

MOORMAN, WHITNEY DANIELLE, M.S. Exploratory Studies into the Therapeutic and Diagnostic Capability of Blood from the Horseshoe Crab, *Limulus polyphemus*. (2020)

Directed by Dr. Christopher Kepley. 74 pp.

Antimicrobial resistance is a major health challenge that causes serious morbidity and mortality worldwide. The overuse of antibiotics has sped up the rise of resistant pathogens and rendered many antibiotics useless. There has been a worldwide push to use antibiotics with more caution, but in order to do so, physicians need quicker diagnostic methods than blood cultures for confirming bacterial infections. Sepsis, which is when the body's response to an infection harms its own tissues and organs, is a fast-acting syndrome. Delaying administration of antibiotics rapidly increases the risk for mortality, forcing physicians to prescribe broad spectrum antibiotics until blood cultures can provide more information. To combat these antimicrobial resistant superbugs, not only do we need a faster way to diagnose them, but we need more diverse methods to fight them.

The horseshoe crab, *Limulus polyphemus*, has an innate property in its blood to coagulate in the presence of LPS at the pico- to nanogram level. The limulus amoebocyte lysate (LAL) assay has been used for decades by the biomedical industry to verify sterility of medical devices. Scientists have attempted to use the LAL assay as a diagnostic test for bacterial infections, with much difficulty over the years.

The overall objective of this research is to investigate the diagnostic and therapeutic potential of blood components from *Limulus polyphemus*. To achieve this goal, the first aim will determine a simple protocol to detect bacteria or bacterial endotoxin in human blood. Preliminary data shows that anticoagulants that are often

found in blood collection tubes will inhibit the LAL assay in the presence of endotoxin. We have demonstrated the ability to overcome these inhibitors by isolation and washing of the red blood cells. The second part of this aim quantifies the detection limits for endotoxin. We performed serial dilutions of pathogen concentrations to determine sensitivity of the LAL assay to bacteria and bacterial endotoxin. We also performed the LAL assay using *E. coli*. Our results demonstrate the ability to detect bacteria and bacterial endotoxin in human blood samples.

The second aim of this research focused on the therapeutic potential of the horseshoe crab blood and its components. To achieve this, bioassays on different fractions and preparations of hemolymph were tested against two strains of *Staphylococcus aureus* to see if any fraction of the blood has bioactivity. We have found that antimicrobial activity was not observed in the hemolymph, plasma, and amoebocytes of the horseshoe crab blood. Further studies are needed to investigate isolated antimicrobial peptides and hemocyanin from amoebocytes for testing.

EXPLORATORY STUDIES INTO THE THERAPEUTIC
AND DIAGNOSTIC CAPABILITY OF BLOOD
FROM THE HORSESHOE CRAB,
LIMULUS POLYPHEMUS

by

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A Thesis Submitted to
the Faculty of The Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Greensboro
2020

Approved by

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To Ben and Aiden for your continuous love and support through all of my endeavors.

Thank you for being by my side in this journey.

APPROVAL PAGE

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ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Christopher Kepley for his guidance and support over the last several years. I would also like to thank my committee members, Dr. Eric Josephs and Dr. Kristen Dellinger for their guidance through my experiments and thesis. I would also like to thank Dr. Josephs for supplying the bacteria for my LAL assay experiments.

I would like to thank Dr. Nadja Cech and Manead Khin for their invaluable assistance and training for my experiments and for the use of their lab. I would also like to thank Dr. Anthony Dellinger, Dr. Rachel Tinker-Kulberg and Kepley Biosystems Inc. for their advice, support, and assistance throughout my experiments.

TABLE OF CONTENTS

	Page
LIST OF TABLES	vii
LIST OF FIGURES	viii
 CHAPTER	
I. INTRODUCTION	1
1.1. Sepsis	1
1.2. Antimicrobial Resistance	3
1.3. Natural Antimicrobial Products	4
II. LITERATURE REVIEW	6
2.1. Gram-Negative Bacteria	6
2.2. The Uses of the Horseshoe Crab (<i>Limulus polyphemus</i>)	7
2.3. LAL Interaction with Blood.....	10
2.4. Components of Horseshoe Crab (HSC) Blood	12
III. DIAGNOSTIC CAPABILITIES OF THE LAL ASSAY TO DETECT BACTERIAL ENDOTOXIN IN HUMAN BLOOD SAMPLES	15
3.1. Introduction.....	15
3.2. Methods.....	15
3.2.1. Blood Sample Collection	15
3.2.2. LAL Assay	16
3.2.3. Effect of Anticoagulants on LAL Assay.....	16
3.2.4. Location of LPS in Blood	17
3.2.5. Isolating Red Blood Cells and Bound LPS.....	18
3.2.6. LAL Assay with <i>E. coli</i>	21
3.3. Results.....	23
3.3.1. Effect of Anticoagulants on LAL Assay.....	23
3.3.2. Location of LPS in Blood	25
3.3.3. Isolating Red Blood Cells and Bound LPS.....	26
3.3.4. LAL Assay with <i>E. coli</i>	33
3.4. Discussion.....	34
3.4.1. Effect of Anticoagulants on LAL Assay.....	34
3.4.2. Location of LPS in Blood	35
3.4.3. Isolating Red Blood Cells and Bound LPS.....	36

3.4.4. LAL Assay with <i>E. coli</i>	38
3.5. Conclusion and Future Perspectives	39
IV. THERAPEUTIC CAPABILITIES OF HORSESHOE CRAB BLOOD AGAINST <i>STAPHYLOCOCCUS AUREUS</i>	40
4.1. Introduction.....	40
4.2. Methods.....	40
4.2.1. Antimicrobial Assays.....	40
4.2.2. HSC Plasma	42
4.2.3. Antimicrobial Activity of HSC Blood Components	43
4.2.4. Effect of Detergent on Antimicrobial Properties of HSC Blood.....	43
4.2.5. Inductive Coupled Plasma Analysis of HSC Plasma.....	44
4.3. Results.....	45
4.3.1. HSC Plasma	45
4.3.2. Antimicrobial Activity of HSC Blood Components	47
4.3.3. Effect of Detergent on Antimicrobial Properties of HSC Blood.....	49
4.3.4. Inductive Coupled Plasma Analysis of HSC Plasma.....	50
4.4. Discussion	51
4.4.1. HSC Plasma	51
4.4.2. Antimicrobial Activity of HSC Blood Components	52
4.4.3. Effect of Detergent on Antimicrobial Properties of HSC Blood.....	52
4.4.4. Inductive Coupled Plasma Analysis of HSC Plasma.....	53
4.5. Conclusion and Future Perspectives	54
REFERENCES	56

LIST OF TABLES

	Page
Table 3.1. Treatment for Each Sample in Determining the Effect of Anticoagulants on the LAL Assay	17
Table 3.2. Sample Conditions for LAL Assay After RBC Isolation	19
Table 3.3. Conditions to Determine the Sensitivity of RBC Isolation Procedure on the LAL Assay (50-0.05 EU/ml)	20
Table 3.4. Conditions to Determine the Sensitivity of RBC Isolation Procedure on the LAL Assay (50-3.125 EU/ml)	21
Table 3.5. Treatments for Samples Performing LAL Assay Using Whole Bacteria Added Prior to RBC Isolation	22
Table 3.6. Treatments for Samples Performing LAL Assay Using Whole Bacteria Added After RBC Isolation	23
Table 4.1. Copper Concentration of HSC Plasma Samples Analyzed by ICP	51

LIST OF FIGURES

	Page
Figure 2.1. Coagulation Cascade Reaction in the Horseshoe Crab	8
Figure 2.2. Large and Small Granules Located Inside the Amoebocytes of the Horseshoe Crab.....	14
Figure 3.1. Combined Results from the LAL Assay with Sodium Citrate Vacutainers	24
Figure 3.2. Combined Results from the LAL Assay with Lithium Heparin Vacutainers.....	24
Figure 3.3. Samples After Centrifugation for Serum and Plasma Separation.....	25
Figure 3.4. Combined Results from LAL Assay on Serum and Plasma.....	26
Figure 3.5. Combined Results of Samples with No Anticoagulant	27
Figure 3.6. Combined Results of Lithium Heparin Samples	28
Figure 3.7. Combined Results of Sodium Citrate Samples	28
Figure 3.8. Combined Results of Control Samples Run in EFW	29
Figure 3.9. Combined Results of Samples with LPS Added Prior to the Washing and RBC Isolation Procedure	30
Figure 3.10. Combined Results of Samples with LPS Added After the Washing and RBC Isolation Procedure	30
Figure 3.11 Combined Results of Titration Control Samples Run in EFW	31
Figure 3.12. Combined Results of LPS Titration with LPS Added Prior to Washing Protocol.....	32
Figure 3.13. Combined Results of LPS Titration with LPS Added After the Washing Protocol	32
Figure 3.14. Combined Results of LAL Assay with Varying Concentrations of <i>E. coli</i> Added Prior to the Washing Protocol	33

Figure 3.15. Combined Results of LAL Assay with Varying Concentrations of <i>E. coli</i> Added After the Washing Protocol	34
Figure 4.1. Sample 96-Well Plate Layout for Bioassay Screening of HSC Blood and Components Against <i>S. Aureus</i> Strains, SA1199 and AH1263	42
Figure 4.2. Bacterial Growth of SA1199 and AH1263 in HSC Plasma	46
Figure 4.3. Bacterial Growth of SA1199 and AH1263 in HSC Plasma and Copper Standard	46
Figure 4.4. Bacterial Growth of SA1199 and AH1263 in Different Fractions of HSC Blood	48
Figure 4.5. Bacterial Growth of SA1199 and AH1263 in HSC Plasma and Triton x-100	49
Figure 4.6. Standard Concentration Curve for Copper by ICP	50

CHAPTER I

INTRODUCTION

1.1. Sepsis

Sepsis is a condition that arises when the body's response to an infection injures its own tissues and organs. It is a major public health concern and the incidence of sepsis continues to rise, making it one of the most common reasons for hospitalization, with an estimated 1.7 million cases annually in the United States (1). One study spanning five years found that 265,000 Americans die as a result of sepsis each year, and 1 in 3 patients who die in a hospital have sepsis (1). According to the Global Sepsis Alliance, sepsis affects 27 to 30 million people every year, 7 to 9 million die, which accounts for one death every 3.5 seconds. Septicemia was the most expensive condition treated in the United States in 2013, costing hospitals 23.7 billion dollars which will likely continue to increase as the population ages (2). It has been shown that early interventions, such as appropriate antibiotic therapy, improve survival rate for septic patients, making early diagnosis critical (3,4). One study showed a linear increase in the risk of mortality for each hour antibiotic administration was delayed from one to six hours (3). With such a small window of opportunity, physicians tend to prescribe broad spectrum antibiotics which contributes to the rise of antimicrobial resistant (AMR) strains. Both inaccurate diagnosis and the over-prescribing of antibiotics have largely been the result of a lack of reliable and rapid diagnostic procedures.

Even amongst the healthcare community, the definition of sepsis is not well agreed upon, contributing to lack of a quick diagnosis for the patient. In fact, at the recent International Consensus Definitions for Sepsis and Septic Shock meeting, sepsis was defined as a “life-threatening organ dysfunction caused by a dysregulated host response to infection” (5). The goal of this updated definition was to help facilitate earlier recognition. Along with the new a definition of sepsis, the International Consensus also defined that organ dysfunction can be represented by an increase in the Sequential Organ Failure Assessment score of 2 points or more (5). Patients that presented with a respiratory rate of 22/min or greater, systolic blood pressure of 100 mm Hg or less, or altered mental status were identified with suspected infection (5). There currently does not exist a standard diagnostic test for sepsis but instead physicians use these clinical criteria for diagnosis. Prompt administration of antibiotics is recommended and blood cultures should be taken (3,6). However, the current recommendation on incubation periods for routine blood cultures is five days (7). Research has shown that the full 5 days may not be necessary, but even this research recommends a three day incubation period (8). By the time a patient may be considered septic, a physician draws blood, a lab runs the culture, and several days have passed that a patient cannot afford to wait for antibiotics. New methods are emerging, such as the use of mass spectrometry to identify bacterial membrane lipids from patient samples or polymerase chain reaction to detect pathogens (9,10). Two platforms based on matrix-assisted laser desorption/ionization time-of-flight have been approved by the Food and Drug Administration (FDA) for

clinical use (9). This method is quicker than blood cultures, but not cost efficient for hospitals to use.

1.2. Antimicrobial Resistance

According to the Centers for Disease Control (CDC), AMR is one of the greatest global health challenges of modern time (11). The rise of antimicrobial resistance occurs when microbes (bacteria and fungi) are able to survive when they are under the influence of an antimicrobial agent (12). There are several ways for bacteria to become antibiotic-resistant, the main method is through selective pressure. Selective pressure occurs when not all of the bacteria exposed to an antibiotic are susceptible and the surviving bacteria continue to multiply (12,13). This creates a bacterial population that is resistant to the antibiotic it was exposed to during the initial infection. Overuse of antibiotics helps speed up selection for resistant bacteria. In recent years, AMR has become a bigger problem as the discovery of new antibiotics has slowed drastically, while antibiotic use is rising (14,15). The CDC estimates that each year, 47 million courses of antibiotics are prescribed unnecessarily in the United States alone (14). This overuse of antibiotics only quickens the emergence of AMR strains and antibiotics lose their effectiveness. In turn, key medical procedures such as gut surgery, caesarean sections, and joint replacements could become too dangerous to perform. It will also impact the ability of chemotherapy and organ transplants to be successful (12).

Since antibiotics are used in crops, animals, and people, the selection of resistant organisms can occur in each of these populations. For example, some types of antibiotic-resistant germs can spread person to person. “Nightmare bacteria” such as carbapenem-

resistant *Enterobacteriaceae* can also survive and grow in sink drains at healthcare facilities and spread to patients and to the environment through the wastewater (16–18). *Candida auris* is an emerging fungus that is especially pernicious in its global threat as it is often multidrug-resistant, difficult to identify with standard laboratory methods, is resistant to most disinfecting protocols for hospital settings, and has caused outbreaks in healthcare settings (19). In animals, resistant microbes can be spread between animals and people through food or contact with animals as in the case of the multi-drug-resistant *Salmonella* Heidelberg bacteria which can make chickens, cattle, and people sick (20–22). Lastly, antibiotic-resistant microbes ubiquitous in the environment such as *Aspergillus fumigatus*, a common mold, can make people with weak immune systems sick. In 2015, azole resistant *A. fumigatus* was reported in collected isolates from patients around the United States (23). The report also found that United States crop fields were treated with fungicides that are similar to antifungals used in human medicine (23,24).

1.3. Natural Antimicrobial Products

According to the CDC, no new major classes of antibiotics were approved for gram-negative infections between 1962 and 2000 (11). With this slow development of antibiotics and growing populations of pathogens that have developed resistance to antimicrobials, novel products are sorely needed. Researchers have looked to nature for inspiration, such as using extracts and oils from plants. There is a push right now to discover naturally derived antibacterial agents with a novel mechanism of action (25,26). Natural products, such as essential oils from plants, have been investigated for their antimicrobial properties against varying pathogens. Rosemary and clove, whether alone

or in combination, exhibited antimicrobial properties against gram-positive bacteria, gram-negative bacteria, and fungi (27). Another study investigated cinnamon, Chinese chive and Corni fructus against bacteria, mold and yeasts (28). The group found that all three harbored antimicrobial activity when tested using a disc diffusion method.

Scientists are not only looking to plants as possible sources, but antimicrobial peptides in organisms are being looked at with new eyes for their therapeutic potential. The plasma from one species of horseshoe crab (HSC), *Carsinoscorpius rotundicauda*, has been shown to be bioactive against gram-positive and gram-negative strains of bacteria (29). The hemocyanin contained in HSC blood has also been investigated for potential antimicrobial activity (30,31).

With a rapidly growing population of antimicrobial resistant strains of pathogens, rapid diagnostic tools are desperately needed for more accurate diagnosis of bacterial infections. These studies explored the diagnostic and therapeutic potential of the blood from the HSC, *Limulus polyphemus*. While others have investigated individual proteins and peptides from HSC blood, we will look at the larger components, such as amoebocytes and plasma. Our hypothesis is HSC blood can be used to detect bacterial infections in human blood samples and that it harbors antimicrobial properties.

CHAPTER II

LITERATURE REVIEW

2.1. Gram-Negative Bacteria

Species of bacteria can be classified using several methods such as their appearance (size and shape), gram staining, and whether they are aerobic or anaerobic. A gram stain is a quick test to classify bacteria as either gram-negative or gram-positive. Gram-positive bacteria have a thick peptidoglycan layer that traps the crystal violet dye and will stain the cell purple (32). Gram-negative bacteria, by contrast, have a thin peptidoglycan layer that will not retain the crystal violet stain and the cell will turn red (32). Gram-negative bacteria also contain lipopolysaccharides (LPS) in their cell membranes. LPS is made of three major components: hydrophilic polysaccharides, hydrophobic lipid A, and O-antigen repeats (33). The lipid A portion is responsible for the majority of the bioactivity of the endotoxin.

The bioactivity of LPS, or endotoxin, can stimulate Toll-like receptor 4, which is found on several types of immune cells such as macrophages and dendritic cells (33). In macrophages, LPS will cause polyclonal B cell activation (34). LPS will also induce the release of proinflammatory cytokines, such as tumor necrosis factor and interferon- γ (34). Activation of these cells not only induce a non-specific immune response, but a high concentration of LPS can induce fever, increase heart rate and possibly lead to septic shock (33).

Since the symptoms of bacterial infections do not differentiate between gram-negative and gram positive, samples must be characterized in the lab (35). The gram stain can quickly distinguish between gram-positive and gram-negative, but further testing is required to identify the organism for better treatment. For this, blood cultures and urine samples are the most common samples collected (32). Other samples, depending on the type of infection suspected, can be used such as tissue or other sterile fluids. Several different detection methods are used in the laboratory to identify the bacterial or fungal species. These different methods include: cultures, antibody detection, antigen detection, and microscopy (32).

2.2. The Uses of the Horseshoe Crab (*Limulus polyphemus*)

The horseshoe crab (HSC), *Limulus polyphemus*, has an innate property in its blood to coagulate in the presence of LPS at the pico- to nanogram level (36). This endotoxin is found in the outer membrane of Gram-negative bacteria (37). In HSCs, detection of LPS induces the amoebocytes to degranulate and activates the zymogen Factor C to the active form of Factor C'. This Factor C' activates Factor B into Factor B'. The Factor B' proenzyme then activates the proclotting enzyme into the clotting enzyme. The last step in this cascade converts coagulogen into a coagulin gel clot, therefore trapping the invading bacteria (Figure 2.1). The cascade can also be activated by 1-3 β -D-glucan. This activates Factor G into Factor G' which then activates the proclotting enzyme into the clotting enzyme (36,37).

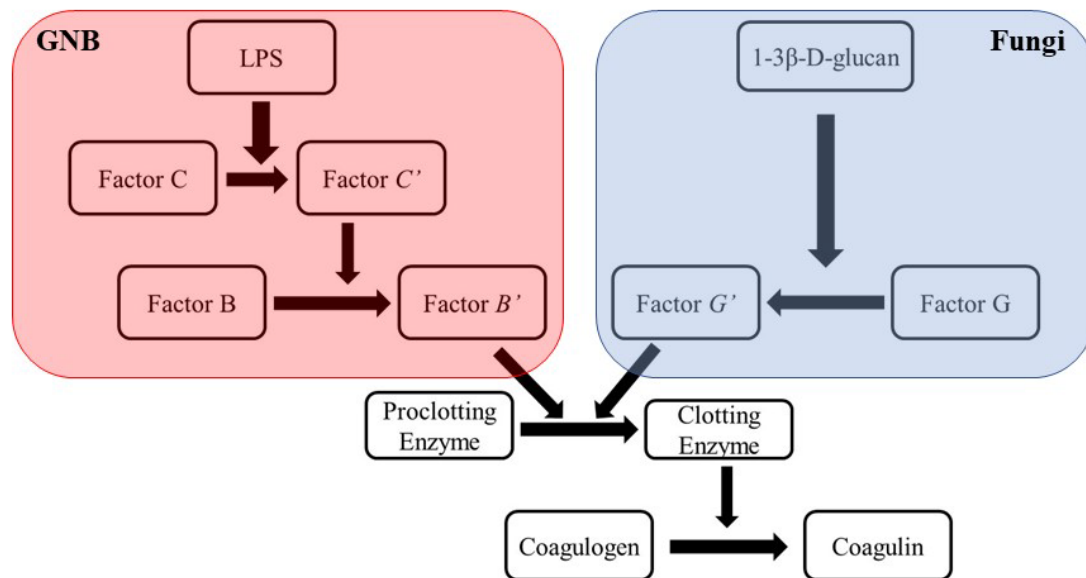


Figure 2.1. Coagulation Cascade Reaction in the Horseshoe Crab.

Levin and Bang discovered this cascade in the 1950's and 1960's and the FDA approved use of the HSC-derived limulus amoebocyte lysate (LAL) assay in the 1970's (37–39). The LAL assay is widely used by the pharmaceutical industry to test intravenous drug products for potential contamination (37,39–41). It is also used to test medical products, such as implantable devices for possible endotoxin concentrations to ensure sterility (37,41,42).

Of course, to obtain the LAL needed the HSC must be caught and transported to facilities to harvest their blood. The HSC are bled and then placed back in their ecosystems. However, transport of the HSCs for blood collection has a high mortality rate (10-30%) and disrupts their spawning (41). It is estimated that between the mortality rate and the HSCs that are sold as bait after bleeding, approximately 130,000 HSCs are killed by the biomedical industry every year (43). Restrictions were placed to regulate the

number of HSCs harvested, which has aided their population from dropping rapidly. For this reason, research has turned to replicating the key factor in the amoebocytes that causes coagulation. The recombinant factor C (rFC) test uses a cloned version from a HSC, thereby eliminating the need to repeatedly bleed the HSC (41). The proenzyme will activate when it encounters trace amounts of endotoxin. The activated rFC will then serve as a catalyst to hydrolyze a synthetic substrate. This will then form a quantifiable product that can be measured by both fluorometric and colorimetric assays (37). Although rFC would allow more testing without impacting the HSC population, the United States Pharmacopeia (USP) recently decided to not include this synthetic testing in the chapter of endotoxin testing standards. The USP believes that rFC does not have the same level of evidence as compared to LAL (44).

HSCs have not only been used in the medical industry, but are also harvested for bait in commercial fishing, particularly for whelk and eel pots (41). In the late 1990s, commercial harvest of the HSC increased, reaching a peak of almost six million pounds in 1997 (45). Over the years, regulations and limitations on HSC harvesting, such as those from the Atlantic States Marine Fisheries Commission (ASMFC), have been put in place. In 2018, the total for bait landings came in below quota with a total harvest of 658,589 crabs (45). New innovations in bait gear have allowed fishermen to more effectively catch eel and whelk using less bait.

With HSCs being harvested for bait and biomedical use, their population began to dwindle. The ASMFC established a Horseshoe Crab Management Board in 1998 to help conserve HSCs at a sustainable level to ensure their role in the coastal ecosystems. Limits

were put in place on the number of HSCs each state was allowed to harvest. Some states also have limitations or bans on the harvest of female HSCs (46). For the biomedical industry, states must report the number of crabs collected, crabs rejected, crabs bled and characterize mortality (46). However, the crabs that are bled and then subsequently used as bait are counted against state quotas for the commercial fishing industry but not included in biomedical use quotas (46). Data from ASMFC does not address the number of HSCs that would fall in this category. Post-bleeding mortality rates were analyzed using literature and estimated at 15% (46). However, many of the studies analyzed did not implement biomedical best practices. The 1998 FMP set a mortality threshold that if exceeded, would prompt the board to consider action. The threshold was exceeded in 2018, as it has been for the last 11 of 12 years (46).

The LAL assay has been the gold standard for endotoxin detection in the biomedical industry for decades, but HSCs were also previously used as lab animal models for other studies. Research on the HSCs eyes have allowed for a better understanding on vision mechanisms, such as phototransduction (47,48). LAL has also been used for research into bacterial detection in human blood, but with mixed results (38,39,49–51).

2.3. LAL Interaction with Blood

There are numerous obstacles that have prevented accurate and simple blood testing using the LAL assay. The assay can either be suppressed or activated by components in whole blood, plasma or serum (38,39). These proteins that interfere must be removed before the assay can accurately detect endotoxin levels. Blood collection

methods can also affect the outcome of the LAL assay as many blood collection tubes contain an anticoagulant. For example, blood collection tubes can contain a sodium citrate solution or have another anticoagulant, such as ethylenediaminetetraacetic acid or heparin, spray coated on the interior of the tube.

There is an inherent issue with these anticoagulants inside the tubes which can interfere with the LAL assay. Addition of heparin to the lysate prior to the addition of the gram-negative bacteria inhibited the reaction and formation of a gel-clot (49). Heparin, a glycosaminoglycan, prevents clotting by inhibiting thrombin in the coagulation pathway (52). Thrombin is responsible for the activation of several components in the cascade, the most important being the conversion of fibrinogen to fibrin which ultimately forms a clot (52). The addition of salt, such as sodium chloride, has been shown to prevent heparin from inhibiting coagulation in the assay (49).

Sodium citrate prevents the formation of a gel-clot through chelation of calcium in the bloodstream (53). Calcium is released by platelets and binds to phospholipids, this then provides a surface for coagulation factors to assemble (54). It is an essential component in the coagulation cascade, without it, the blood is unable to clot. Citrate has prevented the LAL assay from initiating a gel-clot in the presence of LPS in previous studies (50). Addition of calcium chloride has been shown to inhibit the chelation by citrate and improve sensitivity of the LAL assay (50).

With both anticoagulants, methods have been proposed to overcome their inhibitory effect. However, findings are inconsistent with one another, leading to difficulty in using LAL to detect endotoxin in blood samples. Results from studies that

use salt to overcome heparin vary widely. Tsuji and Steindler looked into several different salts and differing concentrations to determine what would best work to overcome the inhibitory nature of heparin. Magnesium (at 50 mM) resulted in the most sensitive LAL reaction, with calcium providing only partial gelation (51). A more recent study by Solaimanian et al. found that 10 mM concentration of magnesium had the optimal recovery of endotoxin (55). Another study found that sodium chloride could be used to recover gel formation, but the concentrations varied (49). These varied results could be the result of different LAL batches, different preparation methods or experimental design variations.

2.4. Components of Horseshoe Crab (HSC) Blood

Hemocyanin is a high molecular weight protein found in the blood of several species of mollusks and arthropods, such as the HSC (30,56,57). It is hexameric and consists of functionally heterogeneous subunits which are made up of three domains (30,57,58). The primary function of hemocyanin is oxygen transport through the hemolymph (30,56,57). *Limulus polyphemus* has the largest known arthropod hemocyanin and contains 48 binding sites for oxygen (57). More recently, hemocyanin has also been implicated in several other physiological and homeostatic processes, such as hormone transport, molting and protein storage (30,57).

Hemocyanin also contains a pair of deoxygenated copper (Cu I) atoms in the second domain (30,57). Upon oxygenation, the copper becomes Cu (II), which imparts the blue color of the HSC blood. Copper was recognized as the first metallic antimicrobial agent by the Environmental Protection Agency in 2008 (59,60). Currently,

copper is being used in the treatment of drinking water and research has been conducted using copper surfaces in hospital settings (60). Copper is thought to act on microbes through contact killing, however, the exact mechanism of action is still unknown (59). Copper has been recognized as an antibacterial, antifungal, and antiviral agent whether it be used as a surface or particle (59).

Recent studies have reported that hemocyanin can convert its function from binding oxygen to phenoloxidase-like activity (30,57,61). Phenoloxidase is a copper-containing protein that is involved in the synthesis of melanin and also plays a role in the primary immune response of arthropods, such as the HSC (30,57). Hemocyanin can be activated by several different factors, *in vitro* and *in vivo* (30). *In vitro*, hemocyanin has been activated by sodium dodecyl sulfate micelles and phosphatidylserine liposomes (30,57,61,62). *In vivo*, the activation of hemocyanin phenoloxidase activity is not well understood. One study found that treatment with fatty acids and phospholipids caused phenoloxidase activity (63). However, the fatty acids and phospholipids were dissolved in ethanol, which is a known inducer of phenoloxidase, so it is difficult to determine what caused the activity (57).

Polyphemusins are a family of antimicrobial peptides located inside the smaller granules (Figure 2.2) of the HSC hemocyte (64). These peptides are comprised of a chain of 18 amino acid residues with a disulfide bond (64,65). There are three different members of this peptide family: polyphemusin I, II, and more recently III (65). Polyphemusins have a high affinity for LPS and have been found to disrupt the membranes of gram-negative and gram-positive bacteria (65). They have also shown to

inhibit fungal growth (66), degrade biofilms (67), inhibit tumor cell growth (68), and inhibit HIV cell fusion (69).

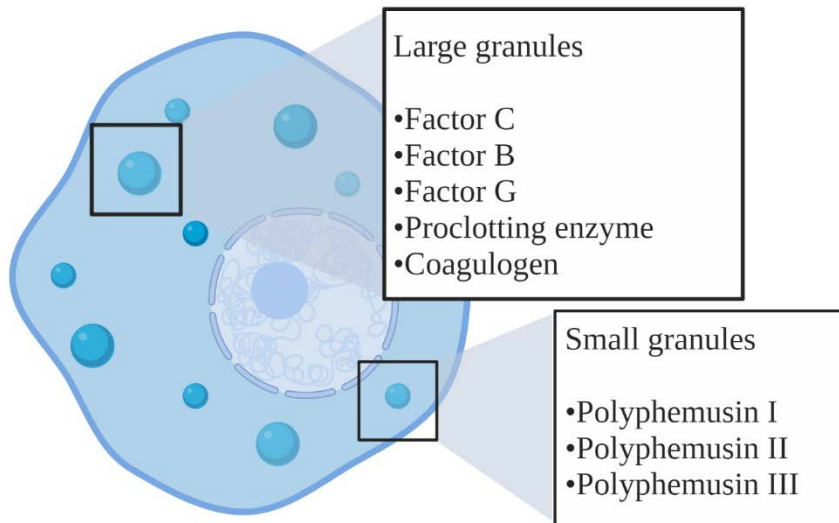


Figure 2.2. Large and Small Granules Located Inside the Amoebocytes of the Horseshoe Crab. Created with BioRender.com

CHAPTER III

DIAGNOSTIC CAPABILITIES OF THE LAL ASSAY TO DETECT BACTERIAL ENDOTOXIN IN HUMAN BLOOD SAMPLES

3.1. Introduction

Detecting LPS in human blood samples comes with several challenges. The main obstacle is in the preparation of the sample for testing and how anticoagulants affect the process of detection. To assess this, several anticoagulants were selected to determine their effect on the LAL assay. Next, a method was developed to rid blood samples of inhibiting proteins, thus allowing the blood sample to accurately detect endotoxin in the blood. The effects of anticoagulant on this isolation method was also investigated. The final step in this aim was investigate the ability of the LAL assay to detect a bacterial infection with whole bacteria rather than just the LPS that starts the pathway. Activation of Factor C is initiated by LPS, which is normally embedded into the membrane of gram-negative bacteria. Lyophilized LPS was added to blood samples in most of the experiments, but the last one uses bacteria, *Escherichia coli*, from a culture for the source of LPS.

3.2. Methods

3.2.1. Blood Sample Collection

All blood was collected using aseptic techniques by certified phlebotomists, Dr. Christopher Kepley and Dr. Anthony Dellinger. Collection and use of human blood were covered by approved IRB 13-0276. The area around the blood draw region was cleaned

with ethanol wipes and collected via venipuncture into various types of BD vacutainer blood collection tubes. Participants did not donate blood more than twice a month and no more than 10 mL was taken at one time.

3.2.2. *LAL Assay*

The following protocol was used for all of the LAL assays. LAL (with a sensitivity range of 0.05-0.1 EU/ml), purchased from Sigma Aldrich, was prepared with endotoxin-free water (EFW) as per the manufacturer's directions. Once sample preparation was complete, LAL was added to the blood sample and incubated at 37°C for one hour. Upon completion of incubation period, samples were visually assessed for gel-clot formation and clot integrity by inversion of the sample.

3.2.3. *Effect of Anticoagulants on LAL Assay*

Two different anticoagulants, sodium citrate and lithium heparin, were tested to determine what effect they had on the effectiveness and sensitivity of the LAL assay. Sodium citrate tubes contain 0.3 ml sodium citrate solution, while lithium heparin tubes are spray coated at 37 USP. Sodium citrate is believed to chelate calcium in the blood, which is an integral part in the coagulation cascade. Lithium heparin inhibits thrombin, which activates several factors in the cascade.

Blood was collected as described using previous methods and collected into either a sodium citrate tube or lithium heparin tube, purchased from Fisher Scientific. All tubes were then gently inverted to properly mix and prevent coagulation. 50 µl of each sample were then aliquoted into pyrogen-free glass tubes, followed by the addition of

1.25 μ l of the endotoxin standards. 50 μ l of LAL were then added to each of the samples and the previously described protocol was used to perform the LAL assay.

There were three different controls used in this experiment. An endotoxin control, in EFW, was used to ensure that the LAL would form a clot in the presence of endotoxin. A second control was used to ensure that there was no clotting of blood with LPS present without the addition of LAL. The final control did not have LPS added to ensure that the technique was endotoxin free and did not form a clot. Endotoxin standards were made from a stock endotoxin, at a concentration of 4000 EU/ml, using a 10-fold serial dilution. The standards of endotoxin tested in both anticoagulants were: 50, 5.0, 0.5, and 0.05 EU/ml (Table 3.1).

Table 3.1. Treatment for Each Sample in Determining the Effect of Anticoagulants on the LAL Assay.

Concentration	Blood (μ l)	LPS Volume (μ l)	LAL (μ l)	EFW (μ l)
Endotoxin Control	0	1.25	50	50
LAL Control	50	1.25	0	50
0 EU/ml	50	0	50	0
50 EU/ml	50	1.25	50	0
5 EU/ml	50	1.25	50	0
0.5 EU/ml	50	1.25	50	0
0.05 EU/ml	50	1.25	50	0

3.2.4. Location of LPS in Blood

To test where LPS localizes in the blood, blood was collected using the aseptic technique previously described and distributed into three different types of tubes. The first tube contained a clot activator, also purchased from Fisher Scientific, to separate serum. The other two types contained sodium citrate and lithium heparin to separate

plasma. Two samples for each condition were tested, one with LPS added and another without LPS as a negative control. The samples spiked with LPS were at the same concentration of 50 EU/ml. All samples were then incubated at room temperature for 30 minutes. Samples were then centrifuged at 21°C and 1200 rcf for 10 minutes. After centrifugation, 50 µl were removed from either the plasma or serum layer and tested in a LAL assay using the previously described method.

3.2.5. Isolating Red Blood Cells and Bound LPS

Blood samples were collected using the aseptic techniques previously described and aliquoted into either sodium citrate tubes, lithium heparin tubes or blood collection tubes with no anticoagulant. Endotoxin, at a concentration of 50 EU/ml, was added to samples and allowed to incubate for 15 minutes at room temperature. Samples were then centrifuged at 2000 rcf for 10 minutes at 4°C and the supernatant was discarded. Samples were then washed in 0.9% NaCl for a total volume of 1 ml and gently resuspended. Samples were centrifuged again at the previous conditions and lysed in EFW. All samples were then rotated for 20 minutes at 4°C. After rotation, samples were centrifuged one final time at 3000 rcf for 10 minutes at 4°C. 100 µl samples were then pulled from each condition and used in a LAL assay as previously described. Conditions for each tube can be found in Table 3.2. For each condition (no anticoagulant, sodium citrate or lithium heparin), there was one sample with no endotoxin added as a negative control, one with endotoxin at 50 EU/ml that was washed, one with endotoxin at 50 EU/ml that was unwashed, and one that had the endotoxin added after the washing step as a positive

control. Control samples were tested in EFW to ensure no contamination of the LAL assay and that the assay itself worked.

Table 3.2. Sample Conditions for LAL Assay After RBC Isolation.

Treatment	Anticoagulant	Endotoxin	Washed	Cell condition	Blood (µl)	LAL (µl)
1	None	≤50 EU/ml	No	lysed	100	100
2	None	≤50 EU/ml	Yes	lysed	100	100
3	None	0 EU/ml	Yes	lysed	100	100
4	None	2.5 µl of 4 EU/ml	Yes	lysed	100	100
5	Sodium Citrate	≤50 EU/ml	No	lysed	100	100
6	Sodium Citrate	≤50 EU/ml	Yes	lysed	100	100
7	Sodium Citrate	0 EU/ml	Yes	lysed	100	100
8	Sodium Citrate	2.5 µl of 4 EU/ml	Yes	lysed	100	100
9	Heparin	≤50 EU/ml	No	lysed	100	100
10	Heparin	≤50 EU/ml	Yes	lysed	100	100
11	Heparin	0 EU/ml	Yes	lysed	100	100
12	Heparin	2.5 µl of 4 EU/ml	Yes	lysed	100	100
13	None	50 EU/ml	No	-	-	100
14	None	5 EU/ml	No	-	-	100
15	None	0.5 EU/ml	No	-	-	100
16	None	0 EU/ml	No	-	-	100

The same protocol was used to determine the sensitivity of the method to lower levels of LPS. Solutions were made from an endotoxin stock using a serial dilution. The final concentrations of LPS in the LAL assay were: 50, 5, 0.5, and 0.05 EU/ml. For this experiment, only sodium citrate tubes were used for blood collection. Samples were spiked with LPS solutions either prior to the red blood cell (RBC) isolation method or after as a positive control and to compare gel-clot formation. The same LPS

concentrations were run in EFW rather than blood to ensure the accuracy of the LAL assay (Table 3.3).

Table 3.3. Conditions to Determine the Sensitivity of RBC Isolation Procedure on the LAL Assay (50-0.05 EU/ml).

Samples	Blood Volume (µl)	Endotoxin Volume (µl)	LAL (µl)	Endotoxin-Free Water (µl)	Final Endotoxin Concentration (EU/ml)
1	100	25	70	32.5	≤ 50
2	100	25	70	32.5	≤ 5
3	100	25	70	32.5	≤ 0.5
4	100	25	70	32.5	≤ 0.05
5	100	-	70	32.5	0
6	100	2.5	70	30	50
7	100	2.5	70	30	5
8	100	2.5	70	30	0.5
9	100	2.5	70	30	0.05
10	-	2.5	70	130	50
11	-	2.5	70	130	5
12	-	2.5	70	130	0.5
13	-	2.5	70	130	0.05
14	-	2.5	70	130	0

The experiment was repeated, this time with different concentrations of LPS in order to narrow the sensitivity level of the LAL assay. The final concentrations of LPS in the LAL assays were: 50, 25, 12.5, 6.25, and 3.125 EU/ml. Once again, LPS was added prior to the isolation method and afterwards as positive controls. Another sample was run in EFW to ensure accuracy of the LAL assay. Table 3.4 has the listed conditions for each sample tested.

Table 3.4. Conditions to Determine the Sensitivity of RBC Isolation Procedure on the LAL Assay (50-3.125 EU/ml).

Samples	Blood Volume (μl)	Endotoxin Volume (μl)	LAL (μl)	Endotoxin-Free Water (μl)	Final Endotoxin Concentration (EU/ml)
1	100	25	100	0	≤ 50
2	100	25	100	0	≤ 25
3	100	25	100	0	≤ 12.5
4	100	25	100	0	≤ 6.25
5	100	25	100	0	≤ 3.125
6	100	-	100	0	0
7	100	2.5	100	0	50
8	100	2.5	100	0	25
9	100	2.5	100	0	12.5
10	100	2.5	100	0	6.25
11	100	2.5	100	0	3.125
12	-	2.5	100	100	50

3.2.6. LAL Assay with *E. coli*

A bacteria inoculum of Turbo competent *E. coli*, donated by Dr. Eric Josephs, was grown overnight at 37°C in lysogeny broth with ampicillin. The optical density (OD) was measured at 600 nm. At an OD₆₀₀ of 0.3, the bacteria were plated in serial dilutions to later perform a colony count.

Blood was once again collected using the previously described aseptic techniques into sodium citrate tubes. The aliquots (Samples 1-7) had bacterial dilutions added and were then incubated at room temperature for 15 minutes (Table 3.5). All samples were centrifuged at 13,000 rcf for 10 minutes at 4°C, allowing bacteria and RBCs to pellet. Supernatant was removed, samples were washed with 0.9% NaCl solution and centrifuged again at the same conditions. Supernatant was removed again and all samples

had EFW added to lyse the RBCs. Following this step, all samples were then rotated at 4°C for 20 minutes. 100 µl from Tube 7, which had only LB broth added, was placed into Tubes 8-13. Bacteria were then added to each of the tubes from the serial dilution plates as a control for known bacterial concentration in the samples (Table 3.6). Tubes were then frozen overnight at -70°C and used the next day for the LAL assay. For the LAL assay, all samples were thawed and the LAL assay was run on 100 µl of each sample as previously described. Another control was used, running the LAL assay in 100 µl of EFW rather than blood to ensure the accuracy of the test.

Table 3.5. Treatments for Samples Performing LAL Assay Using Whole Bacteria Added Prior to RBC Isolation.

Samples	Blood Volume (ml)	Bacterial dilution volume (µl)	Concentration in Blood (CFU/ml)	Concentration in LAL Assay (CFU/ml)	Calculated OD
1	1	25	1,250,000	625,000	0.5×10^{-2}
2	1	25	125,000	62,500	0.5×10^{-3}
3	1	25	12,500	6,250	0.5×10^{-4}
4	1	25	1,250	625	0.5×10^{-5}
5	1	25	125	62.5	0.5×10^{-6}
6	1	25	12.5	6.25	0.5×10^{-7}
7	1	25	0	0	0

Table 3.6. Treatments for Samples Performing LAL Assay Using Whole Bacteria Added After RBC Isolation.

Samples	Blood Volume (μl)	Bacterial dilution volume (μl)	Concentration in LAL Assay (CFU/ml)
8	100	2.5	625,000
9	100	2.5	62,500
10	100	2.5	6,250
11	100	2.5	625
12	100	2.5	62.5
13	100	2.5	6.25
14	100 μl EFW	2.5	625,000

3.3. Results

3.3.1. *Effect of Anticoagulants on LAL Assay*

The effect of sodium citrate and lithium heparin anticoagulants was investigated on the LAL assay in blood. Both anticoagulants inhibited the formation of a gel-clot during the LAL assay.

In the sodium citrate experiment, none of the conditions with the varying concentrations of LPS formed gel-clots after 1 hour of incubation at 37°C (Figure 3.1). At the highest concentration of LPS, 50 EU/ml, there was a weak clot formation but did not withstand inversion of the tube. The endotoxin control in EFW, did form a firm gel-clot. The second control, with no LAL, did not form a gel-clot. The third control, with no LPS, did not form a gel-clot. All controls had the expected results. From 50 EU/ml to 0.5 EU/ml, there was a small blood clot formed by the LAL in the RBC portion while the fluid portion remained as such. It appears that the LAL was able to clot a portion of the sample, but not a complete gel-clot as was necessary to pass this assay.

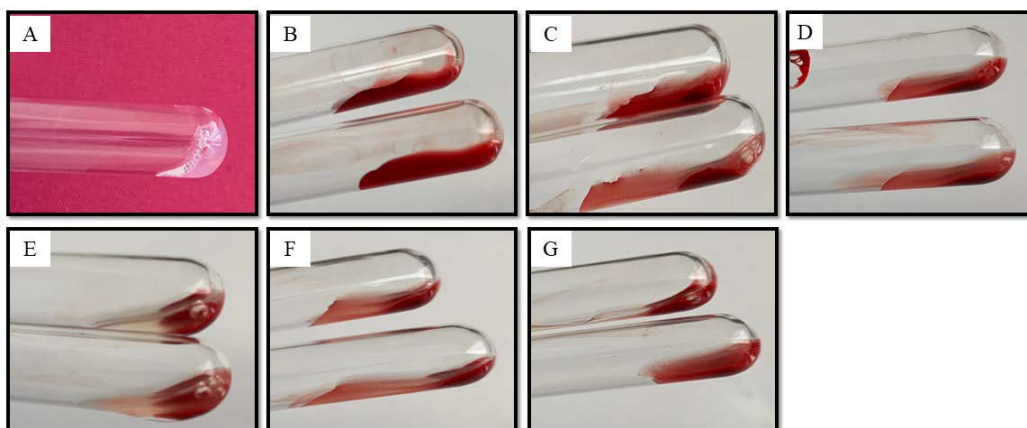


Figure 3.1. Combined Results from the LAL Assay with Sodium Citrate Vacutainers. **A)** 50 EU/ml LPS control in EFW formed a firm clot within ten minutes of incubation. **B)** No LAL control did not form a clot. **C)** No LPS control did not form a clot. **D)** 50 EU/ml LPS formed a weak clot after incubation. **E)** 5 EU/ml LPS, **F)** 0.5 EU/ml LPS, and **G)** 0.05 EU/ml LPS did not form clots.

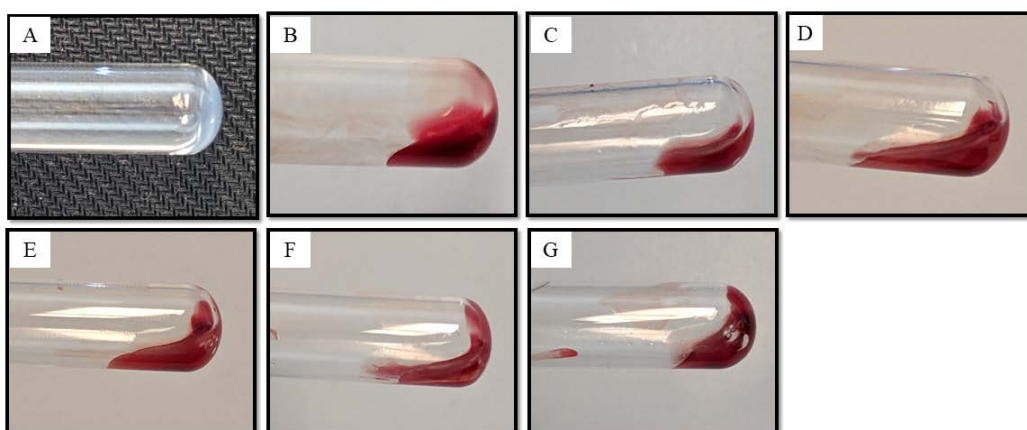


Figure 3.2. Combined Results from the LAL Assay with Lithium Heparin Vacutainers. **A)** 50 EU/ml LPS control in EFW formed a firm clot within ten minutes of incubation. **B)** No LAL control did not form a clot. **C)** No LPS control did not form a clot. **D)** 50 EU/ml LPS, **E)** 5 EU/ml LPS, **F)** 0.5 EU/ml LPS, and **G)** 0.05 EU/ml LPS did not form clots.

For lithium heparin, the controls had the same results as those previously found in the sodium citrate experiment. The endotoxin control in EFW did form a gel-clot, once again, after approximately 10 minutes of incubation time. None of the LPS

concentrations caused gel-clot formation (Figure 3.2). Unlike sodium citrate, even the highest concentration of LPS did not induce even a weak clot formation.

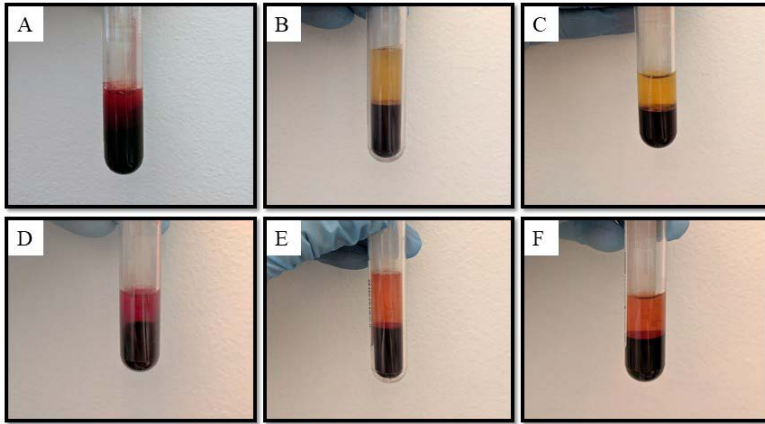


Figure 3.3. Samples After Centrifugation for Serum and Plasma Separation. **A-C)** control samples with no LPS in no anticoagulant, sodium citrate, and lithium heparin, respectively. **D-F)** 50 EU/ml LPS added to samples prior to separation in no anticoagulant, sodium citrate, and lithium heparin, respectively.

3.3.2. Location of LPS in Blood

After the 30-minute incubation period at room temperature, the tubes with no anticoagulant added did clot, as expected. The samples in sodium citrate or lithium heparin tubes did not clot due to the presence of the anticoagulants. Tubes with no anticoagulant did show separation of serum after centrifugation, but the results were more visually apparent in the sample with LPS added (Figure 3.3). The sodium citrate and lithium heparin samples had clear separation of plasma from the RBCs. In the samples with LPS added, the plasma was red in color compared to the yellow of the non-LPS samples. The LAL assay was then conducted on all samples, but no treatment produced a firm gel-clot (Figure 3.4).

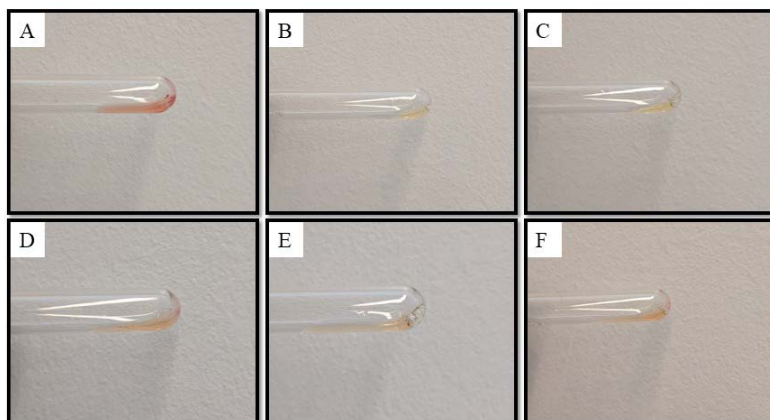


Figure 3.4. Combined Results from LAL Assay on Serum and Plasma. **A-C)** control samples with no LPS in no anticoagulant, sodium citrate, and lithium heparin, respectively. **D-F)** 50 EU/ml LPS added to samples prior to separation in no anticoagulant, sodium citrate, and lithium heparin, respectively. No firm gel-clots were formed in any of the samples.

3.3.3. Isolating Red Blood Cells and Bound LPS

Samples that were not washed with the NaCl solution formed a firm gel-clot in all treatments: no anticoagulant, sodium citrate and lithium heparin. Samples that were spiked with LPS prior to the isolation procedure, to better mimic in vivo conditions, and then washed with NaCl solution also formed a firm gel-clot. These samples in all treatments remained clotted for a longer time period than the unwashed samples. The positive controls in all treatments, LPS added after the washing and isolation procedure, formed a firm gel-clot as well. The negative control in all treatments, no LPS addition, did not form gel-clots as expected. Overall, the samples with no anticoagulant (Figure 3.5) had the weakest clot integrity, followed by lithium heparin (Figure 3.6), and sodium citrate (Figure 3.7) having the strongest gel-clots. All samples that formed firm gel-clots were able to withstand 180° inversion without movement of the clot. Endotoxin controls

were also run at the same time in EFW at concentrations of 50, 5, 0.5, and 0 EU/ml (Figure 3.8). The three highest concentrations did form firm gel-clots, as expected. As in the previous experiment, 50 EU/ml formed a firm gel-clot within ten minutes but softened after one hour of incubation.

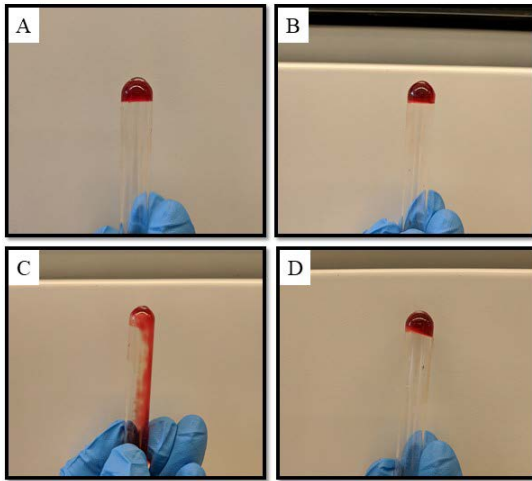


Figure 3.5. Combined Results of Samples with No Anticoagulant. **A** and **B** were spiked with LPS to give a concentration of ≤ 50 EU/ml and both samples formed firm gel clots. **A**) left unwashed and **B**) washed with 0.9% NaCl to rid of any further inhibitors left after removal of plasma. **C**) not spiked with any LPS as a negative control and did not form a clot as expected. **D**) spiked with 4 EU/ μ l LPS after the removal of plasma as a positive control and formed a clot as expected.

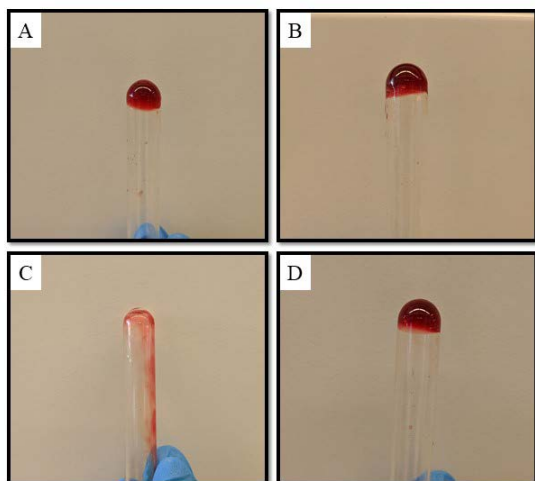


Figure 3.6. Combined Results of Lithium Heparin Samples. **A** and **B** were spiked with LPS to give a concentration of ≤ 50 EU/ml and both samples formed firm gel clots. **A**) left unwashed and **B**) washed with 0.9% NaCl to rid of any further inhibitors left after removal of plasma. **C**) not spiked with any LPS as a negative control and did not form a clot as expected. **D**) spiked with 4 EU/ μ l LPS after the removal of plasma as a positive control and formed a clot as expected.

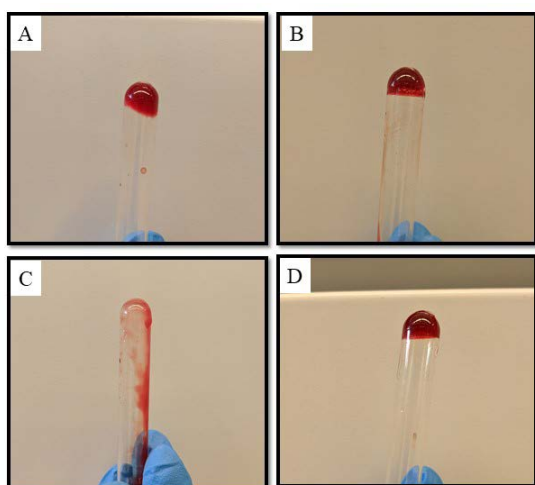


Figure 3.7. Combined Results of Sodium Citrate Samples. **A** and **B** were spiked with LPS to give a concentration of ≤ 50 EU/ml and both samples formed firm gel clots. **A**) left unwashed and **B**) washed with 0.9% NaCl to rid of any further inhibitors left after removal of plasma. **C**) not spiked with any LPS as a negative control and did not form a clot as expected. **D**) spiked with 4 EU/ μ l LPS after the removal of plasma as a positive control and formed a clot as expected.

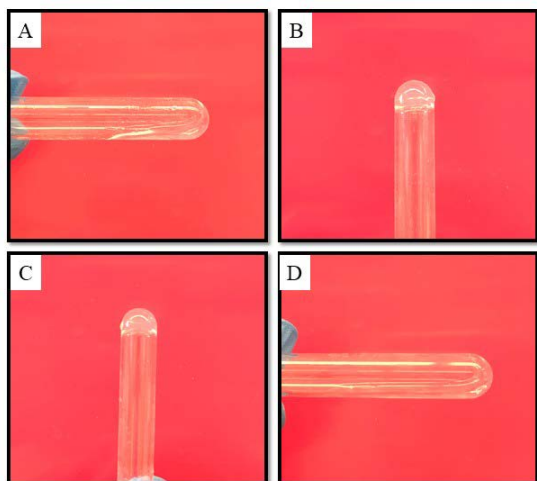


Figure 3.8. Combined Results of Control Samples Run in EFW. **A)** 50 EU/ml, **B)** 5 EU/ml, **C)** 0.5 EU/ml, and **D)** 0 EU/ml of LPS.

The next step was to investigate the sensitivity of the LAL assay using this isolation and washing protocol. For this, the concentration of LPS in the samples were: 50, 5, 0.5, 0.05 and 0 EU/ml. The sample that was treated with 50 EU/ml prior to isolation and washing formed a firm gel-clot after the hour of incubation. The sample with 5 EU/ml added formed a weak gel-clot that could not withstand inversion and the other three samples did not form gel-clots (Figure 3.9). The samples that had LPS added after the washing procedure did form gel-clots at 50, 5 and 0.5 EU/ml (Figure 3.10). Positive control samples run at the same LPS concentrations but in EFW formed firm gel-clots at 50, 5 and 0.5 EU/ml as well (Figure 3.11).

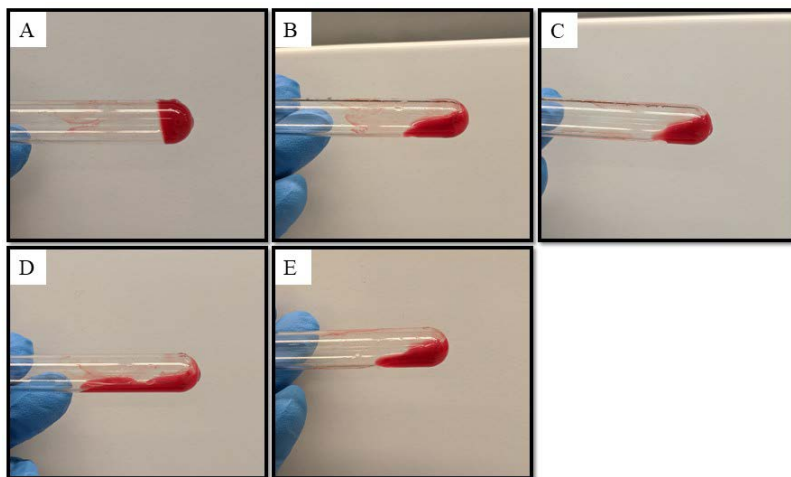


Figure 3.9. Combined Results of Samples with LPS Added Prior to the Washing and RBC Isolation Procedure. **A)** 50 EU/ml, **B)** 5 EU/ml, **C)** 0.5 EU/ml, **D)** 0.05 EU/ml, and **E)** no LPS added.

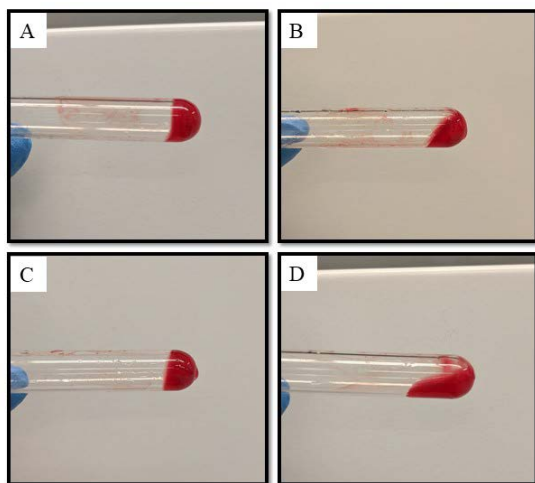


Figure 3.10. Combined Results of Samples with LPS Added After the Washing and RBC Isolation Procedure. **A)** 50 EU/ml, **B)** 5 EU/ml, **C)** 0.5 EU/ml, and **D)** 0.05 EU/ml LPS added.

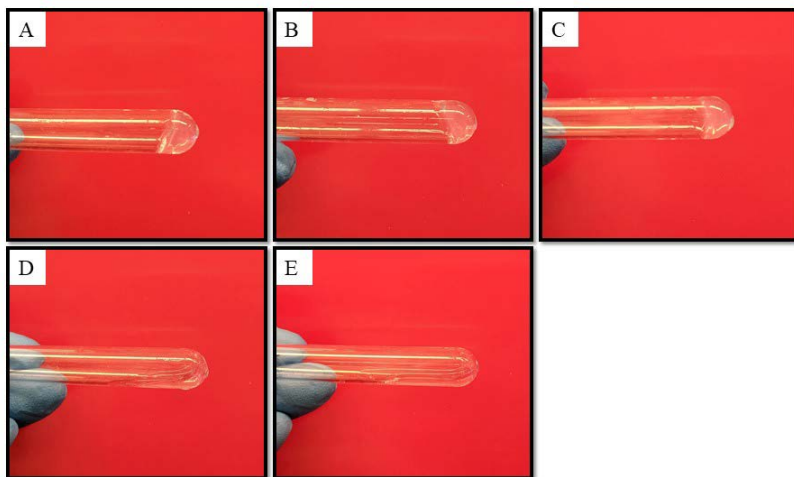


Figure 3.11 Combined Results of Titration Control Samples Run in EFW. **A)** 50 EU/ml, **B)** 5 EU/ml, **C)** 0.5 EU/ml, **D)** 0.05 EU/ml, and **E)** no LPS added.

The experiment was repeated with different concentrations of LPS, in order to narrow down the sensitivity of the LAL assay. LPS concentrations in the assay tested were: 50, 25, 12.5, 6.25, 3.125, and 0 EU/ml. For samples that were treated with LPS prior to washing, firm gel-clots were formed at 50, 25, and 12.5 EU/ml. The sample with 6.25 EU/ml formed a weak gel-clot that was unable to withstand inversion. The other two samples did not form a gel-clot at all (Figure 3.12). The samples with LPS added after washing, showed similar results. Firm gel-clots were formed at 50 and 12.5 EU/ml (Figure 3.13). At 25 EU/ml, a weaker gel-clot formed, but it was unable to withstand inversion. The positive control, run in EFW at 50 EU/ml, formed a firm gel-clot.

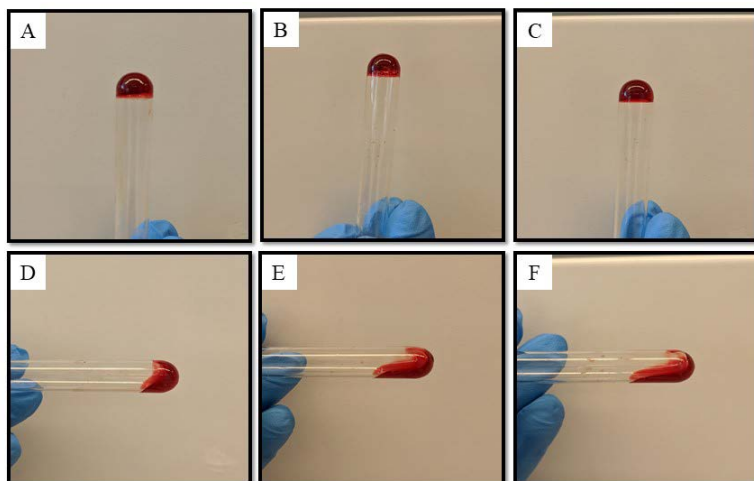


Figure 3.12. Combined Results of LPS Titration with LPS Added Prior to Washing Protocol. **A)** 50 EU/ml, **B)** 25 EU/ml **C)** 12.5 EU/ml, **D)** 6.25 EU/ml, **E)** 3.125 EU/ml, and **F)** no LPS. **Samples A-C** formed firm gel clots that could withstand 180-degree inversion. **Samples D and E** formed softer clots that did not withstand inversion. **Sample F**, as the negative control, did not clot.

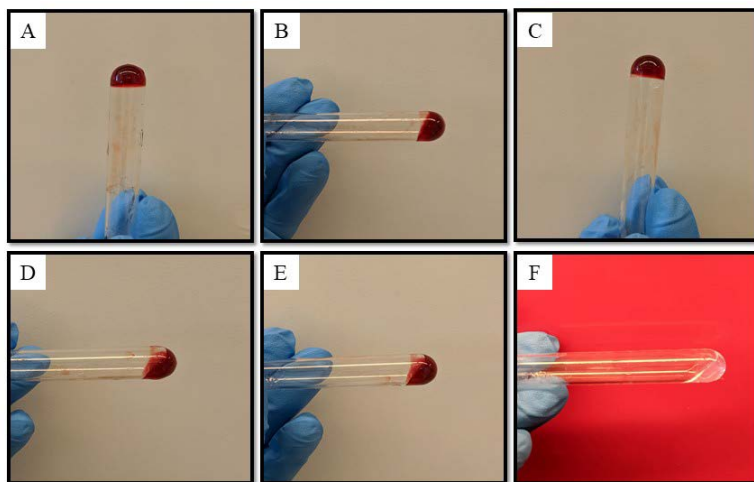


Figure 3.13. Combined Results of LPS Titration with LPS Added After the Washing Protocol. **A)** 50 EU/ml, **B)** 25 EU/ml, **C)** 12.5 EU/ml, **D)** 6.25 EU/ml, and **E)** 3.125 EU/ml, and **F)** 50 EU/ml LPS in EFW. **Samples A and C** formed firm gel clots that could withstand 180-degree inversion. **Samples B, D and E** formed softer clots that did not withstand inversion. **Sample F**, as the positive control, did form a firm gel-clot within ten minutes and softened after the hour of incubation.

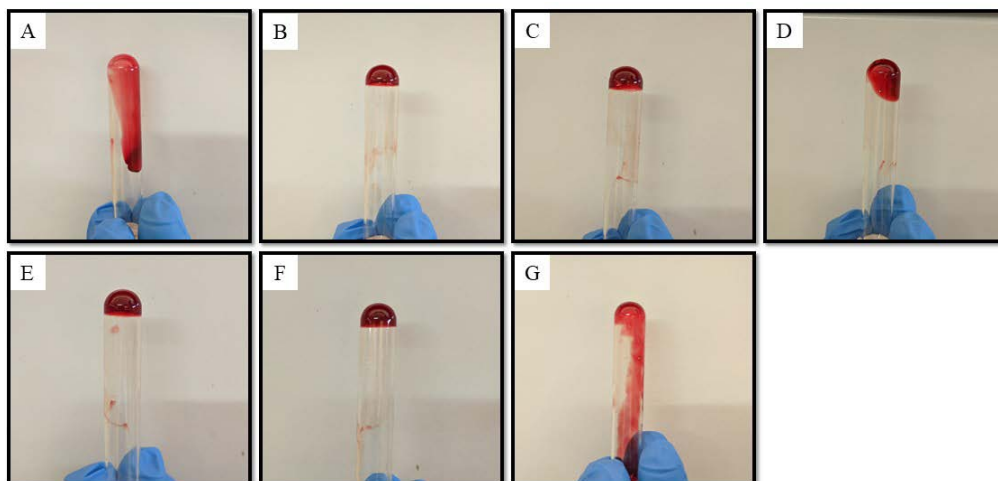


Figure 3.14. Combined Results of LAL Assay with Varying Concentrations of *E. coli* Added Prior to the Washing Protocol. **A)** 6.25×10^5 CFU/ml, **B)** 6.25×10^4 CFU/ml, **C)** 6.25×10^3 CFU/ml, **D)** 6.25×10^2 CFU/ml, **E)** 6.25×10^1 CFU/ml, **F)** 6.25×10^0 CFU/ml, and **G)** 0 CFU/ml. **Samples B, C, E, and F** formed firm gel clots that could withstand 180-degree inversion. **Samples A and D** formed softer clots that did not withstand inversion. **Sample G**, as the negative control, did not clot.

3.3.4. LAL Assay with *E. coli*

The plates made from the dilutions of *E. coli* had a higher CFU/ml than expected. The negative control, with broth alone, had a bacterial count of 0 CFU/, as expected. All other plates had a lawn of bacteria, preventing an accurate colony count. Concentrations are an estimate of what was expected and do not reflect accurate colony counts. LAL assay results were not consistent in clot formation as in previous experiments. For the samples that had the addition of bacteria prior to washing, the highest concentration sample only had a weak gel-clot formation that could not withstand inversion. The next two samples, with a 10-fold and 100-fold lower concentration, did form firm gel-clots. There was gel-clot formation in all samples run with bacteria added prior to washing (Figure 3.14). The integrity of two clots ($625,000$ and 625 CFU/ml) were weaker than the

other samples that were treated prior to washing. For the positive controls, samples that had bacteria added after washing, similar results were found (Figure 3.15). Two samples (6,250 and 62.5 CFU/ml) did not form firm clots that could withstand inversion. All other samples with that treatment formed firm gel-clots. The negative control did not form a gel-clot, while the sample conducted in EFW with the highest bacteria concentration did form a gel-clot.

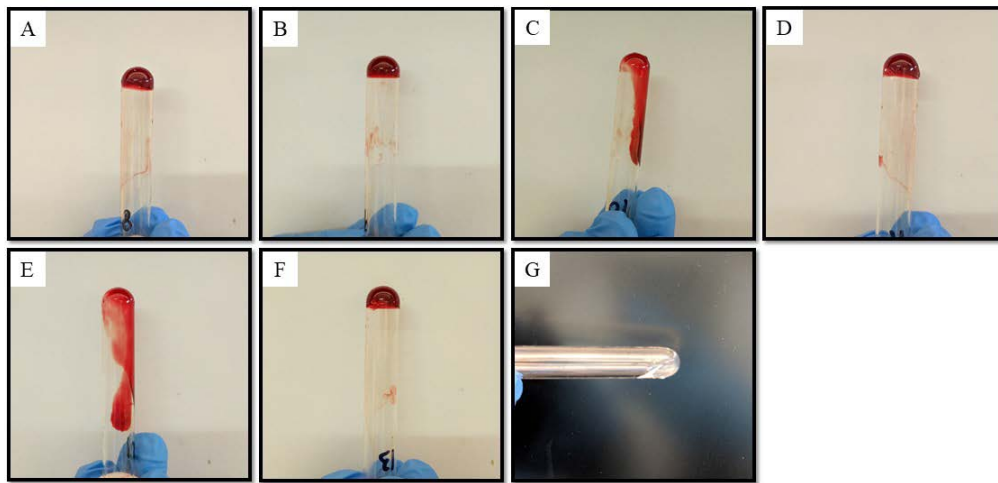


Figure 3.15. Combined Results of LAL Assay with Varying Concentrations of *E. coli* Added After the Washing Protocol. **A)** 6.25×10^5 CFU/ml, **B)** 6.25×10^4 CFU/ml, **C)** 6.25×10^3 CFU/ml, **D)** 6.25×10^2 CFU/ml, **E)** 6.25×10^1 CFU/ml, **F)** 6.25×10^0 CFU/ml, and **G)** 6.25×10^5 CFU/ml in EFW. **Samples A, B, D and F** formed firm gel clots that could withstand 180-degree inversion. **Samples C and E** formed softer clots that did not withstand inversion. **Sample G**, as the positive control, did form a firm gel-clot within ten minutes and softened after the hour of incubation.

3.4. Discussion

3.4.1. Effect of Anticoagulants on LAL Assay

In both experiments, the anticoagulants prevented gel-clot formation as expected. Sodium citrate chelates calcium in the blood by binding it in a nonionized form (70). In

coagulation, the calcium binds to phospholipids, giving a surface for coagulation factors to bind to (54). The LAL may have activated the coagulation cascade; however, without calcium, the pathway is incomplete. The highest LPS concentration did form a weak gel-clot, but it still was not enough to overcome the inhibition by sodium citrate.

With lithium heparin, none of the LPS concentrations were able to overcome inhibition from heparin. Heparin acts by binding to antithrombin, which in turn inhibits thrombin and the interaction of several clotting factors (70). Thrombin activates several coagulation factors and platelets as well as conversion of fibrinogen to fibrin (52). The positive control conducted in EFW did form a firm gel-clot after 10 minutes of incubation in both experiments, showing that the LAL assay was working.

Based on the observations seen in these results, this assay will not be effective unless the anticoagulants are inhibited. Salts, such as calcium chloride, have been found to overcome the inhibition and allowed clot formation (49–51). Salt addition or removal of the anticoagulant prior to testing blood for bacterial LPS would allow for more accurate detection of infection.

3.4.2. Location of LPS in Blood

To determine the location of LPS in the blood, serum or plasma were separated for testing. Finding the location of LPS would allow for more effective isolation and thus yield more accurate results. The two samples that were collected in vacutainers not containing an anticoagulant clotted prior to centrifugation, therefore allowing for separation of serum from the rest of the blood. The serum layers in these samples were red rather than a yellow color, suggesting hemolysis.

For the samples collected in sodium citrate and lithium heparin vacutainers, the samples that did not have LPS added prior had a yellow-colored plasma as expected. However, the samples with LPS added had a red color to the plasma, once again suggesting hemolysis (Figure 3.3). Incubation of blood with LPS has been found to increase free hemoglobin concentration and diminish RBC integrity (71). It is believed that, in vitro, LPS interacts directly with the cell membrane, and causes hemolysis (71).

The negative results observed in all samples (Figure 3.4) suggests that there is not enough free floating LPS to cause clot formation. This suggests that LPS mostly binds to the RBCs. A previous study had conducted the LAL assay on plasma samples from healthy donors and hospitalized patients (39). Healthy donors had plasma separated and then spiked with LPS. Hospitalized patients had symptoms of septic shock. In both cases, LPS was high enough in the plasma to cause a positive result. The goal of these experiments is to detect infection at an earlier stage. At this point in an infection, LPS appears to be mostly bound to the RBCs.

3.4.3. Isolating Red Blood Cells and Bound LPS

Centrifuging blood samples after the addition of endotoxin resulted in a clearer supernatant, unlike the previous experiment. The washing step with NaCl also yielded clear supernatant after centrifuging suggesting that there was no hemolysis. However, after adding EFW to lyse RBCs, there was no clear supernatant or pellet formed from centrifuging the samples. This suggests that there was complete hemolysis in all of the samples. The samples with sodium citrate (Figure 3.7) did result in much clearer supernatant after separation, suggesting that this anticoagulant would be the ideal one to

use for a diagnostic LAL assay. The clots formed in these samples during the LAL assay also withstood inversion the longest. Supernatant from samples without anticoagulant were slightly red (Figure 3.5), hinting at a partial hemolysis prior to the lysis step.

For the LAL assays conducted, the formation of firm gel-clots in all treatments, with the exception of samples with no LPS, shows that this method for isolation of RBCs can lead to positive results for LPS detection. Washing samples with 0.9% NaCl did lead to stronger clot formation by ridding the samples of more serum or plasma inhibitors. Clot formation in sodium citrate samples was superior to those in lithium heparin, while those without anticoagulant were the weakest gel-clots. The positive control sample that was run in EFW at 50 EU/ml (Figure 3.8) did form a firm gel-clot after ten minutes of incubation, as seen in previous experiments. After the full hour of incubation, the clot softened, unlike the same concentration in blood. It has been previously found that hemoglobin enhances the activity of LPS and acts as an LPS binding protein (34). This correlates with our findings of superior clot formation in blood samples compared to the positive controls run in EFW.

The next experiment investigated the sensitivity of the LAL assay using the previous RBC isolation. The 5 EU/ml had weak clot formation that could not withstand inversion, so the endpoint for sensitivity is above that concentration (Figure 3.9). For comparison, the positive controls that had LPS added after the isolation procedure formed firm gel-clots down to 0.5 EU/ml (Figure 3.10). This would suggest that less than 10% of LPS remains bound to the RBCs during this isolation method. However, less LAL was

used in this experiment as with previous and forthcoming experiments. LAL concentration may not have been at the optimal level for the experiment.

To further determine the threshold for the LAL assay, we investigated the sensitivity ranging from 3.125-50 EU/ml. The weaker gel-clot formation at 6.25 EU/ml and lack of clot formation at 3.125 EU/ml does correlate with our previous experiment (Figure 3.12). 100 µl of LAL was used in this experiment, unlike the previous, but results were similar. The positive control of 50 EU/ml in EFW (Figure 3.13) showed the same results as in previous experiments, thus strengthening the idea that hemoglobin enhances LPS activity.

3.4.4. LAL Assay with *E. coli*

Isolation and washing of RBCs in NaCl solution resulted in clear supernatant, once again suggesting that there was no hemolysis in these steps. The sample with broth only in blood did not clot (Figure 3.14). This shows that the broth was adequately washed away in the protocol, therefore not causing a false positive on the LAL assay. Certain samples did not form firm clots as expected. Unlike previous experiments, blood samples were frozen after the isolation and washing procedure but prior to the LAL assay. Freezing and the subsequent thawing of RBC samples would cause hemolysis. While lysis may aid in the release of endotoxin and clot formation, it also ruins the integrity and morphology of the RBCs (70). RBCs can be frozen and thawed safely through cryopreservation, using a protectant, such as glycerol (72). However, RBC morphology can still be significantly altered after using glycerol (72). It has also been shown that cryostorage can affect the amount of hemoglobin in the sample (72). The loss of

hemoglobin would greatly affect the clot formation in the LAL assay as seen in samples run in EFW at the same high concentration of their blood counterparts. For this protocol, blood samples were rapidly frozen, which has been shown to deteriorate blood samples (70). Proper freezing of blood samples requires a much more controlled rate of freezing than was given to our blood samples. A future study could either perform the LAL assay directly following the isolation procedure, or use a different cryopreservation method as described by Pallotta et al (72).

While results were not consistent, the formation of firm gel-clots in most samples show the possibility of detecting bacterial infections at an earlier stage. While freezing samples is possible for this method, a different approach would need to be used. Fresh blood samples yield more consistent results and would need to be used for a repetition of this experiment.

3.5. Conclusion and Future Perspectives

This set of experiments has provided evidence that it is possible to detect bacterial LPS in human blood samples. These findings are consistent with other studies (38,39,49–51,55), but they also highlight the difficulty of obtaining consistent results. Quantifying clots with the use of a chromogenic LAL assay would allow for more detailed results as well. The protocol used in these experiments can be used in further studies to better determine the amount of LPS that remains bound to RBCs. Future research can also investigate whether this same method can be used to detect 1-3 β -D-glucan from fungi. These studies can be used to lead to a diagnostic device that would more rapidly detect bacterial infections and minimize the use of broad-spectrum antibiotics.

CHAPTER IV

THERAPEUTIC CAPABILITIES OF HORSESHOE CRAB BLOOD AGAINST *STAPHYLOCOCCUS AUREUS*

4.1. Introduction

With the growing spread of AMR, natural products have been investigated for their antimicrobial properties. HSC blood contains antimicrobial peptides and hemocyanin which have been studied as a potential source for new therapeutics (30,57,64,65,73). HSC plasma was tested against a gram-positive bacterium using the broth microdilution method. The next step was to test different portions of HSC blood to see what specifically might impart antibacterial activity. The last assay tested whether detergent induced phenoloxidase activity in hemocyanin or killed the bacteria itself. The final part of this aim focused on the concentration of copper in HSC plasma samples to see if there was a correlation between copper concentration and antibacterial activity.

4.2. Methods

4.2.1. Antimicrobial Assays

The following protocol was used for all antimicrobial assays conducted, sample preparation of the material to be tested varied and can be found in following sections. The first step for the assays was to prepare a bacteria inoculum. For all experiments, a laboratory strain of *Staphylococcus aureus* (SA1199), and methicillin-resistant *S. aureus* (AH1263) were used. Bacterial strains were provided by Dr. Nadja Cech. One colony of

each strain was grown in Mueller Hinton broth (MHB) and incubated overnight at 37° and 250 rpm for 18-24 hours.

The following day, bacteria were revived by pouring the inoculum into a fresh tube and adding fresh MHB. Bacteria were once again incubated at 37° and 250 rpm for 2 hours. Following the incubation, the OD was read at 600 nm and used to calculate the concentration of bacteria added to reach a final dilution of 5 mg/ml. Samples were added to a 96-well plate in triplicate and under sterile conditions. Plates were then secured with tape and incubated at 37° and 250 rpm for 18-24 hours.

On the final day, plates were removed from incubation and OD was read at 600 nm using a Synergy H1 microplate reader (BioTek). The OD₆₀₀ values were used to determine the inhibition of growth. The average OD₆₀₀ from each treatment was used for evaluation and statistical analysis, which was performed in Prism.

Dimethyl sulfoxide (DMSO) was used as a negative control, as it will not inhibit the growth of *S. aureus*. Levofloxacin, an antibiotic, was used as a positive control for all experiments. Broth was also plated alone to ensure there was no contamination of the media. For all treatments, there were test wells which included the sample, bacteria and broth. There were also blank wells which included the sample and broth alone, which would allow a direct comparison of growth between treatment and no treatment. Figure 4.1 shows a sample 96-well plate layout for all bioassays. All wells contained the same total volume of 250 µl. DMSO was at 100 µg/ml final concentration while levofloxacin was at 10 µg/ml final concentration.

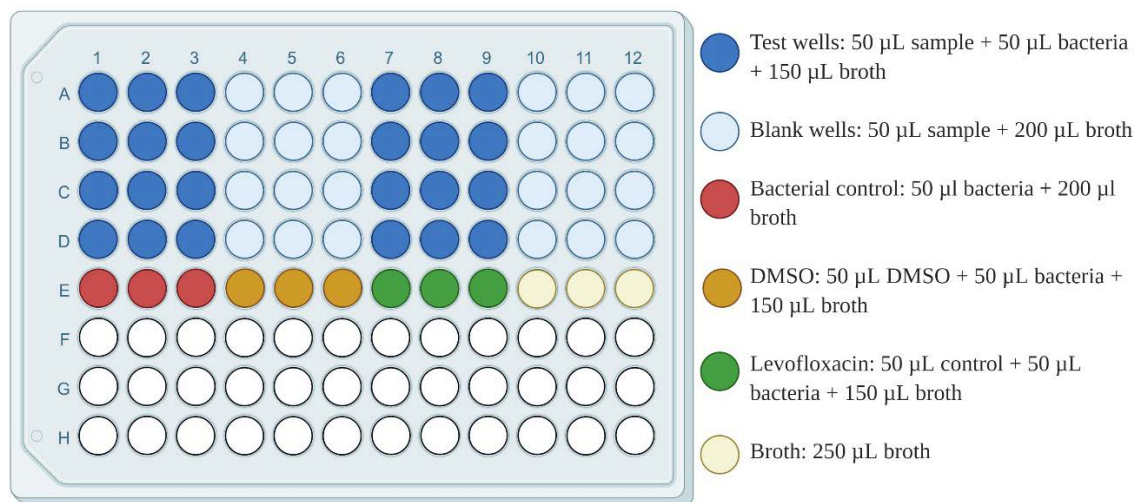


Figure 4.1. Sample 96-Well Plate Layout for Bioassay Screening of HSC Blood and Components Against *S. Aureus* Strains, SA1199 and AH1263. Created with BioRender.com

4.2.2. HSC Plasma

All HSC blood samples were donated by Kepley Biosystems Incorporated. The first step was to investigate HSC plasma against SA1199 and AH1263. Samples from four different HSCs were diluted in DMSO to bring the concentration of hemocyanin to 5 mg/ml in the plasma. Dilution calculations were based on the hemocyanin concentration provided by KBI. All samples were tested at 100 µg/ml and 10 µg/ml final concentration of hemocyanin in the wells. Controls were tested as previously described in the section above. The experiment was repeated a second time with the same HSC plasma samples. A copper sulfate stock was also tested to determine if the copper would have bioactivity against *S. aureus* at the same concentration as the plasma samples.

4.2.3. Antimicrobial Activity of HSC Blood Components

For this experiment, blood was collected from one HSC and the blood was separated into different components: hemolymph, plasma, and amoebocytes. These different components were then tested for bioactivity using the same antimicrobial assays as previously described.

The first treatment was whole hemolymph, the blood was collected and used untreated. The second treatment was whole hemolymph with 1% detergent, Triton x-100, added. The third condition was HSC plasma only, the blood was centrifuged to isolate the plasma for testing. The fourth treatment was intact amoebocytes that were separated from plasma through centrifugation and then resuspended in 0.5 ml of 3% NaCl. The final two treatments were amoebocytes that were lysed in different conditions. The first condition was amoebocytes lysed in 0.5 ml of EFW. In the second condition, amoebocytes were physically agitated until they lysed. After preparation, all samples were maintained on ice until plating. These treatments were tested at undiluted and then at a 100-fold dilution of the sample.

4.2.4 Effect of Detergent on Antimicrobial Properties of HSC Blood

This experiment focused solely on the effect of the detergent, Triton x-100, on the bioactivity of HSC blood against bacteria. Four samples of 1 ml of blood were collected from four different HSCs. To each sample, 10 µl of detergent were added to bring the concentration of detergent to 1% in solution. Samples were maintained on ice until plating. For this experiment, another control was added of just the detergent with bacteria

and MHB. Treatments were once against treated undiluted and at a 100-fold dilution of the sample in broth.

4.2.5 Inductive Coupled Plasma Analysis of HSC Plasma

To determine the quantity of copper found in the plasma of HSC blood, inductive coupled plasma optical emission spectroscopy (ICP-OES) was conducted. The same plasma that was used for the initial bioassays was used in the ICP analysis to see if there was any correlation between the amount of copper and bioactivity. For ICP analysis, the samples must be digested in an acid, typically nitric acid, hydrochloric acid or a combination of the two, known as aqua regia. Standards are made from a stock solution of the element under investigation. These standards are then run first to create a calibration curve, which is then used to determine the intensity. All standards and samples are run in triplicate and the average is used for calculations.

To digest the HSC plasma, samples were diluted 100-fold in EFW and gently mixed. To each sample, 70% nitric acid was added to bring the final concentration of acid in the solution to 2%. Samples were incubated overnight at room temperature. The following day, samples were filtered using a 0.22 μm membrane. All samples were clear with no particulates. Copper standards were made using a stock copper solution. Standards ranged from 0-5 ppm based on the expected copper concentration provided by KBI. Standards were also run in 2% nitric acid.

A scatterplot was made from the standard values to find a best fit equation. This equation was then used to calculate copper concentration in samples. All scatterplots and mathematical analysis were completed in Excel.

4.3. Results

4.3.1 HSC Plasma

To be considered bioactive against a bacterial strain, the percent growth of bacteria needs to be less than that of the negative control, in this case, DMSO. The second criterion is that the sample needed to inhibit more than 50% bacterial growth (74). The first bioassay was performed with HSC plasma ($n = 4$). None of the HSC plasma samples were considered bioactive against SA1199 at either 100 $\mu\text{g/ml}$ or 10 $\mu\text{g/ml}$. Results were also analyzed using a one-way ANOVA, comparing all HSC plasma samples at 100 $\mu\text{g/ml}$ against one another. There was no statistically significant difference between HSC samples. The average from each HSC was then used to run an unpaired t-test against DMSO at a p-value of 0.05 (Figure 4.2a). HSC plasma, although not considered bioactive, is significantly different from DMSO values ($p=0.0002$). HSC plasma #27 met criteria to be considered bioactive against strain AH1263 at 100 $\mu\text{g/ml}$ only. None of the other samples inhibited growth enough to be bioactive. The same statistical analysis was used for the results from testing against AH1263 (Figure 4.2b). HSC plasma was again significantly different from DMSO ($p=0.0276$).

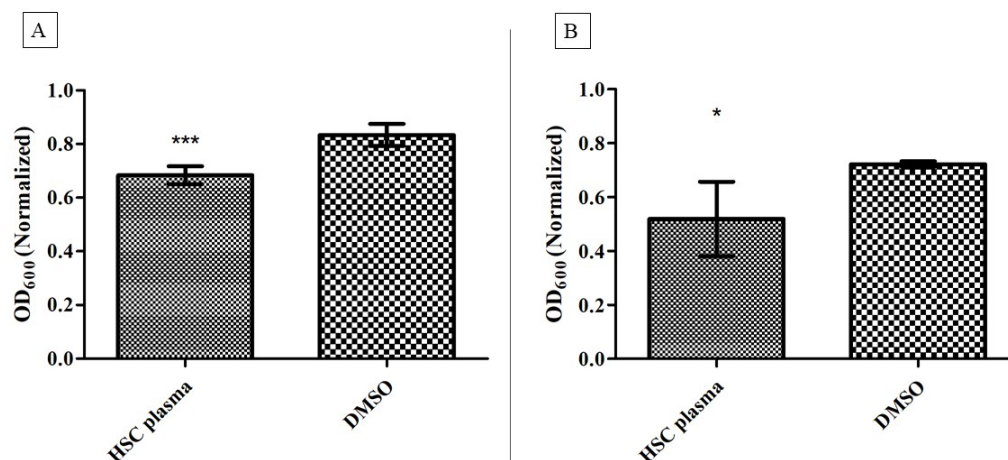


Figure 4.2. Bacterial Growth of SA1199 and AH1263 in HSC Plasma. **A)** SA1199 and **B)** AH1263 tested in HSC plasma (n=4) were compared to growth in DMSO. Optical density was measured at 600 nm after 24 hours 37°C and 250 rpm. An unpaired t-test was performed, ***p = 0.0002, *p = 0.0276.

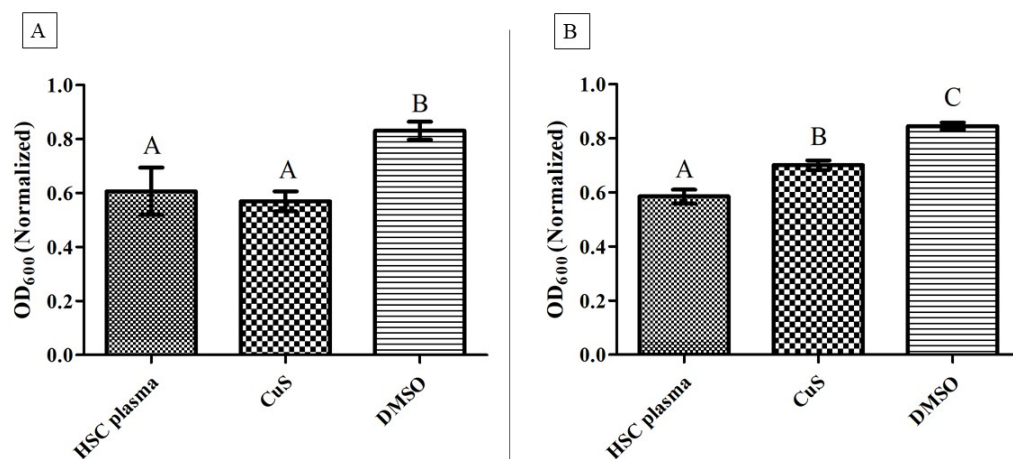


Figure 4.3. Bacterial Growth of SA1199 and AH1263 in HSC Plasma and Copper Standard. **A)** SA1199 and **B)** AH1263 tested in HSC plasma (n=4), and CuS were compared to growth in DMSO. Optical density was measured at 600 nm after 24 hours 37°C and 250 rpm. One-way ANOVA with multiple comparisons (post hoc Tukey test) was performed. Treatments that do not share a letter significantly differ from one another.

The second repetition of this experiment yielded similar results. The outlier, HSC plasma #27, did not show bioactivity in either strain at either dosage this time around. The sample with CuS alone did not show any bioactivity against either strain of *S. aureus*. No significant difference was found among the HSC plasma samples ($n = 4$) from one another using a one-way ANOVA. HSC plasma samples were then combined and used in a one-way ANOVA against CuS and DMSO, and there was a significant difference in treatments ($p=0.0027$) (Figure 4.3a). Post hoc testing was conducted using the Tukey test at a significance level of 0.05. The HSC plasma and CuS were not significantly different from one another. However, DMSO was significantly higher than the other two treatments ($p < 0.01$). The one-way ANOVA for results against AH1263 were significant ($p < 0.0001$) (Figure 4.3b). Using the same post hoc testing, all treatments were significantly different from one another ($p < 0.0001$).

4.3.2. Antimicrobial Activity of HSC Blood Components

In the experiment investigating different fractions of HSC blood, samples were tested undiluted and at 100-fold dilution in MHB. DMSO was used as a negative control at 100 $\mu\text{g/ml}$ again. Levofloxacin continued to be the positive control at a concentration of 10 $\mu\text{g/ml}$. Undiluted hemolymph samples, with the addition of 1% Triton x-100, showed bioactivity against both strains of *S. aureus*. It also exhibited bioactivity against SA1199 at the 100-fold dilution in MHB.

The one-way ANOVA from SA1199 testing yielded a p-value of < 0.0001 (Figure 4.4a). Post hoc testing, using the Tukey test, found that hemolymph treated with 1% Triton x-100 was significantly different from all of the other conditions, including the

negative control of DMSO. DMSO was found to be significantly different from all of the treatments. The p-value for these treatments was <0.01 . Other than hemolymph with Triton x-100, none of the other conditions significantly varied from one another.

For the bioassay against AH1263, the one-way ANOVA came back with a p-value of <0.0001 (Figure 4.4b). For the post hoc testing, the p-value for significance was <0.05 . Untreated hemolymph was significantly different from hemolymph with 1% Triton x-100 added and from DMSO, but not other treatment. Hemolymph with Triton x-100 was significantly different from DMSO. Plasma alone and amoebocytes lysed in EFW were significantly different from DMSO as well.

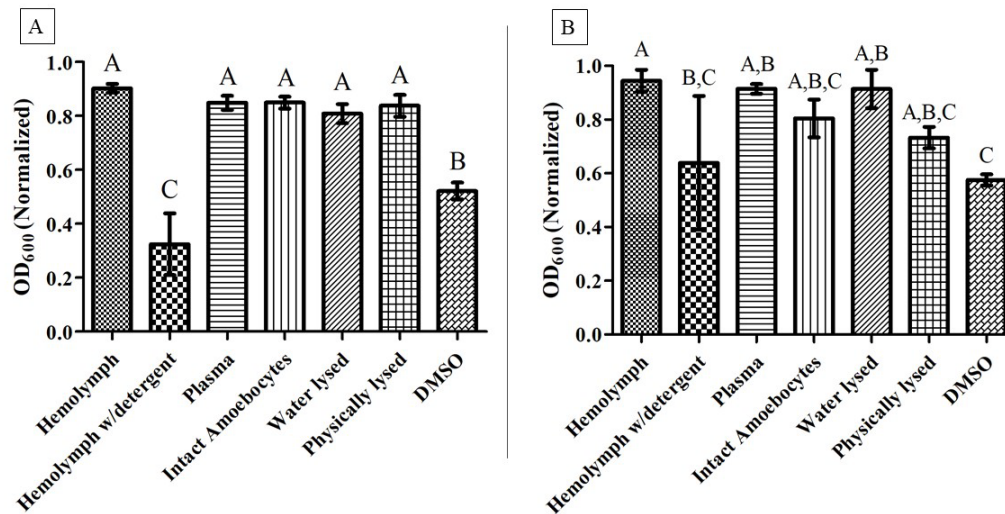


Figure 4.4. Bacterial Growth of SA1199 and AH1263 in Different Fractions of HSC Blood. **A)** SA1199 and **B)** AH1263 tested in different fractions of HSC blood were compared to growth in DMSO. Optical density was measured at 600 nm after 24 hours 37°C and 250 rpm. One-way ANOVA with multiple comparisons (post hoc Tukey test) was performed. Treatments that do not share a letter significantly differ from one another.

4.3.3. Effect of Detergent on Antimicrobial Properties of HSC Blood

Since detergent has been found to induce phenoloxidase activity in hemocyanin, the addition of detergent to hemolymph was investigated further. Undiluted, all four HSC samples showed bioactivity against SA1199 and AH1263. There was no bioactivity seen at the 100-fold dilution in either strain. A detergent control was also used at the same concentrations to determine if the activity was solely due to the detergent. The detergent showed bioactivity against both strains when undiluted as well, but not at the 100-fold dilution.

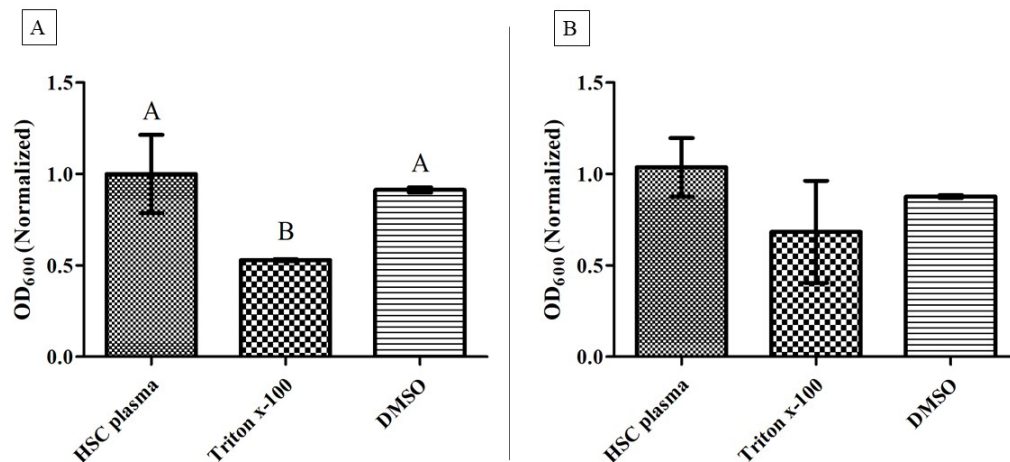


Figure 4.5. Bacterial Growth of SA1199 and AH1263 in HSC Plasma and Triton x-100. **A)** SA1199 and **B)** AH1263 tested in HSC plasma and Triton x-100 were compared to growth in DMSO. Optical density was measured at 600 nm after 24 hours 37°C and 250 rpm. One-way ANOVA with multiple comparisons (post hoc Tukey test) was performed. Treatments that do not share a letter significantly differ from one another.

HSC plasma samples were not found significantly different from one another. The average from each HSC was then used in a one-way ANOVA against Triton x-100 and DMSO using the 100-fold dilutions. The treatments were significantly different from one

another ($p = 0.0082$) (Figure 4.5a). Post hoc testing was conducted using the Tukey test and found Triton x-100 to be significantly different from HSC plasma and DMSO ($p < 0.05$). HSC plasma and DMSO did not vary significantly from one another. The same method was used to test results from AH1263, but treatments were not found to be significantly different in either the one-way ANOVA or in post hoc testing (Figure 4.5b).

4.3.4. Inductive Coupled Plasma Analysis of HSC Plasma

Copper standards were run to create a standard curve for the unknown samples (Figure 4.6). Standards were at: 0, 0.15625, 0.3125, 0.625, 1.25, 2.5, and 5 ppm. A best fit line was determined ($R^2 = 0.9998$). This equation was then used to calculate the copper concentration in each sample at the 100-fold dilution. The four samples ranged from 309 ppm to 977 ppm (Table 4.1).

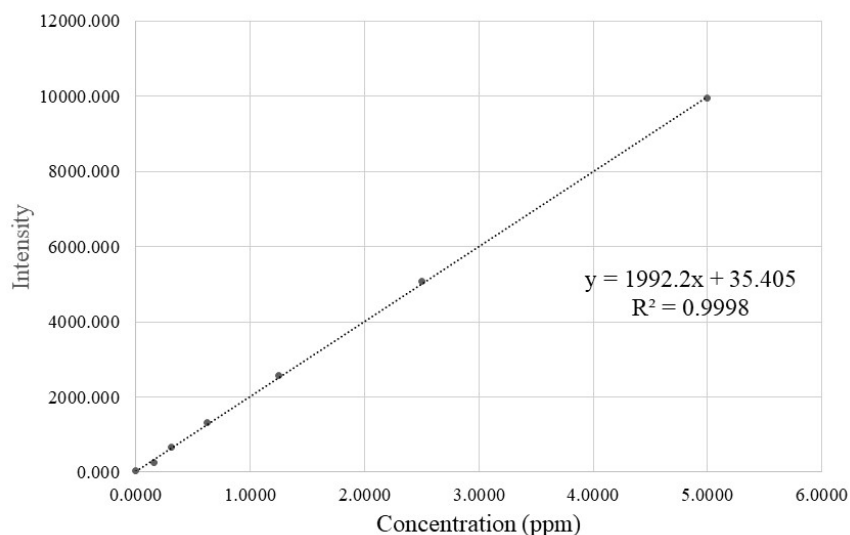


Figure 4.6. Standard Concentration Curve for Copper by ICP.

Table 4.1. Copper Concentration of HSC Plasma Samples Analyzed by ICP.

HSC plasma	Copper Concentration (ppm)
#8	926.32
#19	977.66
#25	737.44
#27	309.82

4.4. Discussion

4.4.1 HSC Plasma

The broth microdilution method is the most accurate to determine the minimum inhibitory concentration (MIC) (75). The Clinical and Laboratory Standard Institute (CLSI) outlines a standard protocol for this method (76). The MIC is the lowest concentration of the agent being tested that inhibits visible growth of the bacteria (75). Bacterial growth amongst the HSC plasma samples did not vary significantly ($p>0.05$) another in the one-way ANOVA, showing that there is no difference between HSC subjects. The HSC plasma did show inhibition significantly lower (SA1199: $p=0.0002$, AH1263: $p=0.0276$) than the negative control in both strains of both experiments. Samples were diluted to have the hemocyanin concentration at 100 $\mu\text{g/ml}$, but this may not be what imparts the antimicrobial activity that has been observed in HSC blood. Copper at the same concentration was significantly lower than DMSO in both strains ($p<0.01$), but only significantly different from HSC plasma when tested against AH1263 ($p<0.0001$). The copper, may not be the contributing factor to the antimicrobial activity. Although copper is used as an antimicrobial agent, there are many factors that affect its efficacy. The copper is more effective at higher copper concentrations and when it comes into direct contact with the bacteria (60). The two copper ions located in the hemocyanin

domain may not have the contact or concentration necessary to induce antimicrobial activity against bacteria.

4.4.2. Antimicrobial Activity of HSC Blood Components

The next step was to determine whether a different fraction of HSC blood contained antimicrobial properties. Detergent, such as Triton x-100, has been shown to induce phenoloxidase activity of hemocyanin (30). Amoebocytes contain large and small granules, with the small granules containing the antimicrobial peptide, polyphemusin, inside (65,66). All treatments, with the exception of hemolymph with detergent added, were not significantly different from one another, but significantly higher bacterial growth compared to the negative control (SA1199: $p < 0.0001$). These treatments actually increased bacterial growth, leading to a suspected contamination. However, the blank wells with only the sample treatment and broth did not exhibit bacterial growth. All other controls (DMSO, levofloxacin, and broth) had expected OD readings as seen in previous experiments, once again ruling out possible contamination.

Hemolymph with detergent was significantly lower than DMSO ($p < 0.01$), as well as meeting criteria for bioactivity against SA1199. Triton x-100 has also been shown to lyse cells (77), so the lack of bacterial growth may not have been inhibited, but killed in that treatment.

4.4.3. Effect of Detergent on Antimicrobial Properties of HSC Blood

Based on the previous findings, we wanted to determine whether the lack of bacterial growth was due to detergent enhancing antimicrobial activity of HSC plasma or due to cell death by detergent. Four different HSCs were used for statistical analysis only

after determining that there was no significant difference in HSC samples. Against SA1199, Triton x-100 showed significantly lower bacterial growth compared to the other treatments ($p < 0.05$), while HSC plasma did not vary from the negative control. It appears that the bacteria are being killed by the detergent through lysis rather. Such as seen by Tanihara et al. in human fibroblasts (77), the antimicrobial peptides may be preventing cell lysis by Triton x-100, hence the higher bacterial growth.

4.4.4. Inductive Coupled Plasma Analysis of HSC Plasma

Values for copper concentration came back higher than expected values and were thus determined using a standard curve. Copper concentration does not appear to correlate to bioactivity as there was no significant difference amongst HSC samples in the bioassays ($p > 0.05$) despite a large variance in copper concentration. Higher copper concentration did not correlate to a lower bacterial growth, as may be expected. This data further supports the idea that the copper is either not in the right form or does not have great enough access to bacteria to impart antibacterial properties to hemocyanin. For example, studies have shown that copper has a greater antimicrobial efficacy at concentrations greater than or equal to 55% (60). Although 90% of the total plasma protein consists of hemocyanin (78), total plasma protein concentration can vary amongst horseshoe crabs (78). One study found that mean protein concentration among their wild-type horseshoe crabs and caught horseshoe crabs varied from $3.2\text{--}9.7\text{ g dL}^{-1}$ (78). Additionally, the form of copper can also heavily impact its efficacy as an antimicrobial. Copper nanoparticles have shown greater antimicrobial activity compared to insoluble copper at the same concentration (60). Hemocyanin contains deoxygenated copper atoms,

but it has been found that greater antimicrobial activity is found in higher oxidation states of copper (60). This would suggest that oxygenated hemocyanin would have greater antimicrobial activity than deoxygenated hemocyanin due to the greater oxidation state of copper. Other studies suggest that the direct contact of copper with bacteria can cause membrane damage (60). With copper ions located within the subdomains of hemocyanin, it is unlikely that the copper would have the access to bacteria necessary for contact killing. These experiments also only investigated the potential of HSC blood on *S. aureus*, a gram-positive bacterium. Future studies are needed to investigate these effects on gram-negative bacteria and fungi as the immune system of HSCs is set off by these microbes. LPS and 1-3 β -D-glucan cause degranulation of amoebocytes (36), which in turn releases the antimicrobial peptides found in the granules. This degranulation, while causes clotting to prevent spread of bacteria, may also be inhibiting bacterial growth through the polyphemusins.

4.5. Conclusion and Future Perspectives

In these studies, we have demonstrated that the copper in hemocyanin had no significant effect on the antimicrobial properties of the HSC blood. The antimicrobial assays conducted in these sets of experiments show the difficulty in determining antimicrobial activity of a compound. With the copper and antimicrobial peptides in the HSC blood, the potential for bioactivity is there, however, it is easily interfered with. The antimicrobial activity of isolated polyphemusins would be the next step in evaluation of HSC blood. For example, polyphemusins could be isolated to test their impact on bacterial growth. A synthetic version of the peptide could be engineered, testing in

isolation would better indicate antimicrobial activity. Another avenue to explore would be the phenoloxidase-like activity of hemocyanin. This would require finding the best reagent to stimulate the phenoloxidase-like activity that does not kill or inhibit bacterial growth itself. Isolation of hemocyanin would also allow for more accurate testing. Some suggest that the antimicrobial peptides activate the hemocyanin and others that the hemocyanin is the source of antimicrobial peptides (30). Studying both polyphemusin and hemocyanin individually and in conjunction with one another may provide a clearer picture into the mechanism of action and the source of antimicrobial activity contained in HSC blood.

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