND	Negative
6 hr- No spike	36.1	Negative	ND	Negative
8 hr- No spike	37.1	Negative	ND	Negative
0 hr- Spike	35.9	Negative	ND	Negative
2 hr- Spike	32.0	Positive	40.3	Positive
4 hr- Spike	28.5	Positive	36.1	Positive
6 hr- Spike	25	Positive	32.1	Positive
8 hr- Spike	19.1	Positive	26.5	Positive

* ETGA positive ≤ 35.24 Ct

† gsPCR positive = detectable Ct by SMART Cyclor software

ND = No Ct determined by SMART Cyclor software

Table 1 The ETGA and *S. aureus*-specific qPCR data obtained from simulated BSI experiments (presented in Figure 5) were assembled. A positive or negative result was assigned to each sample using the model system threshold value pre-determined in Figure 3.

Sample ID	ETGA Ct	ETGA Result*	gsPCR Ct	gsPCR Result [†]
0 hr- No spike	37.5	Negative	ND	Negative
2 hr- No spike	36.8	Negative	ND	Negative
4 hr- No spike	37.1	Negative	ND	Negative
6 hr- No spike	36.7	Negative	ND	Negative
8 hr- No spike	36.5	Negative	ND	Negative
0 hr- Spike	35.9	Negative	ND	Negative
2 hr- Spike	32.4	Positive	37.4	Positive
4 hr- Spike	30.1	Positive	37.8	Positive
6 hr- Spike	26.6	Positive	35.7	Positive
8 hr- Spike	20.9	Positive	30.8	Positive

* ETGA positive ≤ 35.24 Ct

[†] gsPCR positive = detectable Ct by SMART Cyclor software

ND = No Ct determined by SMART Cyclor software

Table 2 The ETGA and *E. coli*-specific qPCR data obtained from simulated BSI experiments (presented in Figure 6) were assembled. A positive or negative result was assigned to each sample using the model system threshold value pre-determined in Figure 3.

Table 3. Assembled *C. albicans* time course results

Sample ID	ETGA Ct	ETGA Result*	gsPCR Ct	gsPCR Result†
0 hr- No spike	36.7	Negative	ND	Negative
6 hr- No spike	37.0	Negative	ND	Negative
10 hr- No spike	36.7	Negative	ND	Negative
26 hr- No spike	35.7	Negative	ND	Negative
0 hr- Spike	34.8	Positive	ND	Negative
6 hr- Spike	33.5	Positive	34.5	Positive
10 hr- Spike	32.0	Positive	33.8	Positive
26 hr- Spike	25.0	Positive	24.3	Positive

* ETGA positive ≤ 35.24 Ct

† gsPCR positive = detectable Ct by SMART Cyclor software

ND = No Ct determined by SMART Cyclor software

Table 3 The ETGA and *C. albicans*-specific qPCR data obtained from simulated BSI experiments (presented in Figure 7) were assembled. A positive or negative result was assigned to each sample using the model system threshold value pre-determined in Figure 3.

3.4 Discussion

Herein, we set out to assess the feasibility of using ETGA methodology to detect the presence of microbes within spiked blood culture samples via measurement of endogenous microbial DNA polymerase activity. To our knowledge, detection of microbes in blood culture via this approach has never been attempted and therefore represents a novel methodology for rapid detection of BSI. However, an obvious theoretical barrier to detection of BSI via ETGA stemmed from the fact that blood culture samples contain DNA polymerase-harboring HC, such as leukocytes and thrombocytes (Figure 1). In this study, we first confirmed that erythrocyte-depleted HC represent a source of DNA polymerase-dependent background signal and therefore must be controlled sufficiently to enable sensitive detection of microbes via ETGA. When bearing in mind our previous ETGA methodology characterization studies,²⁹ we estimated that the average Ct value obtained from triplicate preparations of lysed HC

(presented in Figure 2D) is less than or equal to the Ct value that can be expected from a single bacterial CFU. Taken together, these data suggested that the differential cell lysis procedure was effective at reducing the HC-dependent background signal, thus potentially enabling detection of low amounts of microbes within a blood culture sample.

On a separate, but mentionable note, previous reports suggest that monitoring DNA polymerase activity in HC is of clinical interest.^{36,37} Therefore, the capability of the ETGA assay to quantitatively measure DPE activity from 'non-lysed' HC thus represents another potentially important research and/or clinical utility for this methodology.

After demonstrating effective reduction of HC-derived background signal, we assessed the ability of the ETGA blood culture assay to detect a panel of clinically prevalent bacteria. The results from these experiments demonstrated that the ETGA signal from the spiked blood culture aliquots were proportionate to CFU input and easily distinguishable from non-spiked aliquots. These results further verified that the selectivity of the differential cell lysis procedure is sufficient to prevent significant contribution of HC-derived DNA polymerase activity, but gentle enough to still enable ETGA-mediated detection of clinically prevalent Gram positive and Gram negative bacteria. These results also reinforced the potential universality of our novel approach and to our knowledge represent the first example of detection of bacteria from blood culture samples via measurement of their endogenous DNA polymerase activity. When considered together, the established sensitivity of the core ETGA assay,²⁹ and the effective reduction of background signal from HC via differential cell lysis, suggested that the ETGA blood culture assay has the potential to enable detection of microbes within blood culture samples earlier than traditional blood culture methodology.

To test the feasibility of this hypothesis, we simulated BSI and analyzed for positivity via four independent methodologies: 1.) traditional blood culture TTD (BACTEC 9050), 2.) CFU plating, 3.) gsPCR of genomic DNA and 4.) ETGA blood culture assay time course for TTD.

Staphylococcus aureus (Gram-positive bacteria), *E. coli* (Gram-negative bacteria) and *C. albicans* (yeast) were chosen for growth monitoring experiments due to their high prevalence among clinical BSI isolates.^{8,24,38} The BACTEC TTD values obtained from our simulated bacterial BSI are similar to a range previously determined from clinical samples³⁹ and therefore support the

potential clinical translatability of our experimental conditions. Contrary to the lengthy times required for BACTEC detection, the ETGA blood culture assay detected both *S. aureus* and *E. coli* from sample aliquots taken after only 2 hours of incubation. This result is also impressive from a bacterial load perspective, as our experimental inoculums represent a starting bacterial concentration of approximately 2.5 CFU per mL in the blood culture bottle, which simulates an original concentration of approximately 10 CFU per mL of whole blood. Also, considering that up to 50% of whole blood samples from patients with confirmed BSI have been found to contain microbial concentrations ≥ 10 CFU/mL,⁴⁰ our data suggest that the ETGA blood culture assay has potential to reduce the BSI TTD of clinical samples with similar microbial concentrations. Gene specific PCR analysis was also run from the same lysates used to generate ETGA data and confirmed the presence and relative amounts of *S. aureus* and *E. coli* in the simulated BSI samples analyzed via amplification of their genomic DNA. Furthermore, these data also suggest that nucleic acid-based microbial species identification and resistance mechanism interrogation can be performed subsequently to, or simultaneously with, ETGA blood culture assay analysis; assuming that the bacterial concentration within the analyzed blood culture aliquot is within the limit of detection for the particular nucleic acid-based assay being employed. Since microbial identification is required for guiding optimal antimicrobial susceptibility testing, these data further demonstrate the potential value added if the ETGA blood culture assay were to eventually be integrated within a routine clinical laboratory workflow.

Bloodstream infections caused by *Candida* species are also a significant cause of patient morbidity and mortality;⁴¹ thus we felt it was important to test the feasibility of detecting simulated BSI using *C. albicans*. As stated before, simulated Candidemia has been previously shown to exhibit inconsistent blood culture positivity when spiked at approximately 1000 CFU per bottle and can require extended incubation times.^{34,35} Thus, *C. albicans* spikes were performed here at slightly above 1000 CFU per bottle in an effort to minimize nonlinear growth of *C. albicans* during feasibility studies. Although our *C. albicans* CFU spike level is likely higher than what would be observed from a typical clinical sample at the time of draw, it still translates into only 60 CFU in the initial ($t = 0$) 0.75mL blood culture aliquot (confirmed by

plating in Figure 7C); thus, suggesting that the ETGA blood culture assay also has potential to detect the presence of fungi with impressive sensitivity. ETGA blood culture assay lysates also contained analyzable *C. albicans* genomic DNA, which further demonstrates that our procedure is amenable to simultaneous and/or subsequent bacterial and fungal species identification using PCR-based methodologies as well.

In its current manual form, the entire ETGA blood culture assay procedure requires approximately 2 hours turnaround time to obtain a result. Thus, 2 hours must be added to the amount of time each blood culture sample spent in the BACTEC incubator prior to being aliquotted in order to obtain a more realistic ETGA TTD for comparison the to continuous-monitoring blood culture system. Therefore, for the simulated bacteremias presented herein, the actual ETGA TTD totaled 4 hours versus the corresponding continuous-monitoring blood culture TTD of 12 hours, yielding a 3-fold reduction in TTD. Considering the liquid handling, centrifugation steps, and requirements of contamination control, it would likely be difficult to integrate the current manual form of the ETGA blood culture assay procedure into today's busy clinical laboratory workflow. However, it is reasonable to expect that the 2 hours required for the current non-optimized manual ETGA blood culture assay procedure could be cut in half via ETGA automation where lengthy centrifugation and liquid handling steps could be replaced with more rapid filtration steps, as well as robotic manipulations. Automation of ETGA could also control variations associated with human manipulation and provide random-access capabilities; all of which could strengthen the potential for the ETGA assay to be utilized as a BSI screening tool within the clinical setting. Once BSI is confirmed by ETGA, existing sample lysates could be subsequently interrogated by nucleic acid-based assay for species identification and antimicrobial resistance mechanisms, thus shortening the overall time required for initiation of data driven antimicrobial susceptibility testing and therapy.

In conclusion, we have demonstrated feasibility of a novel approach for detecting the presence of microbes within spiked blood culture samples via selective measurement of their DNA polymerase extension activity. The collective data generated from our simulated BSI experiments also strongly suggest that the ETGA blood culture assay has the potential to reduce the TTD for clinical BSI caused by either bacteria or fungi, when compared to traditional

methods. By detecting the presence of microbes via the highly conserved biochemical activity of DNA polymerase extension, the ETGA blood culture assay protocol potentially represents a bona fide universal microbe screening method and could prove useful for rapidly detecting BSI derived from a more diverse range of microbial species than other methodologies such as 'universal' PCR assays (e.g. those that detect 16S sequences). Since ETGA sample lysates also provide analyzable microbe-derived nucleic acids, species identification and antimicrobial susceptibility mechanisms can be readily interrogated from existing ETGA-positive samples. In addition to reduced TTD of true BSI positives, the ETGA blood culture assay may have potential to predict 'true BSI negative' blood cultures much earlier than current blood culture systems; which could reduce the time and costs of unnecessary hospital stay. To this end, we are currently planning to assess the performance of the ETGA blood culture assay in the clinical setting. We are also considering additional procedural and reagent modifications aimed at detecting microbes directly from whole blood. By eliminating the need for culture, such modifications of the ETGA procedure could further reduce the TTD of BSI.

3.5 Supplemental Data

Fig. S1 - Schematic overview of the ETGA blood culture assay procedure

Fig. S2 Parallel gene specific PCR for *S. aureus* derived from sample lysates used to generate Figure 2C

Table S1 - Microbial strains and growth media

Microorganism	ATCC #	Liquid Growth Media	Solid Growth Media
<i>Escherichia Coli</i>	25922	Brain Heart Infusion	Brain Heart Infusion
<i>Klebsiella pneumoniae</i>	700721	Brain Heart Infusion	Brain Heart Infusion
<i>Pseudomonas aeruginosa</i>	10145	Brain Heart Infusion	Brain Heart Infusion
<i>Enterobacter cloacae</i>	13047	Brain Heart Infusion	Brain Heart Infusion
<i>Acinetobacter baumannii</i>	15308	Brain Heart Infusion	Brain Heart Infusion
<i>Enterobacter aerogenes</i>	15038	Brain Heart Infusion	Brain Heart Infusion
<i>Enterococcus faecalis</i>	51299	Brain Heart Infusion	Brain Heart Infusion
<i>Enterococcus faecium</i>	19434	Brain Heart Infusion	Brain Heart Infusion
<i>Streptococcus pyogenes</i>	19615	Brain Heart Infusion	Brain Heart Infusion
<i>Streptococcus agalactiae</i>	13813	Brain Heart Infusion	Brain Heart Infusion
<i>Streptococcus pneumoniae</i>	6303	Resuspended Colony	Blood Agar
<i>Staphylococcus epidermidis</i>	12228	Brain Heart Infusion	Brain Heart Infusion
<i>Staphylococcus aureus</i>	25923	Brain Heart Infusion	Brain Heart Infusion
<i>Candida albicans</i>	18804	YM	YM

Table S2 - Complete Blood Counts (CBC) and BACTEC results from healthy donor panel

Donor #	Age	Sex	WBC (1x10 ³ /μL)	RBC (1x10 ⁶ /μL)	Platelets (1x10 ³ /μL)	BACTEC Result
1	42	M	6.5	5.15	239	Negative
2	24	M	7.3	5.35	306	Negative
3	40	M	5.3	4.91	180	Negative
4	48	F	6.5	4.04	288	Negative
5	25	F	4.6	4.32	171	Negative
6	68	M	5.6	4.71	227	Negative
7	39	F	4	4.25	225	Negative
8	68	F	5.6	4.7	264	Negative
9	58	F	4.4	4.61	256	Negative
10	33	M	8.7	5.33	198	Negative
11	54	M	5.7	4.92	190	Negative
12	24	F	6.4	4.53	297	Negative
13	27	F	6.4	4	223	Negative
14	52	M	5.1	4.45	214	Negative
15	36	M	6.7	4.95	256	Negative

WBC Reference Interval = 4.0 -10.5 x 10³/μL

RBC Reference Interval = 4.10 - 5.60 x 10⁶/μL

Platelets Reference Interval = 140 - 415 x 10³/μL

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Chapter 4

Measurement of Microbial DNA Polymerase Activity Enables Detection and Growth Monitoring of Microbes from Clinical Blood Cultures

Daniel R. Zweitzig, Nichol M. Riccardello, John Morrison, Jason Rubino, Jennifer Axelband, Rebecca Jeanmonod, Bruce I. Sodowich, Mark J. Kopnitsky, S. Mark O'Hara

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Abstract

Surveillance of bloodstream infections (BSI) is a high priority within the hospital setting. Broth-based blood cultures are the current gold standard for detecting BSI, however they can require lengthy incubation periods prior to detection of positive samples. We set out to demonstrate the feasibility of using enzymatic template generation and amplification (ETGA)-mediated measurement of DNA polymerase activity to detect microbes from clinical blood cultures. In addition to routine-collected hospital blood cultures, one parallel aerobic blood culture was collected and immediately refrigerated until being transported for ETGA analysis. After refrigeration holding and transport, parallel-collected cultures were placed into a BACTEC incubator and ETGA time-course analysis was performed. Of the 308 clinical blood cultures received, 22 were BACTEC positive, and thus were initially selected for ETGA time course analysis. The ETGA assay detected microbial growth in all 22 parallel-positive blood cultures in less time than a BACTEC incubator and also yielded genomic DNA for qPCR-based organism identification. In summary, feasibility of detecting microbes from clinical blood culture samples using the ETGA blood culture assay was demonstrated. Additional studies are being considered towards development of clinically beneficial versions of this methodology.

4.1 Introduction

Bloodstream infections (BSI) can cause sepsis, which is associated with significant patient morbidity and mortality [1,2]. The health care costs related to sepsis are also high. In fact, a recent study estimated that the annual economic burden associated with BSI care in the United States is \$17 billion [2]. It should therefore not be surprising that surveillance and detection of BSI is of high priority within the hospital setting [3].

Broth-based blood culture is the current gold standard test for detecting BSI and is a prerequisite to additional downstream microbiology testing aimed at guiding appropriate therapy [4-8]. This approach is problematic, as sepsis can rapidly progress over the course of hours, while blood culture results can take days to confirm the presence of a hematopathogen [3,8,9]. Since delays in administration of proper antimicrobials have been shown to increase morbidity and mortality rates [10], patients are often prescribed empiric broad spectrum antimicrobial therapy with the hope of narrowing the antibiotic coverage once the pathogen is identified to the genus or species level. Unfortunately, this practice contributes to suboptimal therapy and the increasing problem of multidrug-resistant organisms through selection pressure and may also contribute to superinfections [11,12].

To address these issues, numerous rapid nucleic acid tests (NAT) have been developed with the goal of reducing the time to confirmation of BSI as well as pathogen identification (ID) and antimicrobial susceptibility determination [13-15]. Despite showing promise, adoption of NAT within the clinical microbiology laboratory has been met with resistance for numerous reasons including high costs and the inability of NAT to distinguish live microbes from circulating nucleic acid [16]. Also, studies have revealed that the broad range detection of microbes via NAT, such as 16S-targeted PCR, may not represent bona fide universal detection assays due to sequence variability across species [17-19].

We recently developed a novel methodology that enables rapid, sensitive, and universal detection of viable microbes via enzymatic template generation and amplification (ETGA)-mediated measurement of endogenous DNA polymerase activity [20]. We subsequently used a differential cell lysis procedure in combination with ETGA (referred to hereafter as the ETGA Blood Culture Assay) to enable detection of microbes 3 times faster than a continuous

monitoring blood culture incubator during simulated BSI experiments [21]. Herein, we set out to verify that the ETGA Blood Culture Assay could also detect the presence and growth of microorganisms in clinical blood culture samples.

4.2 Materials and Methods

4.2.1 Hospital Setting and Patient Enrollment

Saint Luke's Hospital and Health Network Internal Review Board (IRB), Bethlehem PA, approved this study protocol, identification number, SLHN 2011-04. Patients were screened for enrollment at a Level 1 community trauma center with a 37-bed Intensive Care Unit (ICU), a 45-bed Emergency Department (ED), and an annual census of 75,000. Adult patients (age 18 or older) in the ED and ICU were eligible for inclusion if they presented with signs and symptoms suggestive of serious bacterial infection and if their clinical evaluation included blood cultures at the discretion of the enrolling physician. Upon determination of eligibility, subjects or their health care proxies were approached to volunteer for participation; written informed consent was obtained prior to enrollment.

4.2.2 Clinical Blood Culture Sample Collection and Subsequent

Handling

Upon voluntary written consent enrollment, patients underwent a routine blood culture collection procedure as per hospital protocol. Blood culture protocol typically consists of two sets of blood culture bottles (2 aerobic BD-cat# 442192 and 2 anaerobic BD-cat# 442191) collected using aseptic technique. Blood was collected simultaneously into a third aerobic blood culture bottle which was designated only for parallel BACTEC and ETGA blood culture assay analysis (no analyses from this specimen were used in clinical decision making).

Hereafter, the third parallel-collected aerobic culture bottle will be referred to as the ETGA-bottle. All 4 hospital culture bottles were incubated in BD BACTEC™ automated blood culture system (BD Company, Franklin Lakes, NJ) in the hospital lab. A hospital blood culture was defined as positive using criteria outlined by the Centers for Disease Control [22], and the causative microorganism was subsequently identified according to standard clinical microbiology procedures. The ETGA-bottle was routinely drawn last in the draw sequence,

blind coded, stored in a refrigerator, and transported twice-weekly 50 miles to our laboratory for independent BACTEC and ETGA analysis.

4.2.3 BACTEC and ETGA Time Course Analysis of Clinical Blood

Cultures

At the initiation of this study, ideal resources were not available for around-the-clock time course ETGA monitoring of the ETGA-bottle within the hospital laboratory. Therefore, the ETGA-bottle was refrigerated prior to being transported to our laboratory for ETGA analysis. Although refrigeration is not recommended by CLSI [23], recent studies have shown that microbial growth is unaffected after limited refrigeration of blood culture samples [24]. After being transported from the hospital, the ETGA-bottle was placed into a BACTEC 9050 incubator. One milliliter aliquots were aseptically removed in a time course manner using a 3 mL syringe (21ga. Needle) and were immediately refrigerated to enable synchronization of the ETGA sample preparation procedure. For BACTEC-positive ETGA-bottles, blood culture time course aliquots were promptly removed from refrigeration holding and 0.75 mL was subjected to the ETGA blood culture assay procedure (described below). One hundred micro-liters from each of the remaining blood culture aliquots (non-processed) were also immediately plated onto blood agar for parallel CFU monitoring. After completion of the ETGA blood culture assay procedure, sample lysates were frozen at -20°C to enable subsequent nucleic acid analysis. For BACTEC-positive ETGA-bottles, microbe identification (derived from the corresponding hospital blood cultures) were retrospectively obtained from the hospitals' microbiology laboratory.

4.2.4 The ETGA Blood Culture Assay Procedure

The ETGA Blood Culture Assay consists of a differential cell lysis procedure followed by measurement of microbe-derived DNA polymerase extension activity via ETGA. A detailed description of the core ETGA technology and subsequent development of the ETGA Blood Culture Assay have been recently described [20, 21]. Briefly, 0.75 mL of blood culture is transferred to a 1.5 mL tube already containing 250 μL of 1% Triton X-100. The sample tube is then capped, inverted four times to mix, and incubated for 5 minutes at room temperature. After spinning at $8000 \times g$ for 3 minutes, the supernatant is poured off into a waste container

and the tube is then inverted onto a plastic-backed lab wipe to drain any residual liquid. Using an extended pipette tip, 1 ml of 5 mM NaOH is added and subsequently pipetted up and down ten times to resuspend blood debris. The sample tube is then capped, incubated for 5 minutes at room temperature, and spun at 8000 x g for 3 minutes. The supernatant is poured off into a waste container and the tube is then inverted onto a plastic-backed lab wipe to drain any residual liquid. Next, 0.6 mL of a Tris-based wash buffer is added, pipetted up and down 5 times to resuspend the sample, and simultaneously transferred to pre-labeled bead mill lysis tube. All beadmill lysis tubes are then spun at 8000 x g for 3 minutes. After spinning, supernatants are carefully removed using a 1 mL pipette and DNA polymerase extension activity is assayed as described previously [20, 21].

4.2.5 Gene specific qPCR Assays

The primers and probes for the *S. aureus* and *E. coli*-specific assays have been previously reported [20]. The primer and probe sequences for the *S. agalactiae* and *S. epidermidis* assays are as follows: *S. agalactiae* - Forward primer 5' -TCGCATTTTAGATCCATTTC- 3', Reverse primer 5' -GCTCTATCAGTTGGTTTTAAATCAGG- 3', Probe 5' -CAATTAAAGCTCAAGTTAACGATGTAAAGGCA 3'. *S. epidermidis* - Forward primer 5' -CAACTCGATGCA AATCAGCAA- 3', Reverse primer 5' - GAACCGCATAGCTCCCTGC- 3', Probe 5' - TACTACGCTGGTGGAACTCAAA TCGTTATCG- 3'. The qPCR reactions were prepared as follows: 300nM forward and reverse primers, 100nM hydrolysis probe, 15µL Light Cyclor 480 Probes Master (Roche Applied Sciences cat# 04902343001), nuclease free water (Life Technologies, cat# AM9932) to a volume of 26µL, and 4µL of sample. The samples were analyzed on a Cepheid Smart Cyclor II (Sunnyvale, CA) using the following protocol: 95°C for 5 minutes, and then 45 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. Fluorescence was measured during the 60°C stage of each cycle.

4.3 Results and Discussion

4.3.1 BACTEC Results and Corresponding Microbial ID

In this report, we set out to assess the feasibility of detecting microbes from clinical blood culture samples via selective measurement of their DNA polymerase extension activity using ETGA. A schematic diagram of the proposed experimental approach is presented in Figure 1.

Figure 1. A schematic overview of the study design is presented.

Initially, 308 ETGA-bottles were collected and subjected to BACTEC analysis. Of these, 22 yielded positive BACTEC results (Table 1). The microbial ID for each of these 22 samples was obtained retrospectively from the parallel-collected blood culture samples that were analyzed by the hospital laboratory, and are also presented in Table 1. The most prevalent organisms found to be associated with the BACTEC-positive samples were *E. coli* and *Streptococcus sp.*, followed by *Staphylococcus sp.* Consistent with previous observations, these species are common among BSI-causing microorganisms [14].

4.3.2 Growth Monitoring of Clinical Blood Cultures via the ETGA Blood Culture Assay

As mentioned, the primary goal of this study was to assess the feasibility of using the ETGA blood culture assay to detect the presence and growth of microbes within clinical blood cultures. Towards this goal, ETGA blood culture assay time course analysis was performed for each of the 22 ETGA-bottles that were called positive by the BACTEC instrument. The ETGA assay time course analysis data for these samples were assembled into 5 separate groups and are presented in Figures 2A-E. A horizontal red line was incorporated into each graph, representing a positivity threshold cycle threshold (Ct) value of 35.24 that was previously established from ETGA Blood Culture Assay analysis of blood cultures obtained from healthy donors [21]. Despite being determined previously, the 35.24 threshold Ct marker is included as a visual reference within the time course graphs to help differentiate microbial growth from typical blood culture sample background noise. As shown in Figures 2A-E, the ETGA blood culture assay sensitively detected the presence and growth of microbes within all 22 of the ETGA-bottles. The ETGA Ct values and parallel CFU plating data for these samples also correlate well with one another (Tables S2-S6). Of note, the growth curve derived from sample ZSL-229 dipped below the positivity threshold at the 6 hour time point (Figure 2C). This atypical growth curve behavior correlates well with the CFU fluctuation and/or mixed microbial population observed from parallel plating analysis of sample aliquots taken from the corresponding ETGA-bottle (Figure S1).

Figure 2. ETGA time course monitoring of clinical blood cultures (A-E) BACTEC-positive ETGA-bottles that underwent time course monitoring were separated into 5 groups and linear plots were generated using their respective ETGA Blood Culture Assay Ct values versus the time each culture bottle spent within the BACTEC instrument. A horizontal red line, representing a previously determined ETGA blood culture assay positivity threshold, is also included in each plot as a visual reference. The sample ID numbers are also included to the right of the curves in each graph and can be used to locate the corresponding microbial ID and BACTED TTD values using Table 1. (F) Linear plots are also presented from ETGA time course analysis of 15 BACTEC-negative ETGA-bottles.

Taken together, these preliminary time course studies suggest that the ETGA blood culture assay has potential to reduce the Time-to-Detection (TTD) of microbes from clinical samples,

when compared to standard BACTEC analysis (Table S1). In fact, ETGA detected 100% (4 of 4) of the samples containing *S. aureus* at time zero (Figure 2B). Also, considering the range of bacterial species present (Table 1), these data support the potential universality of detecting microbes from clinical samples via the highly conserved biochemical activity of DNA polymerase extension.

Sample ID	Hospital Microbe ID	BACTEC Result (TTD hours)
4	E. coli	Positive (11.67)
12	E. coli	Positive (10.83)
39	E. coli	Positive (14.83)
63	E. coli	Positive (10.67)
183	E. coli	Positive (13.17)
254	E. coli	Positive (10.67)
256	E. coli	Positive (11.00)
208	S. aureus	Positive (12.33)
258	S. aureus	Positive (10.67)
273	S. aureus	Positive (8.33)
190	S. aureus	Positive (15.17)
255	CoNS	Positive (22.67)
165	CoNS	Positive (26.83)
5	Beta h strep - Group A	Positive (11.00)
210	Beta h strep - Group C	Positive (11.83)
217	Beta h strep - Group B	Positive (13.33)
289	Beta h strep - Group G	Positive (19.83)
86	Proteus	Positive (13.33)
114	Abiotrophia/granulicatella	Positive (30.67)
229	Alpha haemolytic strep	Positive (22.67)
95	S. pneumoniae	Positive (14.33)
301	S. pneumoniae	Positive (14.83)

Table 1. BACTEC TTD values are presented for the ETGA-bottles. Microbial ID’s provided by parallel analyses of the corresponding cultures at the Hospital’s clinical microbiology laboratory are also presented.

Furthermore, when considering these clinical samples and previously published data obtained during assay development studies [20, 21], ETGA has detected without fail more than 30 different microbial species.

In addition to the 22 positive samples, time course aliquots from 15 BACTEC-negative ETGA-bottles were subjected to ETGA blood culture assay time course analysis. As shown in Figure 2F, all of the ETGA blood culture assay signals derived from the 15 negative clinical samples remained below the positivity Ct threshold, and are thus consistent with the previously determined healthy donor threshold [21]. Therefore, these results demonstrate that the growth curves presented in Figures 2A-E are specific for microbial growth and not reflective of artifactual changes in assay background signal due to the time that these negative blood cultures spent in refrigeration and/or in the BACTEC incubator. For more accurate clinical diagnostic positivity/negativity and ETGA TTD determinations, a rigorous threshold value will need to be determined statistically using ETGA assay signals derived from a larger number of negative clinical blood culture samples that had not been subjected to atypical handling such as refrigeration.

4.3.3 Nucleic Acid-based Microbial Identification from ETGA lysates

In addition to DNA polymerase activity, we previously demonstrated that ETGA lysates from simulated BSI also contain microbe-derived nucleic acid, and thus are readily available for subsequent microbial identification via rapid NAT such as qPCR [21]. To determine the feasibility of parallel NAT analysis of clinical blood cultures, we retrospectively interrogated the ETGA lysates (generated from 13 of the 22 BACTEC-positive ETGA-bottles) for the genomic DNA of *S. aureus*, *E. coli*, *S. epidermidis*, and *S. agalactiae*, based upon availability of the corresponding in-house gene specific qPCR (gsPCR) assays. The gsPCR ID results presented in Table 2 agree with the Hospital microbiology laboratory's independent ID, and demonstrate that the ETGA blood culture assay procedure produced lysates from clinical samples that are suitable for rapid parallel nucleic acid-based species identification.

Sample ID	Hospital Microbe ID	<i>S. aureus</i> Assay Result	<i>E. coli</i> Assay Result	<i>S. epidermidis</i> Assay Result	<i>S. agalactiae</i> Assay Result
4	<i>E. coli</i>	Negative	Positive	Negative	Negative
12	<i>E. coli</i>	Negative	Positive	Negative	Negative
39	<i>E. coli</i>	Negative	Positive	Negative	Negative
63	<i>E. coli</i>	Negative	Positive	Negative	Negative
183	<i>E. coli</i>	Negative	Positive	Negative	Negative
256	<i>E. coli</i>	Negative	Positive	Negative	Negative
254	<i>E. coli</i>	Negative	Positive	Negative	Negative
208	<i>S. aureus</i>	Positive	Negative	Negative	Negative
190	<i>S. aureus</i>	Positive	Negative	Negative	Negative
258	<i>S. aureus</i>	Positive	Negative	Negative	Negative
273	<i>S. aureus</i>	Positive	Negative	Negative	Negative
217	Streptococcus-group B	Negative	Negative	Negative	Positive
165	<i>S. epidermidis</i>	Negative	Negative	Positive	Negative

Table 2. Thirteen of the ETGA Blood Culture Assay Sample lysates were analyzed by 4 different qPCR assays specific for genomic DNA targets of *S. aureus*, *E. coli*, *S. epidermidis*, and *S. agalactiae*.

4.4 Summary

Herein, we demonstrated the feasibility of using the ETGA blood culture assay to rapidly detect the presence and growth of microbes within clinical blood cultures. Together, we feel that these results merit additional studies aimed at performing time course analysis of clinical blood cultures within the hospital laboratory setting, towards a more realistic assessment of the ETGA blood culture assay’s capability to be used as a rapid BSI screening tool. However, in its current

manual form, it would be difficult to incorporate the ETGA blood culture assay into the busy workflow of the modern clinical microbiology laboratory. To this end, experiments are underway to further simplify the ETGA blood culture assay and make it more amenable to automation. If achieved, automation could potentially reduce the TTD of BSI. Also, an ETGA lysate-coupled NAT approach could enable much faster organism identification times and initiation of data-guided antimicrobial susceptibility testing faster than traditional methods. The importance of this proposed approach is supported by the mounting evidence that rapid identification of BSI leads to lower morbidity and mortality, translating into reduced length of hospital stay and associated cost savings [25]. We are also interested in evaluating procedural modifications that would enable ETGA-mediated detection of microbes directly from whole blood and blood products.

4.5 Supplemental Data

Figure S1 – ETGA assay curves and available parallel plating images

Table S1 - ETGA versus BACTEC Time-to-Detection (TTD) comparisons

Sample ID	Microorganism	First Positive Time point (hours) †	ETGA Procedure Time (hours)	Total ETGA TTD (hours)	BACTEC TTD	Fold TTD Reduction
4	E. coli	4	2	6	11.67	1.95
12	E. coli	4	2	6	10.83	1.81
39	E. coli	4	2	6	14.83	2.47
63	E. coli	4	2	6	10.67	1.78
183	E. coli	7	2	9	13.17	1.46
256	E. coli	4	2	6	10.67	1.78
254	E. coli	4	2	6	11.00	1.83
208	S. aureus	0	2	2	12.33	6.17
190	S. aureus	0	2	2	10.67	5.34
258	S. aureus	0	2	2	8.33	4.17
273	S. aureus	0	2	2	15.17	7.59
255	CoNS	4	2	6	22.67	3.78
165	CoNS	14	2	16	26.83	1.68
95	Streptococcus	4	2	6	14.33	2.39
5	Streptococcus	4	2	6	11.00	1.83
301	Streptococcus	0	2	2	14.83	7.42
210	Streptococcus	4	2	6	11.83	1.97
217	Streptococcus	6	2	8	13.33	1.67
289	Streptococcus	4	2	6	19.83	3.31
229	Streptococcus	7	2	9	22.67	2.52
86	Proteus	4	2	6	13.33	2.22
114	Abiotrophia	4	2	6	30.67	5.11
Average Fold TTD Reduction =						3.2

Table S2 – Corresponding ETGA and plating data for Figure 2A

Sample ID	Time Point (hr)	ETGA Ct Value from 0.75 mL sample prep	CFU from parallel 0.1 mL sample plating
ZSL-00004	0	NEG	0
	4	32.4	26
	8	19.8	TNTC
	9	25.2	TNTC
	10	15.6	TNTC
ZSL-00012	0	36	0
	4	35.1	2
	7	24	500
	8	21.5	~1000
	10	18.6	TNTC
ZSL-00039	0	36.2	0
	4	34.2	0
	6	29.3	21
	8	24.7	500
	10	18.7	TNTC
ZSL-00063	0	35.7	0
	4	26	500
	6	22.5	TNTC
	7	21.4	TNTC
	8	18.1	TNTC
ZSL-00183	0	36	0
	4	35.9	0
	6	35.3	4
	7	32.9	4
	8	26.9	200
ZSL-00256	9	22.6	TNTC
	0	37.2	2
	4	35	19
	6	27.3	200
	10	21.6	TNTC
ZSL-00254	0	NEG	0
	4	29.2	86
	6	23.4	500
	8	17.6	TNTC
	10	16.9	TNTC

TNTC = Too Numerous to Count

Table S3 – Corresponding ETGA and plating data for Figure 2B

Sample ID	Time Point (hr)	ETGA Ct Value from 0.75 mL sample prep	CFU from parallel 0.1 mL sample plating
ZSL-00190	0	35.1	0
	4	33.7	9
	6	29.7	200
	8	22.9	TNTC
	10	17.7	TNTC
ZSL-00258	0	33.3	3
	4	24.3	500
	6	18	TNTC
	8	16.5	TNTC
ZSL-00273	0	26.4	500
	4	18.1	TNTC
	6	21.2	TNTC
ZSL-00208	0	33.8	4
	4	28.2	500
	6	25.5	TNTC
	8	20	TNTC
	10	15.9	TNTC

TNTC = Too Numerous to Count

Table S4 – Corresponding ETGA and plating data for Figure 2C

Sample ID	Time Point (hr)	ETGA Ct Value from 0.75 mL sample prep	CFU from parallel 0.1 mL sample plating
ZSL-00095	0	35.6	0
	4	34.4	1
	6	29	21
	7	26.4	127
	9	23.2	TNTC
	10	19.8	TNTC
ZSL-00005	0	35.6	0
	4	33.9	TNTC
	8	22.8	TNTC
ZSL-00210	0	37.7	0
	4	27.5	TNTC
	6	19.9	TNTC
	8	16	TNTC
ZSL-00229	0	36.6	3
	4	34.6	1
	6	NEG	2
	7	34.2	1
	10	31.6	32
	14	24	TNTC
ZSL-00217	0	38.5	0
	4	37.7	2
	6	33.6	133
	8	29.2	TNTC
ZSL-00289	0	37.1	0
	4	34.2	0
	6	34.1	4
	10	33	TNTC
ZSL-00301	0	34.8	0
	4	33.4	8
	6	32.7	304
	8	29.2	TNTC
	10	25.8	TNTC
	12	28	TNTC

TNTC = Too Numerous to Count

Table S5 – Corresponding ETGA and plating data for Figure 2D

Sample ID	Time Point (hr)	ETGA Ct Value from 0.75 mL sample prep	CFU from parallel 0.1 mL sample plating
ZSL-00255	0	35.8	0
	4	34.4	0
	10	32.3	76
	14	23.1	TNTC
	18	19.8	TNTC
	21	18.1	TNTC
ZSL-00165	0	36.7	0
	4	35.5	0
	10	36.5	0
	14	28.9	299
	18	22.6	TNTC
	22	15.2	TNTC

TNTC = Too Numerous to Count

Table S6 – Corresponding ETGA and plating data for Figure 2E

Sample ID	Time Point (hr)	ETGA Ct Value from 0.75 mL sample prep	CFU from parallel 0.1 mL sample plating
ZSL-00086	0	35.3	2
	4	31.4	500
	8	24	TNTC
	9	22.6	TNTC
ZSL-00114	0	35.7	ND*
	4	33	ND*
	6	29.3	ND*
	8	26.7	ND*
	10	23.6	ND*
	11	22.3	ND*
	12	19	ND*

* None determined due to no growth on blood agar plates.

TNTC = Too Numerous to Count

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Chapter 5

A Novel Approach for Rapid Detection of Bacterially Contaminated Platelet Concentrates via Sensitive Measurement of Microbial DNA Polymerase Activity

Daniel R. Zweitzig, Nichol M. Riccardello, John M. Pester,
Rebecca Jeanmonod, Mark J. Kopnitsky, S. Mark O'Hara.

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Abstract

BACKGROUND: Transfusion of bacterially contaminated platelet concentrates (PC) can result in serious health consequences for the affected patient. Prior to being released from blood banking facilities, PC are routinely screened for bacterial contamination by culture-based tests. However, culture-based PC-screening methodologies require extended holding and incubation periods, and are prone to false negative results due to sampling error. Screening PC closer to the time of transfusion using rapid point-of-issue (POI) tests represents an alternative approach; however, FDA-approved assays generally suffer from a lack of sensitivity.

STUDY DESIGN AND METHODS: Presented herein is the feasibility of a novel approach towards rapid, sensitive, and universal detection of bacterially-contaminated PC via selective measurement of microbial DNA polymerase activity. This approach is achieved using a differential cell lysis procedure in combination with enzymatic template generation and amplification (termed ETGA-PC Assay).

RESULTS: Serial dilution spiking experiments revealed an approximate sensitivity of 30-200 CFU/mL (average = 85 CFU/mL) for *S. epidermidis*, *S. aureus*, *E. coli*, and *K. pneumoniae*. An additional 22 clinically relevant strains of bacteria were also detected below 200 CFU/mL after spiking into PC aliquots. Furthermore, the ETGA-PC Assay was able to accurately monitor the presence and growth of 7 clinically relevant bacterial species that were spiked into PC units.

CONCLUSION: Together, the data presented here demonstrate that the ETGA-PC Assay is a feasible approach for rapid and sensitive detection of bacterially contaminated PC.

Experiments, aimed at simplification and/or automation of the assay procedure, are underway.

5.1 Introduction

Transfusion of blood components such as red blood cell concentrates and platelet concentrates (PC) is an important part of patient care around the world. Due to numerous factors, blood components are at risk of containing a variety of infectious agents, including viruses and bacteria.¹ Despite significant reductions to the risk of transfusion-related viral infections,^{2,3} bacterial contamination remains a persistent problem for blood banking facilities and hospitals.⁴⁻⁸ In particular, PC represent the highest risk among all blood components, with bacterial contamination rates as high as 1 in 1000 to 3000 units.⁶⁻¹⁰ Since millions of PC units are transfused annually, many patients are therefore at risk of acquiring life threatening transfusion-related illnesses, such as sepsis.⁶

In a continued effort to reduce these risks, the American Association of Blood Banks (AABB) adopted a new standard in 2004 recommending the implementation of measures to detect and limit bacterial contamination of PC.¹¹ To meet this mandate, blood banking facilities have implemented procedures such as improved skin disinfection, diversion of the initial blood draw, and bacterial detection tests to screen for contaminated PC.¹² Currently, broth-based blood culture is the most commonly used test for screening PC prior to release from blood banking facilities,^{13,14} and its implementation has been associated with a reduced risk of transfusion-related fatalities.¹⁵ However, culture-based testing doesn't always detect PC units containing low levels of bacteria, resulting in release of contaminated units (i.e. false negatives).¹⁶ Since PC are stored at 22-24° C prior to transfusion, bacteria are able to proliferate to dangerous levels within contaminated units missed by culture-based tests. Unfortunately, such scenarios have resulted in transfusion of contaminated PC and have been associated with fatal septic reactions.^{15,17} In addition to these safety risks, pre-release testing by culture-based methodologies presents logistical challenges to blood banking facilities. For example, the testing procedures associated with these methodologies can require up to 48 hours of holding and incubation prior to releasing PC for transfusion.^{9,18} Since PC have a shelf life of only 5 days from the time of collection, such delays limit their availability for transfusion. Together, the aforementioned limitations associated with pre-release testing have sparked the development

of rapid point-of-issue (POI) assays for detection of bacterially-contaminated PC just prior to transfusion.^{9,19}

We recently reported the characterization of a novel enzymatic template generation and amplification (ETGA) methodology that enables rapid, highly sensitive, and universal detection of viable microbes via measurement of endogenous DNA polymerase extension activity.²⁰ In a subsequent study, we demonstrated that a differential cell lysis procedure, followed by ETGA analysis, enabled reduced Time-to-Detection (TTD) of simulated bloodstream infections when compared to a continuous monitoring blood culture instrument.²¹ Herein, we set out to determine the feasibility of using ETGA to detect bacterially-spiked PC. Since anucleated cells such as platelets are known to contain mitochondrial DNA polymerase,²² early experiments were aimed at evaluating and controlling PC-derived ETGA background signal via a modified differential cell lysis procedure. The goal of subsequent experiments was to assess the ability of a modified differential cell lysis-ETGA procedure (referred to hereafter as the ETGA-PC Assay) to sensitively detect bacterially-spiked PC.

5.2 Materials and Methods

5.2.1 Source of Platelets

Three types of PC were used in this study. Single donor whole blood derived non-leukoreduced (WBNLR) PC were purchased from Innovative Research (Novi, Michigan). Apheresis non-leukoreduced (ANLR) PC were purchased from Biological Specialty (Colmar, PA). Innovative Research and Biological Specialty received informed consent from all donors prior to collecting PC. Apheresis leukoreduced (ALR) PC were collected at Miller Keystone Blood Center (Bethlehem, PA) for use at St. Luke's University Hospital (Bethlehem, PA) and transported to our laboratory after their five-day expiration period. St. Luke's University Hospital and Health Network Internal Review Board (IRB), Bethlehem PA, approved this study protocol.

5.2.2 ETGA PC-Assay Procedure

The ETGA PC-Assay consists of a differential cell lysis procedure followed by sensitive measurement of DNA polymerase extension activity. We previously described a hematopoietic cell lysis procedure that enabled reduction of the DNA polymerase signal associated with 0.75

mL aliquots of whole blood-innoculated blood culture bottles.²¹ For the present study, we modified this procedure for PC as follows. Briefly, 0.5 mL PC aliquots were added to microfuge tubes containing 0.5 mL of a detergent mix (1% Triton X-100/Tween-20/Saponin) in an effort to begin lysing platelets. Tubes were then capped, inverted four times to mix, and incubated at room temperature for 5 minutes. Samples were then spun at 8000 x g for 3 minutes. The supernatant was poured off into a waste container, and the opening of each tube was tapped onto a plastic-backed wipe to remove any residual liquid. The pellets were then resuspended in 5 mM sodium hydroxide by pipetting up and down ten times in an effort to complete platelet lysis and inactivate extracellular DNA polymerase activity. Samples were incubated at room temperature for 5 minutes, and spun at 8000 x g for 3 minutes. The supernatant was poured off into a waste container, and the opening of each tube was tapped onto a plastic-backed wipe to remove any residual liquid. Next, 0.6 mL of a Tris-based wash buffer was added to each tube, pipetted up and down five times, simultaneously transferred to a beadmill lysis tube, and spun at 8000 x g for 3 minutes to concentrate bacteria. After spinning, the supernatant was removed and DNA polymerase activity was measured by ETGA (see below).

5.2.3 Measurement of DNA polymerase activity via ETGA

ETGA is a highly sensitive method that links microbial DNA polymerase activity to qPCR amplification. Complete details regarding measurement of DNA polymerase activity via ETGA (including oligonucleotide sequences, buffer formulations, and qPCR conditions) have been previously reported.²⁰ Briefly, 50 µL of a DNA polymerase extension (DPE) reaction mix is added to a beadmill tube containing pre-concentrated microbes. The core components of the DPE mix are a synthetic DNA substrate, a dNTP mix (dATP, dCTP, dTTP, dGTP), Tris-buffer, and magnesium sulfate. The beadmill tube containing the DPE mix is vortexed at 2800 rpm for 6 minutes using a 2 mL disrupter attachment (Scientific Industries) in order to lyse the microbes and release endogenous DNA polymerase. After disruption, the beadmill tubes are transferred to a 37° C heat block and are incubated for 20 minutes, during which the synthetic DNA substrate is modified by microbial-derived DNA polymerase. Samples are then transferred to 95° C for 5 minutes to inactivate bacterial DNA polymerase and subsequently spun at 12k x g for

30 seconds. Four microliters from each beadmill tube is then transferred to a qPCR reaction, and positivity is determined automatically by the thermocycler's software.

5.2.4 Assessment of PC-derived Background Signal

To assess the level and DNA polymerase-dependence of PC-derived background signal, 0.5 mL of PC (n = 6 aliquots) were pelleted at 8000 x g, resuspended in 0.5 mL sterile saline (150 mM), and simultaneously transferred to beadmill tubes. Beadmill tubes were spun at 8000 x g for 3 minutes, and the supernatant was carefully removed. The DNA polymerase activity was measured via standard ETGA methodology in three of the six beadmill tubes. Since dideoxyCTP has been previously demonstrated to block DNA polymerase extension of the ETGA assay substrate,^{20,21} a DPE reaction mix (containing dATP, dGTP, dTTP and **dideoxyCTP**) was added to the remaining 3 bead mill tubes in an effort to demonstrate that any observed assay background signal is specific for DNA polymerase, and not other enzymatic activities associated with PC lysates. Complete methodological details regarding assessment of DNA polymerase-dependence of ETGA signal via dCTP versus ddCTP have been previously published.^{20,21}

5.2.5 Reduction of PC-derived background signal by a Selective Cell

Lysis procedure

For background reduction experiments, 0.5 mL aliquots of WBNLR, ANLR, and ALR PC were subjected to the ETGA-PC Assay procedure as described above. For each unit, an additional 0.5 mL aliquot was washed once with saline as described above, and was included as a Non-lysed Control (NLC). The NLC was included for each assayed PC unit in an effort to demonstrate the potential ETGA background signal for each PC aliquot when not subjected to the differential cell lysis portion of the of the ETGA-PC assay. For each incoming PC unit, sterility was verified by innoculating 8 mL of PC into a blood culture bottle and also by plating 100 µL of PC onto blood agar. Each PC-innoculated blood culture bottle was continuously monitored by a blood culture incubator for 5 days before designating as negative.

5.2.6 Spiking of serially diluted bacteria into PC aliquots

Staphylococcus epidermidis (ATCC# 12228), *S. aureus* (ATCC# 25923), *E. coli* (ATCC# 25922), and *K. pneumoniae* (ATCC # 700721) were used for serial dilution spiking studies due to the

established prevalence of these species among contaminated PC units associated with transfusion-related fatalities.^{9,23} From a fresh plate of each bacterial species, colonies were suspended in 3ml saline until an OD₆₂₅ = 0.100 ± 0.010 was achieved. This OD reading yielded bacterial stocks at approximately 1E8 CFU/mL. Dilutions were then made in saline to obtain stock concentrations of 1E6 CFU/mL, 1E5 CFU/mL, 2E4 CFU/mL, 1E4 CFU/mL, and 5E3 CFU/mL. Next, 0.99 mL of PC were added to 6 sterile microfuge tubes. Ten microliters of each bacterial stock was added to five of these tubes, resulting in spiked PC stocks targeted at 1E4 CFU/mL, 1E3 CFU/mL, 200 CFU/mL, 100 CFU/mL, and 50 CFU/mL. Ten microliters of sterile saline was added to the final tube of PC, designated as the No Spike Control (NSC). Next, 0.5 mL of each spike level, and the NSC, were subjected to the ETGA-PC Assay procedure as described above. One hundred microliters of each PC stock were also plated to determine the approximate CFU levels within spiked samples and to confirm the sterility of the NSC. For each of the four bacterial species, serial dilution spikes were performed as described above using n = 3 different ANLR PC units and n = 3 different ALR PC units, totaling 6 independent experimental replicates. Stocks of 22 additional bacterial species (see Table 3 for microbial ID's) were prepared in saline and spiked into PC aliquots at 3 target-input levels around the 200 CFU/mL level. All spikes were compared to a donor-matched NSC. One hundred microliters of PC stocks (spiked and non-spiked) were also plated in parallel to determine the approximate CFU-input levels and to confirm the sterility of non-spiked PC.

5.2.7 Analysis of Bacterial Growth within PC Units

Immediately prior to spiking bacteria, a 0.5 mL aliquot of PC was subjected to the ETGA-PC Assay to determine the baseline background level for each respective unit. For analysis of bacterial growth in PC units, the panel of four microorganisms (listed above) was expanded to include *P. aeruginosa* (ATCC# 27853), *S. marcescens* (ATCC# 29021), and *B. cereus* (ATCC# 11778). Except for *B. cereus*, stock solutions of each bacterial species were prepared as described above. For *B. cereus*, 2 colonies from a freshly grown plate were inoculated into brain heart infusion (BHI) broth and incubated at 30 ° C with shaking until reaching an OD₆₀₀ of 1.00 ± 0.10. Using stock solutions of each bacterial species (n = 7 total), a target of 100 CFU was spiked into each PC unit. The actual amount of input CFU was determined by plating analysis of

the stock bacterial suspensions. Once spiked, the PC unit was placed into a PC incubator (Helmer, PC900) and incubated at 22° C with shaking. During incubation, aliquots of PC were removed and immediately analyzed by the ETGA-PC Assay after 24, 48, 72, 144, and 168 hours of incubation. In an effort to monitor the CFU levels, parallel plating was performed on serial dilutions of each PC aliquot taken during the time course incubation.

5.3 Results

5.3.1 Characterization and Reduction of PC-Derived ETGA Assay

Background Signal

In this study we set out to determine the feasibility of using ETGA to detect bacterially-contaminated PC. A basic schematic diagram of the proposed approach is presented in Figure 1.

Fig. 1 Schematic of a novel approach for detecting contaminated PC. Figure 1 depicts a schematic overview of the ETGA-PC Assay. First, platelets are lysed via a differential cell lysis procedure, leaving intact bacteria. Bacterial DNA polymerase is then exposed to a synthetic DNA substrate during a beadmill-induced microbial lysis. During a 37° C incubation, bacterial DNA polymerase then modifies the synthetic DNA substrate. The modified DNA substrate is detected by qPCR, and indicates the presence of bacterial contamination.

Since platelets are a potential source of DNA polymerase activity,²² we first set out to verify that 0.5 mL aliquots from a sterile PC unit were detectable by the ETGA assay. As shown in Figure 2, triplicate analysis of non-lysed PC aliquots contributed significant amounts of ETGA assay background signal. In contrast, ETGA assay signal was undetectable from triplicate parallel-preparations of non-lysed PC aliquots that were incubated with a dNTP mix containing dideoxyCTP instead of dCTP (Figure 2).

Fig. 2 Characterization of PC-derived background signal. ETGA Assay signals from non-lysed PC aliquots were compared using DNA polymerase extension reactions containing standard nucleotides (dATP, dGTP, dATP and dCTP) versus a nucleotide mix containing (dATP, dGTP, dATP and **dideoxyCTP**).

Next, we assessed the ability of a differential cell lysis procedure to reduce the amount of background signal associated with 0.5 mL aliquots taken from WBNLR, ANLR, and ALR PC units. As shown in Figures 3A-C, the differential cell lysis procedure effectively reduced the ETGA assay signals associated with all three types of PC units, relative to the respective NLC's. In addition to the PC units presented in Figure 3A-C, $n = 6 \times 0.5$ mL aliquots from WBNLR ($n = 3$ units), ANLR ($n = 7$ units), and ALR ($n = 34$ units) were subjected to the ETGA-PC assay. In Figure 3D, a compilation of the background signals (obtained from all PC units analyzed) is presented, relative to a representative NLC signal. An experimental positivity threshold was established using the assembled background data, and is presented in Figure 3D. This positivity

threshold was used as an objective assay result criteria for subsequent bacterial-spiking experiments.

Fig. 3 Assessment and reduction of ETGA assay background derived from different types of PC units. (A-C) ETGA-PC assay signal was determined for whole blood non-leukoreduced (WBNLR), apheresis non-leukoreduced (ANLR), and apheresis leukoreduced (ALR) PC units. A non-lysed PC control was included to determine the potential background signal associated with each respective unit. (D) ETGA-PC Assay signals derived from 47 PC units (multiple replicates per unit), were combined into one graph. A positivity threshold was subsequently generated by taking the highest endpoint fluorescence value obtained from the background curves.

Parallel blood culture analysis of an 8 mL inoculation from each PC unit was also performed and yielded negative results after a 5 day incubation period.

5.3.2 Detection of Clinically Relevant Bacteria from Spiked PC aliquots

After demonstrating that the differential cell lysis procedure was effective at reducing PC-derived background signal, we set out to verify that it was also compatible with detection of DNA polymerase activity from clinically relevant bacterial species via ETGA. Two Gram positive (*S. aureus* and *S. epidermidis*) and two Gram negative (*E. coli* and *K. pneumoniae*) bacterial

species were initially chosen for spiking experiments due to their established prevalence among contaminated PC units.^{9,23} The ETGA-PC Assay was performed on bacterially-spiked PC aliquots from three independent ANLR and ALR units as described in the materials and methods section, and a representative set of ETGA-qPCR curves (along with the positivity threshold) is presented for each of the four bacterial species in Figures 4A-D.

Fig. 4 Examples of ETGA-PC Assay curves from bacterial spikes. (A-D) Four different bacterial species were serially diluted and spiked into PC aliquots. Examples of the ETGA-PC Assay results are presented for each bacterial species versus non-spiked PC aliquots. In each graph, the positivity threshold is included as a visual reference.

Complete data sets from ETGA-PC Assay analysis of serially diluted bacterial spikes into ANLR and ALR PC units is presented in Table 1 and Table 2 respectively. Together, the serial dilution spiking experiments revealed an approximate sensitivity of 30-200 CFU/mL (average = 85 CFU/mL) for *S. epidermidis*, *S. aureus*, *E. coli*, and *K. pneumoniae*.

Strain	CFU Targeted per 0.5 mL of PC*	CFU Assayed- Unit A	Result-Unit A	CFU Assayed- Unit B	Result-Unit B	CFU Assayed- Unit C	Result-Unit C
E. coli	5000	2800	Positive	5000	Positive	4500	Positive
E. coli	500	280	Positive	500	Positive	450	Positive
E. coli	100	56	Positive	100	Positive	90	Positive
E. coli	50	28	Positive	50	Positive	45	Positive
E. coli	25	14	Positive	25	Positive	23	Positive
K. pneumoniae	5000	4500	Positive	2800	Positive	3450	Positive
K. pneumoniae	500	450	Positive	280	Positive	345	Positive
K. pneumoniae	100	90	Positive	56	Positive	69	Positive
K. pneumoniae	50	45	Positive	28	Positive	34	Negative
K. pneumoniae	25	23	Negative	14	Positive	17	Negative
S. aureus	5000	4500	Positive	11000	Positive	3330	Positive
S. aureus	500	450	Positive	1100	Positive	333	Positive
S. aureus	100	90	Positive	220	Positive	67	Positive
S. aureus	50	45	Positive	110	Positive	34	Positive
S. aureus	25	23	Positive	55	Positive	17	Negative
S. epidemidis	5000	3000	Positive	4000	Positive	3500	Positive
S. epidemidis	500	300	Positive	400	Positive	350	Positive
S. epidemidis	100	60	Positive	80	Positive	70	Positive
S. epidemidis	50	30	Positive	40	Negative	35	Positive
S. epidemidis	25	15	Positive	20	Positive	18	Positive

* Multiply by two to obtain CFU/mL

Table 1. ETGA-PC Assay Results from Serial Dilution Spiking of Bacteria into Apheresis-Non-Leukoreduced PC via the ETGA

Strain	CFU Targeted per 0.5 mL of PC*	CFU Assayed- Unit A	Result-Unit A	CFU Assayed- Unit B	Result-Unit B	CFU Assayed- Unit C	Result-Unit C
E. coli	5000	4150	Positive	4000	Positive	3650	Positive
E. coli	500	415	Positive	400	Positive	365	Positive
E. coli	100	83	Positive	80	Positive	73	Positive
E. coli	50	42	Negative	40	Positive	37	Positive
E. coli	25	21	Positive	20	Positive	19	Negative
K. pneumoniae	5000	5000	Positive	3500	Positive	4700	Positive
K. pneumoniae	500	500	Positive	350	Positive	470	Positive
K. pneumoniae	100	100	Positive	70	Positive	94	Positive
K. pneumoniae	50	50	Positive	35	Positive	47	Positive
K. pneumoniae	25	25	Negative	18	Positive	24	Positive
S. aureus	5000	8750	Positive	4250	Positive	5850	Positive
S. aureus	500	875	Positive	425	Positive	585	Positive
S. aureus	100	175	Positive	85	Positive	117	Positive
S. aureus	50	88	Positive	43	Negative	59	Positive
S. aureus	25	44	Positive	22	Negative	30	Positive
S. epidemidis	5000	3600	Positive	5000	Positive	1800	Positive
S. epidemidis	500	360	Positive	500	Positive	180	Positive
S. epidemidis	100	72	Positive	100	Positive	36	Positive
S. epidemidis	50	36	Negative	50	Positive	18	Negative
S. epidemidis	25	18	Positive	25	Negative	9	Negative

* Multiply by two to obtain CFU/mL

Table 2. ETGA-PC Assay Results from Serial Dilution Spiking of Bacteria into Apheresis-Leukoreduced PC via the ETGA

We also tested the ability of the ETGA-PC Assay to detect an additional 22 clinically relevant bacterial species from PC aliquots spiked with microbes at levels close to 200 CFU/mL. As shown in Table 3, the ETGA-PC Assay was able to detect all 22 bacterial species at or below this level.

Bacterial Species	ATCC #	Lowest level of CFU detected per 0.5 mL of PC
<i>S. marcescens</i>	29021	28
<i>P. aeruginosa</i>	27853	34
<i>Y. enterocolitica</i>	9610	60
<i>K. oxytoca</i>	700324	30
<i>B. cereus</i>	11778	100
<i>B. subtilis</i>	6633	60
<i>P. acnes</i>	6919	95
<i>C. perfringens</i>	10543	65
<i>S. pyogenes</i>	19615	85
<i>S. pneumoniae</i>	6303	125
<i>E. cloacae</i>	13047	30
<i>E. aerogenes</i>	15038	25
<i>S. lugdunensis</i>	43809	25
<i>S. enterica</i>	700720	60
<i>E. faecalis</i>	51299	25
<i>S. bovis</i>	35034	35
<i>S. warneri</i>	27839	50
<i>C. flavescentis</i>	10340	26
<i>S. agalactiae</i>	13813	33
<i>M. luteus</i>	381	15
<i>A. baumannii</i>	15308	50
<i>E. faecium</i>	19434	50

Table 3. Additional 22 clinically relevant bacterial strains detected by the ETGA-PC Assay

5.3.3 Monitoring of Bacterial Growth within PC units

Next, we set out to determine if the ETGA-PC Assay could monitor the growth of 7 different bacterial species within PC units. To start, we determined the approximate CFU/mL within each of the spiked-PC units by dividing the number of CFU spiked (obtained from plating analysis) by the volume of PC contained within each unit. The resultant CFU/mL values for each of bacterially-spiked units are shown in the lower right portion of the Figure 5 graph, and are all less than 1 CFU/mL. In Figure 5, the qPCR-Ct values obtained from ETGA-PC Assay analysis of PC aliquots were plotted versus the time for which each unit was incubated. The ETGA-PC

Assay results and CFU/mL values (determined by plating analysis) are also presented in table format directly below the line plots (Figure 5).

Fig. 5 Time course analysis of bacterially-spiked PC units. PC units were spiked with seven different bacterial species and ETGA-PC assay and CFU plating analysis were performed on aliquots in a time course fashion. The resultant ETGA assay Ct values were plotted versus the time that each unit spent in a PC incubator prior to aliquot removal. In the lower right portion of the time course plot, the starting CFU/mL values are shown for each of the spiked PC units. The ETGA-PC Assay and CFU plating results from the time course experiments are also presented in table format directly below the time course plots.

5.4 Discussion

In this report, we assessed the feasibility of a novel approach toward sensitive and universal detection of bacterially contaminated PC via measurement of bacterially-derived DNA polymerase activity (Fig. 1). Since anucleated cells such as platelets have been established to

contain mitochondrial DNA polymerase,²² we hypothesized that PC might yield ETGA assay background signal. As suspected, early feasibility experiments presented herein showed that PC contribute significant DNA polymerase-dependent ETGA assay background signal and therefore need to be reduced to enable detection of DNA polymerase activity from spiked-bacteria. To this end, we demonstrated that a differential cell lysis procedure could effectively reduce the ETGA assay background signal derived from three different types of PC. We then compiled the background data from additional PC units and generated an experimental positivity threshold. In subsequent experiments, ETGA-PC Assay signals derived from PC aliquots containing serial dilutions of four clinically relevant bacteria were distinguishable from donor-matched PC aliquots that had not been spiked with bacteria. In fact, when using the experimental positivity threshold as an objective measure, these preliminary experiments demonstrated that the ETGA-PC assay can detect as little as 30 CFU/mL of bacteria within several of the spiked PC aliquots. As shown in Tables 1 and 2, we did notice that some bacterial species were not consistently detected at less than 100 CFU/mL when spiked into PC units derived from different donors. This inconsistent detection at low levels is likely reflective of PC donor-dependent bacterial recovery differences associated with the ETGA-PC assay sample preparation procedure. Considering this, more replicates are certainly needed to determine a true limit-of-detection (LOD) value for each bacterial species, however, when spiked into six aliquots of PC (representing three independent ALR and ALNR units), our data suggest that the ETGA-PC Assay is able to consistently detect ≤ 200 CFU/mL of *E. coli*, *K. pneumoniae*, *S. aureus*, and *S. epidermidis*. When compared to the claimed-LOD values for current FDA-approved POI tests,^{19,24,25} the ETGA-PC Assay could represent approximately a 50-300 fold improvement in sensitivity. Furthermore, independent studies have shown that one of the FDA-approved POI assays is prone to failure when challenged with clinically relevant gram negative bacterial species that were spiked into PC at levels well above the claimed LOD, thus raising concerns regarding its reliability.²⁶⁻²⁸

In addition to some commonly encountered contaminants, a wide range of bacterial species have been isolated from contaminated PC units.^{8,9,29,30} It is therefore imperative that a POI test is not only sensitive, but also universal in nature. To this end, we also set out to verify that the

ETGA-PC Assay is able to detect a range of clinically relevant bacterial species. Our experiments showed that the ETGA-PC Assay is also able to sensitively detect the presence of an additional 22 clinically relevant bacterial species. These data further support the notion that measurement of DNA polymerase activity represents a bona fide universal detection approach. We felt that it was also important to assess the ability of the ETGA-PC Assay to monitor the growth of bacteria within PC units incubated under standard conditions. These experiments effectively demonstrated that the ETGA-PC Assay can sensitively detect the presence of bacteria during incubation of spiked PC units. Similar to previous studies,^{26,31} *S. epidermidis* is prone to slow growth within platelet units, and in our experiments, it was not detected by plating or ETGA until after 144 hours of incubation. Except for the 24-hour time point taken from the PC unit spiked with *K. pneumoniae*, all of the plating results are in agreement with the ETGA-PC Assay results. The subsequent 48-hour time point for this unit yielded a strong ETGA-PC Assay positive signal, which was consistent with the high levels of CFU determined by parallel plating analysis.

Considering the feasibility data presented herein, we feel that the ETGA-PC Assay has potential to be used as a POI test for rapid detection of bacterially-contaminated PC units close to the time of transfusion. To our knowledge, this is first time that microbes have been detected from PC via measurement of their endogenous DNA polymerase activity and therefore represents a truly novel approach. In its current manual form, the ETGA-PC Assay requires approximately 2 hours to obtain results for a batch of six samples, 90 minutes of which are spent in sample incubations and PCR thermocycling. Experiments are underway to further simplify the assay, reduce the overall time-to-result, and/or make the procedure more amenable to automation.

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Chapter 6

Feasibility of a Simple Microsieve-Based Immunoassay Platform

Daniel R Zweitzig, Arjan G Tibbe, Ai T Nguyen, Cees JM van Rijn,
Mark J Kopnitsky, Kathleen Cichonski, Leon WMM Terstappen

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Abstract

The intrinsic properties of silicon microsieves, such as an optically flat surface, high overall porosity, and low flow resistance have led to an increasing number of biotechnology applications. In this report, the feasibility of creating a microsieve-based immunoassay platform was explored. Microsieves containing 5 μm pores were coupled with poly-acrylic acid chains, and then mounted into a plastic holder to enable rapid reagent exchanges via a wicking mechanism. The mounted microsieves were coated with infectious disease-related antigens at [2.5 $\mu\text{g/mL}$ and 25 $\mu\text{g/mL}$], [20 $\mu\text{g/mL}$ and 50 $\mu\text{g/mL}$], and [20 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$] to facilitate detection of serum-derived human antibodies against Rubella (3-day measles), *B. burgdorferi* (Lyme disease), or *T. pallidum* (syphilis) respectively. The prototype microsieve-based immunoassay platform was able to distinguish positive control sera containing antibodies against Rubella, *T. pallidum*, and *B. burgdorferi* from negative control sera with similar qualitative results as FDA-approved ELISA tests. Testing of a WHO IgG syphilitic standard at 0.3, 0.15, 0.075, 0.0375, and 0.01875 IU/mL demonstrated that the *T. pallidum* microsieve assay is able to distinguish disease-specific IgG signal from background signal at similar, and possibly lower, levels than the corresponding ELISA. The *T. pallidum* microsieve assay prototype also differentiated positive clinical serum samples from negative donor samples; and the results were in good agreement with ELISA ($R^2 = 0.9046$). These feasibility studies demonstrate the potential for utilizing microsieves, along with a reagent wicking device, as a simple diagnostic immunoassay platform.

6.1 Introduction

Membrane-based filtration of cells and smaller biomolecules is a commonly utilized technique throughout various fields of biotechnology [1, 2]. Specialized microfiltration membranes termed microsieves have been described, and consist of a thin silicon nitride membrane layer harboring uniform pores, resting atop of a mono crystalline silicon support [3]. In addition to silicon nitride microsieves, more recently-described polymeric microsieves have been fabricated to contain pores with a wide range of diameters, at varying densities [3, 4, 5]; thus representing promising tools for various biomedical applications. For example, several studies have demonstrated that microsieves are more effective at capturing and imaging circulating tumor cells derived from matrices such as whole blood, relative to other filter types. [6-8] Microsieves have also been developed to contain specialized pores that facilitate isolation, fluorescent imaging, and subsequent nucleic acid-based analysis of single tumor cells [9]. In addition to eukaryotic cell capture, microsieves are beginning to show utility within the field of microbiology. In one such study, microsieves were used as a novel microbial co-cultivation and microscopic analysis platform [10]. Furthermore, covalent coupling of antibodies to the microsieve surface enabled efficient capture and imaging of *Salmonella enterica* from different sample matrices [11]. Considering the usages of microsieves such as blood filtration and microbial enrichment, numerous infectious disease-related diagnostic applications can be envisioned for microsieves that are based upon direct capture and analysis of cells from biological matrices. However in addition to directly capturing or visualizing a disease-causing agent, confirmation of a current or past infection can also be made by indirect detection of biomolecules that are elevated within a patient's serum due to a pathogen-induced immunological or physiological response. Such serologic detection of bacterial and viral-specific biomolecules is a mainstay of clinical practice; however, newer rapid diagnostic devices offer the potential to improve patient care and improve workflow [12]. Herein we describe a simple microsieve-based immunoassay platform and present feasibility experiments aimed at demonstrating its potential for infectious disease serology applications.

6.2 Materials and Methods

6.2.1 Microsieves and reagent wicking apparatus

The microsieves utilized in these studies were 5 mm x 5 mm square, with a silicon nitride filter membrane thickness of 1 μm . The pores within the microsieve were circular, with a diameter of 5 μm . Further details describing the fabrication of such microsieves have been previously described. [3-5] Poly-acrylic acid (PAA) chains were covalently attached to the microsieve surface by first coating with 3-aminopropyltrimethoxysilane, followed by PAA (average $M_w \sim 250$ kDa). Each 5 x 5 mm PAA-coated microsieve was subsequently mounted into a plastic holder, which was fabricated to slide in and out of a custom wicking device. The wicking device was designed to facilitate buffer exchanges during the microsieve immunoassay procedures via contacting the bottom of the microsieve with an absorbent cube.

6.2.2 Covalent coupling of proteins to PAA-microsieves

A 30 μL drop of deionized water was placed onto the microsieve surface, incubated for 15 minutes, and removed by wicking. Next, 4.6 mg of *N*-hydroxysuccinimide (NHS) and 25 mg of Ethyl-(dimethylaminopropyl) carbodiimide (EDC) were each dissolved in 800 μL of 50 mM MES buffer (pH 4.5). A 30 μL drop of freshly made EDC/NHS solution was placed onto the microsieve surface, incubated for 30 minutes, and the microsieve surface was rinsed with 5 mM acetic acid (pH 5.5). Next, the protein of interest was diluted to the desired coating concentration in 5 mM acetate buffer (pH 5). A 30 μL drop of the diluted protein was then added to the microsieve surface, incubated for 1 hour, and the microsieve surface was rinsed with deionized water. Next, a 30 μL drop of 100 mM ethanolamine (pH 8.5) was added to the microsieve surface, incubated for 30 minutes, and rinsed with phosphate buffered saline (pH 7.4). To provide additional blocking, a 30 μL drop of Stabilguard reagent (Surmodics, Eden Prairie MN) was added to the microsieve surface, incubated for 15 minutes, and removed by wicking through the bottom. Another 30 μL drop of Stabilguard reagent was added to the microsieve surface, incubated for 15 minutes, and removed by wicking through the bottom just prior to initiating the microsieve immunoassay procedure.

6.2.3 BSA-biotin and HRP-streptavidin model experiment

BSA-biotin was purchased from Thermo Fisher Scientific (cat#29130), and BSA was purchased from Sigma-Aldrich (cat# B6917). Both BSA-biotin and BSA were covalently coated onto the surface of different microsieves at a concentration of 0.1 mg/mL according the procedures described above. Streptavidin-HRP was purchased from Thermo Fisher Scientific (cat#21130), and was diluted in an HRP-conjugate diluent (Stabilzyme HRP, Surmodics). To perform the assay, a 30 μ L drop of 1:20,000-diluted streptavidin-HRP was added to the surface of the microsieve and incubated for 20 minutes. Using a top-to-bottom wicking mechanism, the microsieve was then washed 4 times using 100 μ L of phosphate buffered saline containing 0.5 % Tween-20 (PBST). After washing, assay signal was detected via luminol reagent as described below.

6.2.4 Human IgG detection model experiment

Goat anti-human IgG (gamma) and goat anti-human IgM (mu) were purchased from American Qualex (San Clemente, CA), and covalently coated onto the surface of separate microsieves at a concentration of 0.1 mg/mL according the procedures described above. Normal human serum was diluted 1:21 in phosphate buffered saline containing 0.1 % Tween-20 and 0.1 % BSA. A 30 μ L bead of diluted serum was added to the microsieve surface and incubated for 20 minutes. Using a top-to-bottom wicking mechanism, the microsieve was then washed 4 times using 100 μ L of PBST. Next, a 30 μ L drop of anti-human IgG conjugate (goat anti-human IgG (Fc) conjugated to HRP, American Qualex diluted 1:10,000 in conjugate diluent) was added to the microsieve surface and incubated for 20 minutes. Using a top-to-bottom wicking mechanism, the microsieve was then washed 4 times using 100 μ L of PBST. After washing, assay signal was detected via luminol as described below.

6.2.5 Coating of infectious disease-specific antigens on PAA-microsieves

The three antigens utilized were: Rubella (Rubella Grade IV Antigen, Meridian Life Science), *B. burgdoferi* (VIsE, Diarect), and *T. pallidum* (p17, Ross Southern Laboratories). Antigens were covalently coated onto the microsieve surface using the procedures described above. For the initial feasibility assessment, antigens were coated at a final concentration of [2.5 μ g/mL and 25

µg/mL] for Rubella, [20 µg/mL and 50 µg/mL] for *B. burgdorferi*, and [20 µg/mL and 100 µg/mL] for *T. pallidum*.

6.2.6 General microsieve immunoassay procedure, signal detection, and quantitation

After antigens were coated onto the microsieve surface, reagents from three ELISA test systems were utilized to perform the microsieve-based immunoassay procedures. The reagents were sample diluents, wash buffers, and HRP-conjugates, and were taken directly from the following ELISA test systems: Rubella (cat# 9Z9801G), *B. burgdorferi* (cat# 3Z9661), and *T. pallidum* (cat# 3Z7611G); which are all manufactured by ZEUS Scientific Inc. (Branchburg, NJ). To begin the immunoassay procedure, serum samples were diluted in assay-specific sample diluents, then a 30 µL drop of diluted serum was added to the microsieve surface and incubated for 20 minutes. Using a wicking mechanism, the microsieve was then washed 4 times using 100 µL of ELISA wash buffer. Next, a 30 µL drop of assay-specific HRP-conjugate was added to the microsieve surface and incubated for 20 minutes. Using a wicking mechanism, the microsieve was then washed 4 times using 100 µL of ELISA wash buffer. After washing, a 30 µL drop of freshly mixed Femtoglow luminol reagent (cat# SHRPE21008DT, Michigan Diagnostics, Royal Oak MI) was added to the microsieve surface, incubated for 1 minute, then the microsieves were placed into a dark box and imaged using a Coolsnap HQ2 CCD camera (Photometrics, Tucson AZ) at various exposure times. Captured images were opened in ImageJ software and quantified using the 'analyze' tool.

6.2.7 Serum samples, controls, and standards

Positive and negative controls used for the initial testing of *T. pallidum*-positive, *B. burgdorferi*-positive, and Rubella-microsieve assays were taken directly from the ELISA kits mentioned above. The world health organization (WHO) syphilitic IgG standard was purchased from the National Institute for Biological Standards and Controls (cat # 05/122, Hertfordshire, UK), and was serially diluted in ELISA sample diluent prior to testing. Residual serum samples submitted for routine syphilis testing in the hospital setting were purchased from New York Biologics (Southampton, NY). These samples were collected under an IRB-approved protocol and were fully de-identified prior to purchase. Samples previously determined to be positive (n =5) or negative (n = 5) using

an anti-*T. pallidum* IgG ELISA (ZEUS Scientific Inc., cat # 3Z7611G) were selected for testing on the microseive-based *T. pallidum* assay.

6.3 Results and Discussion

6.3.1 Overview of the microseive-based immunoassay approach

Despite the vast number and variety of diagnostic immunoassays that have been developed over the past six decades, the core methodology often still relies on the following common principles: i. Immobilization of a specific capture antigen or antibody to a solid support ii. Incubation of a sample matrix containing the target biomolecule with the immobilized capture antigen/antibody iii. Separation of the captured target biomolecule from unbound reactants iv. Detection of the captured target biomolecule. [12, 13] Several studies have demonstrated the utility of microsieves for capturing and imaging cells [6-11], however to our knowledge no data currently exists regarding the use of these filters for detecting disease-relevant antibodies directly from human serum. In this report, we assessed the feasibility of a unique immunoassay platform that utilizes a microseive as the solid support onto which disease-specific antigens were covalently coated. The microsieves were mounted within a customized plastic holder to enable a simple wicking mechanism that facilitates buffer exchanges during the immunoassay procedure. A basic overview of this approach can be viewed in Figure 1. Panel A shows an image of the micosieve holder containing a 5 x 5 mm microseive. Each microseive harbors 29,500 pores that are each 5 μm in diameter. Adjacent is a representative Scanning Electron Microscopy (SEM) image of a section of the microseive. Panel B illustrates the covalent coupling of antigens to the microseive, and Panel C the generalized immunoassay procedure.

Figure 1

(A) Images depicting a poly-acrylic acid coated 5 x 5 mm microsieve mounted within a plastic holder. The scanning electron micrograph of the microsieve surface highlights the shape and spacing of the 5µm pores. (B) Schematic presentation depicting a general overview of how disease-specific capture biomolecules are covalently coated to the microsieve surface (C) Simple wicking apparatus that enables reagent transfer during the microsieve-based immunoassay.

6.3.2 Microsieve-based immunoassay model experiments

To begin feasibility studies, two basic approaches were employed. In the first approach, a BSA-biotin conjugate was covalently linked to the microsieve surface, and a streptavidin-HRP conjugate was assayed. As shown at the bottom of Figure 2A, microsieves coated with BSA-biotin were effective at producing chemiluminescent signal when assayed with streptavidin-HRP, whereas microsieves coated in parallel with only BSA did not; thus demonstrating that the signal was specific to the biotin-streptavidin interaction. After demonstrating that a basic biotin-

streptavidin interaction was capable of being performed and detected on the microsieve surface, we next set out to model a slightly more complex multi-step binding approach aimed at capturing human IgG. In this second approach, an anti-human IgG antibody was covalently linked to the microsieve surface and human serum was assayed. As shown at the bottom of Figure 2B, after incubation with anti-Human IgG-HRP conjugate, microsieves coated with anti-human IgG were able to detect IgG derived from normal human serum. However, microsieves parallel-coated with anti-human IgM did not detect any serum-derived IgG signal after incubation with the same anti-IgG-HRP conjugate; once again demonstrating that the signals derived from this model system experiment were specific to the targeted biomolecule. Together, these experiments demonstrate that the mounted microsieve matrix enables targeted detection of biomolecules, and is not subject to significant background chemiluminescence signal.

Figure 2
(A) Microsieve model experiment using BSA-biotin and streptavidin-HRP (B) Microsieve model experiment aimed at detecting endogenous human IgG

6.3.3 Microsieve-based detection of various infectious disease-related biomolecules

Since their inception during the late 1950's, immunoassays have become a widely utilized method; and in particular, have demonstrated significant diagnostic utility within the infectious disease testing arena [13,14]. After demonstrating basic feasibility via the model system experiments described in Figure 2, the next series of experiments were aimed at performing infectious disease-related immunoassays using microsieves as the solid support, and assessing whether they could distinguish positive from negative serum samples. To this end, we chose to coat microsieves with antigens that would enable detection of the following infectious disease-related antibodies: anti-*Rubella* IgG, anti-*B. burgdorferi* IgG/IgM, and anti-*T. pallidum* IgG. As shown in Figure 3A-C, the microsieves with viral or bacterial antigen were able to detect elevated levels of antibodies from positive control sera, relative to negative control sera at multiple antigen coating concentrations.

Figure 3

Images of chemiluminescent signals derived from negative and positive samples for the following microsieve-based immunoassays (A) Rubella (B) *B. burgdorferi* (C) *T. pallidum*

To better represent how the microsieve data relates to a more common immunoassay, RLU values were quantified and plotted relative to Optical Density values generated from the same samples using the respective comparator ELISA test systems (Figure 4A-C). These data

demonstrate that several different antigens are able to be coupled to the microsieve surface in a confirmation that is suitable for specific binding to antibodies from human serum, without excessive background signal from negative serum.

Figure 4
(A-C) Chemiluminescent signals from microsieve-based immunoassays were quantified and are presented along with corresponding ELISA data from the same samples.

6.3.4 Testing of a WHO standard using the *T. pallidum* microsieve immunoassay

Syphilis infection remains a persistent healthcare problem worldwide, and early detection is helpful for reducing patient morbidity (15). With this in mind, the *T. pallidum* microsieve immunoassay prototype was chosen for additional feasibility experiments. Serial dilutions of a syphilitic WHO IgG standard were assayed using the *T. pallidum* microsieve immunoassay prototype, as well as an FDA-approved ELISA. In Figure 5A, chemiluminescent images from analysis of the WHO standard are presented. Quantitation of the corresponding RLU values show that the microsieve-based assay can readily distinguish down to 0.01875 IU/mL from background signal (Figure 5B). The same dilutions of the WHO standard that were analyzed by ELISA turned negative after a 1:8 dilution (0.0375 IU/mL) (Figure 5B), suggesting that the microsieve immunoassay may be slightly more sensitive at detecting IgG antibody.

Figure 5
(A) Chemiluminescent signals from microsieve-based immunoassay analysis of syphilitic WHO IgG standard. The IU/mL of the WHO standard dilutions are presented below each respective chemiluminescent image. (B) A table is presented to compare the RLU values derived from microsieve-based assay analysis of the WHO standard relative to parallel analysis using an FDA-approved ELISA.

6.3.5 Testing of clinical samples using the *T. pallidum* microsieve immunoassay

Next, feasibility testing of the *T. pallidum* IgG microsieve immunoassay was continued by assaying clinical serum samples submitted for routine syphilis testing. As shown in Figure 6A, microsieve-based immunoassay signals obtained from ELISA-positive clinical samples were clearly distinguishable from an equal number of ELISA-negative samples. The ELISA kit positive control was also distinguishable from the negative control when simultaneously assayed by the microsieve assay, demonstrating that the immunoassay reagents used within the experiment were performing as expected. The RLU values determined from microsieve analysis were also in good agreement with corresponding O.D. values derived from ELISA ($R^2 = 0.9046$) (Figure 6B). Despite the relatively small number of samples tested, these data show that the microsieve immunoassay is able to detect endogenous anti-*T. pallidum* IgG from ELISA-positive clinical serum samples with signals that are clearly distinguishable from ELISA-negative samples.

Figure 6
(A) Chemiluminescent images are presented from microsieve-based analysis of ELISA kit controls, as well as ELISA-negative and ELISA-positive clinical serum samples. (B) A scatter plot is presented depicting the RLU values from microsieve analysis of clinical samples versus the corresponding O.D. values obtained from ELISA.

Future experiments will be aimed at incorporating simultaneous detection of syphilitic IgM antibodies, as well as non-treponemal antigens (e.g. RPR), and intra-assay controls towards producing a more clinically utilizable version of a microsieve-based *T. pallidum* assay.

6.4 Conclusions

We have demonstrated feasibility for utilizing microsieves, in combination with a reagent wicking device, as a unique and simple immunoassay platform capable of detecting various infectious disease-related antibodies from human serum. Additional feasibility experiments for *T. pallidum* show that microsieve-based infectious disease immunoassays have potential to achieve clinically relevant levels of sensitivity and specificity. Future work will be aimed at adapting the platform

for multiplex biomarker detection using whole blood; collectively towards developing rapid, simple, and adoptable diagnostic assays. To achieve this, several variations of the microsieve-based immunoassay are being explored. For example, larger 10 mm x 10 mm microseives have been developed, which offer increased surface area for spotting numerous antigens and controls (See supplemental Figure 1 for schematics). These larger microsieves containing spotted antigens and controls are also compatible with the reagent wicking device, and thus are amenable to a simple assay procedure. Also, due to measuring chemiluminescence, the microsieve-based immunoassay platform may be compatible with a simple imaging system such as a smartphone.

6.5 Supplemental Data

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Chapter 7

Distinguishing Septic from Normal Donors by detection of sPLA₂-IIA from Human Plasma Using a Microsieve-Based Immunoassay

Daniel R Zweitzig, Arjan G Tibbe, Mark J Kopnitsky, Kathleen Cichonski, Leon WMM Terstappen

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Abstract

Bloodstream infections that progress to septic shock are responsible for hundreds of thousands of deaths each year, and are associated with significant healthcare costs. Recent studies have shown that a member of the secreted phospholipase protein family, termed sPLA2-IIA, may play a role during the innate immune response to bacterial infections, and is elevated in the plasma of septic patients. In this report, the feasibility of a simple microsieve-based sPLA2-IIA detection immunoassay was explored. Microsieves containing 5 μm pores were covalently coupled with a sPLA2-IIA-specific monoclonal antibody at 0.1, 1.0, and 10 $\mu\text{g}/\text{mL}$ and then assayed with plasma-based positive and negative controls to determine the optimal coating concentration. Recombinant sPLA2-IIA was then serially diluted to a final concentration of 200, 100, 50, 25, 12.5, and 6.25 ng/mL and tested alongside a non-spiked sample to estimate the detection limit of the prototype assay. Recombinant sPLA2-IIA was also spiked into serum, EDTA-plasma, and Lithium-Heparin plasma, in an effort to evaluate assay performance when analyzing these sample matrices. The preliminary limit of detection studies suggest that the microsieve assay is able to distinguish approximately 6-12 ng/mL of sPLA2-IIA from a non-spiked sample. When compared to an immunoassay diluent, the microsieve assay also yielded acceptable percent recoveries for each of the three sample matrices spiked with clinically significant levels of sPLA2-IIA. The sPLA2-IIA microsieve assay prototype also clearly distinguished five samples from septic patients from five normal donor samples, and the results were in good agreement with a comparator ELISA test system ($R^2 = 0.9347$).

7.1 Introduction

Bloodstream infections that progress to sepsis and septic shock are a major cause of patient morbidity and mortality for hundreds of thousands of people each year (1,2). The economic burden of sepsis is significant, and is estimated to be around \$17 billion per year within the United States alone (1,2). Currently, blood culture testing is performed for patients suspected of having a bloodstream infection that may progress to septic shock, in an effort to confirm the presence of a microorganism. (3,4). Despite the widespread usage of blood cultures, the clinical utility is somewhat limited since the technique can take up to several days to confirm the presence of a bloodstream infection, while patients can progress from sepsis to septic shock over the course of hours (3-5). Furthermore, not all patients who develop sepsis yield positive blood culture results (6, 7). Emerging nucleic acid amplification methodologies have been developed to enable more rapid and sensitive detection of hematopathogens; however, the presence of circulating microbe-specific nucleic acid does not necessarily indicate that a given patient will become septic (7). Considering the shortcomings of the aforementioned methodologies, development of new tests aimed at detecting and/or monitoring the presence of endogenous sepsis-induced physiological biomarkers is warranted (8-10)

Secreted phospholipase A₂ (sPLA₂) proteins are calcium-dependent extracellular enzymes that catalyze the hydrolysis of phospholipids into pro-inflammatory molecules (11). A notable member of this protein family, termed sPLA₂-IIA, is suspected to play a role in the innate immune response to systemic bacterial infections, and has been shown to exhibit potent bactericidal properties (12-15). In addition, sPLA₂-IIA levels are markedly elevated in the plasma of patients with sepsis, relative to normal donors (15-17); further suggesting that this biomarker plays a role in the innate immune response. Other groups have shown that increased sPLA₂-IIA plasma levels are to distinguish bacterial from viral infections (18, 19). We recently demonstrated the feasibility of a simple 'antigen-down' microsieve-based immunoassay platform, capable of detecting infectious disease-related antibodies from human serum (20). This initial antigen-down approach required incubation of a serum sample to capture infectious disease-specific antibodies, followed by a second incubation with an HRP-conjugated anti-human antibody (a.k.a. 'two step immunoassay'). In this report, we set out to

assess the feasibility of creating a ‘one step’ microsieve-based dual monoclonal antibody immunoassay with sufficient sensitivity for distinguishing normal donors from septic patients via detection of sPLA2-IIA levels.

7.2 Materials and Methods

7.2.1 Microsieves and reagent wicking apparatus

For the sPLA2-IIA studies described herein, 5 mm x 5 mm square silicon nitride microsieves containing 5 μm pores were utilized. A description of the poly-acrylic acid (PAA) coating, plastic microsieve holder, and reagent wicking device were described previously (20).

7.2.2 Antibodies and recombinant protein

Two monoclonal antibodies (clones 9C8 and 6G2) and a full length recombinant sPLA2-IIA protein were obtained from Aterovax (Paris, France).

7.2.3 Assessment of anti-sPLA2-IIA monoclonal antibody coating concentrations

A detailed protocol for covalently coupling proteins to the surface of PAA-microsieves was described previously (20). In this study, the same procedure was adapted for covalently coupling an anti-sPLA2-IIA monoclonal antibody (clone 9C8). Briefly, the antibody was initially diluted to 0.1, 1.0, and 10 $\mu\text{g}/\text{mL}$ in 5 mM acetate buffer (pH 5.0). A 30 μL drop of the diluted antibody was then added to the pre-activated microsieve surface, incubated for 1 hour, then rinsed with deionized water. Next, a 30 μL drop of 100 mM ethanolamine (pH 8.5) was added to the microsieve surface, incubated for 30 minutes, and rinsed with phosphate buffered saline (pH 7.4). To provide additional blocking, a 30 μL drop of Stabliguard reagent (Surmodics, Eden Prairie MN) was added to the microsieve surface, incubated for 15 minutes, and removed by wicking through the bottom. Another 30 μL drop of Stabliguard reagent was added to the microsieve surface, incubated for 15 minutes, and removed by wicking through the bottom just prior to initiating the microsieve immunoassay procedure. To assess the optimal coating concentration, plasma-based positive and negative controls were taken from a sPLA2-IIA ELISA test system (ZEUS Scientific,

ACC6301I), and assayed according to the 'one step' microsieve immunoassay procedure described below.

7.2.4 'One step' microsieve immunoassay procedure, signal detection, and quantitation

A ready-to-use conjugate solution containing HRP-coupled to anti-sPLA2-IIA antibody clone 6G2 and wash buffer were taken from an existing sPLA2-IIA ELISA test system (ZEUS Scientific, ACC6301I) and utilized for the microsieve-based immunoassay procedures. To begin the immunoassay procedure, plasma samples were diluted 1:11 in the conjugate solution, then a 30 μ L drop of diluted plasma was added to the antibody-coated microsieve surface and incubated for 30 minutes. Using a simple wicking mechanism (20), the microsieve was then washed 4 times using 100 μ L of the ELISA kit wash buffer. Next, a 30 μ L drop of freshly mixed Femtoglow luminol reagent (cat# SHRPE21008DT, Michigan Diagnostics, Royal Oak MI) was added to the microsieve surface, and they were placed into a dark box and imaged using a Coolsnap HQ2 CCD camera (Photometrics, Tucson AZ) at various exposure times. Captured images were opened in ImageJ software and quantified using the 'analyze' tool.

7.2.5 Preliminary limit of detection experiment

Recombinant sPLA2-IIA was serially diluted in ELISA conjugate diluent to stocks with final concentrations of 200, 100, 50, 25, 12.5, and 6.25 ng/mL. A 30 μ L drop of each stock was added to the surface of a microsieve that was pre-coated with sPLA2-IIA specific antibody, and the immunoassay was performed as described above.

7.2.6 Sample matrix evaluation

Blood from a single donor was drawn into three different types of collection tubes [Becton Dickinson - Serum: tube cat #367977, EDTA-plasma: tube cat#366450, Lithium-heparin: tube cat#367880]. Serum and plasma were prepared from each tube type according to the manufacturer's recommendations. Recombinant sPLA2-IIA was spiked into each matrix, along with an ELISA conjugate diluent control, to a final concentration of 25 ng/mL. Spiked and non-spiked matrices, and the diluent control, were subsequently assayed in parallel according to the microsieve immunoassay procedures described above.

7.2.7 Analysis of plasma samples from normal and septic patients

Lithium-heparin plasma samples from healthy donors (n = 5) and septic patients (n = 5) were obtained from Bioreclamation Inc. (Westbury, NY). For each sepsis sample, the causative microorganism was also provided by the vendor. All samples were collected as de-linked remnant specimens, thus protecting donor anonymity. Each sample was tested using the sPLA2-IIA microsieve assay according to the procedures described above. The same samples were also tested using an sPLA2-IIA-specific ELISA (ZEUS Scientific, ACC6301I) according to the procedures outlined in the associated product insert.

7.3 Results and Discussion

7.3.1 Overview of the sPLA2-IIA microsieve immunoassay

Monitoring the clinical progression from a bloodstream infection to the potentially fatal pathophysiological condition of septic shock is an important part of emergency and intensive care medicine (3, 4). Several endogenous physiological sepsis biomarkers have emerged in recent years, and have the potential to offer useful diagnostic and prognostic information (8-10). Among these putative biomarkers, sPLA2-IIA has gained increasing attention due to its intrinsic bactericidal activity, as well the observed increase in plasma levels within septic patients (12-18). We recently demonstrated for the first time the utilization of a simple microsieve-based immunoassay platform for detection of serum-derived antibodies during a traditional 'two step' incubation procedure (20). In this report, we assessed the feasibility of producing a microsieve-based dual monoclonal antibody sandwich immunoassay for detection of human sPLA2-IIA, using a single incubation procedure. A schematic of the approach is illustrated in Figure 1. Panel A shows an image of the microsieve holder containing a 5 x 5 mm silica nitride microsieve coated with an sPLA2-IIA-specific monoclonal antibody. Panel B illustrates the simple immunoassay procedure that utilizes a capillary-based reagent transfer mechanism.

Figure 1 - (A) Images depicting a poly-acrylic acid coated 5 x 5 mm microsieve mounted within a plastic holder, as well as the antibody coating **(B)** Schematic presentation depicting a general overview of sPLA2-IIA microsieve immunoassay procedure

7.3.2 Antibody coating optimization and sensitivity assessment

Feasibility assessment of the sPLA2-IIA immunoassay was initiated by evaluating assay signal from microsieves coated with an anti-sPLA2-IIA monoclonal antibody at various concentrations. As shown in Figure 2A, the maximum signal obtained from assaying a plasma-based positive control sample was derived from the microsieve coated with 10 $\mu\text{g/mL}$ of antibody. A plasma-based negative control sample did not yield any noticeable signal from sieves coated with any of the three antibody concentrations (Figure 2A). Thus, an antibody coating concentration of 10 $\mu\text{g/mL}$ was chosen for subsequent experiments. To begin assessing the detection limit of the prototype assay, recombinant sPLA2-IIA was serially diluted and assayed versus a non-spiked control. This experiment revealed that the microsieve-based sPLA2-IIA assay was able to distinguish signal derived from samples spiked at approximately 6-12 ng/mL of protein from a non-spiked sample (Figure 2B). Recent internal studies using a commercially available ELISA suggest that a clinically significant plasma level cut off for sPLA2-IIA is around 25-50 ng/mL (manuscript in preparation); thus, the ability of the prototype microsieve assay to readily

distinguish 6-12 ng/mL of sPLA2-IIA from background signal, suggest that it may possess sufficient sensitivity.

Figure 2 - (A) Images of chemiluminescent signals are presented from testing of plasma-based controls using microsieves coated with various anti- sPLA2-IIA antibody concentrations (B) Images of chemiluminescent signals are presented from testing of serially diluted recombinant sPLA2-IIA protein, using microsieves coated with 10 $\mu\text{g/mL}$ of antibody

7.3.3 Sample matrix evaluation

After evaluating detection of sPLA2-IIA from sample diluent, the next experiment was aimed at determining if there are any apparent differences in recovery from various sample matrices. To this end, recombinant sPLA2-IIA was spiked into three different sample matrices at a clinically relevant level, in parallel with sample diluent. Spiked and non-spiked matrices, as well as sample diluent, were subsequently assayed using the prototype microsieve assay. Figure 3A shows the assay signals that were obtained from each spiked sample, and also reveals that non- spiked samples did not exhibit any apparent background signal. The relative light unit (RLU) values from each spiked matrix were also quantified, and the percent recovery values relative to parallel-

spiked sample diluent were 96.3%, 101.1%, and 91.7% for Serum, EDTA-plasma, and Lithium Heparin-plasma respectively. A graphical representation of the percent recoveries for each matrix is presented in Figure 3B.

Figure 3 - (A) Images of chemiluminescent signals are presented from testing of the indicated sample matrices that had been spiked with clinically relevant amounts of recombinant sPLA2-IIA (25 ng/mL), versus non-spiked controls (B) Chemiluminescent signals from microsieve-based analysis of the various spiked sample matrices were quantified and percent recovery values were calculated relative to spiked sample diluent

Together, the data from this spike and recovery experiment suggest that all three sample matrices tested are all compatible with the sPLA2-IIA microsieve assay; however, future experiments that evaluate patient-matched matrices containing endogenous sPLA2-IIA protein would be beneficial.

7.3.4 Testing of clinical sepsis samples using the sPLA2-IIA microsieve immunoassay

Sepsis is a deadly condition associated with a dramatic and damaging physiological response to an infection. Improper diagnosis and ineffective monitoring of this condition can delay proper treatment, or conversely, can foster improper administration of antimicrobials; thus, further development of methods to detect sepsis-specific biomarkers is warranted (10). Detection of sPLA2-IIA in plasma has been shown to offer diagnostic and prognostic utility (15-19), however, simple methods detect its presence lacking. To this end, the sPLA2-IIA microsieve assay was evaluated using plasma samples derived from patients diagnosed with sepsis, versus plasma from normal donors. As shown in Figure 4A, microsieve assay signals derived from septic patients were clearly distinguishable from those derived from normal donors. A plasma-based positive and negative control were included in parallel, and confirmed that the assay reagents were performing as expected (Figure 4A). In Figure 4B, the causative microorganism for each septic patient is also listed, and revealed that sPLA2-IIA is elevated in response to infection from a surprisingly diverse set of bacteria, as well as a *Candida* species.

A

B

Sepsis Patients	
<u>Sample ID</u>	<u>Associated Microorganism</u>
1	<i>Staphylococcus hominus</i>
2	<i>Escherichia coli</i>
3	<i>Streptococcus pneumoniae</i>
4	<i>Candida parapsolosis</i>
5	<i>Staphylococcus aureus</i>

Figure 4 - (A) Chemiluminescent signals from microsieve-based immunoassay analysis of plasma samples derived from sepsis patients versus normal donors are presented (B) The causative microorganism associated with each septic patient is presented

Next, the RLU values associated with each microsieve assay signal were quantified and are presented in Figure 5A. In addition to microsieves, the same normal and septic samples were tested using a quantitative sPLA2-IIA ELISA test system, and a graph of the resultant ng/mL values is presented in Figure 5B. In an effort to compare the microsieve and ELISA data, a scatter plot comparing RLU versus ng/mL values was subsequently generated and regression analysis was performed. As shown in Figure 5C, the R^2 value of 0.9347 demonstrates that the data obtained from the two different methodologies are in good agreement and support the notion that the prototype microsieve assay possesses sufficient analytical sensitivity to distinguish septic from normal patients.

A

B

C

Figure 5 - (A) Chemiluminescent signals from microsieve-based analysis of septic and normal donors were quantified and graphed (B) The ng/mL values derived from ELISA analysis of septic and normal donors were graphed (C) A scatter plot was generated using microsieve RLU values versus the ELISA ng/mL data, and regression analysis was performed

This agreement with ELISA is impressive, since the ELISA test system requires two independent one hour incubations at 37°C, totaling about 2.5 hours of total assay time; whereas the microsieve assay only requires one core incubation of 30 minutes at room temperature, totaling about 40 minutes of total assay time. It should also be noted that both the microsieve and ELISA

data revealed that sPLA2-IIA was highly elevated in response to a *Candida* infection. To our knowledge, elevation of sPLA2-IIA plasma levels in response to a fungal infection has not been previously reported, and suggests that this biomarker's clinical diagnostic utility may not be limited to bacterial infections.

7.4 Conclusion

We have demonstrated, for the first time, the feasibility of utilizing microsieves, in combination with a wicking device, as a simple platform for detecting sPLA2-IIA from human plasma. Furthermore, this early prototype of the microsieve-based sPLA2-IIA assay was able to effectively distinguish plasma derived from clinically diagnosed septic patients from that of normal donors with a nearly identical capacity as a commercially available ELISA test system. Future experiments will be aimed at generating a version of the sPLA2-IIA microsieve assay that can utilize whole blood as the sample matrix, in an effort to better enable point of care utility. Experiments aimed at spotting multiple antibodies, antigens and/or controls onto the microsieve surface are also underway.

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Chapter 8

Pilot Experiments: Measurement of the Sepsis

Biomarker sPLA₂-IIA from Whole Blood

Daniel Zweitzig, Arjan Tibbe, Leon Terstappen

8.1 Introduction

Direct and indirect detection of infectious disease agents often relies upon the measurement of circulating biomarkers from a patient's blood. Many traditional diagnostic methods, such as ELISA, require separation of whole blood into plasma or serum prior to analysis (1). In the first step of this process, blood is collected into vacuum tubes containing different types of anticoagulants for preparing plasma or specialized gels for preparing serum (1). Regardless of the tube type, preparation of plasma or serum typically requires transportation to a diagnostic laboratory, followed by centrifugation to separate the blood components. Depending upon the setting, the sample transportation and blood separation steps can take up to several hours to occur (2). These time-consuming blood processing procedures are acceptable when a patient's life is not immediately at risk; however, a more rapid turn-around time is desired when serious clinical conditions such as bloodstream infections are suspected. Consequently, an interest in the development of rapid point of care (POC) diagnostics for this purpose continues to grow (3, 4). Some desirable features of a typical POC test include: 1.) The ability to utilize complex matrices such as whole blood 2.) Relatively low cost 3.) Easy to use 4.) Relatively low time to result 5.) Acceptable sensitivity and accuracy relative to existing reference methods (3, 4). In chapter 7 of this thesis the feasibility of detecting the sepsis biomarker, sPLA2-IIA, using a simple microsieve-based immunoassay was presented. Despite achieving acceptable sensitivity, and correlation to an existing ELISA test system, these studies were all performed using human plasma that had been previously prepared from whole blood. In this chapter, pilot experiments aimed at modifying the microsieve-based immunoassay for rapid and sensitive detection of sPLA2-IIA from whole blood are presented; all towards moving this approach closer to a POC format. A concept for adapting the microsieve-based immunoassay into a POC cartridge is also discussed.

8.2 Materials and Methods

Attempts at detecting sPLA2-IIA from whole blood via the microsieve-based immunoassay closely mimicked the procedures described in Chapter 7 that were used for plasma. For all the following experiments, 5 mm x 5 mm square, poly-acrylic acid (PAA)-coated, silicon nitride microsieves containing 10 μm pores were utilized. A description of the PAA coating, coupling of the antibody to the microsieve surface, the plastic microsieve holder, and reagent wicking device were described in chapters 6 and 7. However, an overview of the specific procedures and reagent modifications used to adapt the assay for use with whole blood can be found within the following sections.

8.2.1 First Attempt at Detecting sPLA2-IIA from Whole Blood (Venous Collection) Using the Microsieve-Based Immunoassay

Anti-sPLA2-IIA monoclonal antibody (clone 9C8) was covalently coated onto the microsieve surface at a concentration of 10 $\mu\text{g}/\text{mL}$ as described previously. Blood from an apparently healthy donor was collected into two EDTA vacutainer tubes (BD, cat# 366450). Plasma was prepared from one of the tubes by centrifuging at 3000 rpm for 10 minutes at 4° C. Recombinant sPLA2-IIA protein was spiked at a final concentration of 500 ng/mL into a master stock of donor-matched plasma and whole blood. Each master stock was diluted in its respective matrix to create additional sample stocks at 100 ng/mL and 20 ng/mL. All spiked samples, as well as non-spiked matrix controls, were diluted 1:5 in an ELISA sample diluent containing 0.06 $\mu\text{g}/\text{mL}$ of an anti-sPLA2-IIA monoclonal antibody (clone 6G2) conjugated to HRP. Thirty microliters of each diluted sample were added to the surface of microsieves that had been pre-coated with antibody, then incubated at room temperature for 30 minutes. After the incubation, the microsieves were washed twice with 0.5 mL of ELISA washer buffer. After washing, luminol reagents were mixed at a 1:1 ratio, then added to the surface of each microsieve. Assay signals were subsequently captured and quantitated as described in chapters 6 and 7.

8.2.2 Optimization and Repeat Attempt at Detecting sPLA2-IIA from Whole Blood (Venous Collection) Using the Microsieve-Based Immunoassay

All experimental procedures for the second whole blood experiment were the same as the first, except for the following variations: 1.) The anti-sPLA2-IIA monoclonal antibody (clone 9C8) was coated onto the sieve surface at a concentration of 100 µg/mL (i.e. 10-fold higher than before), 2.) Only whole blood from an EDTA collection tube was utilized (i.e. no plasma was prepared in parallel), 3.) Whole blood was spiked at 100 ng/mL, 50 ng/mL, and 25 ng/mL 4.) The concentration of the anti-sPLA2-IIA conjugate (clone 6G2) was 16.7-fold higher than the first experiment (i.e. 1 µg/mL), 5.) An additional luminol substrate (ThermoFisher, SuperSignal, cat # A38555) was used in parallel with the standard Femptoglow substrate. Spiked blood samples were assayed in parallel with non-spiked blood according to the procedures described above.

8.2.3 Finger Stick Collection and Microsieve Flow-Through Attempt

Microsieves were coated with 100 µg/mL of anti-sPLA2-IIA monoclonal antibody (clone 9C8) as described previously. A finger stick was performed using a 30-gauge lancet (BD, 325773) via a spring-loaded device according to the manufacturer's recommended procedures.

Approximately 15 µL of blood was removed from the finger surface via capillary action using a micropipette (Hemosense, Microsafe, cat # 200235) and dispensed onto the microsieve surface. Two drops of sample diluent containing 1 µg/mL of anti-sPLA2-IIA conjugate (clone 6G2) were also subsequently added to the sieve surface. The blood/diluent mixture was then incubated at room temperature for 30 minutes. Wicking of the blood sample was attempted.

8.2.4 Detection sPLA2-IIA from Whole Blood (Finger Stick Collection) Using the Optimized Microsieve-Based Immunoassay

Microsieves were coated with 100 µg/mL of anti-sPLA2-IIA monoclonal antibody (clone 9C8) as described previously. Sample diluent containing 1 µg/mL of anti-sPLA2-IIA conjugate (clone

6G2) was supplemented with K3-EDTA to a final concentration of 2 mg/mL. Three separate stocks of diluent/conjugate/EDTA solutions were spiked with sPLA2-IIA to a final concentration of 100 ng/mL, 50 ng/mL, and 25 ng/mL respectively. Two drops of from each spiked stock of sample diluent/conjugate/EDTA solution, as well as a non-spiked stock (approximately 30 μ L each) were added to the surface of the antibody-coated microsieves. A finger stick was performed, as described above, and 15 μ L blood samples were dispensed directly into the diluent/conjugate/EDTA solution on the microsieve surfaces. The blood/diluent mixture was then incubated at room temperature for 25 minutes. After incubation, the microsieves were washed twice with 0.5 mL of ELISA washer buffer via wicking. After washing, luminol reagents were mixed at 1:1 ratio, then added to the surface each sieve. Assay signals were subsequently captured and quantitated as described in chapters 6 and 7.

8.2.5 Evaluation of Reproducibility from Whole Blood (Finger Stick Collection) Using the Optimized Microsieve-Based Immunoassay

All procedures for this whole blood experiment were the same as described in the previous experiment, except for the following variations: 1.) Sample diluent/conjugate/EDTA solution was only spiked to a final concentration of 25 ng/mL, 2.) Four replicates of the 25 ng/mL spiked diluent were assayed in parallel with four replicates of non-spiked diluent, along with blood derived from finger sticks.

8.3 Results and Discussion

Measurement of sPLA2-IIA levels from blood samples represents a promising approach for distinguishing septic from non-septic patients (5). One published study, utilizing an ELISA test comprised of the same sPLA2-IIA antibodies used in this thesis, demonstrated that an assay cutoff value of 25 ng/mL yields the optimal clinical sensitivity and specificity (6). However, all published reports related to measuring sPLA2-IIA for this purpose, including the studies presented within Chapter 7 of this thesis, were derived from experiments using serum or plasma. Since detecting sepsis should ideally occur as quickly as possible, development of

assays that measure sPLA2-IIA directly from whole blood is of interest; especially since such an option is not commercially available at this time. Consequently, we set out to assess the ability of the microsieve-based sPLA2-IIA immunoassay to utilize whole blood as the sample matrix. Since 25 ng/mL represents a clinically significant cutoff value, the preliminary goal was to ensure that the whole blood microsieve assay could discriminate samples containing sPLA2-IIA at that level, from non-spiked samples. In an initial experiment, spiked sPLA2-IIA was assayed from whole blood versus donor-matched plasma, using the same microsieve-based immunoassay reagent formulations as those presented in chapter 7. As shown in Figure 1, whole blood samples spiked with sPLA2-IIA yielded lower signals relative to donor-matched plasma, as well as a higher background signal; thus, demonstrating that performance of the existing assay conditions is insufficient when utilizing this sample matrix.

Figure 1 – An initial attempt at microsieve-based immunoassay detection of spiked sPLA2-IIA recombinant protein from EDTA-plasma and donor-matched whole blood (venous collection), versus non-spiked samples

Considering the data presented in Figure 1, an additional experiment was designed towards improving detection of sPLA2-IIA from whole blood. This follow up experiment included some modifications to the original sPLA2-IIA microsieve immunoassay conditions; such as increasing the antibody coating concentration, increasing the HRP-antibody conjugate concentration, and evaluating an alternative luminol reagent. The data from this follow up experiment revealed an

improved ability to distinguish clinically relevant levels of sPLA2-IIA (i.e. 25 ng/mL) from whole blood versus a non-spiked donor-matched sample (Figure 2).

Figure 2 – (A) Detection of recombinant sPLA2-IIA from whole blood (venous collection) after making modifications to antibody concentrations within the microsieve-based immunoassay, as well as parallel evaluation of a different luminol substrate. (B) Quantification and graphical representation of the corresponding RLU values is presented.

Although improved detection of sPLA2-IIA from whole blood was achieved via reagent modifications (Figure 2), the first two experiments described above were performed using samples derived from a venous collection of whole blood into vacuum tubes containing anticoagulant. However, collection of blood via less complex methods, such as a finger stick, is more amenable to a true POC approach. Therefore, a few additional experiments were performed towards assessing the ability of the microsieve immunoassay to utilize whole blood collected via finger sticks. In a preliminary experiment, blood collected via finger stick was added to the surface of the microsieve, followed by a few drops of the same sample diluent

used in the experiments associated with Figures 1 and 2. After a short incubation, an attempt was made to pass the blood through the microsieve via capillary action; however, the sample did not flow through. It was suspected that the sample did not pass through the microsieve due to blood coagulation during the incubation. Thus, an anticoagulant was added to the assay sample diluent, and the experiment was repeated. As shown in Figure 3, after adding an anticoagulant to the sample diluent, the microsieve assay was able to clearly distinguish clinically relevant levels of sPLA2-IIA from finger stick blood samples, relative to a non-spiked control sample. It should be noted that in previous experiments using venous blood, sPLA2-IIA was spiked directly into the whole blood, then diluted with a larger volume of sample diluent. However, in this finger stick experiment, the sample diluent was spiked with sPLA2-IIA, thus resulting in a larger volume of spiked sample being assayed; all of which explains a more robust signal at the 25 ng/mL level relative to the venous blood experiments.

Figure 3 - Sensitive detection of spiked sPLA2-IIA recombinant protein from whole blood derived from finger stick samples using the optimized microsieve-based immunoassay

After showing that addition of anticoagulant to the microsieve assay sample diluent enabled sensitive detection of sPLA2-IIA from finger stick samples, an additional experiment was setup to assess the reproducibility of the new assay conditions. This preliminary experiment demonstrated that the assay can reproducibly distinguish low levels of sPLA2-IIA from non-spiked samples (Figure 4).

Figure 4 – The reproducibility of detecting sPLA2-IIA at clinically relevant levels from whole blood derived from finger stick samples using the optimized microsieve-based immunoassay

Despite being able to sensitively detect sPLA2-IIA from whole blood derived from a finger stick, the newly optimized microsieve-based immunoassay still relies upon addition of reagents such as wash buffer and luminol via manual pipetting. Therefore, additional assay adaptations were considered towards bringing the approach even closer to a POC format. In Figure 5A, images are presented for a simple reagent delivery and removal device, containing a pre-mounted microsieve, that could better-meet the POC criteria described previously. This device was originally developed and successfully utilized for human cell capture, imaging, and enumeration from various bodily fluids Figure 5B.

Figure 5 – (A) Image of a simple POC staining device containing a pre-mounted microsieve **(B)** Images related to the how the POC device can be used for capture and staining of leukocytes.

In the new immunoassay application proposed herein, the microsieve surface could be pre-coated with an anti-sPLA2-IIA capture antibody as described previously (or other biomolecules if desired), then mounted into the device. In all the experiments to date, the chemiluminescent detection substrate consisted of a two-part luminol reagent. However, other single reagent detection approaches could be considered towards simplifying the last step of the procedure. For example, if the anti-sPLA2-IIA antibody (clone 6G2) is conjugated to a luciferase enzyme, instead of HRP, then a single detection reagent such as D-luciferin could be utilized for detection. To begin running the POC assay, a drop of blood from a finger stick collection could be added to the microsieve surface, followed by two drops of an assay diluent containing anti-sPLA2-IIA conjugated to luciferase, then incubated for 15-20 minutes. The remaining assay procedures could be carried out via a few simple turns of the device as outlined within Figure 6, however, a brief overview of how the cassette might function is provided as follows. After incubating a diluted blood sample for 15-20 minutes to allow formation of an antigen/antibody

complex, the cassette is turned to the second position, during which the bottom of the microsieve contacts an absorbent cube. The diluted blood sample then moves through the microsieve surface into the absorbent cube via capillary action. The cassette is turned to the third position and ruptures a blister pack containing wash buffer. Upon rupture, the wash buffer passes up through the microsieve surface to resuspend residual undesired blood components, as well as excess anti-sPLA2-IIA detection conjugate. The cassette is then turned to the fourth position, during which the bottom of the microsieve contacts an absorbent cube to remove the wash buffer via capillary action. Finally, the cassette is turned to the fifth and ruptures a blister pack containing D-luciferin substrate, followed by chemiluminescent signal detection.

Figure 6 – Schematic overview of how the proposed sPLA2-IIA POC device could function

Alternatively, the anti-sPLA2-IIA antibody (clone 6G2) could be conjugated to various fluorophores, thus obviating the need for a detection substrate altogether. However, the drawback of the fluorescent detection approach would be the need for more specialized equipment, such as the cytometer presented in Figure 5.

8.4 Conclusions

In this chapter, several pilot experiments were presented for detecting the sepsis biomarker sPLA2-IIA from whole blood using the microsieve-based immunoassay. After making modifications to the existing assay procedures and reagents, sPLA2-IIA could be reproducibly detected from whole blood samples, at clinically relevant levels. The ability to sensitively and reproducibly detect this protein biomarker directly from whole blood moved the microsieve-based approach closer to being utilizable as a POC assay. To our knowledge, this is the first time sPLA2-IIA has been detected directly from whole blood. Furthermore, a concept was also presented for a microsieve-based POC device that could enable sequential delivery of sPLA2-IIA reagents in a more simplistic and controlled fashion.

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Summary and Outlook

Detection of infectious disease agents, and/or the physiological responses they induce, plays an important role in protecting public health. This thesis began by providing a historical overview of the various ways in which microorganisms, and the infections they cause, are detected (Chapter 1). In Chapter 2, a novel methodology was presented for universally detecting microorganisms via sensitive measurement of endogenous DNA polymerase activity (termed ETGA). By circumventing the reliance upon recognition of specific nucleic acid sequences, ETGA offers significant potential to detect a much broader range of pathogens in normally sterile fluids relative to pre-existing PCR-based methods. Chapter 3 demonstrated how the ETGA assay could be combined with a selective cell lysis procedure to enable sensitive detection of microorganisms from spiked blood culture samples. This model system study revealed that the ETGA approach can reduce the time-to-detection of blood culture samples spiked with clinically relevant pathogens, relative to traditional detection methods. In Chapter 4, the potential utility of the ETGA-blood culture assay approach was evaluated in collaboration with a local hospital using samples derived from patients suspected of having bloodstream infections. This study confirmed that the ETGA-blood culture assay can reduce the time-to-detection of samples containing a variety of patient-derived hematopathogens. In addition to blood culture samples, the ETGA assay in combination with a modified selective cell lysis procedure was also evaluated for the ability to sensitively detect microorganisms within platelet concentrate units. This approach was presented in Chapter 5, and it showed that the assay could sensitively detect a range of clinically-relevant bacteria that had been spiked into platelet concentrate units. Thus, the ETGA-based platelet screening assay represents a promising approach for sterility screening of blood components prior to transfusion.

Despite achieving sensitive and universal detection of microorganisms from complex biological matrices, the ETGA assay applications presented in chapters 3-5 required manual sample processing procedures to eliminate hematopoietic cell-derived DNA polymerase activity. These sample processing procedures consisted of multiple rounds of selective lysis and centrifugation steps, aimed at retaining intact microorganisms while removing lysed hematopoietic cell

fractions; all of which require over 30 minutes of hands-on time to perform. Since hands-on sample processing procedures are undesirable in a modern clinical laboratory setting, more automatable upstream ETGA assay procedures were explored. These preliminary studies were not included in the main thesis, but will be discussed briefly below.

Filtration is one approach already being used for automating the isolation of microorganisms from biological samples, prior to nucleic acid amplification. For example, in the Cepheid Xpert system (discussed in Chapter 1), biological samples collected via swabbing are eluted into an assay buffer, then passed through a filter to collect microorganisms and simultaneously discard unwanted sample matrix components. The captured microorganisms are then lysed via sonication and the liberated nucleic acid is amplified/detected via qPCR (1-3). Given the success of this approach, we began evaluating additional selective lysis procedures that would enable filtration of blood culture samples through small pores (i.e. 0.1- 0.5 μM) to capture microorganisms. Once captured on the surface of the filter, the microorganisms could then theoretically be lysed or eluted to initiate ETGA analysis (Figure 1).

Figure 1 – Schematic overview of selective cell lysis followed by filter capture of microbes

Utilization of microsieves as the filter type was desirable, since directly lysing and/or eluting microbes from the two-dimensional filter surface was anticipated to be more efficient than doing so from more complex, three-dimensional filter matrices, such as PVDF membranes.

In follow-up studies not presented in the main chapters of this thesis, updates to the selective lysis procedures such as treatment with DNase and Trypsin were attempted; all towards trying to improve the filterability of complex biological samples. These preliminary studies also assessed the viability of bacteria spiked into samples undergoing the various lysis conditions. In Figure 2, a representative experiment is presented for blood culture samples. In this experiment, samples that had been treated with surfactants (with or without DNase) clogged 0.1 μ M pore spin filters, while samples treated with surfactants plus Trypsin were able to pass through the filters more effectively (Figure 2A). These experiments also revealed that the various lysis conditions did not grossly affect the viability of bacteria, thus maintaining feasibility of the approach (Figure 2B).

A

B

Figure 2 – (A) Leukocyte-derived genomic DNA degradation and filterability (0.1 μ M PVDF filter spin column) were assessed for blood culture samples after incubation with various selective cell lysis reagents (B) Images of colony forming units derived from plating blood culture samples spiked with *S. aureus* bacteria onto brain heart infusion agar

The most effective lysis procedures (numbered 11 and 12 in Figure 2) were repeated on freshly collected blood culture samples and the filterability of those samples was subsequently evaluated using microsieves containing 0.45 μ M pores. Despite being successfully filtered

through spin columns, none of the lysed blood culture samples or untreated controls passed through the microsieves (data not shown). Thus, exploration and/or consideration of alternative approaches that would be amenable to automating the ETGA assay approach are warranted in the future. Notably, the ETGA technology was recently licensed to a company for continued assay automation and sepsis detection-related development efforts. In a recent published report, this company demonstrated an expansion of the assay's clinically utility by using it to confirm negative blood cultures earlier than existing methods, and presented an automated sample processing procedure (4). Other possible future applications for ETGA include sterility screening for pharmaceuticals, therapeutic biologics, environmental samples, and filterable beverages, as well as antimicrobial susceptibility testing (AST). Notably, in a separate published study not included in this thesis, we demonstrated the feasibility of utilizing the ETGA assay as a rapid and universal molecular AST method (5).

In chapter 6, the focus of the thesis shifted from directly detecting microorganisms via ETGA, to indirectly detecting the presence of infectious disease agents via measurement of serologic biomarkers produced by the host immune response. To achieve this, development of a microsieve-based immunoassay procedure was described. During these feasibility studies, the microsieve assay was shown to detect clinically relevant levels of antibodies against infectious disease agents such as Rubella (German Measles), *Borrelia burgdorferi* (Lyme Disease), and *Treponema pallidum* (Syphilis). In Chapter 7, this approach was expanded upon by the development of a microsieve-based immunoassay for detecting the sepsis-induced serologic biomarker sPLA2-IIA. The sPLA2-IIA assay also achieved clinically relevant analytical sensitivity and could clearly distinguish sepsis patients from apparently healthy controls. Chapter 8 presents pilot experiments aimed at optimization of the sPLA2-IIA microsieve assay for utilization of whole blood as the sample matrix. After making a few adjustments to the sample diluent, as well as the antibody coating concentration, these studies demonstrated an improved ability of the assay to sensitively detect sPLA2-IIA spiked into whole blood. Furthermore, towards the end of Chapter 8, a concept for adapting the sPLA2-IIA microsieve assay into a POC device was described. In this proposed approach, blood samples could be added to the surface of the microsieve, then the remainder of the assay protocol could be easily performed by

simply rotating the position of the device a few times to deliver and remove internally-housed reagents.

Other modifications to the microsieve-immunoassay POC device could be considered in the future towards improving potential utility. For example, instead of coating the entire surface of the microsieve with a single biomolecule, spotting multiple antigens or antibodies towards multi-analyte detection would be an interesting approach to explore. Furthermore, spotting of multiple biomarkers could enable incorporation of intra-assay controls and calibrators for monitoring performance and determining accurate results. Some preliminary attempts at spotting proteins onto the microsieve surface, followed by signal detection via immunoassay have already been attempted (right side of Figure 3A). However, additional studies are needed to further develop and optimize the approach. Also, detection of chemiluminescent signals via simple devices such as a cell phone represent a concept to explore (Figure 3B).

Figure 3 - (A) Concept of the POC device mounted with a microsieve containing numerous antigen/antibody spots for running a multiplex immunoassay presented. Preliminary image from preliminary antigen spotting experiment is also presented to the right. (B) Concept of adapting the POC device to accommodate signal detection via a smartphone camera is presented.

While completing this thesis the COVID-19 pandemic has affected the world, with the causative agent being identified as the SARS-CoV-2 pathogen. This pandemic has necessitated the development of numerous vaccines and therapies; however, it has also sparked the development of various nucleic acid-based and serology-based diagnostic approaches. As mentioned previously, indirect detection of infection via serology-based measurement of disease specific antibodies requires pathogen specific antigens. The SARS-CoV-2 virus contains several biomolecules that are candidate antigens for serology assays, which include a nucleocapsid protein, surface spike proteins, an envelope protein, a membrane protein, and hemagglutinin esterase. To date, the nucleocapsid and spike proteins are the most widely utilized for developing serologic assays intended for detection of SARS-CoV-2 specific IgG, IgM, and IgA antibodies. Theoretically, the microsieve-based immunoassay approach could be

applied to simultaneously detect antibodies to various SARS-CoV-2 antigens from a single drop of whole blood.

In addition to detecting soluble disease-specific antigens or antibodies from blood, another possible application of the microsieve POC device would be for capturing infectious-disease related cells. In one such approach, antibodies specific to a bacterium could be coated onto the surface of microsieves containing larger pores (i.e. 5 μM or 10 μM), then cells would be captured in an antibody-dependent fashion while the samples are passing through the membrane (Figure 4).

Figure 4 - Overview of a proposed approach for capturing and staining bacteria using the microsieve POC device.

In fact, feasibility of microsieve-based capture of heat-killed *Salmonella* bacteria from sample matrices such as milk has already been demonstrated (6). Additional studies (not included in the main chapters of this thesis) were also performed to begin evaluating the ability of antibody-coated microsieves to capture live *Salmonella* bacteria from more complex biological matrices. In these experiments, the feasibility of antibody-dependent capture of bacteria from

complex matrices such as whole blood or platelet rich plasma were demonstrated, however, the recovery of bacteria was relatively poor (Figure 5).

Figure 5 – *S. enterica* bacteria were spiked into PBS or whole blood samples, then passed through antibody-coated microsieves. Representative images are presented for FITC-stained bacteria on the surface of microsieves used to filter both spiked-matrices.

Thus, further optimization and experimentation would be required to more accurately assess the potential of this approach to be considered clinically utilizable. Another potential utility for the POC device would be to capture and image immune cells via surface markers upregulated/modified/exposed in response to an infectious-disease process. For example, CD64 is upregulated on the surface of neutrophils in response to infections (7), and one study demonstrated that that measuring it via flow cytometry can distinguish septic from normal donors (8). Considering this, the microsieve-based POC device could represent a simple alternative to flow cytometry for measuring infection-related biomarkers such as CD64 via capture and staining.

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Short Summary

There is an ongoing need for improved methods to detect microorganisms and the infections they cause. This thesis described new molecular and serologic approaches related to detection of infectious disease agents, as well as other biomolecules upregulated by the host immune response. Notably, the ETGA assay described in Chapter 2 represents a truly novel biochemical method for detecting microorganisms that had never been described prior. By combining the ETGA assay with selective cell lysis procedures, several clinically relevant applications were also demonstrated. In addition to ETGA, a novel microsieve-based immunoassay approach was described. Preliminary studies using this simple immunoassay procedure demonstrated clinically relevant sensitivity for detecting infectious disease-specific antibodies, as well as the sepsis biomarker sPLA2-IIA. Towards the end of the thesis, numerous follow-up studies were also proposed, which offer potential for continued advances within the field of infectious disease diagnostics.

Short Summary (in Dutch)

Er is een voortdurende behoefte aan verbeterde methoden om micro-organismen en de infecties die ze veroorzaken te detecteren. Dit proefschrift beschrijft nieuwe moleculaire en serologische benaderingen gerelateerd aan detectie van infectieuze ziekteverwekkers, evenals andere biomoleculen die door de immuunrespons van de gastheer worden gereguleerd. Opmerkelijk is dat de ETGA-test beschreven in hoofdstuk 2 een echt nieuwe biochemische methode voor het detecteren van micro-organismen vertegenwoordigt die nog nooit eerder was beschreven. Door de ETGA-test te combineren met selectieve cellysisprocedures, werden ook verschillende klinisch relevante toepassingen aangetoond. Naast ETGA werd een nieuwe op microzeven gebaseerde immunoassaybenadering beschreven. Voorlopige studies met behulp van deze eenvoudige immunoassay-procedure toonden klinisch relevante gevoeligheid voor het detecteren van infectieziekte-specifieke antilichamen, evenals de sepsis biomarker sPLA2-IIA. Tegen het einde van het proefschrift werden ook talrijke vervolgstudies voorgesteld, die potentieel bieden voor verdere vooruitgang op het gebied van diagnostiek van infectieziekten.

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