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Assessment of motility and hemolytic activity in clinical isolates of Acinetobacter baumannii from University of Kentucky hospital, Lexington, KY

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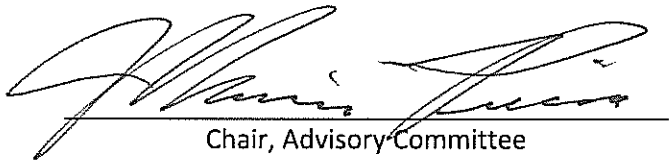

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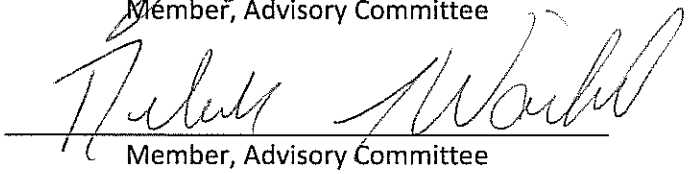

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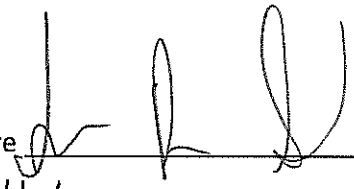
Signature 

Date 11/11/2013

Assessment of motility and hemolytic activity in clinical isolates of *Acinetobacter baumannii* from University of Kentucky hospital, Lexington, KY

By

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Bachelor of Science
Eastern Kentucky University
Richmond, Kentucky
2011

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DEDICATION

To my parents
my brother, sister-in-law, and niece,
and all my co-workers at the TRC/UTC
for their unwavering support.

ACKNOWLEDGMENTS

I would like to express my thanks to my family for their understanding and patience during those times when there was nothing left for me to give. They always pushed me to keep going and trying. I would also like to thank all the people who were by my side through the daily struggles who graciously allowed me to talk, both, at them and with them. I could not have completed this project without every ounce of support.

My thesis would have been impossible without the help of the University of Kentucky Medical Center members who helped provide me with *Acinetobacter baumannii* isolates. A special thanks goes out to Julie Ribes for helping coordinate the collection process and Barry Jacobs for being my contact at the UK Medical Center.

I would also like to thank my thesis committee, Dr. Marcia Pierce, Dr. Rebekah Waikel, and Dr. Bill Staddon, for their guidance.

Abstract

Acinetobacter baumannii is a pathogen rising in notoriety worldwide due to outbreaks linked to multi-drug resistant strains. Research is currently focused on identifying virulence factors, which may contribute to increased ability to cause human disease, such as hemolytic activity and surface motility. The aim of this study was to determine the presence of these two virulent traits in clinical isolates. Forty-eight clinical isolates were recovered from University of Kentucky hospital in Lexington, KY. No hemolytic activity was observed for any of the isolates. Evidence of surface motility was observed in 13 isolates. The brand and concentration of media used allowed for better observation of motility. There is potentially a multifactorial component to virulence not examined in this study, which contribute to increased ability of *A. baumannii* to cause disease. Preliminary statistical tests did not indicate a relationship between surface motility and multi-drug resistance or being part of a complex of *A. calcoaceticus-A. baumannii.* The lack of results indicates a need for further research to be performed on *A. baumannii* to further classify virulence factors and examine the potential for a multifactorial component resulting in its virulence.

TABLE OF CONTENTS

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

FIGURES PAGE

CHAPTER 1

LITERATURE REVIEW

The history of the *Acinetobacter* genus dates back to 1911 with the isolation of the first strains of an organism named *Micrococcus calcoaceticus* by researcher M.W. Beijerinck (24). This organism was named because it appeared as small spherical balls in the calcium acetate-containing medium (24). Several species similar to this organism were described throughout the next 40 years, but were associated with more than 15 different genera (12, 17, 24). Throughout the years the different genera and species classifications included *Diplococcus mucosus, Alcaligenes haemolysans, Achromobacter anitatus, and Achromobacter musosus* (24). In 1954, a proposal was made to separate the motile from the nonmotile microorganisms within the genus *Achromobacter* (24). A 1968 paper published by Baumann et al. concluded that several of these species belonged to a single genus and proposed the name of *Acinetobacter* meaning "unable to move" (9, 24). At the time it was concluded further sub-classification based on phenotypic characteristics was not possible (24). The scientific community did not readily accept this new genus as demonstrated by the three year gap between description and official recognition. Official acknowledgement of *Acinetobacter* was made by the Subcommittee on the Taxonomy of *Moraxella* and Allied Bacteria in 1971 and recognized only a single species (18). This species was initially named *A. calcoaceticus* and included organisms previously referred to by the epithet "*anitratum"* and *"lwoffi"* (18).

The use of DNA-DNA hybridization by Bovet and Grimont in 1986 led to the description of an additional 12 distinct species of *Acinetobacter* (24). Other significant taxonomic modifications have been made over the last 27 years, describing additional species (21, 24). Different methods have been used to describe species, leading to conflicting reports regarding the number of species within the *Acinetobacter* genus. Different sources have listed the number of species in the genus as being 23 (17), 25 (12), 27 (21), 31 (24), or 32 (14, 28). Anton Peleg, a researcher who has published several different papers on *Acinetobacter* and *A. baumannii*, cites 27 validly named species within the genus and 9 DNA-DNA hybridization groups (25). As of 2008, only 17 of these species had been officially named (28). These species include, but are not limited to: *A. pittii, A. nosocomialis, A. lwoffii, A. junii, A. haemolyticus, A. calcoaceticus,* and *A. baumannii* (25).

In general, the free-living saprophytes of the *Acinetobacter* genus are considered to be ubiquitous pathogens in nature (12, 24, 28). *Acinetobacter johnsonii, A. lwoffii,* and *A. radioresistens* can be found as part of the normal human and animal skin flora, as well as in some spoiled foods (21). The widespread nature of the genus falsely suggests that the species *baumannii* is ubiquitous in nature (21). While *A. baumannii* has been isolated from soil, vegetables, and surface water through enrichment, reports of isolation should be carefully considered to ensure identification to the species level uses current validated methods that are more robust and accurate (21, 24). Some strains of the bacteria have also been isolated from small-size living organisms such as ticks, body lice, human body louse, and fleas (2, 28).

Very few species of the *Acinetobacter* genus are known to consistently cause clinical disease. A*. baumannii*, *A. nosocomialis* and *A. pittii* are the only species identified as being clinically relevant in the last few years (25). Many members of the genus are known to comprise a part of the normal human skin flora (24, 25). Rates of skin colonization by the different species of the genus have been reported to differing degrees. These differences can be attributed to different research methods, as well as differences in the methods used to identify the bacteria. Results with colonization rates on the low end of the spectrum are thought to be the result of noise (25).

Phenotypic laboratory tests can lead to misclassification and misidentification of *Acinetobacter baumannii*. There is difficulty in identifying, without question, genetically closely related species no matter the genus (28). Misidentification of *A. baumannii* as gram-positive occurs due to difficulty in the destaining process (24). This would lead to the incorrect conclusion that the isolate belongs to a different species of bacteria (24). It is very difficult to separate *A. baumannii* from *A. calcoaceticus, A. pitti* (formerly known as genomic species 3), and *A. nosocomialis* (formerly known as species 13tu) using phenotypic laboratory tests (25, 28). When these species cannot be separated they are referred to as the *A. calcoaceticus-A. baumannii* complex (12, 24, 25,28). Significantly less information is available regarding the non-*baumannii* species compared to the *baumannii* species, adding to the difficulty of the separating process (25). There is also a lack of identifiable traits that may cause one species of the genus to be more equipped to cause human outbreaks (21, 25). Some epidemiologists have concluded there is an overestimation of the prevalence of medical issues related to the

bacteria due to the difficulty in identification and separation from the complex (17). The clinical significance of *Acinetobacter baumannii* has also been called into question, in part due to the difficulty in determining if clinically isolated cultures are the result of skin colonization or from an infection (17).

The genome of *Acinetobacter baumannii* ranges from 3.2 Mb to 3.9 Mb depending upon the particular isolate analyzed (1, 11, 28). Despite its small-sized core genome, *A. baumannii* should be taken as a serious clinical threat due to its large accessory genome containing numerous antibiotic resistance genes. Horizontal gene transfer can be utilized by the bacterium to acquire new genetic material through plasmids, integrons, and transposons (6). Plasmids have been associated with the transfer of antibiotic resistance genes more than transposons and integrons within the *Acinetobacter* genus (20). The bacterium also has the capability of rearranging existing genes allowing for evolution of virulence factors such as resistance to antibiotics. A retrospective study performed in the UK found a rise in carbapenem resistance from 0%, in 1998, to 55%, in 2006, in *A. baumannii* species causing bacteremia (31). Crude mortality rates for bloodstream infections resulting from *A. baumannii* have been estimated to range between 28 and 43 percent (21). *A. baumannii* has been described as an organism threatening the current antibiotic era due to existence of pandrug-resistant strains (24).

While researchers acknowledge a lack of information regarding virulence factors, some have been identified and need to be described further. These factors include, but are not limited to: siderophore-mediated iron acquisition systems, biofilm

formation, capsule formation, quorum-sensing, surface proteins, and expression of genes regarding acquisition of essential nutrients (21, 25). Desiccation, hemolytic activity, and surface motility have also been identified as potential virulence factors (2). Some of these virulence factors, such as surface motility, have a lot of conflicting research regarding their properties.

The pathogenesis of *Acinetobacter baumannii* has been linked to its ability to evade the bactericidal activity of host serum. In particular, one study found the mortality of patients correlated with the serum resistance of *A. baumannii* (32). The ability to resist the bactericidal activity of serum was linked to acquisition of a surface protein named factor H (32). The study acknowledged other research that found factor H did not bind to the surface of the bacteria (32). The conflicting information regarding whether or not factor H binds to the surface of the bacteria could be the result of the different strains of the bacteria used for each study or it could be the result of other unknown factors such as differing laboratory conditions. A phospholipase D and transposon mutant in a gene for penicillin binding protein 7/8 have also been linked to reduced serum resistance (2).

A number of hemolysin-related genes and two phospholipase C (plc) genes have also been found in all of the strains sequenced, despite *A. baumannii* historically being classified as non-hemolytic (2, 28). This classification may be linked to the type of blood used as well as the assay method performed. Hemolysis has never been observed on sheep blood agar, but some evidence of hemolysis on horse blood agar exists (2, 24). Liquid assays have shown to be more sensitive at showing the hemolytic activity (2).

Even though the sensitivity is higher using liquid assays the hemolytic activity is significantly lower than that observed in species of bacteria known to be hemolytic.

Despite *A. baumannii* being historically described as being non-motile, researchers have also examined the role of motility in the pathogenesis of the bacterium due to the presence of several genes related to motility (8). Genomic analysis has revealed a lack of flagellar genes (27). Type IV pilus apparatus and pilus assembly genes have been identified within the *A. baumannii* genome. Type IV pilus assembly protein genes identified within the *A. baumannii* genome are pilQ, pilO, pilN, pilW, and pilM (2, 25, 27). Type IV pilus biogenesis protein genes pilB and pilJ have also been identified (2, 25). Fimbrial biogenesis genes fimT, pilB, and pilZ have also been found within the genome of *A. baumannii* (2, 25, 27). pilU, pilI, and pilT genes, which are responsible for pilus retraction through twitching motility, have also been identified within the genome (25, 27). The *A. baumannii* strain M2, lacking a functional pilT gene, was found to have a 54% reduction in motility compared to strains with a functional gene (27). Twitching motility represents a significant component in overall motility (27).

A single conclusion regarding the motility, or lack thereof, has not been drawn due to conflicting results. Differences in laboratory conditions can play a part in not allowing for observation of motility in all clinical isolates studied (8, 22). Studies have shown that most movement tends to occur on the surface of semi-solid media and decreases as the concentrations of agar increase (8, 22). A study of the M2 strain of *A. baumannii* found robust surface motility on Luria-Bertani (LB) broth modified with 10 g tryptone, 5 g yeast extract, and 5 g NaCl with 0.2-0.4% concentration agar plates, while

higher concentrations of agar showed less evidence of motility (8). Similar to results observed in other bacteria, differences in evidence of surface motility have been observed depending on the brand of media used (8). When the test was performed using the modified LB broth base described above with Difco agar media inhibition was observed at 0.35%, while inhibition of motility was observed at 0.5% when Eiken agar was used (8). Inhibition of motility occurs at higher concentrations of agar because the media is harder than at lower concentrations. Eiken agar is thought to have compounds that promote motility of bacteria and/or increase the "wettability" allowing the bacteria cells to spread easier (8). Specific compounds in EIken agar, which promote spreading of the bacteria, have not been identified.

The patterns of motility observed have been shown to differ within and between different strains (8, 22). Branching patterns of motility have been observed in the M2 strain of *A. baumannii* using Difco media with a modified LB broth base (8). Evenly distributed patterns of migration from the point of inoculation have been observed in the M2 strain using Eiken media with a modified LB broth base (8). Other strains tested in the same study found varying patterns of motility ranging from simple concentric rings to complex flower-like patterns as well as a few strains with no signs of motility as shown below in Figure 1 (8). A translucent zone observed just ahead of the advancing colony was thought to be a secreted surfactant (8¸ 33). Further studies are needed to determine if motility differs by using different mechanisms, if the capacity of the organism to sense environmental cues differs, or if other factors are in play.

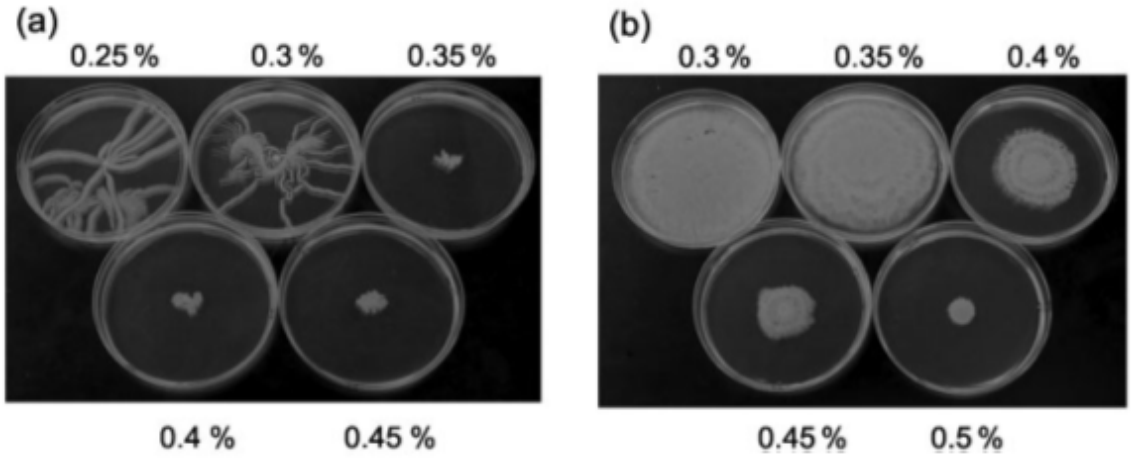

Figure 1: Motility on agar plates adapted from Source: Clemmer, K.M. R.A. Bonomo & P.N. Rather. 2011. Genetic analysis of surface motility in *Acinetobacter baumannii*. Microbiology 157: 2534–2544.

Motility on Difco Bacto agar (a) and Eiken agar (b) at the percentages indicated. Branching pattern of motility shown on (a) and concentric rings of motility on (b).

A. baumannii has also been studied to determine how well it can resist drying out or dessication. Desiccation has been studied because it has potential to be a major contributing factor to the persistence of *A. baumannii* in hospital environments. The survival times of *A. baumnnii* are significantly longer than other species within the *Acinetobacter* genus and are comparable to those of *Staphylococcus aureus* (2). A study by Jawad et al. found the mean survival time of 22 studied strains to be 27.29 days by looking at well-defined hospital-outbreak related strains and sporadic isolates from hospitalized patients in the same geographic area (16). Another study by Giannouli et al. looked at several different distinct genotypes of international clonal lineages I-III and found survival times ranged from 16 to 96 days (13). Strains belonging to genotypes ST1, ST78, and ST25, all part of international clonal lineage I, were found to have higher

survival times ranging from 75 to 89 days (13). A typical reference strain, ATCC 19606, has been shown to resist desiccation for less than 29 days in the Giannouli et al. (13) study and 6 days in the Jawad et al. (16). The differences in survival times can be attributed to different strategies used by the strains, differences in research protocol, unknown factors, or a combination of the above. Desiccation is another example of the lack of information known about *A. baumannii.* Further research is needed to better describe and determine virulence factors such as desiccation.

International clonal lineages have been studied to determine if differences exist in the virulence factors between the strains. Each international clonal lineage has a central, predominant genotype with a very few single locus variants (30). It has been found these international epidemic clonal lineages have selective advantage for causing disease over non-epidemic strains, although the reasons are still unknown (30). One particular study of international clonal lineages found regional differences in regards to aminoglycoside-resistance genes. Strains from the Czech Republic were determined to have a limited number of resistant genes and integron structures when compared to strains from other European countries (23). Differences in antibiotic usage and local availability of resistance genes, to acquire through lateral gene transfer, were considered factors for these differences (23). International clonal lineages I and II from the Czech Republic were found to share all resistance genes except one as well as integron regions (23). The lack of differences of aminoglycoside-resistance genes in the two clonal lineages examined indicate the possibility that strains occupying one

geographical region may share gene pools more readily through horizontal gene transfer than strains in differing geographical regions (23).

The ability for bacteria to acquire iron from their environment is essential to growth and inability to acquire iron in iron-poor environments leads to death (2). Hosts can defend themselves against potential bacterial infections by reducing the amount of free extracellular iron concentrations. One reason *Acinetobacter baumannii* has been able to infect numerous patients is due to the different strategies developed to collect iron from the environment. Several different mechanisms for iron-uptake have been observed and examined through studies of *A. baumannii* strains living in different environments. Direct contact between the bacteria and hemoglobin, such as in the gut of a host, allow the bacteria to aggregate and destroy the cells releasing the iron into the environment. This allows for quick uptake of the iron as exhibited in the SDF strain isolated from a human body louse (2, 28). Other systems involve the use of high affinity molecules released outside of the cells to collect the iron. *A. baumannii* strains AYE and ATCC 19606 use the acinetobactin sideophore to chelate iron by competing with host iron-binding proteins (28, 33). A genetic analysis of ATCC strain 17978 revealed two independent siderophore-mediated iron acquisition systems acquired through horizontal gene transfer and transposition (33).

Despite the official genus name of *Acinetobacter* being relatively new, the history of the genus dates back to organisms originally classified as belonging to other genera. A particular species of the genus, *A. baumannii,* has been known to cause human disease throughout the world. Despite some conflict regarding the extent of

clinical significance researchers agree more information regarding the species is needed as it is a valid clinical concern. One focus of research regarding this particular species is the examination of virulence factors potentially responsible for increases in human disease. Motility, hemolytic activity, desiccation, and ability to acquire iron from the environment are among the virulence factors studied. Thus far, there is conflicting research regarding the significance of each potential virulence factor and further research is needed to further describe each factor.

CHAPTER 2

INTRODUCTION

The 23 species found in the *Acinetobacter* genus include environmental organisms, all of which can be human pathogens although most are not associated with human disease. The most prevalent and worrisome clinical species worldwide is *Acinetobacter baumannii,* which has been identified as the source of multiple outbreaks and is emerging globally as a troublesome pathogen (14, 24). According to the CDC, approximately 80% of infections caused by the *Acinetobacter* genus are specific to the *baumannii* species (7). Strains of *A. baumannii* are isolated in up to 1% of nosocomial infections (28). *A. baumannii,* a gram-negative, glucose non-fermentative, non-motile, non-hemolytic, catalase-positive, oxidase-negative aerobic coccobacillus, is frequently found as an opportunistic pathogen in patients with mechanical ventilation, urinary or respiratory catheters (5, 12, 14, 16, 17, 24). Critically-ill patients and patients with openings in their skin and respiratory tract are those most often afflicted by *A. baumannii* (24).

The majority of infections are hospital-acquired or nosocomial infections. Researchers have seen a rise in infections in long-term care facilities, such as nursing homes, and in wounded military personnel (21). Infection with *A. baumannii* can occur through contact with contaminated hospital personnel or by exposure to contaminated hospital equipment (21, 29). Evidence has shown the bacterium can colonize implanted removable devices, such as catheters, arterial pressure monitoring devices, and

respiratory equipment (16, 21). Dry environmental objects, such as mattresses, pillows, and remotes, have also been implicated as a method of transmission (16).

While mortality rates are largely unknown, evidence supports prolonged hospital stays in ICUs leading to poorer outcomes in afflicted patients and increased attributable mortality (19, 21). Hospital-acquired pneumonia is the most commonly observed clinical presentation (14, 21). Bloodstream infections, urinary tract infections, hospital-acquired meningitis, wound infections, and bone infections are also clinical syndromes due to infections with *A. baumannii* (14, 16, 21). Intensive care units experience outbreaks which are difficult to control and quick to spread; the source of infections may be difficult to identify in these outbreaks (14).

In the past, *A. baumannii* was considered to be a pathogen of low virulence, meaning it was thought to have a lowered ability to infect and cause disease (14). In the past, most studies involving *A. baumannii* were based on describing the outbreaks, source of outbreaks, risk factors, and outcomes to help improve therapeutic treatment (21). Several epidemiological studies have shown the bacteria can survive in harsh hospital environments as well as cause disease outbreaks. Recognition that limited knowledge exists regarding the organism's pathogenicity and virulence factors have caused many researchers to question the status quo. Virulence factors have been recently identified for *A. baumannii,* although relatively few were identified (2, 24). Limited data has been collected concerning their function to date (2, 24). These factors include, but are not limited to, resistance to desiccation, the ability to evade the bactericidal activity of blood serum, resistance to iron starvation, biofilm formation, and

motility (2, 32). In addition to phenotypic virulence factors, genes which influence iron-uptake, biofilm formation, and quorum sensing have been found to differ between species in the *Acinetobacter* genus (14, 28).

Previously isolated strains of *Acinetobacter baumannii* have shown differences in their ability to lyse blood cells as well as differences in motility (2). Resistant (outbreak related) and antibiotic susceptible (non-outbreak related) isolates have been shown to exhibit limited hemolytic activity in liquid medium using defibrinated horse blood, but not using defibrinated sheep blood (2). Surface motility levels have been shown to differ between multi-drug resistant and antibiotic susceptible isolates (2, 21). Differences in motility have also been seen within clinical isolates as not all strains have displayed motility under laboratory conditions (21).

PURPOSE OF RESEARCH

The objective of this study was to identify the virulence factors present in clinical isolates of *Acinetobacter baumannii* collected from the University of Kentucky Hospital in Lexington, KY. The virulence factors to be assessed in this study included hemolytic activity (using two different methods) and surface motility. The ability of *A. baumannii* to persist in hospital environments, as well as to develop multi-drug resistance, increases the likelihood of the species becoming endemic in local hospitals. Identifying the characteristics of *A. baumannii* isolates will allow hospital staff to make informed decisions about patients who have risk factors for *A. baumannii* infection.

CHAPTER 3

MATERIALS AND METHODS

COLLECTION AND STORAGE OF ISOLATES

Isolate ATCC 19606 was ordered from Microbiologics to act as a control. The clinical isolates for this study were obtained from the University of Kentucky Medical Center. A total of 50 isolates were collected from the medical center and mailed overnight on TSA slants; only 48 isolates were recovered. Sixteen of the isolates obtained were previously determined to be multi-drug resistant. Nine isolates were determined to be a complex of *A. calcoaceticus-A. baumannii* . Four isolates were a complex of *A. calcoaceticus-A. baumannii* and multi-drug resistant. More information about the isolates can be found in Appendix A, Table 2: Characteristics of bacterial strains used in this study. On the day the isolates arrived, they were transferred from the TSA slants onto Luria-Broth (LB) plates and incubated for 24 hours at 35°C. All isolates were harvested and then stored in a -80°C freezer in one milliliter of 10% serum-sorbitol solution. Prior to each test, the isolates were cultured onto LB plates using isolation streaks and incubated for 24 hours at 35°C. Each procedure was performed three different times.

HEMOLYTIC ACTIVITY

Hemolytic activity of the isolates was determined using both an agar plate method and a liquid hemolytic assay. Activity for the plate assay was determined by incubating 5 µL of bacteria-containing saline, normalized to an $OD_{600} = 1.00 \pm 0.1$, on

Columbia agar with 5% defibrinated horse blood for 18 hours at 35°C. Zones of clearance were used to determine whether hemolytic activity was present or not. *Staphylococcus aureus* was used as a control to indicate a positive reading. Bouvet et al. determined *A. baumannii* ATCC 19606 to be non-hemolytic and thus this isolate was used a negative control (4).

The liquid hemolytic activity assay was performed by incubating one colony of the isolate, grown on a LB plate as described above, for 3 hours at 37°C with gentle agitation in Tryptic-Soy broth (TSB) with 1% defibrinated horse blood. Prior to the horse blood being added to the TSB the blood cells were washed with phosphate buffer solution (PBS) and centrifuged for 10 minutes at 1000 g and 4°C. Excess liquid was removed and discarded. The process of washing the horse blood was performed three separate times. After incubation, the TSB containing bacteria mixture was centrifuged for 20 minutes at 1000 g and 4°C. The supernatant was removed and the OD_{600} was determined. Prior to each OD_{600} reading, the spectrometer was set to zero using sterile distilled water. The percentage (P) of blood lysis was determined using the Antunes equation $P = (X-B)/(T-B)X100$ by subtracting the OD_{600} of sterile distilled water (B) from the value of the supernatant (X) and dividing by the difference of the OD_{600} of distilled water (B) and TSB with horse blood(T) (2).

SURFACE MOTILITY

Surface motility was determined by stab inoculating petri dishes containing surface motility agar with 1% TTC. Sterile saline containing bacteria was normalized to $OD_{600} = 1.00 \pm 0.1$ prior to stab inoculation. Plates were incubated at 35°C for 18 hours.

Motility was determined to be positive or negative based on the amount of red indication from the TTC.

CHAPTER 4

RESULTS

HEMOLYTIC PLATE ASSAY

There were no zones of clearance indicating hemolytic activity for any of the 48 isolates tested. This hemolytic test was also performed on a negative control (ATCC 19606) and positive control (*Staphylococcus aureus*). Figure 2 shows a representative sample indicating a lack of hemolytic activity and the positive control indicating the presence of hemolytic activity.

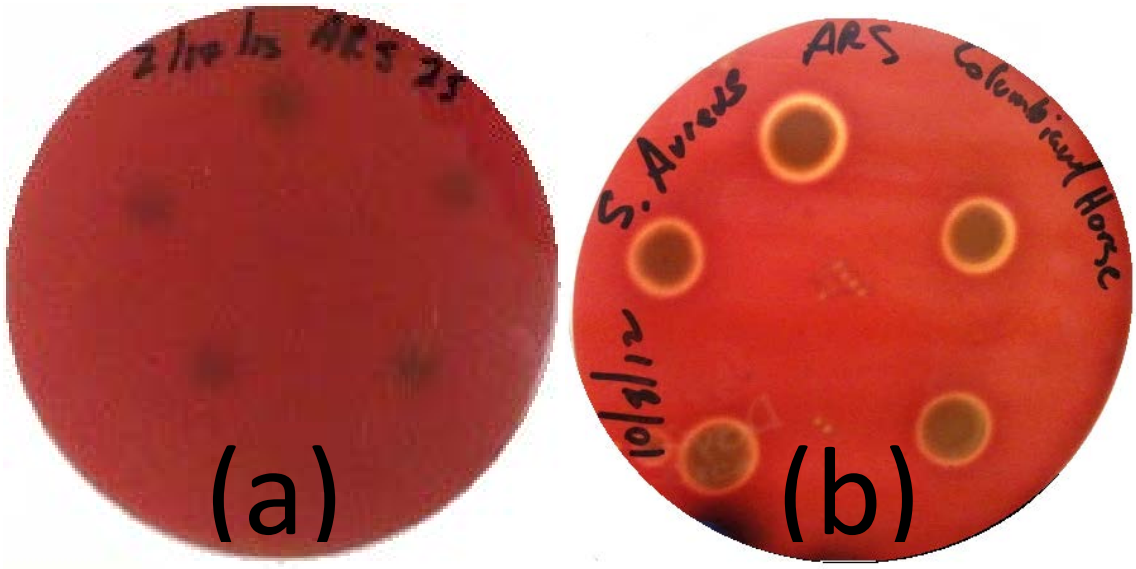

Figure 2: Hemolytic-negative and hemolytic-positive representative plates.

(a): A representiave sample of *Acinetobacter baumannii* including the negative control, ATCC 19606. This plate shows no zones of clearance indicating a lack of presence of hemolytic activity.

(b): *Staphylococcus aureus* was used as a positive control of hemolytic acitivy. Note the zones of clearance indicated by the lighter circles surrounding the darker circles.

HEMOLYTIC LIQUID ASSAY

The average OD_{600} of the isolates ranged between 0.018 and 0.136. Percentage of blood cells lysed differed from 2% to 12%. The negative control, ATCC 19606, had an average OD_{600} of 0.052 while the positive control, *S. aureus,* had an average OD_{600} of 0.606. The negative control had an average of 6.501% of blood cells lysed, while the negative control had an average of 75.320% of blood cells lysed. Differences in the average percentage of blood cells lysed by isolate are illustrated in Figure 3 and Figure 4. Differences in the average OD_{600} by isolate are illustrated in Figure 5 and Figure 6.

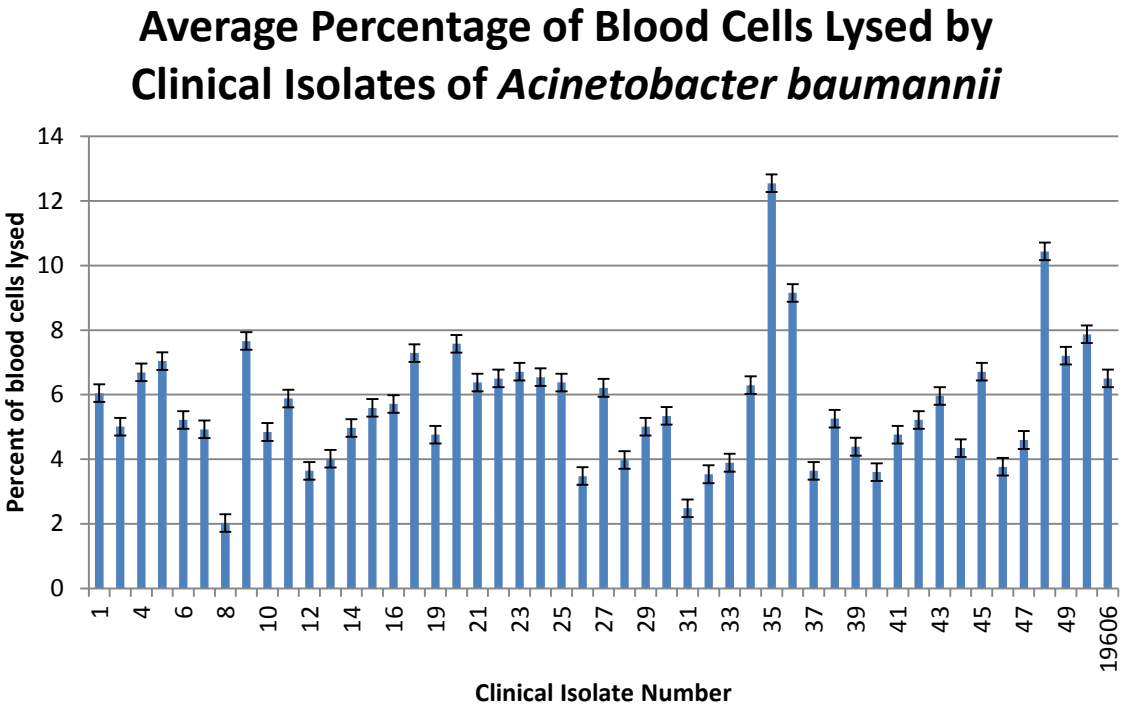

Figure 3: Average Percentage of Blood Cells Lysed in Supernatant without a Positive Control.

This graph shows the average percentage of three assays for each isolate of blood cells lysed. The Positive Control is not represented in this figure to show how the isolates compared to each other.

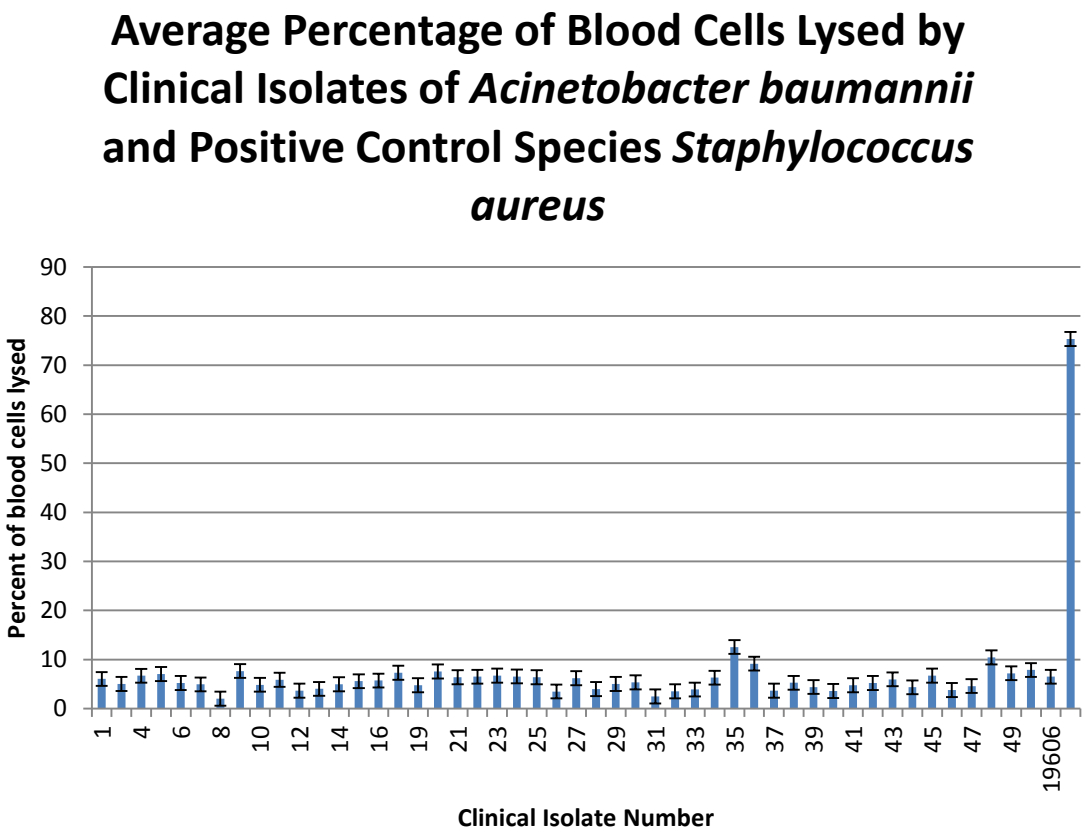

Figure 4: Average Percentage of Blood Cells Lysed in Supernatant with a Positive Control Species *Staphylococcus aureus.*

This graph shows the average percentage of three assays for each isolate of blood cells lysed including the Positive Control species *Staphylococcus aureus,* located on the far right of the graph. This graph is intended to show how the percentage of blood cells lysed by *Acinetobacter baumannii* compared to the Positive Control.

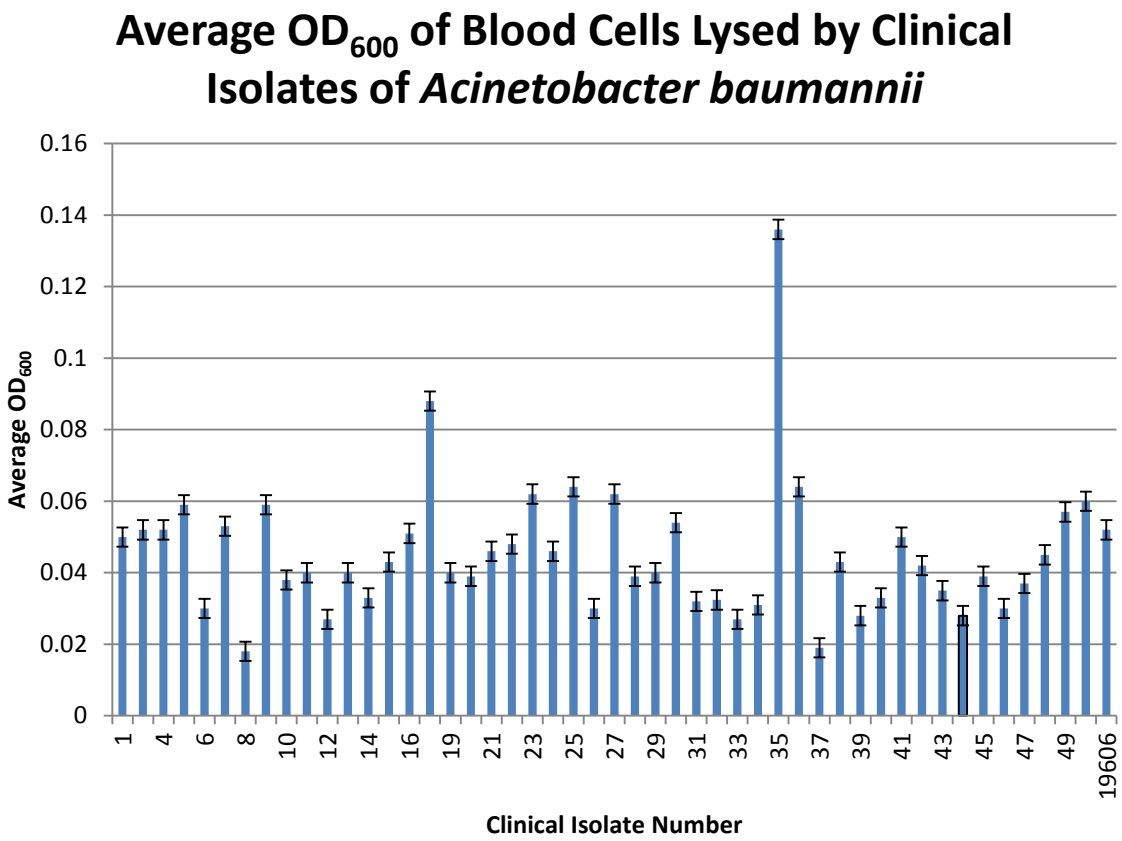

Figure 5: Average OD_{600} of Supernatant without a Positive Control.

This graph shows the average OD_{600} of three assays for each isolate without a Positive Control. This graph is intended to show how the average OD_{600} compared to the other isolates.

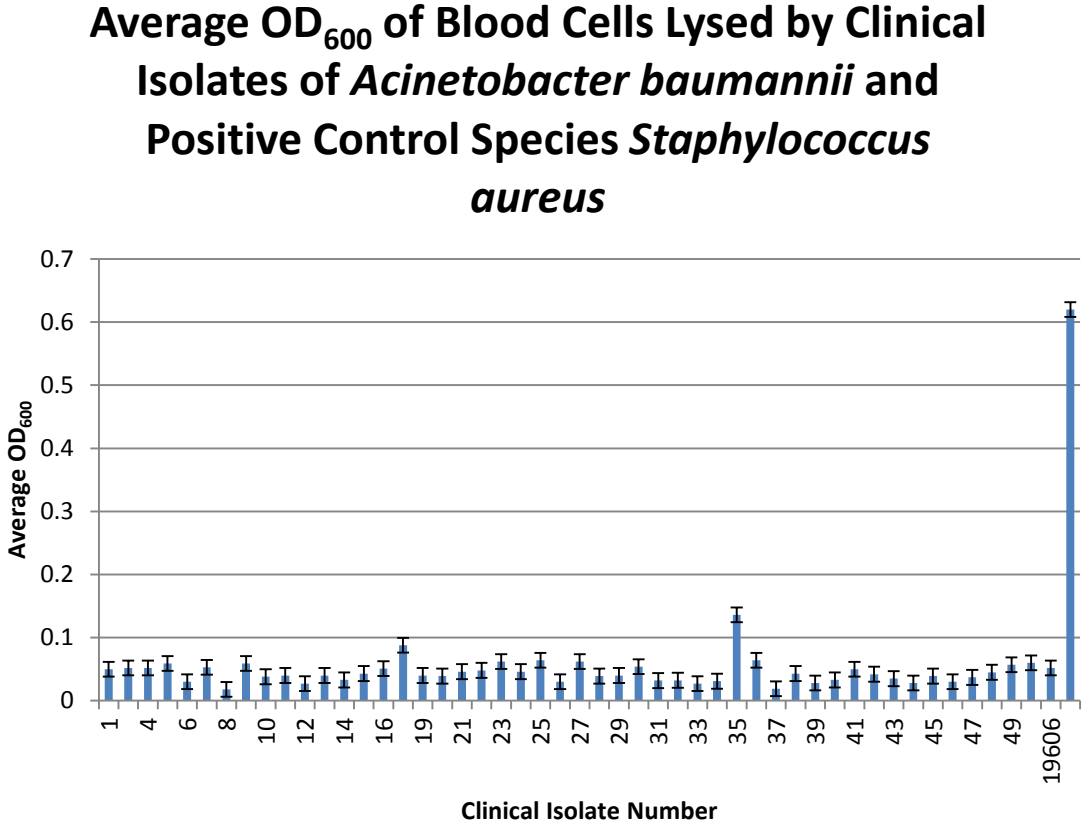

Figure 6: Average OD_{600} of Supernatant including a Positive Control Species *Staphylococcus aureus.*

This graph shows the average OD_{600} of three assays for each isolate, including a Positive Control, *Staphylococcus aureus*. This graph is intended to show how the average OD_{600} of each isolate compared to the Positive Control species *Staphylococcus aureus.*

SURFACE MOTILITY

Of the 48 clinical isolates and one reference strain (ATCC 19606) tested, only 13 clinical isolates had evidence of surface motility. 36 clinical isolates and reference strain ATCC 19606 did not show motility. Figure 7 shows a representative sample determined to be positive for surface motility and a representative sample determined to be negative for surface motility. Table 1 shows the motility results for all strains tested. Figure 8 and Figure 9 show the differences in count of isolates by motility and

multi-drug resistance and complex of *A. calcoaceticus-A. baumannii.* Figure 10 and Figure 11 are statistical results showing the relationship between motility and multi-drug resistance and complex of *A. calcoaceticus-A. baumannii.*

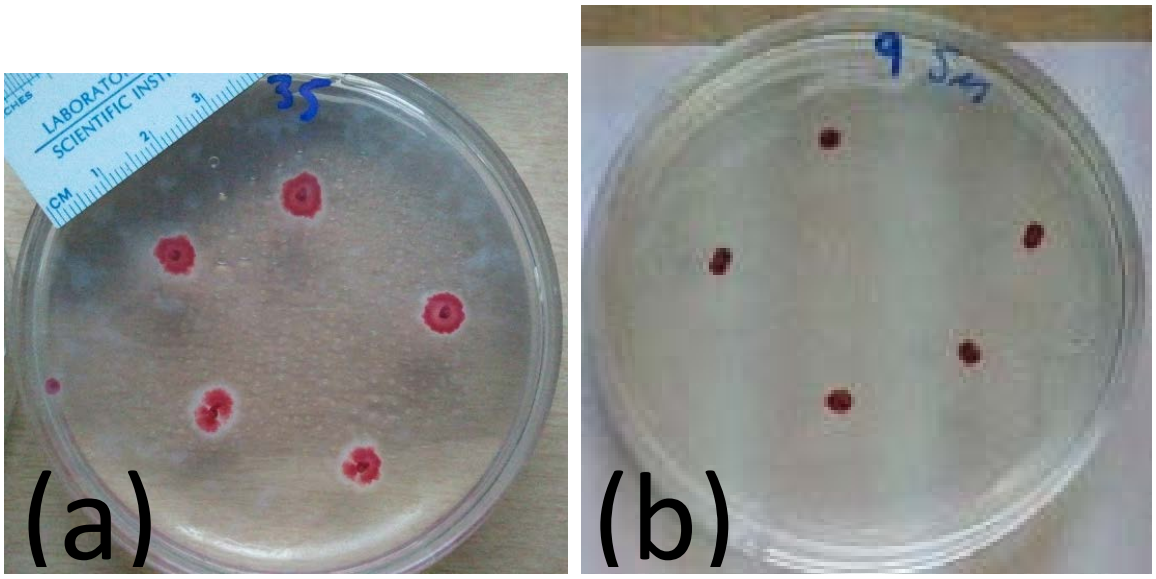

Figure 7: Surface motility-negative and surface motility-positive representative plates.

(a): This plate shows a representative sample, isolate 35, which was determined to have surface motility. The bright red spots are from an indicator, TTC, included in the preparation of the surface motility agar. The bright red spots indicate surface motility. Note the spots are larger than the plate on the right indicating no surface motility.

(b): This plate shows a representative sample, isolate 9, which was determined to have no surface motility. Note the lack of bright red spots and smaller diameter of the dull red spots when compared to the plate on the left.

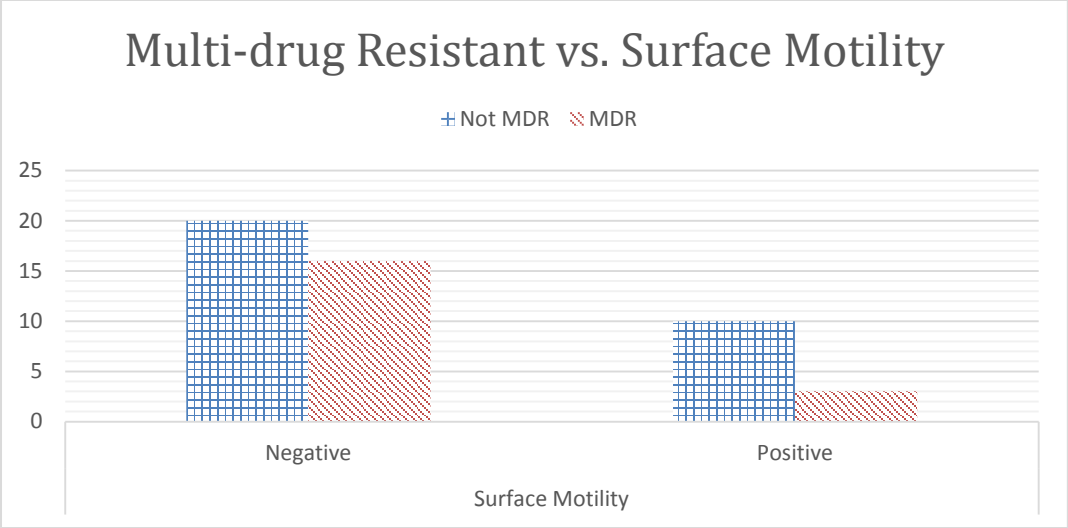

Figure 8: Graph of Multi-drug resistant vs Surface Motility.

This graph shows the count of isolates determined to be positive and negative for surface motility categorized by determination of multi-drug resistance. Note the lack of difference of count for each surface motility characterization within each MDR categorization.

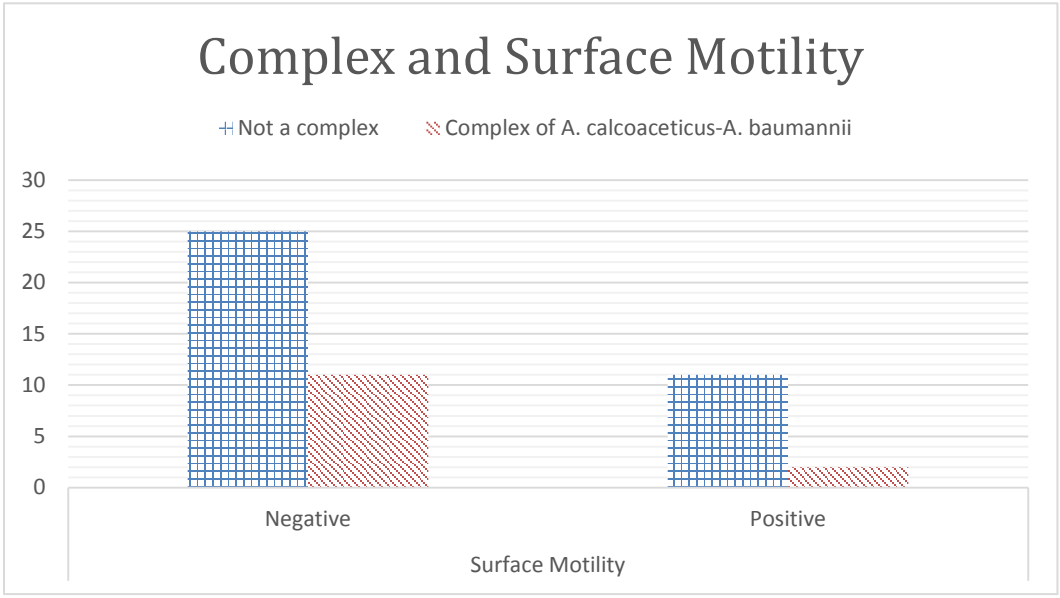

Figure 9: Graph of Complex vs. Surface Motility.

This graph shows the count of isolates determined to be positive and negative for surface motility categorized by determination of whether the isolate was a complex of *A. calcoaceticus-A. baumannii* or not. Note the lack of difference of count for each surface motility characterization within each complex categorization.

Statistical Tests for Multi-drug resistant vs. Surface Motility

	Test Value	p-value
Chi-Square	1.931[a]	0.381
Phi Coefficient	0.195	0.381

a. 2 cells (33.3%) have expected count less than 5. The minimum expect
count is .78.

Figure 10: Statistical Tests for Multi-drug resistant vs. Surface Motility.

This chart shows the statistical test values for the Chi-Square and Phi Coefficient tests performed to determine if a relationship exists for isolates classified as Multi-drug resistant and whether or not they have surface motility. Note the much higher test value when compared to the p-value for the Chi-Square test. Note the much lower test value when compared to the p-value for the Phi Coefficient test.

Statistical Tests for Isolates that are part of a complex vs. Surface Motility

	Test Value	p-value
Chi-Square	1.870[b]	0.393
Phi Coefficient	0.191	0.393

b. 3 cells (50.0%) have expected count less than 5. The minimum expected
count is .51.

Figure 11: Statistical Tests for Complex vs. Surface Motility.

This chart shows the statistical test values for the Chi-Square and Phi Coefficient tests performed to determine if a relationship exists for isolates classified as being part of an *A. calcoaceticus-A. baumannii* complex and whether or not they have surface motility. Note the much higher test value when compared to the p-value for the Chi-Square test. Note the much lower test value when compared to the p-value for the Phi Coefficient test.

Table 1: Surface Motility results for all *A. baumannii* isolates tested broken down by multi-drug resistance and if the isolate was determined to be *A. calcoaceticus-A. baumannii* complex

Strain	Part of a Complex?	Multi-drug Resistant	Motility
1	-	+	+
2	-	-	+
4	-	-	+
5	-	-	-
6	-	-	-
7	-	-	-
8	-	-	-
9	-	-	-
10	+	-	-
11	+	-	-
12	-	+	-
13	-	+	-
14	-	+	-
15	-	+	-
16	+	-	-
18	-	-	-
19	-	-	-
20	-	+	-
21	-	-	+
22	-	-	+
23	-	+	+
24	-	-	-
25	-	-	+
26	-	+	+

Table 1: (continued)

Strain	Part of a Complex?	Multi-drug Resistant	Motility
27	-	+	-
28	-	-	+
29	-	+	-
30	-	+	-
31	+	+	-
32	+	-	-
33	+	+	-
34	+	-	-
35	-	-	+
36	-	+	-
37	+	-	-
38	-	-	-
39	-	-	-
40	+	+	-
41	+	-	-
42	+	-	-
43	+	+	-
44	-	+	-
45	-	-	-
46	-	-	-
47	+	-	+
48	+	-	+
49	-	-	+
50	-	-	-
ATCC 19606	-	-	-

CHAPTER 5

DISCUSSION

Acinetobacter baumannii has emerged as a worldwide pathogen of concern due to the increasing number of outbreaks of disease associated with the bacteria and the arise of multi-drug resistant strains of the bacteria (2, 24). Many different studies attempting to identify, classify, and describe virulence factors have stemmed from this concern. In an attempt to further our understanding of the role of surface motility and hemolytic virulence factors, isolates were acquired for study from the University of Kentucky hospital in Lexington, KY. A total of 50 isolates were collected from different areas of the hospital over a period of 6 months. Only 48 of those isolates were recovered for testing. Reference strain ATCC 19606 was also purchased as a control organism.

In order to determine hemolytic activity two different assay methods were used; one was qualitative while the other was quantitative. Defibrinated horse blood was chosen for both assays as studies have shown hemolysis can be observed on horse blood agar, but not sheep blood agar. The qualitative method involved plating the bacteria onto a blood agar plate and observing the zone of clearance or lack thereof. The qualitative method involved growing the bacteria in a suspension of tryptic soy broth and using the optical density to determine the amount of blood cells lysed. No zones of clearance were observed for any of the clinical isolates for the qualitative method; see Figure 2 to view representative plates of positive and negative hemolytic

results. The lack of hemolytic activity on plates is consistent with the species historically being described as non-hemolytic (2). The quantitative liquid assay showed limited signs of hemolytic activity averaging approximately 6% of blood cells lysed. A strain of *Staphylococcus aureus* was used as a positive control and lysed approximately 75% of the blood cells.

Figure 3 shows the percentage of blood cells lysed by the clinical isolates and the reference strain ATCC 19606. In this figure it appears there is a significant difference in the percent of blood cells lysed; however Figure 4 shows the clinical strains as compared to the positive control. In this figure it is easy to see there is a significant difference between the percent of blood cells lysed by the *A. baumannii* isolates and the positive control, but not within the clinical isolates and reference strain. The significant difference in percentage of blood lysed compared to the positive control led to the conclusion that there was no hemolytic activity for the clinical isolates or the reference strain. Using a limited number of isolates (4), Antunes et al also observed limited hemolytic activity using a liquid assay with an average of approximately 12% of blood cells lysed for all clinical isolates (2). Compared to the Antunes study, this study tested more isolates, 48 isolates versus 4, accounting for the difference, 6% vs. 12%, of average blood cells lysed by all isolates.

Staphylococcus aureus is a gram-positive bacterium known to have hemolytic activity. *S. aureus* damages blood cells by releasing alpha-hemolysins which bind to the surface membrane of host blood cells causing the release of eicosanoids and cytokines and resulting in an inflammatory response (26). Leukocytes and platelets are the most

sensitive components of blood in humans to the alpha-hemolysins (26). Alpha-hemolysins are also responsible for osmotic phenomena, cell depolarization, and loss of ATP (26). Mutants of *S. aureus*, which produce lowered levels of alpha-hemolysins were created in the lab, have shown to have a lowered ability to cause infection in several different animal models (26). Purified versions of the alpha-toxin have been used to show an increased ability to cause infection in the same animal models (26). As a result of alpha-hemolysins, hemolytic activity is shown to be an important virulence factor for *S. aureus.*

A gram-negative bacterial species identified as lacking hemolytic activity is *Klebsiella pneumoniae.* Similar to *A. baumannii*, it is an opportunistic pathogen known to infect patients with an underlying disease. It is ranked second to *Escherichia coli* as a cause of nosocomial gram-negative bacteremia (26). A disadvantage to lacking the ability to lyse blood cells is that the bacteria must develop other methods to cause disease in a host. Capsular antigens, pili, serum resistance, lipopolysaccharide, and siderophores have been identified as virulence factors for *Klebsiella pneumoniae* (3). The capsular antigens create a thick surface surrounding the cell membrane, making phagocytosis by host cells extremely difficult (3). Pili, sometimes referred to as fimbriae, are used to attach the bacteria to the human mucosal surfaces making the first step of infection possible. Siderophores are iron chelators, which compete with host factors for iron, allowing more iron to be available for use by the bacterial cell.

Some of these virulence factors can be neutralized easier than others. Studies have shown cranberry juice is effective at preventing adhesion for enterobacteria in the

gastrointestinal tract (3, 26). The prevention of adhesion has been successful in preventing infection and in the eradication of existing infection in colonized patients. Adhesion is the first step of infection, thus when the bacterial cells cannot adhere to the human host cells then infection does not occur. It would be a greater advantage for bacteria, such as *Acinetobacter baumannii*, to have the ability to lyse blood cells than to have higher adhesive ability because the effects of hemolysis cannot be overcome as easy. As demonstrated by *Staphylococcus aureus,* hemolysis elicits an inflammatory reaction resulting in the death of the invading bacteria in a healthy human host. The inflammatory reaction can cause a cascade of effects that lasts after the bacteria is eradicated, resulting in more damage than is caused by adhesion.

Another potential virulence factor assessed was surface motility. Thirteen of 48 isolates assessed did show evidence of surface motility (Table 1). The concentric rings of motility observed in this study were similar to those observed on Eiken agar by Clemmer et al (see Figure 1 versus Figure 7). The other 35 strains and reference strain ATCC 19606 did not show evidence of surface motility. Signs of motility were unexpected because the name *Acinetobacter* derives from a greek word meaning non-motile and the species is typically described as being non-motile (8). However, despite the historical categorization of being non-motile, other researchers have shown it is very difficult to observe surface motility in laboratory conditions accounting for the signs of motility observed by this study. Surface motility has also been found to be highly variable between several environmental and clinical isolates of *A. baumannii* from different geographic regions (8). Clemmer et al. also found evidence that surface

motility for the M2 strain differs based on the brand and concentration of agar (8). Surface motility was best evidenced on Eiken agar with a concentration of 0.2-0.4% (8). Concentrations above 0.4% allowed for less motility observed (8).

The observations of surface motility in this study are either evidence of actual motility or false positives resulting from the methods used. Surface motility genes could have been acquired by the *A. baumannii* strains through lateral gene transfer from other bacteria found inside the hospital environment. Observation of motility in this study could be attributed to the low concentration (0.2%) of agar. The low concentration of agar and use of TTC indicator may have allowed for low levels of motility to be observed. These levels may not prove statistically significant when compared to bacterial species with known motility. Results were not collected to rule out this possibility. Genetic testing for motility genes would provide more evidence for either of these options, but were not included in the scope of this study.

The unexpected evidence of surface motility is most likely not linked to the complex of *A. calcoaceticus-A. baumannii* (this will be referred to as a complex from this point forward). Similar to *A. baumannii, Acinetobacter calcoaceticus* is not identified as being motile historically. Nine of the 48 recovered isolates for this study were determined to be part of a complex and only 2 of these isolates had evidence of surface motility. The low ratio of complex isolates with evidence of surface motility versus the complex isolates without evidence of surface motility does not indicate a relationship between the two variables. The surface motility observed in this study is possibly the result of acquisition of motility genes from foreign sources.

Specific genes could be identified through whole genome sequencing although this would be time consuming and costly. Whole genome sequencing would allow for the identification of all genes related to a specific virulence factor. This would be especially useful because a gene may be identified that researchers may not expect. It would be more cost effective to use primers to look for specific genes using PCR if there was evidence to support the presence of those genes. There are several different molecular fingerprinting methods that can be used to study the genome of *A. baumannii,* including f-AFLP (fluorescent amplified fragment length polymorphism), RAPD (random amplified polymorphic DNA), PFGE (pulsed-field gel electrophoresis), and REP-PCR (repetitive extragenic palindromic PCR). f-AFLPhas been identified as a way to determine the genetic relatedness of strains with high discriminatory power (10). This test can take 72 to 96 hours to perform. REP-PCR has been identified as a cost-effective efficient method to determine the genetic relatedness of specific strains of *A. baumannii,* requiring only 4 hours to perform (10). REP-PCR was also found to have high discriminatory power and to be a reliable and reproducible method of identifying the relationship of two particular isolates (10), REP-PCR fingerprint analysis can be combined with the use of specialized software, VIGI@ct DiversiLab, to help automate surveillance surveys to identify outbreaks quickly by comparing the fingerprints of multiple isolates at once (10). f-AFLP needs additional software before automated surveillance can occur (10).

Chi-square and phi coefficient statistical tests were performed to determine if a relationship exists between multi-drug resistant strains and surface motility as well as

strains consisting of a complex of *A. calcoaceticus-A. baumannii* and surface motility. Due to the lack of observed hemolysis, these statistical tests were not conducted for the hemolytic activity virulence assays. Similarly, there were no isolates where surface motility was observed that were both multi-drug resistant and were determined to be a complex. Therefore no statistical tests were performed to determine if there was a possible relationship between multi-drug resistant, complex organisms with surface motility. The chi-square test was used to determine if a relationship existed and the phi coefficient determined the strength of the relationship.

The chi-square test result was not statistically significant indicating that no relationship exists between surface motility and multi-drug resistance or being part of a complex of *A. calcoaceticus-A. baumannii* (see Figure 10 and Figure 11). Similarly, the phi coefficient test indicated an extremely poor strength of relationship. Figure 8 and Figure 9 show the lack of differences of count of isolates testing positive and negative for motility against multi-drug resistance and a complex. These tests should only be considered as preliminary results due to the low sample size resulting in no statistical power. In order for either test to have any statistical power there needs to be at least five results for each combination of factors (+surface motility +MDR, +surface motility –MDR, –surface motility +MDR, and –surface motility –MDR). There were only three isolates positive for surface motility and multi-drug resistance. Only two isolates were determined to be positive for surface motility and part of a complex. The probability of type-II error, failure to reject the null hypothesis of no relationship existing, is very high due to the low number of results. The lack of statistical significance does not mean no

relationship exists between these factors; it can only be assumed a potential relationship was not detected in this study. Despite the lack of significance for this study, the idea that a relationship exists should not be disregarded. Further studies should be performed to classify with greater statistical confidence if a relationship does or does not exist.

Highly virulent isolates may not have been detected because this study did not assay for all potential virulence factors. These additional factors have been shown to play a part in virulence for other bacterial species. Iron-uptake is an example of virulence factor that was not assessed. Given that iron is essential for bacterial growth iron acquisition genes, which allow the bacteria to acquire iron in low-iron conditions, would be a significant advantage. Another example would be desiccation. In hospital environments, the ability to survive for long period of times without drying out would allow bacteria on environmental objects to cause disease in more patients.

Another potential reason the results of this study did not find highly virulent isolates is possibly because the multi-factorial aspect of virulence was not taken into account. A multi-factorial approach to virulence means that several different factors all play a role in the pathogenicity. Surface motility, hemolytic activity, increased desiccation times, greater ability to obtain iron from the environment, increased adhesion ability, etc. are not individually responsible for the fact that a particular strain can cause disease more readily than another. The presence of several of these factors account for the overall virulence of an isolate. Specific virulence factor assays may not have statistically higher results when compared with other species known to possess the

virulence factor. The combination of all the slight differences would allow the bacteria to have more opportunities to cause disease. This study did not assess enough virulence factors to be able to determine if there was a multi-factorial component of virulence for these isolates. Future studies should consider the possibility that a combination of virulence factors have a positive and statistically significant relationship with the ability of the species to cause disease.

It is important to remember the small scope of this study means the results or lack of results cannot be applied worldwide. Genetic differences have been described between different *A. baumannii* strains from different epidemic lineages, such as international clonal lineages I-III. The lack of genetic testing in this study is a hindrance in relating the results with other studies due to the lack of information to classify the lineage. For example, isolate data from appendix A shows isolates 21 and 22 were both isolated from the axilla/groin area in the A07A area of the hospital. Genetic testing would have been able to determine if these isolates are in fact the same strain of bacteria or different. The lack of genetic testing also made it impossible to determine the international clonal lineage relationship. This lack of information should be considered when comparing these results to other studies with more thorough testing. These results should only be considered accurate within the hospital. Even within the hospital, these results should be interpreted with caution as the presence of virulent traits can change through time.

Despite the lack of results from this study, *Acinetobacter baumannii* should be considered as a legitimate threat to patient health in the University of Kentucky

hospital. Lateral gene transfer is one method by which this bacterium can become more harmful as time passes. The acquisition of multi-drug resistance is a prime example of how the bacteria can become more harmful. Any acquisition of virulent traits allows for a higher possibility of poorer patient outcomes from infection by this opportunistic pathogen. Hospital staff should be cognizant of the potential for harm and take additional precautions to lower the risk of infection. These precautions include proper sterilization of medical instruments such as catheters and disinfection of environmental objects including pillowcases and remotes. Standard EPA-approved disinfectant or detergent-disinfectant including the use of hypochlorite solutions should be used for disinfection procedures on environmental objects contaminated with *Acinetobacter baumannii* (10, 12, 15). When these precautions fail, microbiologists and epidemiological staff members can utilize the REP-PCR technique and the VIGI@ct DiversiLab software to determine the genetic relationships of isolates and therefore identify outbreak situations earlier (10). After outbreak situations are identified hospital staff can take appropriate measures, such as further disinfection, disposal of potentially contaminated reusable equipment, or even temporary ward closures, to limit the outbreak.

CONCLUSION

Overall, the results of this study and literature review show a need for further research to be conducted on the virulence factors of *Acinetobacter baumanni*. There are conflicting results regarding different factors and whether or not they play a part in the overall virulence of the organism. For example, studies have shown surface motility

to be highly variable. No definite link has been determined as the cause for the variability. Studies should be designed to look at one specific virulence factor using variable laboratory conditions to determine the role of that specific factor. For example, to study surface motility of a particular strain, different types and concentrations of media could be used with the exact same protocol. This will also help determine under what laboratory conditions it is easiest to observe the motility and create a baseline for comparing results. Similar studies could be designed for each virulence factor. The potential for a multi-factorial component to virulence should also be examined thoroughly. One virulence factor alone may not be the determining factor in whether or not a particular strain is virulent. Increased pathogenic potential could be linked to the presence of multiple virulence factors.

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APPENDIX A:

Characteristics of bacterial strains used in this study

Table 2: Characteristics of bacterial strains used in this study

Isolate	MDR or Complex?	Source/Wound Type	Hospital Location
1	MDR	Decubitis Ulcer	3N
2	None	Axilla/groin	A07
3*	MDR	Axilla/groin	1MED
4	None	Axilla/groin	A07J
5	None	Axilla/groin	2MED
6	MDR	Axilla/groin	2MED
7	None	Axilla/groin	Unknown
8	None	Axilla/groin	Unknown
9	None	Axilla/groin	Unknown
10	*A. calcoaceticus-A. baumannii* complex	Axilla/groin	Unknown
11	*A. calcoaceticus-A. baumannii* complex	Chest Tissue	7W
12	MDR	Axilla/groin	2MED
13	MDR	Unknown	Unknown
14	MDR	Unknown	Unknown
15	MDR	Unknown	Unknown
16	*A. calcoaceticus-A. baumannii* complex	Unknown	Unknown
17*	None	Wound	Unknown
18	None	Unknown	Unknown
19	None	Unknown	Unknown
20	MDR	Blood	7E
21	None	Axilla/groin	A07A
22	None	Axilla/groin	A07A
23	MDR	Ischeal Wound	Unknown
24	None	Nares	SSE
25	None	Nares	SSE
26	MDR	Decubitis Ulcer	S4 (ICU)
27	MDR	Decubitis Ulcer	1MED
28	None	Neck Abscess	ER
29	MDR	Rectal	SS3E
30	MDR	Butt	Unknown
31	MDR *A. calcoaceticus-A. baumannii* complex	Catheter tip	Unknown
32	*A. calcoaceticus-A. baumannii* complex	Axilla/groin	Unknown
33	MDR *A. calcoaceticus-A. baumannii* complex	Trach	Unknown
34	*A. calcoaceticus-A. baumannii* complex	Alveolar lauage	Unknown
35	None	Sputum	Unknown
36	MDR	Trach aspirate	Unknown
37	*A. calcoaceticus-A. baumannii* complex	Axilla/groin	Unknown

Table 2: (continued)

Isolate	MDR or Complex?	Source/Wound Type	Hospital Location
38	None	Axilla/groin	Unknown
39	None	Nares	Unknown
40	MDR *A. calcoaceticus-A. baumannii* complex	Bronch wash	Unknown
41	*A. calcoaceticus-A. baumannii* complex	Axilla/groin	Unknown
42	MDR	Coccyx wound	Unknown
43	MDR *A. calcoaceticus-A. baumannii* complex	Axilla/groin	Unknown
44	MDR	Bone	Unknown
45	None	Wound	Unknown
46	None	Wound	Unknown
47	*A. calcoaceticus-A. baumannii* complex	Right leg	Unknown
48	*A. calcoaceticus-A. baumannii* complex	Axilla/groin	Unknown
49	None	Axilla/groin	Unknown
50	None	Axilla/groin	Unknown
***indicates the strain was unable to be recovered**			

APPENDIX B:

Materials

Hema Resource and Supply, Inc. Defibrinated Horse Blood ordered from Hardy Diagnostics

Microbiologics *Acinteobacter baumannii* ATCC 19606

Difco Motility Test Medium