DETERMINING THE INTERACTIONS BETWEEN SERUM PROTEINS OF THE COMPLEMENT SYSTEM AND OUTER MEMBRANE PROTEINS IN AVIAN PATHOGENIC *ESCHERICHIA COLI*

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by

Cindy Botero

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ABSTRACT

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Eschericia coli is a well-known member of the intestinal flora of mammals and birds. However, there exist pathogenic strains capable of causing disease. One strain comes from the O-serogroup of *E. coli,* APEC O2 (+/+), and causes millions of dollars of global losses annually. The elucidation of the mechanisms of complement avoidance in pathogenic strains could potentially provide vital information to understanding bacterial pathogenicity and assist in the future development of a vaccine. The bacterial strains used were APEC O2, an *iss+/bor+* strain, and DH5α, a *iss-/bor+* strain. Mutant strains were created from a knockout of *iss* in APEC O2 (+/+) to create APEC O2Δ*iss* (-/+), and a knockout of the gene *bor* to create DH5αΔ*bor* (-/-). In order to determine how Iss and Bor assist each other in surface exclusion tactics and in serum resistance, each strain was subjected to a complement consumption assay, which measured the amount of complement consumed by each strain, the bactericidal assay, which determined the amount of cell death in response to complement, ELISA, which measured the amount of terminal complement complex remaining after exposure of bacteria to serum, and immunofluorescent microscope visualization, which visually showed the bound C5b-9 terminal complement complex to the outer membrane of the bacterial strains. The results of this study determined that the role the protein Bor has on assisting Iss with serum resistance may not be as significant as previously thought. There may also be something other than the gene *bor* assisting *iss* in the prevention of cell death due to the attachment of membrane attack complex (MAC) on the cell wall. This study expands on our previous

understanding of how proteins of the outer bacterial cell membrane cooperate in order to provide resistance to complement proteins in the immune system.

KEY WORDS: Complement system, Avian pathogenic *Escherichia coli*, Commensal, Pathogenic, C5b-9, Terminal complex, MAC, Membrane attack complex, Immune system, ELISA, Complement consumption assay, *iss, bor, traT,* Bactericidal assay, Immunofluorescence.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

Background

An agent of disease. *Escherichia coli* is a known member of the regular intestinal flora of mammals and birds; however, some strains of *E. coli* are characterized by virulence factors that cause them to become pathogenic, and can contribute to diarrhea and generalized infection in man and in livestock (Vidotto, 1990; Binns, 1979). Avian pathogenic *Escherichia coli* (APEC) is the etiologic agent of colibacillosis in birds, and is responsible for at least five percent of the mortality rate found in the poultry industry, leading to multimillion dollar global losses annually (Fantinatti, 1994; Tyler, 2008). Rodrigues-Siek (2005) hypothesized that some of the virulence factors associated with APEC strains contribute to the survivability of colibacillosis in their natural extraintestinal environments. Colibacillosis caused by APEC begins as a respiratory infection caused by a virus, followed by the opportunistic colonization of the respiratory system by APEC, which then manifests itself in the form of organ lesions, including air sacculitis, cellulitis, pericarditis, and septicemia, all of which can result in increased mortality (Fantinatti, 1994). Johnson *et al.* (2006) showed that there is a link between the APEC strains found in contaminated poultry, extraintestinal pathogenic *E. coli* (ExPEC) causing neonatal meningitis, and uropathogenic *E. coli* (UPEC) causing urinary tract infections (UTIs) in humans (Lynne, 2012); therefore, the understanding of the mechanisms of complement resistance employed by APEC could have an additional potential beneficial impact on human health.

The pathogen: *Eschericia coli.* The most frequently isolated strains of pathogenic *E. coli* found in birds diagnosed with colibacillosis seem to be mainly restricted to a few

O-serogroups. The most common *E. coli* strains in avian species are O1, O2, and O78 (Ike, 1992). The Enteropathogenic strains that cause disease are usually confined along the alimentary tract and contain conjugative plasmids that specify for enterotoxins which enhance their pathogenicity (Binns, 1979). The ColV plasmid ranges in size from 80 to 180 kb, and codes for various functions, including production of the colicin V protein, serum resistance genes, and aerobactin-mediated iron uptake (Johnson, 2002; Ike, 1992). Most of these *E. coli* strains produce colicin V, an antibacterial protein produced by the ColV plasmid (Binns, 1979). Binns *et al.* (1979) demonstrated that there is a correlation between increased pathogenicity and increased serum resistance conferred by the ColV plasmid, which was later confirmed by Wooley *et al.* (1993). Although the ColV plasmid may be responsible for virulence in the *E. coli* strains that contain it, the colicin V protein may not necessarily cause its virulence, but instead virulence is caused by other genes that are present on the ColV plasmid (Binns, 1979). The presence of the ColV plasmid is not the only factor that is required to convert an otherwise benign strain of *E. coli* into an invasive strain, and the actual significance of colicins as a contributing virulence factor is unknown (Binns, 1979).

The complement system. Gram-negative bacteria, such as *E. coli*, are killed by the complement proteins found in serum through either the classical or alternative pathways (Taylor, 1983). The classical pathway begins by the recognition of bacterial surface antigens by certain antibody classes. The predominant natural antibody for enterobacterial species are contained in the IgM antibodies. These antibodies seem to be specific for the O side-chain moiety of the lipopolysaccharide (Taylor, 1983). IgM is capable of attaching to the C1q component of the C1 complex to initiate the complement

cascade. Any prokaryotes with a lipid bilayer membrane exposed to the external environment are potentially susceptible to complement killing. therefore, it is likely that serum resistance is due to the presence of structures at or near the bacterial surface that are capable of interfering with the formation, attachment, or subsequent activity of the membrane attack complex (MAC) (Taylor, 1983). Complement proteins are an incredibly important part of the host innate immune system against invasive bacterial species, which makes the elucidation of the mechanisms employed by bacteria to resist serum vital to the understanding of this area of bacterial pathogenicity (Binns, 1982).

Complement avoidance. A trait that has been found commonly in many APEC isolates is their ability to avoid host defenses. The complement system is one such defense of the host; therefore, a study of the complement-resistance mechanisms that operate in avian *E. coli* may prove beneficial for the identification of a novel virulence trait (Nolan, 2003). The ability of *E. coli* to resist the effects of the host complement system has been shown to contribute to the level of virulence in the bacteria (Wooley, 1993). Nolan (2003) suggested this ability was a distinguishing characteristic useful in differentiating virulent from avirulent isolates, and may play a significant role in the development of colibacillosis in poultry

There are several factors that have been attributed to increased survivability of these virulent *E. coli* strains against the detrimental effects of the complement system in serum, including several outer membrane proteins, O and K-1 capsular antigen, and the presence of a smooth lipopolysaccharide (LPS) layer (Binns, 1982; Wooley, 1993). The bacteria resist complement in serum in one of two ways. The first, is to employ certain proteins that are able to prevent the MAC complex from forming on the organism. An

example of this method is the modulation of hydrophilic structures, which influences the ability of complement to interact with membranes, such as N-acetyl-neuraminic acid (NANA) containing structures, located on the outer surface of bacteria. The removal of these structures results in an increase in the susceptibility of erythrocytes to complement (Taylor, 1983). K-antigens are another exopolysaccharide that possess agglutination-inhibiting activities. These polysaccharides may be impeding the antibody binding, and the resulting attachment of complement to the bacterial surface (Taylor, 1983).

Bacteria may also resist complement by allowing the MAC complex to form on the bacterial surface, but it is somehow rendered impotent. Joiner *et al.* (1982) measured C3 proteins deposited on the surface of pathogenic smooth and commensal rough strains of *S. minnesota*. Twice as many labeled radio C3 proteins were found on the smooth bacteria when compared to the rough bacteria. There was also a depletion in the amount of C5 and C7 in the smooth bacteria, as well as the complete inactivation of C9 after five minutes, when compared to the rough bacteria, which still contained these proteins (Joiner, 1982). This suggested that the MAC complex was being more efficiently and stably deposited on the bacterial surface of the highly susceptible rough bacterial strains. C3 proteins are therefore attaching to the completely resistant strains, and the MAC complex is forming, but it is either failing to insert itself into the bacterial outer membrane, or the inserted complex does not cause any damage to the vital outer or inner membrane structures (Joiner, 1982).

The genes involved. Two genes thought to confer complement resistance include the *traT* gene located on the R6-5 plasmid, and the *iss* gene located on the ColV, I-K94 plasmid (Binns, 1982). The increased serum survival gene, *iss,* is one of the more

common characteristics among APEC strains, and was first described by Binns and colleagues (1979) for its role in providing serum resistance to the ColV plasmid in human isolates. Due to its commonality, the use of *iss*-centric strategies may prove useful in the search of a control of avian colibacillosis (Lynne, 2007). The *iss* gene is able to increase the virulence of *E. coli* strains 100- fold in day old chicks and increases complement resistance 20-fold, suggesting that *iss* containing ColV plasmids may be critically important to pathogenesis in APEC strains (Johnson, 2002). The protein product of the *iss* gene, Iss, has a signal sequence that is characteristic of outer membrane proteins, and occurs much more frequently in isolates associated with colibacillosis than it does in the fecal *E. coli* of healthy birds (Johnson, 2002; Lynne, 2007). The TraT protein, coded by the *traT* gene, is located on the outer membrane, and is thought to help prevent cell-protein interactions through surface exclusion (Taylor, 1983). Iss may work in a similar way to TraT, evidenced by the similar levels of resistance observed between these two proteins, which suggests a common mechanism (Binns, 1982). Binns *et al. (*1982) demonstrated that *traT* and *iss* gene copies that were cloned into the plasmid vector pBR322 confer similar resistance against complement. These two proteins may be acting on the MAC complex in one of two ways: the assembled terminal complex may be unable to cause membrane damage, or the terminal complex is unable to bind to the bacterial membrane at all (Binns, 1982). However, the bacterial consumption of the complement proteins C6, C7, C8, and C9 was the same in the presence and absence of these resistance genes. It is therefore unlikely that they block formation of MAC, but instead block the action of MAC (Binns, 1982). This effect provides evidence that *iss* and *traT* operate at the same place in the complement system by blocking the actions of the

complement system without actually blocking the formation of the terminal complex. Although *traT* is thought to contribute to complement resistance, its contribution to virulence is controversial. Only 2.6% of embryo lethality was explained by the presence or absence of the *traT* gene, and it is found to have a fairly equal distribution among virulent and avirulent strains of avian *E. coli,* demonstrating that *traT* does not significantly contribute to virulence (Wooley, 1993).

One mechanism of resistance to the MAC complex could be due to a failure of amphiphilic MAC complexes to integrate into the hydrophobic domains on the bacterial envelope. This integration of the MAC complex could be intimately related to the degree of fluidity of the membrane. Factors that reduce the fluidity of the outer or cytoplasmic membranes could determine serum resistance (Taylor, 1982). Because the Iss protein is a surface exposed protein on the outer membrane of the bacterial cell (Lynne, 2006), it could potentially be altering the physical properties of the bacterial outer membrane in a way that is preventing the formation of functional terminal complexes.

The *iss* gene is believed to be derived from the lambda gene *bor*, due to a 90% homology in the amino acids of the proteins for which they code (Johnson, 2002; Lynne, 2007). The protein Bor, coded by the gene *bor,* is a lipoprotein of the cell envelope of lambda lysogens of *E. coli,* and is reported to confer serum resistance to the lysogens (Nolan, 2003). Unlike Iss, Bor occurs widely in commensal and pathogenic strains of *E. coli*. Since Iss has a 90% homology with the protein Bor, another outer membrane protein, they could be working in conjunction to increase serum resistance. Strains that had a *bor* deletion also demonstrated a lower resistance to serum (Lynne, 2007). These results demonstrated that both *bor* and *iss* have a role in serum resistance, although *iss*

has a significantly higher role than does *bor*.

CHAPTER II

Introduction

Study aims. Colibacillosis causes millions of dollars in loss to the poultry industry every year. Currently, antimicrobial agents are used to control this disease; however, use of these agents is under significant scrutiny by public health organizations due to fear of resistance by microorganisms. The elucidation of the mechanisms employed by proteins involved in serum resistance could potentially provide vital information to understanding bacterial pathogenicity and assist in the future development of a vaccine that targets these areas of the proteins without affecting commensal strains. Furthermore, due to the link between APEC strains, extraintestinal pathogenic *E. coli* (ExPEC) causing neonatal meningitis, and uropathogenic *E. coli* (UPEC), there is an additional potential beneficial impact on human health.

In this research, the strains that contained either the Iss or Bor proteins, both, or neither were examined for their ability to provide the highest level of serum resistance by assessing how they assist each other in surface exclusion tactics. Strains that contain Bor but not Iss were expected to employ some serum resistance tactics, but not at a significant level. Strains that contain neither Iss nor Bor were expected to be completely susceptible to the effects of serum, and be fully lysed by the MAC terminal complex. A strain that was *iss+/bor-* was not required because by comparing the *iss-/bor+* strains that are either pathogenic mutant or commensal the effects of *iss* can be seen in this manner. Future research can be done to measure how membrane fluidity is impacted by complement proteins in pathogenic and commensal strains of *E. coli* in avian species.

The tests performed were the complement consumption assay, which measures

the amount of complement consumed by each strain, the bactericidal assay, which determines the amount of cell death in response to complement, ELISA, which measures the amount of terminal complement complex remaining after exposure of bacteria to serum, and immunofluorescent microscope visualization, which visually shows the bound C5b-9 terminal complement complex to the outer membrane of the bacterial strains.

Hypotheses. The goal of the proposed research was to determine the amount of complement being consumed and the rate the bacteria are being killed between the four strains, APEC O2 (+/+), APEC O2Δ*iss* (-/+), DH5α (-/+), and DH5αΔ*bor* (-/-), to determine the effectiveness of MAC on bacterial death. This was done by measuring the amount of MAC bound to each bacterial strain, and of unbound MAC for each strain. The hypotheses of this research are:

- Hypothesis 1

  H_A: Iss works in conjunction with Bor in order to provide an increased level of serum resistance than when Iss or Bor are present alone.

  H_0: Iss and Bor do not work together.

- Hypothesis 2

  H_A: Iss and Bor aid the bacteria in serum resistance by employing surface exclusion tactics so the MAC complex is unable to properly bind to the bacterial surface.

H_0: Iss and Bor work by some other mechanism other than surface exclusion.

CHAPTER III

Materials and Methods

A. Determine the amount of complement being consumed and the rate the bacteria are being killed between the four strains, APEC O2 (+/+), APEC O2Δ*iss* (-/+), DH5α (-/+), and DH5αΔ*bor* (-/-), to determine the effectiveness of MAC on bacterial death.

Complement consumption assay. The bacterial strains used were APEC O2, an *iss* +, *bor* + strain, and DH5α, an *iss* -, *bor* + strain. Both these strains were used in the study done by Lynne *et al.* (2007) to examine the effects of the genes *iss* and *bor* on serum resistance in both virulent and non-virulent strains. By using the commensal DH5α (-/+) strain, the chances of the serum being impacted by unknown virulent genes is reduced, and the effects of complement on *iss* and *bor* are more clearly determined because APEC O2Δ*iss* (-/+) and DH5α (-/+) are both *iss* – and *bor* +, making the effects on these two strains comparable. Mutant strains were used from a knock out of the *iss* gene in APEC O2 (+/+) to create APEC O2Δ*iss* (-/+), and a knockout of the *bor* gene from DH5α (-/+) to create DH5αΔ*bor* (-/-). A complement consumption assay was used to measure the amount of complement consumed by each strain. The strains were incubated at 42ºC in 25 ml of LB broth overnight, pelleted at 8,000xg for 10 minutes, and reconstituted in 3.0 ml of PBS. They were diluted to 10^{1}, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions. Chicken serum was added at a 3:1 ratio of bacteria to serum for a total volume of 1 ml, and incubated at 42ºC for 60 minutes to allow for complement consumption. A volume of 0.4 ml of washed sheep erythrocytes that had been sensitized with rabbit anti-erythrocyte immunoglobulin was added, and incubated for 10 minutes at 42ºC for the rest

of the complement to react with the erythrocytes. Any complement that did not bind to the bacteria would bind to the sheep RBCs and lyse them. The reaction was stopped with 1 ml of cold PBS, and centrifuged at 1500xg for five minutes to pellet out the cells and read the hemoglobin in the supernatant. The concentration of released hemoglobin resulting from complement-mediated lysis of erythrocytes was read at 546nm in the spectrophotometer, as described by Jensen *et al.* (1993).

Bactericidal assay. A bactericidal assay determined the amount of cell death in response to complement by measuring viable cell counts. Bacteria were grown for eight hours at 42ºC in 500 ml of LB broth. Either chicken serum or heat inactivated chicken serum was then added at a 1:3 dilution to bacteria. The inactive serum incubation served as the control, and serum was inactivated by heating to 56ºC in a hot water bath for one hour to inactivate the proteins. The mixtures were incubated at 42ºC with shaking, and growth was measured using a spectrophotometer at 30 minutes, one hour, two hour, 12 hour, 24 hour, and 48 hour intervals set to wavelength 750 nm. Serially diluted aliquots of the bacteria and active or heat inactivated serum were grown on nutrient agar plates at 10^{-5}, 10^{-6}, and 10^{-7} dilutions from the bacteria incubated at t = 0, t = 1 hour, t = 2 hour, t = 12 hour, and t = 24 hour, and the colonies were counted. The extent of bacterial killing was measured as the CFU/ml of bacteria with heat inactivated serum minus the CFU/ml of bacteria with unheated serum.

B. Measure the amount of MAC bound to each bacterial strain, and of unbound MAC for each strain.

Enzyme linked immunosorbent assay. In order to measure the amount of the terminal complement complex proteins that were activated initially after incubation with

chicken serum by the bacteria, and after 60 minuters, an indirect ELISA was performed. If less C5b-9 terminal complex was activated initially, then MAC was forming and properly inserting into the membrane. If more was initially activated and more was activated after 60 minutes, then this suggests the MAC was being used up due to surface exclusion tactics. The C5b-9 terminal protein complex protein was tested using the murine monoclonal antibody to Human C5b-9 as the capture antibody. The analysis was carried out in a transparent 96 well polyvinyl chloride (PVC) micro titer plate. Each strain was grown in 25 ml of LB broth overnight at 42˚C with shaking, then aliquoted to 1.0 ml and centrifuged at 1500 x g for 10 minutes to pellet. The pellet was then reconstituted in 1.0 ml of PBS. Chicken serum mixed with human serum at a 1:1 ratio served as the antigen solution. The serum was activated by adding it to each of the bacterial strains at a 1:1 ratio, and then the mixture was incubated at 42ºC for 15 minutes and 60 minutes in a total volume of 1.0 ml. The bacterial/serum solution was then diluted to a 1:3 dilution in PBS. One hundred microliters of each dilution was added to each well, and incubated for 60 minutes at room temperature to allow complete binding. The contents of the plate were then disposed of, and each well was saturated with 1% BSA blocking buffer, and incubated overnight at 4ºC. The plate was then washed three times in PBS, and the primary antibody was added. The primary antibody was murine monoclonal antibody to human C5b-9, diluted to 1:500 in 1% BSA. The ELISA was then incubated at room temperature for 60 minutes so the primary antibody could capture the sample proteins. The plate was washed two times in PBST, and the secondary detection antibody was added, and incubated at room temperature for 60 minutes so the detection antibody could bind to the sample proteins captured by the capture antibody. The

secondary antibody was the goat anti-mouse IgG AP conjugate, diluted to 1:10,000 in PBS. The plate was then washed two times in PBST, and the AP enzyme substrate was added at 100µl to each well. The plate was then incubated at room temperature for 120 minutes. Once the lowest diluted well had changed color, 50µl of stop buffer (2M NaOH) solution was added to each well, and the plate was read on an ELISA plate reader at 405 nm.

Fluorescent microscopy visualization. Each of the strains were visualized by immunofluorescent microscopy using the Nikon Fluorescent Microscope Eclipse E400 to show the amount of bound complement C5b-9 terminal complex to the outer membrane of each of the *iss*$^{+/-}$*/bor*$^{+/-}$ strains. Bacterial cultures were grown overnight in LB broth at 42ºC, pelleted, and reconstituted in 1.0 ml PBS. One hundred microliters of each strain was incubated with 25 µl decomplemented chicken serum, 25µl human serum, and 50µl PBS for 60 minutes at 42 ºC. Chicken serum was decomplemented by incubating in a 56ºC hot water bath for 60 minutes. The human serum provided the C5b-9 source. The control was 200µl of each bacterial strain incubated without serum for 60 minutes at 42ºC. One hundred microliters of the cells were then incubated in 100µl of the primary antibody, diluted 1:100 monoclonal mouse C5b-9 antibody IgG (aE11) in PBS, and incubated for 60 minutes at room temperature. The cells were then pelleted at 1500 x g for 10 minutes, and the supernatant was discarded. The cells were next reconstituted in 100µl goat anti-mouse IgG conjugated with FITC that had been diluted to 1:100 in PBS and incubated for 60 minutes at room temperature in order to see the C5b-9 proteins. One hundred microliters of the cells incubated with serum and 100µl of the cells incubated without serum were incubated with 10-N-nonyl-acridine orange (NAO) that had been

diluted to 1:100 in 70% ethanol, in order to visualize the cardiolipin containing domains on the outer membrane of the *E. coli* strains, and incubated for 60 minutes at room temperature. One hundred microliters of cells were then immobilized using Lugol's iodine, placed on a slide, and mounted on the microscope for observation, using the green light filter AT-GGFP for both NAO staining and FITC linked antibodies. The excitation wavelength for the FITC fluorescence filter was 475nm, and the emission wavelength was 530nm. Pictures were taken using the iPhone 5s camera.

CHAPTER IV

Results

Complement consumption assay. The complement consumption assay measured the amount of complement in serum that was consumed by each strain. The absorbancy measured the amount of released hemoglobin resulting from complement unconsumed by bacteria that then lysed the sheep red blood cells (RBC). The higher the absorbancy, the more hemoglobin had been released and less complement that was used in response to incubation with the strain due to either lysing the strain's bacterial cell wall, or by being excluded from lysing the cell wall. The resulting absorbancy averages from the three trials conducted for each bacterial strain can be seen in Table 1 below. The results for the complement consumption assays showed that APEC O2 Δ*iss* had the highest absorption at 10^1 with OD 0.799, followed by APEC O2 (+/+) at OD 0.666, DH5α (-/+) at OD 0.623, and DH5αΔ*bor* (-/-) at OD 0.405 (Figure 1). Comparing the absorbancy for each strain at the 10^{-3} dilution factor, more complement was consumed on the APEC O2 (+/+) pathogenic strain than the DH5α (-/+) commensal strain. This was not done to a significant degree, however, it was very close, and more trials would need to be conducted in order to confirm this. However, comparing APEC O2 (+/+), which is *iss*+/*bor*+, to the mutant strain APEC O2Δ*iss* (-/+), which is *iss*-/*bor*+ like DH5α (-/+), the difference is not significant, meaning the RBCs were not lysed more in either strain to a significant degree despite one being *iss*-. Also, when comparing the two *iss*-/*bor*+ strains to each other, APEC O2Δ*iss* (-/+) and DH5α (-/+), they are not lysed more in either strain to a significant degree (Table 2). This suggests that more than just the gene *iss* is causing an increase in the consumption of complement. This is confirmed because

the pathogenic *iss*- strain is not consumed significantly more than the *iss*- commensal strain. The gene *bor* may not have an effect on the rate of complement consumption since its presence or absence does not have a significant impact on the amount of complement consumed.

Table 1: Complement Consumption Assay absorbancy averages

Dilution Factor	**APEC O2 (+/+)**	**APEC O2 Δ*iss* (-/+)**	**DH5α (-/+)**	***DH5α* Δ*bor* (-/-)**
10	0.666	0.799	0.623	0.405
1.0	0.366	0.200	0.086	0.088
0.100	0.062	0.070	0.023	0.032
0.010	-0.011	0.040	0.004	0.022
0.001	-0.007	0.001	0.008	0.038

Table 2: ANOVA comparison of absorbancies at 10^{-3} dilution factor for each strain.

Bacterial Strain	**p-value**	**Significant/Not Significant**
APEC O2 (+/+) vs. APEC O2Δ*iss* (-/+)	0.278	Not significant
APEC O2 (+/+) vs. DH5α (-/+)	0.054	Not significant
APEC O2 (+/+) vs. DH5αΔ*bor* (-/-)	0.230	Not significant
APEC O2Δ*iss* (-/+) vs. DH5α (-/+)	0.469	Not significant
APEC O2Δ*iss* (-/+) vs. DH5αΔ*bor* (-/-)	0.914	Not significant
DH5A (-/+) vs. DH5αΔ*bor* (-/-)	0.330	Not significant

Table 3: Standard deviation and standard error of absorbancy at 10^{-3} dilution factor for three trials for each strain.

Bacterial Strain	Standard Deviation	Standard Error
APEC O2 (+/+)	0.0181	0.0105
APEC O2Δ*iss* (-/+)	0.0326	0.0188
DH5α (-/+)	0.0227	0.0131
DH5α Δbor(-/-)	0.0236	0.0136

The lowest percent lysis of the hemoglobin was found in the APEC O2Δ*iss* (-/+) strain, followed by the APEC O2 (+/+) strain, which shows more complement was used

when serum was incubated with the mutant strain than the APEC O2 (+/+) strain. Both of the percent lysis of the pathogenic strains are lower than DH5α (-/+) and DH5αΔ*bor* (-/-), suggesting more complement was used to create MAC terminal complex against the pathogenic strains than the commensal strains, since less RBCs were lysed due to there being less complement left to lyse the RBCs. The highest percent lysis was seen in the commensal strain DH5α (-/+), and the mutant strain DH5αΔbor (-/-), which suggests that less complement was used up when incubated with the DH5α (-/+) and DH5αΔ*bor* (-/-) strains, leaving more left over to lyse the RBCs (Table 4).

The average total lysis minus the initial lysis results, 0.177, and the formula (bacterial absorbancy – true lysis absorbancy / true lysis absorbancy X 100), can be used to give the percent lysis of the RBCs for each strain. The 10^{-3} dilution factor was used to calculate the percent lysis because its results gave the percent lysis that was most comparable between each strain. Therefore, these absorbancies were used to calculate the average percent lysis of the RBCs by each strain (Table 4).

Table 4: Percent lysis of RBC for each strain.

Bacterial Strain	% Lysis
APEC O2 (+/+)	64.97
APEC O2Δ*iss* (-/+)	60.45
DH5α (-/+)	87.01
DH5αΔbor(-/-)	81.92

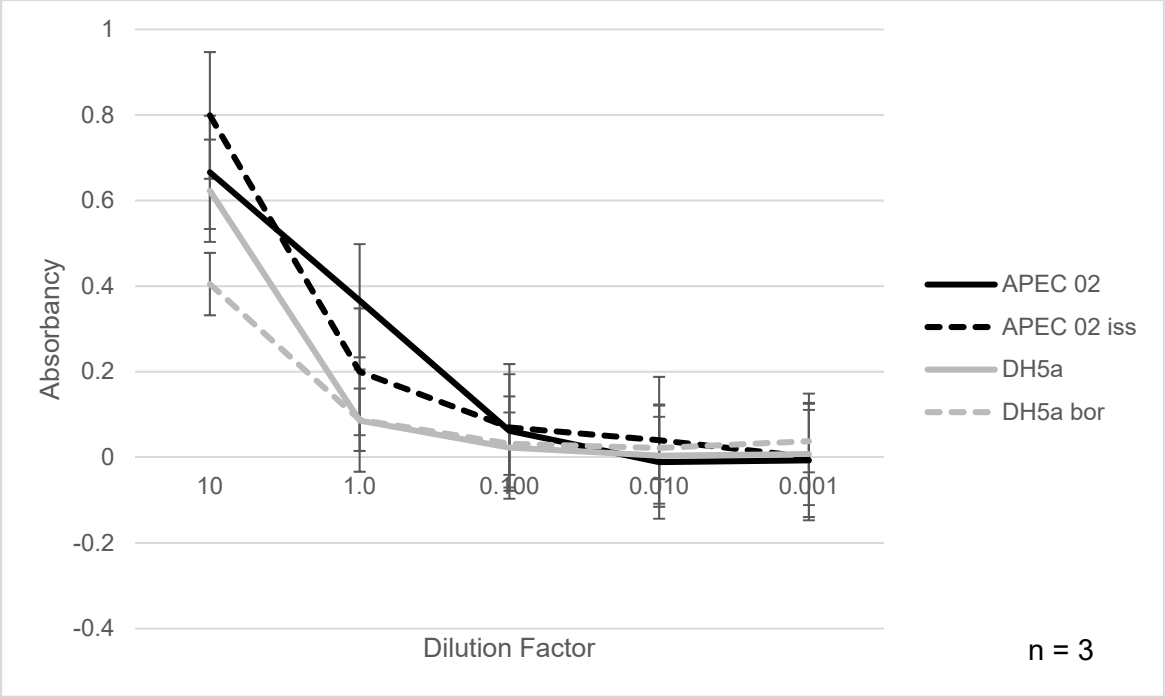

Figure 1: Absorbancy curve of each dilution factor for APEC O2 (+/+), APEC O2Δ*iss* (-/+), DH5α, DH5α Δ*bor*(-/-)

Bactericidal assay. The results for the bactericidal assay absorbancies were averaged from the four trials conducted and compared, and can be seen in Table 4 below. Figure 2 compares the average absorbancies for APEC O2 (+/+), APEC O2Δ*iss* (-/+), DH5α (-/+), and DH5αΔ*bor* (-/-) in active serum (a.) or in their controls, incubation in inactive serum (b.). All strains grow about the same in heat inactive serum, which is expected. In active serum, APEC O2 (+/+) grows more efficiently than does the commensal DH5α (-/+) strain, which is also expected. However, APEC O2Δ*iss* (-/+) grows just as well, if not better than, APEC O2 (+/+) when exposed to active serum. This means that APEC O2Δ*iss* (-/+) is not any less serum resistant, although it is not completely avoiding the effects of inserted MAC, as is supported by the results of the complement consumption assay.

Table 5: Bacterial assay average absorbancy.

	APEC O2 (+/+)		**APEC O2Δiss (-/+)**		**DH5α (-/+)**		***DH5α* Δ*bor* (-/-)**	
Time Point (hours)	**Active Serum**	**Inactive Serum**	**Active Serum**	**Inactive Serum**	**Active Serum**	**Inactive Serum**	**Active Serum**	**Inactive Serum**
0	0.27275	0.30125	0.31125	0.3355	0.25775	0.2865	0.26225	0.284
0.5	0.31975	0.34625	0.355	0.37925	0.30525	0.32225	0.31025	0.322
1	0.42575	0.43525	0.47625	0.47025	0.41325	0.4055	0.42225	0.41425
2	0.62825	0.5975	0.72375	0.62175	0.63475	0.57425	0.651	0.586
12	1.001	0.93525	0.888	0.91075	0.804	0.91325	0.86325	0.89575
24	0.9775	0.8915	1.08325	1.0135	0.73875	0.91225	0.74075	0.88325
48	1.05375	0.91125	1.0655	0.9285	0.6645	0.73325	0.81225	0.67225

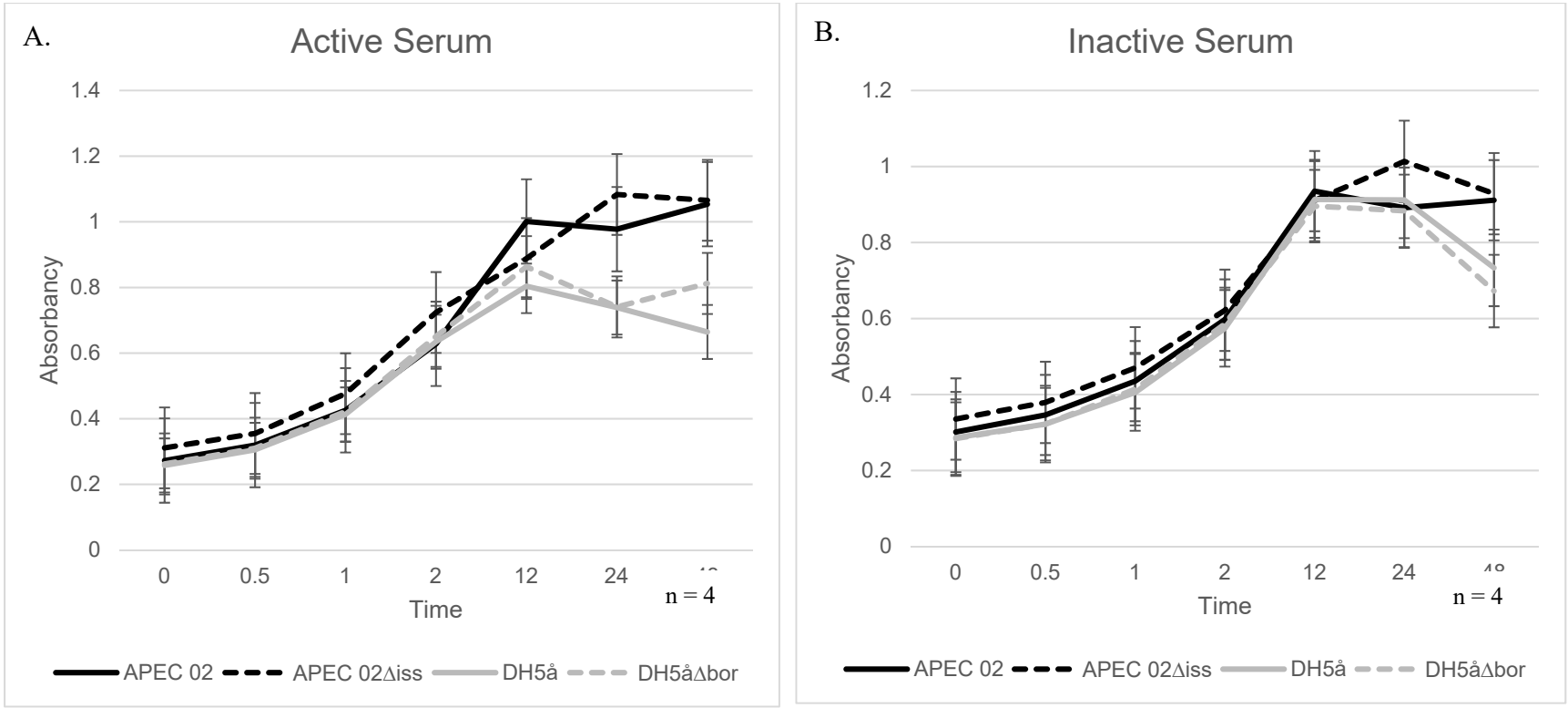

Figure 2: (a.) Absorbancy curve of APEC O2 (+/+), APEC O2Δ*iss* (-/+), DH5α (-/+), DH5αΔbor (-/-) exposed to active serum over time. (b.) Absorbancy curve of APEC O2 (+/+), APEC O2Δ*iss* (-/+), DH5α (-/+), DH5αΔ*bor* (-/-) exposed to heat inactivated serum over time.

Table 6 below lists the total bacterial counts of bacteria that had been serially diluted to 10^{-6} dilution, incubated with either active serum or heat inactivated serum. Figure 3 compares the growth curve of each strain that had been incubated with either serum or heat inactive serum. The growth curve can be seen to drop most dramatically for the pathogenic strain APEC O2 (+/+) when incubated with serum after two hours, then a steady increase in colony forming units (CFU) is observed, at a rate of y = 917x – 284. The mutant strain APEC O2Δ*iss* (-/+) is also seen to steadily increase in CFU number over time at a rate of y = 648x + 241.33, which is similar to the slope of growth of APEC O2 (+/+), although has a slower rate of growth. The growth drop off is less dramatic for the commensal strain DH5α (-/+) and DH5αΔ*bor* (-/-), however, they experience a steady decline in CFU number after two hours, with DH5α (-/+)decreasing with a slope of -440x + 3516, and DH5αΔ*bor* (-/-) decreasing at a steeper rate of y = -534x + 3421.3. This suggests that the commensal strains are unable to grow when incubated with serum, which is expected. The pathogenic strains, however, are able to grow despite having been exposed to serum, although the mutant pathogenic strain APEC O2Δ*iss* (-/+) is less efficient at growing than the APEC O2 (+/+) strain.

Table 6: CFU count for bacterial cells that have been incubated with serum or heat inactive serum for t= 0, 1 hour, 2 hours, 12 hours, and 24 hours.

	APEC O2 (+/+)		**APEC O2Δiss (-/+)**		**DH5α (-/+)**		**DH5αΔ*bor* (-/-)**	
Time Point (hours)	**Active Serum**	**Inactive Serum**	**Active Serum**	**Inactive Serum**	**Active Serum**	**Inactive Serum**	**Active Serum**	**Inactive Serum**
0	4480	2108	2324	3192	3456	2220	3068	3280
1	2888	1220	2236	852	2840	1804	2932	1184
2	774	3276	1016	1804	3084	2960	2484	4420
12	1268	1272	1284	2232	2620	2984	3160	2736
24	2608	2212	2312	1832	2204	1836	1416	1932

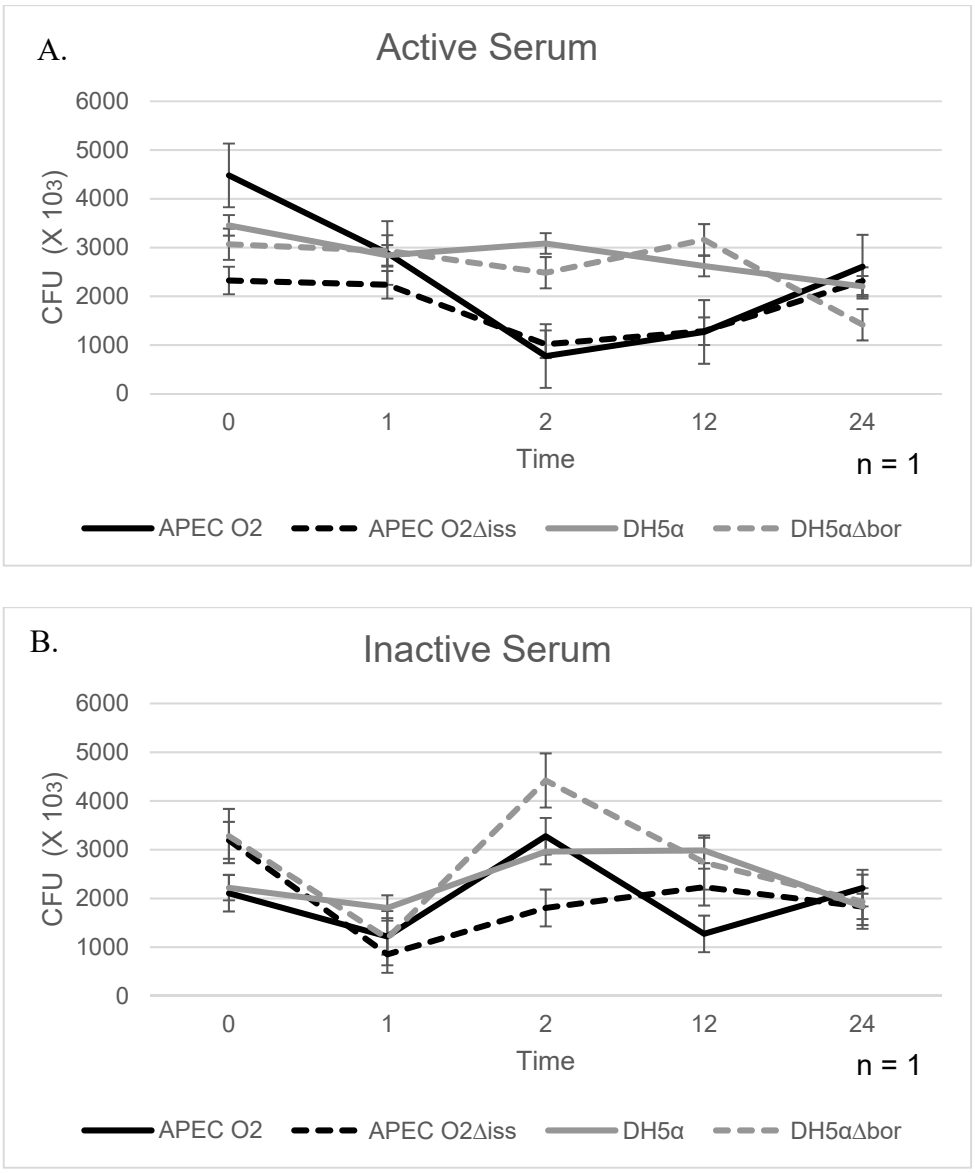

Figure 3: (a.) Growth curve of APEC O2 (+/+), APEC O2Δ*iss* (-/+), DH5α, DH5α Δ*bor*(-/-) exposed to active serum over time. (b.) Growth curve of APEC O2 (+/+), APEC O2Δ*iss* (-/+), DH5α, DH5α Δ*bor*(-/-) exposed to heat inactivated serum over time.

Enzyme linked immunosorbent assay. After determining the protein standard curve for the C5b-9 complement terminal complex, the trendline was calculated using a logarithmic scale of the concentration of protein to the absorbance. The resulting trendline was y=0.0022x + 0.4503 with an R^2 value of 0.86986. The bacteria mixed with

serum was diluted at a 1:3 ratio because this dilution was found to have OD values within the range of the serum dilution standard curve. The absorbancy from the following three trials were then averaged, and the amount of C5b-9 terminal complex protein concentration in each sample was determined using the standard deviation trendline formula (Table 8).

Table 7: Standard deviation and standard error of ELISA absorbancies for three trials for each strain.

Bacterial Strain	Standard Deviation	Standard Error
APEC O2 (+/+) 15min	0.1955	0.1129
APEC O2 (+/+) 60min	0.1150	0.0664
APEC O2Δ*iss* (-/+) 15min	0.2025	0.1169
APEC O2Δ*iss* (-/+) 60min	0.1715	0.0990
DH5α (-/+)15min	0.0456	0.0263
DH5α (-/+)60min	0.0331	0.0191
DH5α Δ*bor*(-/-) 15min	0.0594	0.0343
DH5α Δ*bor*(-/-) 60min	0.0939	0.0543

Table 8: The average C5b-9 terminal complex protein concentration for each strain at 15 minutes and 60 minutes.

Bacterial Strain	Absorbance	Concentration (ng/ml)
APEC O2 (+/+) 15min	0.5915	64.19
APEC O2 (+/+) 60min	0.7107	118.36
APEC O2Δ*iss* (-/+) 15min	0.5767	57.43
APEC O2Δ*iss* (-/+) 60min	0.6595	95.07
DH5α (-/+)15min	0.4739	10.72
DH5α (-/+)60min	0.6011	68.52
DH5α Δ*bor*(-/-) 15min	0.4585	3.74
DH5α Δ*bor*(-/-) 60min	0.6975	112.34

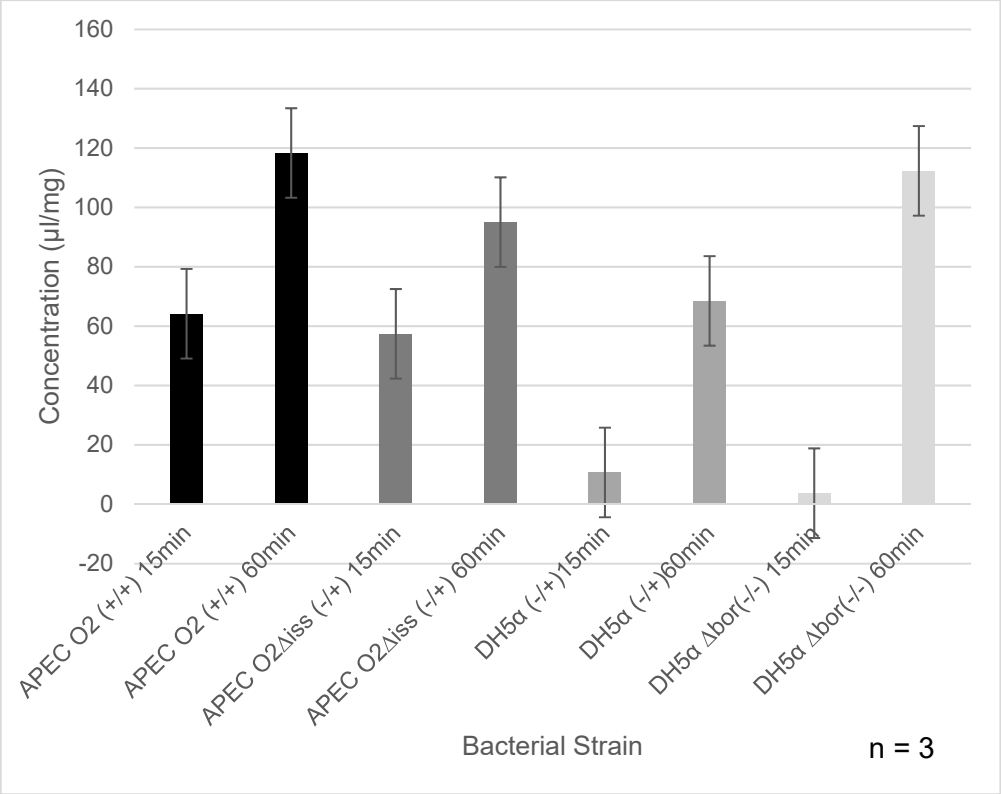

Figure 4: The average C5b-9 terminal complex protein concentration for each strain at 15 minutes and 60 minutes.

Each strain was then compared to determine if the results were statistically significant, using a two-way ANOVA statistical analysis. In order to determine the statistical significance of the differences in concentration of the C5b-9 terminal complex protein at 15 minutes and 60 minutes, each strain had its absorbancy values compared using ANOVA statistical analysis. The p-values for each strain under these conditions can be found in Table 9.

Table 9: Each strain had the values of C5b-9 terminal complex protein concentration at 15 minutes compared to the values of concentration at 60 minutes.

Bacterial Strain	p-value	Significant/Not Significant
APEC O2 (+/+)	0.2528	Not significant
APEC O2Δ*iss* (-/+)	0.5866	Not significant
DH5α (-/+)	0.1087	Not significant
DH5α Δ*bor* (-/-)	0.0089	Significant

These results show that the difference in incubation times between 15 and 60 minutes for each strain is not significant, with the exception of DH5α Δ*bor*(-/-), which has a significant difference in the amount of C5b-9 terminal complex protein when incubated with serum for 60 minutes versus 15 minutes.

Each of the strains were then cross compared to the other strains using ANOVA to determine if the values of C5b-9 terminal protein were significantly different at 15 minutes. The p-value results of this comparison can be found in Table 10.

Table 10: Each strain had the values of C5b-9 terminal complex protein concentration at 15 minutes cross compared to each of the other strains.

Bacterial Strain	p-value	Significant/Not Significant
APEC O2 (+/+) vs. APEC O2Δ*iss* (-/+)	0.9182	Not significant
APEC O2 (+/+) vs. DH5α	0.4105	Not significant
APEC O2 (+/+) vs. DH5αΔ*bor*(-/-)	0.3790	Not significant
APEC O2Δ*iss* (-/+) vs. DH5α	0.2852	Not significant
APEC O2Δ*iss* (-/+) vs. DH5αΔ*bor*(-/-)	0.2552	Not significant
DH5α (-/+)vs. DH5α Δ*bor* (-/-)	0.5788	Not significant

The results shown in Table 8 show that at 15 minutes, the concentration of C5b-9 terminal complement protein is not significantly different, regardless of the strain used.

However, the pathogenic strains at 15 minutes have a higher initial average concentration, even if not to a significant degree, which could be due to the strain being recognized as a pathogenic bacterium, suggesting the complement system will be able to find a target, and they are somewhat distinguishable. However, the commensal strains had the lowest activated concentration of the terminal complex, which is expected, and the pathogenic strains had the highest activated concentration of the terminal complex, which is also expected, even if they are not done so to a significant degree.

Next, each strain was cross compared using ANOVA to determine if the values of C5b-9 terminal protein were significantly different at 60 minutes. The results of this comparison can be found in Table 11.

Table 11: Each strain had the values of C5b-9 terminal complex protein concentration at 60 minutes cross compared to each of the other strains.

Bacterial Strain	p-value	Significant/Not Significant
APEC O2 (+/+) vs. APEC O2Δ*iss* (-/+)	0.5788	Not significant
APEC O2 (+/+) vs. DH5α	0.1167	Not significant
APEC O2 (+/+) vs. DH5α Δ*bor*(-/-)	0.8439	Not significant
APEC O2Δ*iss* (-/+) vs. DH5α	0.4895	Not significant
APEC O2Δ*iss* (-/+) vs. DH5α Δ*bor*(-/-)	0.6626	Not significant
DH5α (-/+)vs. DH5α Δ*bor*(-/-)	0.1192	Not significant

The p-value results in Table 9 show that at 60 minutes, the concentration of C5b-9 terminal complex protein is not significantly different, regardless of what strain is used.

Fluorescent microscopy visualization. Each strain of *E. coli,* (APEC O2 (+/+), APEC O2Δ*iss* (-/+)*,* DH5α, and DH5αΔ*bor* (-/-)), was stained with either 10-N-nonyl-acridine orange (NAO), or primary antibody, monoclonal mouse C5b-9 Ab IgG, and the

secondary antibody, goat anti-mouse IgG conjugated with FITC, and were visualized using an immunofluorescent microscope with a green light filter that had an excitation wavelength of 475nm. The individual bacterial strains were compared within each strain for their appearance when they had been incubated with serum for 60 minutes against strains that had not been incubated with serum.

Figure 5 compares the cellular structure of APEC O2 (+/+) when incubated with serum to APEC O2 (+/+) that has not been incubated with serum, and stained with NAO. Figure 5 (a.) shows the rod shape characteristic of *E. coli* bacterial cells. They are observed to be whole, and without any cellular debris. Figure 5 (b.) shows an increase of cellular clumping, which may be either cellular debris, or only a clumping of cells. Debris of cells could be due to the lysing of the outer bacterial cell wall by complement proteins in the serum, however, clumping of cells could be due to something else present within the serum. Some APEC O2 (+/+) cells, however, are still intact. This suggests that the terminal complex of the complement system is inefficient at lysing the cell wall of APEC O2 (+/+). The shapes of the cells are no longer the same after incubation with serum, and are now smaller and rounder.

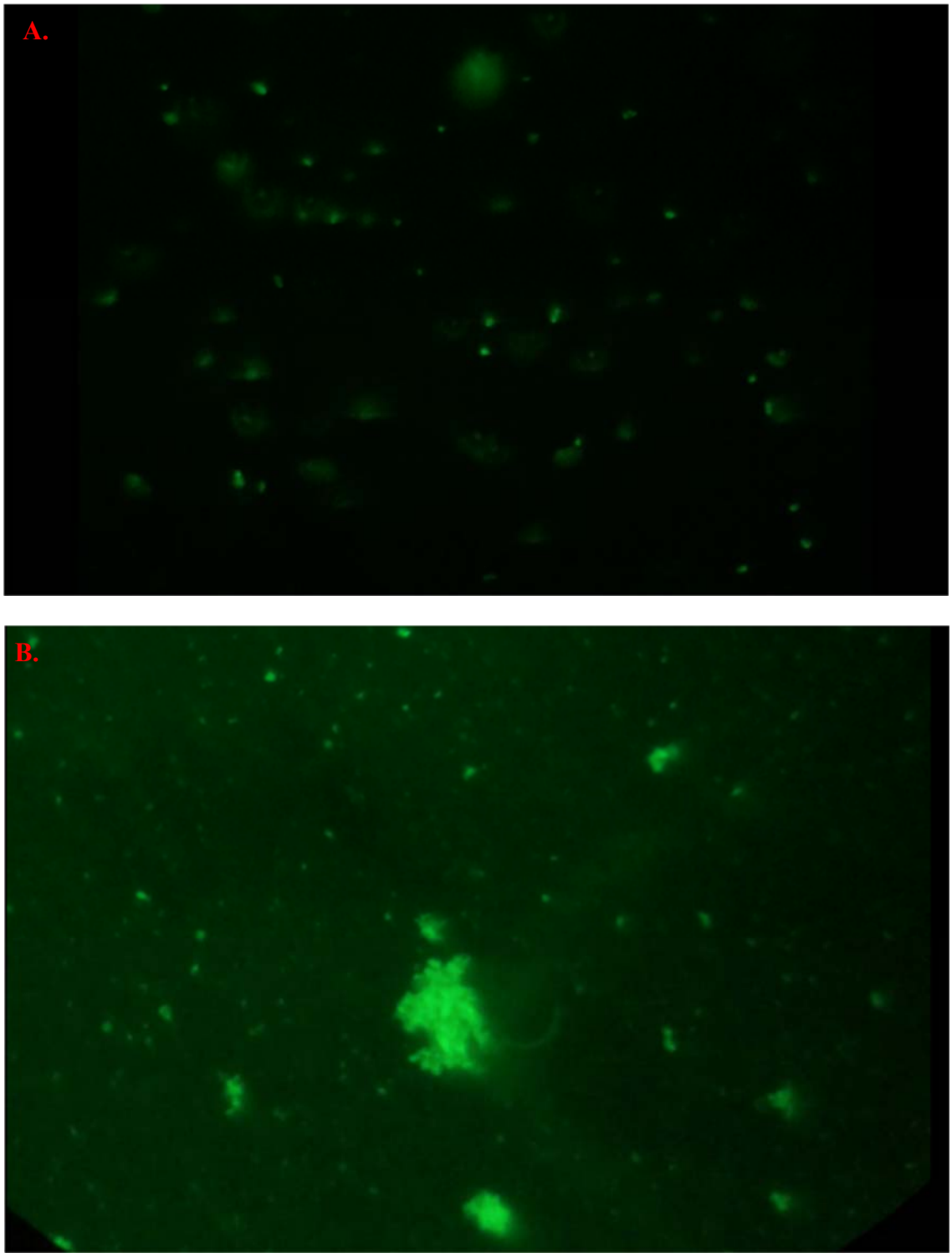

Figure 5: NAO staining of APEC O2 (+/+). (a.) APEC O2 (+/+) without serum. (b.) APEC O2 (+/+) with serum.

Figure 6 and 7 compares the cellular structure of APEC O2Δ*iss* (-/+) when incubated with serum to APEC O2Δ*iss* (-/+) that has not been incubated with serum, stained with NAO. Figure 6 confirms the rod shape appearance of healthy *E. coli* cells, prior to being incubated with serum. Figure 7 shows what is potentially cellular debris. The large, bright spot is a clumping together of the bacterial cells due to exposure to the serum. The spherical shapes may be the shape of the terminal complex, however, they could also be moving due to Brownian motion, or be artifacts due to overstaining. The long strings seen are most likely background. The APEC O2Δ*iss* (-/+) strains appeared to be more sensitive to the terminal complex than the APEC O2 (+/+) strains.

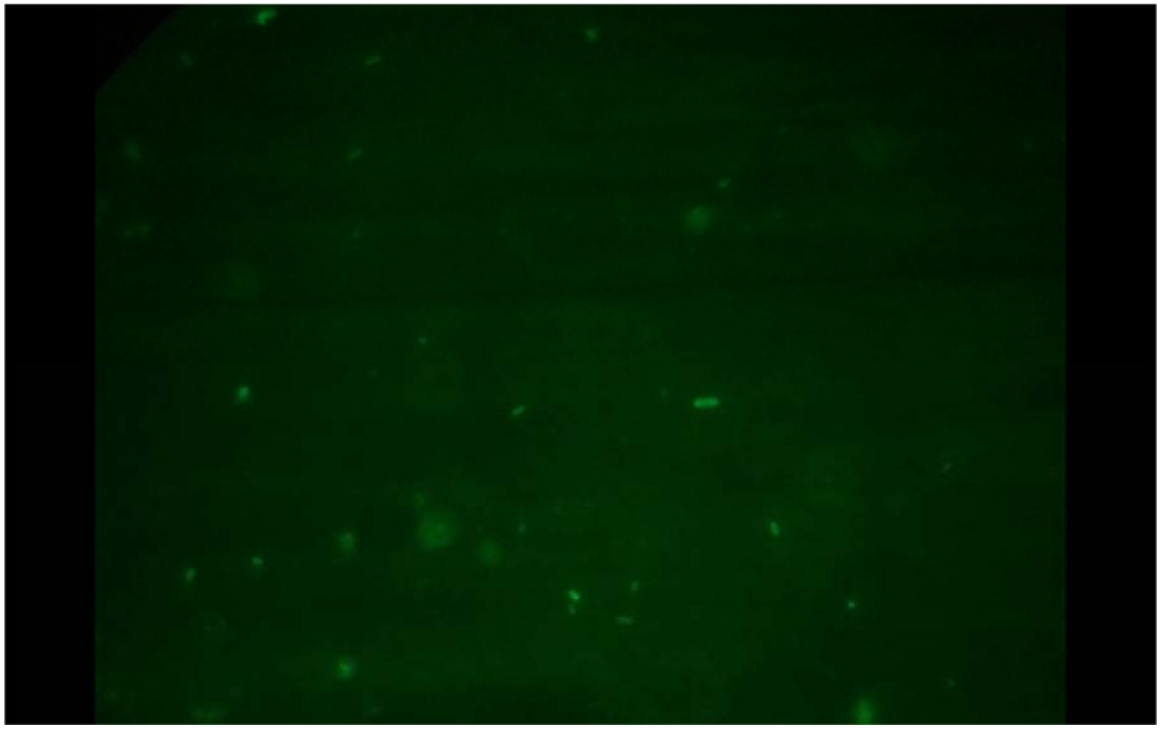

Figure 6: NAO staining of APEC O2Δ*iss* (-/+), incubated without serum.

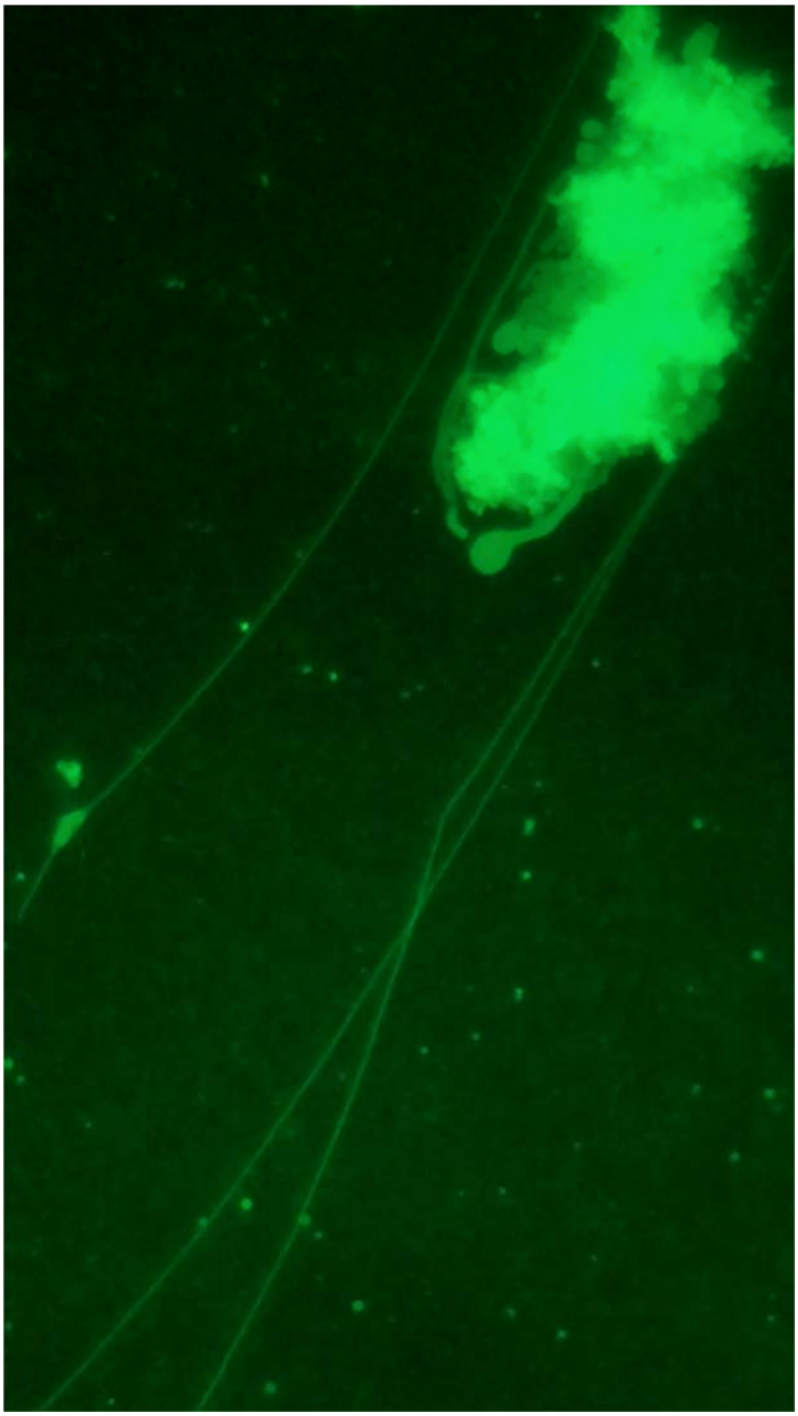

Figure 7: NAO staining of APEC O2Δ*iss* (-/+) incubated with serum.

Figure 8 and 9 compares the cellular structure of DH5α (-/+) when incubated with serum to DH5α (-/+) that has not been incubated with serum, stained with NAO. Figure 8 confirms the rod shape appearance of healthy *E. coli* cells, prior to being incubated with

serum. Figure 9 shows the lysed bacterial cells, and visualizes the cells that are no longer whole, and are now misshapen. This may be a clumping together of the cells, or may be cellular debris due to lysing by the membrane attack complex. There appears to be more cellular debris as a result of incubation with serum in the commensal strain DH5α (-/+) than in the pathogenic strains APEC O2 (+/+) and APEC O2Δ*iss* (-/+).

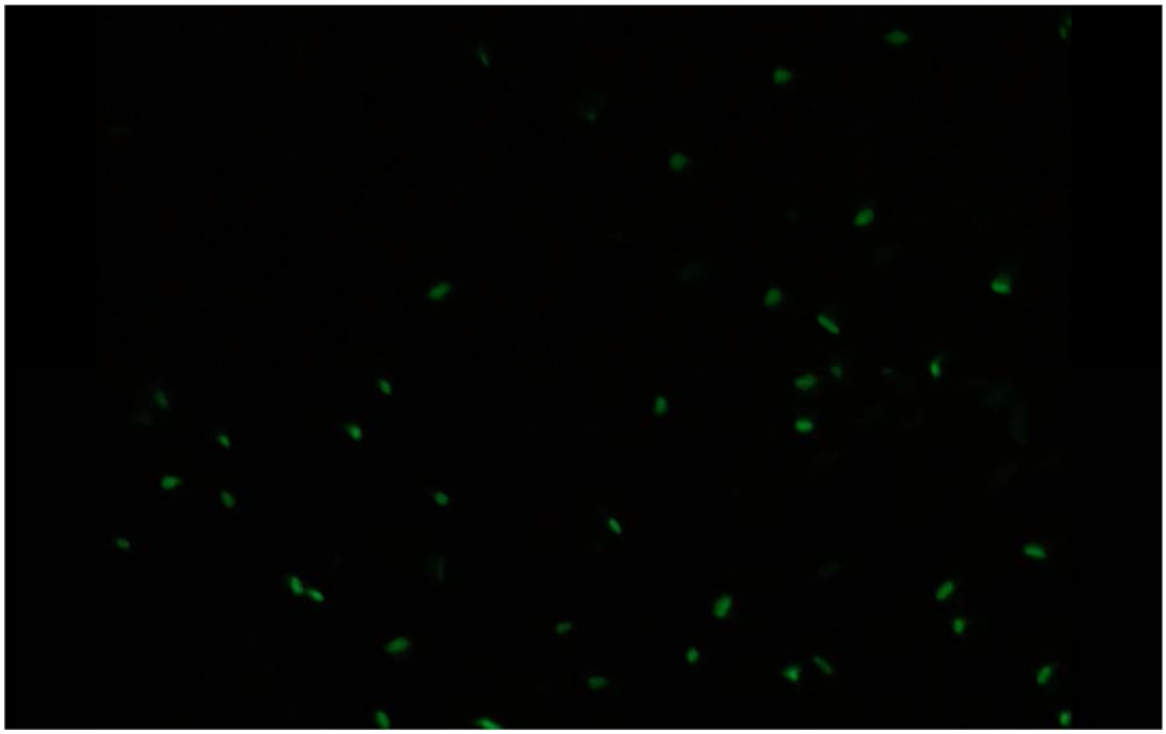

Figure 8: NAO staining of DH5α (-/+) incubated without serum.

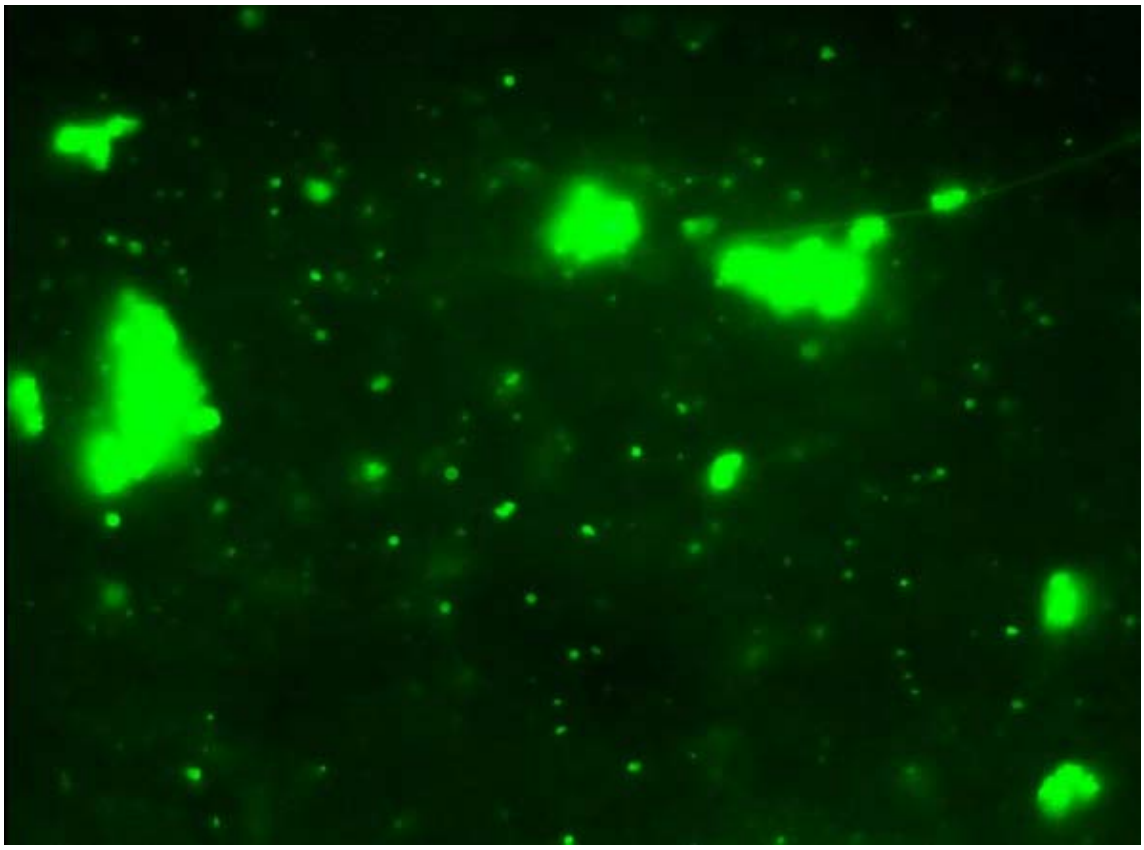

Figure 9: NAO staining of DH5α (-/+) incubated with serum.

Figure 10 compares the cellular structure of DH5αΔ*bor* (-/-) when incubated with serum to DH5αΔ*bor* (-/-) that has not been incubated with serum, stained with NAO. Figure 10 (a.) shows the healthy rod shaped *E. coli* bacterial cells of DH5αΔ*bor* (-/-). Figure 10 (b.) shows that the majority of the DH5αΔ*bor* (-/-) cells have been misshapen after exposure to serum and all that remains is cellular clumping that is potentially cellular debris. The C5b-9 terminal protein complex appears to be the most efficient at affecting the shape of the mutant commensal strain that has had the *bor* gene deleted, DH5αΔ*bor* (-/-)**.**

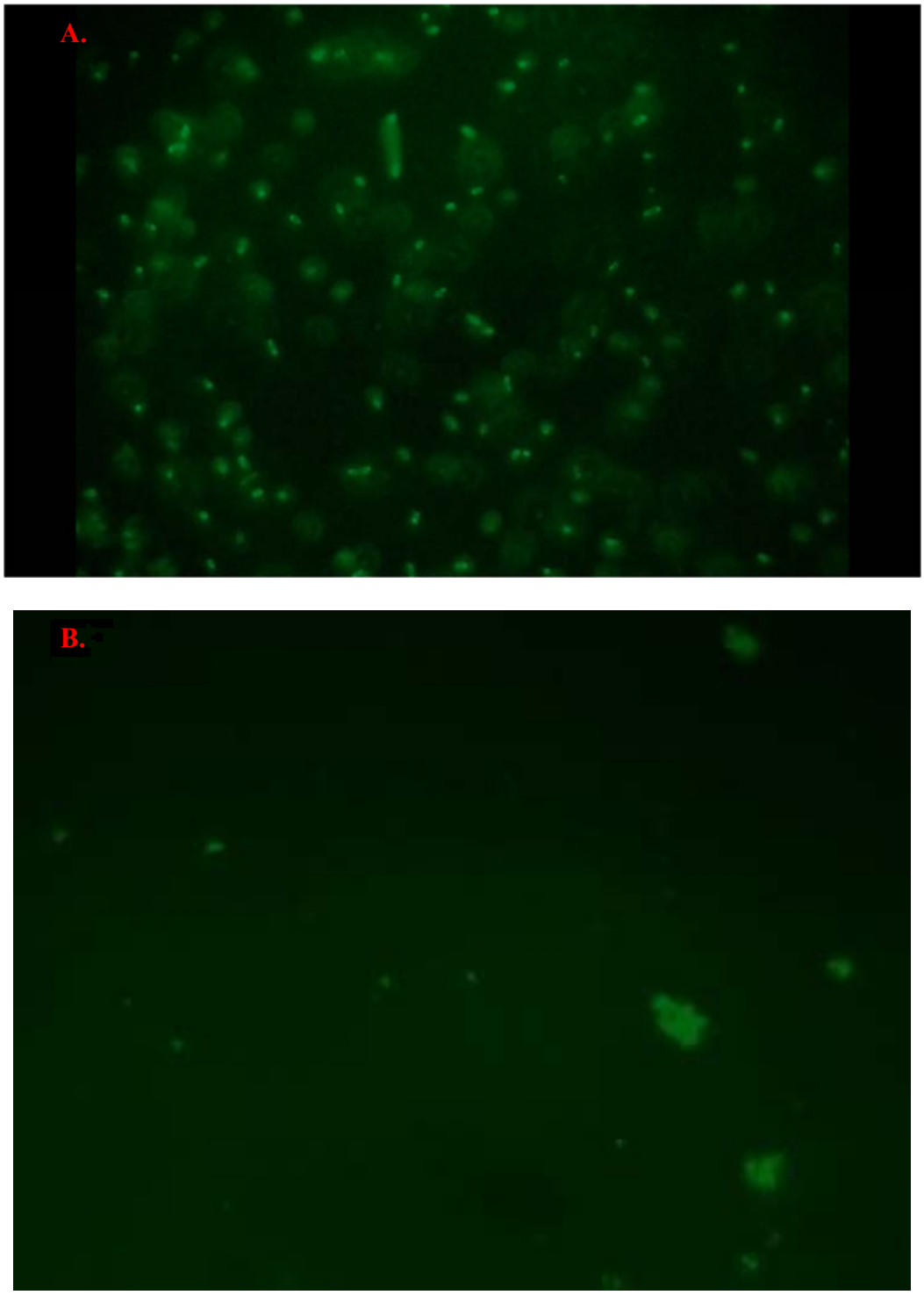

Figure 10: NAO staining of DH5α*Δbor* (-/-). (a.) DH5α*Δbor* (-/-) incubated without serum. (b.) DH5α*Δbor* (-/-) incubated with serum.

Once all bacterial strains were visualized using NAO, each strain was stained using a primary antibody to the C5b-9 terminal protein complex, and a secondary goat anti-mouse antibody that was FITC conjugated. Figure 11 shows the APEC O2 (+/+) cellular debris covered in the C5b-9 terminal protein complex, which suggests that the membrane attack complex was able to attach to this pathogenic strain of *E. coli*. Some of the cells appear completely covered in C5b-9 protein complex, and are still whole. This supports previous research, that MAC is unable to fully insert into the pathogenic strain to lyse the bacterial cell wall. However, the APEC O2 (+/+) strain is not preventing the terminal complex from forming.

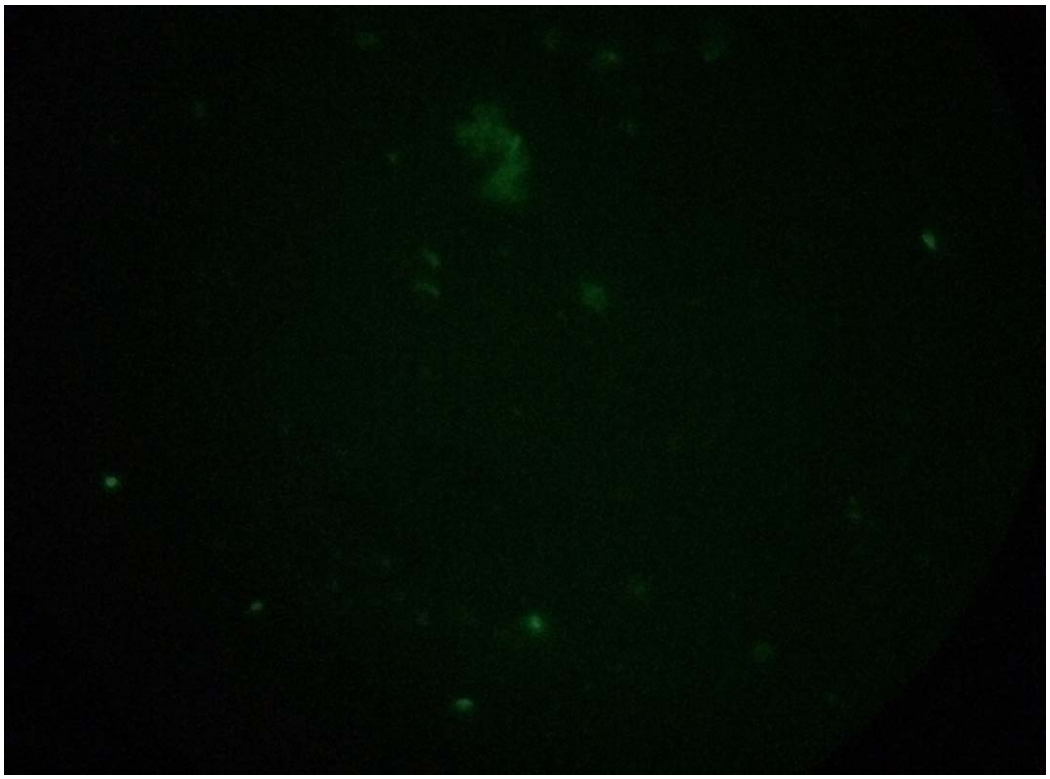

Figure 11: APEC O2 (+/+) with serum, stained with primary antibody to C5b-9, and secondary antibody, conjugated with FITC.

Figure 12 shows the APEC O2Δ*iss* (-/+) cells either lysed or whole. The C5b-9 terminal protein complex is seen attached to the cellular debris, which suggests it is responsible for the alteration of the cell shape seen in Figure 12. However, not all the cells were misshapen. Some glowed with higher intensity indicating a greater amount of terminal complex attached to the outer membrane. This could be due to a resistance to complete insertion of the membrane attack complex into the outer membrane of the APEC O2Δ*iss* (-/+) cell wall, however, this data is qualitative, not quantitative.

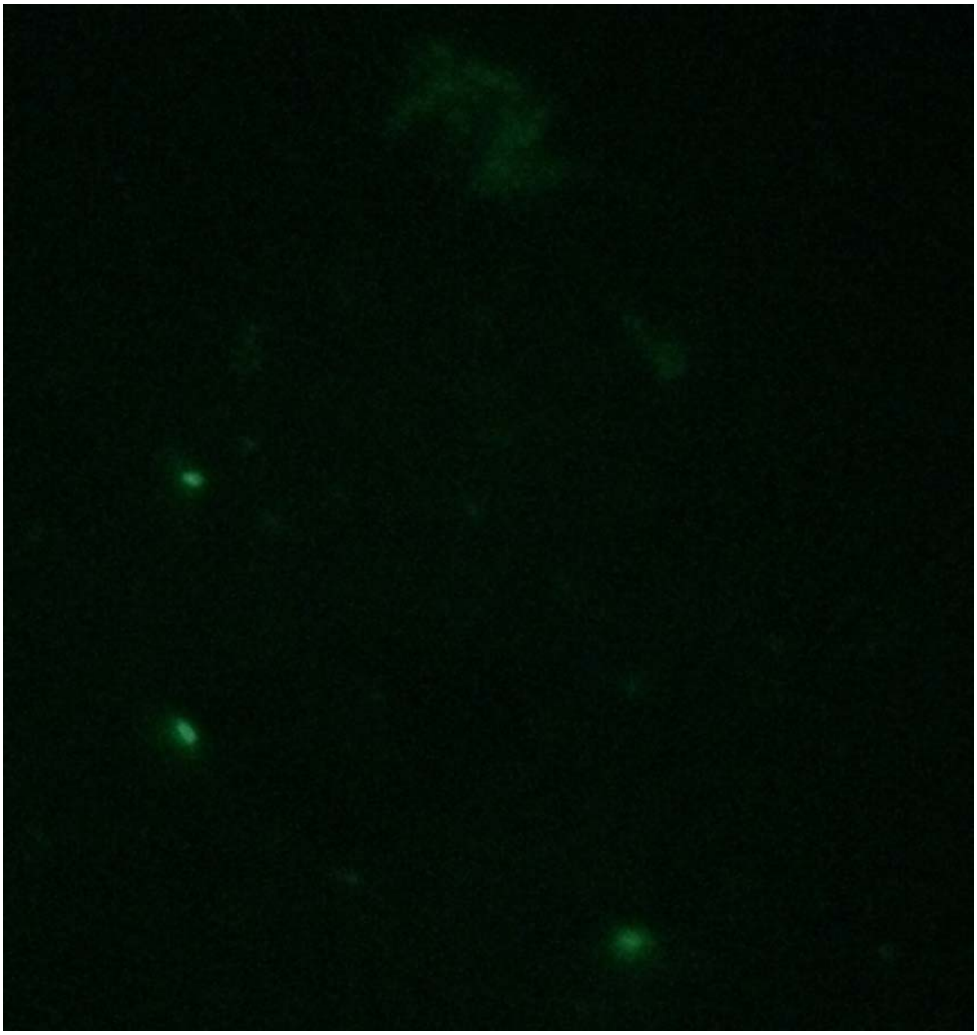

Figure 12: APEC O2Δ*iss* (-/+), with serum, stained with primary antibody to C5b-9, and secondary antibody, conjugated with FITC.

Figure 13 shows the commensal strain DH5α (-/+) that is either still whole or has been misshapen by the C5b-9 terminal protein complex. The terminal complex can be seen attached to the remaining bacterial cells. Also seen is a whole DH5α (-/+) bacterial cell that is completely covered in C5b-9 terminal complex, which appears to be glowing with a higher intensity than the surrounding cells. This suggests the cells have more terminal complex attached to the outer membrane.

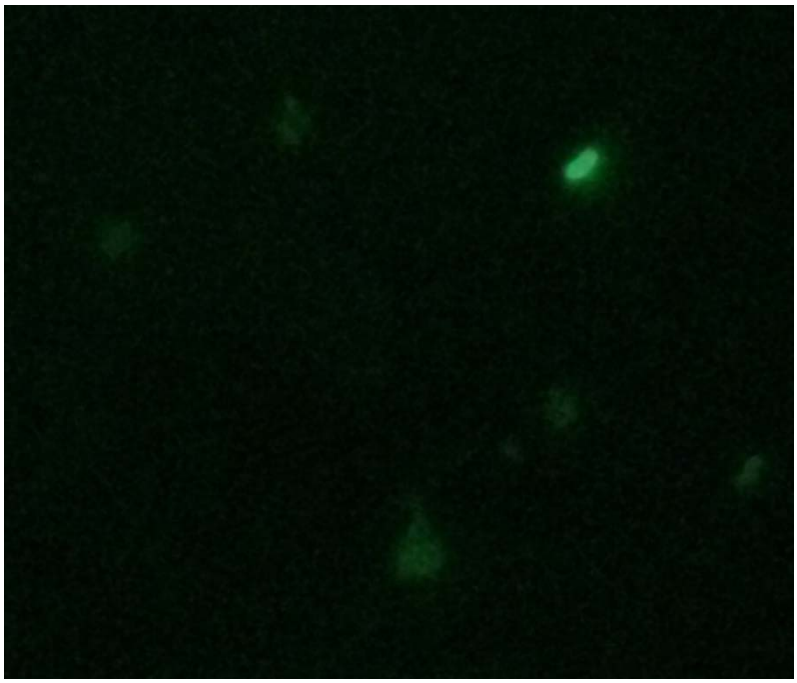

Figure 13: DH5α (-/+) with serum, stained with primary and secondary antibody, FITC linked.

Figure 14 shows the mutant strain DH5αΔ*bor* (-/-) that is either whole or has been misshapen by the C5b-9 terminal protein complex. In Figure 14 (a.), the terminal complex is seen attached to the remaining cell wall of the altered bacterial cells. In Figure 14 (b.), the cell is still whole, however, the double membrane can be seen completely covered in the terminal complex. The cell is seen glowing with greater intensity, and shows more terminal complex on the cell. Because it is seen glowing with greater

intensity than the pathogenic strains, it shows the double membrane. This suggests the protein Bor is not inhibiting the formation of MAC, and may not be preventing the effects of MAC, at least not through surface exclusion tactics.

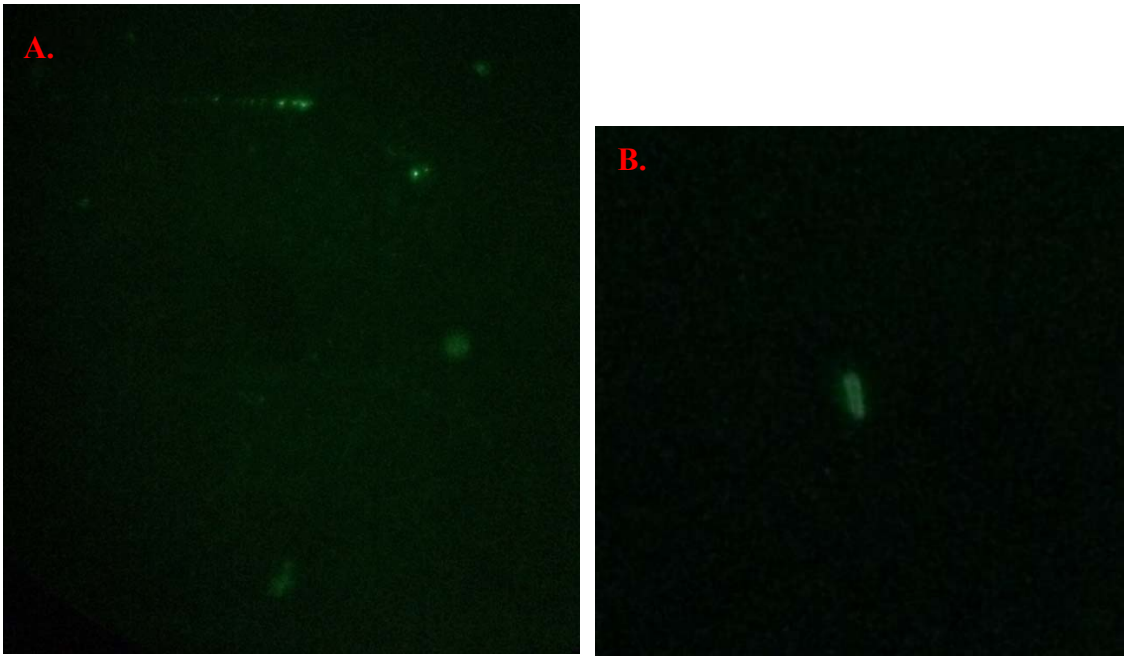

Figure 14: DH5α*Δbor* (-/-) with serum, stained with primary and secondary antibody, FITC linked.

CHAPTER V

Discussion

Avian pathogenic *Escherichia coli* (APEC) is the primary cause of colibacillosis in birds, and has had a devastating impact on the poultry industry as a result. The creation of a vaccine against avian pathogenic *Escherichia coli* (APEC), has been attempted since the 1970s, with elusive results. One gene within APEC, *iss*, has been demonstrated to play a vital role in bacterial pathogenicity in *E. coli*, however, the protein product of the gene *iss* has shown to be too small a target to create a suitable vaccine against it (Lynne, 2007). Understanding the mechanisms in which these pathogenic strains employ serum resistance from their hosts could lead to the discovery of a novel protein target that can be used in conjunction with Iss in order to assist in the future creation of a vaccine.

This study attempted to answer these two hypotheses: Iss works in conjunction with Bor in order to provide an increased level of serum resistance than when Iss or Bor are present alone, and Iss and Bor aids the bacteria in serum resistance by employing surface exclusion tactics so the MAC complex is unable to properly bind to the bacterial surface. The first part of this experiment used the results from the bacterialcidal assay and the complement consumption assay in order to determine the amount of complement being consumed and the rate the bacteria are being killed between the four strains to show the effectiveness of MAC on bacterial cell death.

Since the two strains that are *iss-/bor+*, APEC O2Δ*iss* (-/+) and DH5α (-/+), have wildly differing cell death rates in the bactericidal assay (y=0.888x vs. -0.0698x), this implies that there is something other than *iss* that is helping to contribute to serum resistance in the pathogenic strains. This conclusion is supported by the complement

consumption assay since the APEC O2Δ*iss* (-/+) strain consumed more complement than the DH5α (-/+) strain (60.45% lysis vs 87.01% lysis). Furthermore, the DH5αΔ*bor* (-/-) strain did not have significantly more complement consumed (81.92% lysis) and did not experience a significantly higher cell death rate (y= -0.0255x) than the commensal strain in the complement consumption assay and the bactericidal assay respectively. Therefore, the role the gene *bor* has on assisting *iss* with serum resistance may not be as significant as previously thought.

The second part of this experiment used the results of the bactericidal assay and complement consumption assay, as well as ELISA, and was supported by visualization using immunofluorescence in order to measure the amount of MAC bound to each bacterial strain, and of unbound MAC for each strain. Since the two strains that are *iss-/bor+,* APEC O2Δ*iss* (-/+) and DH5α (-/+), have differing amounts of activating terminal complex (57.43 mg/ml vs 10.72 mg/ml), consumed complement (60.45% lysis vs. 87.01% lysis), and amount of cell death (y=0.0888x vs y= -0.0698x), this implies that something other than the gene *bor* is assisting *iss* in the prevention of cell death due to the attachment of MAC on the cell wall.

Because the terminal complex is formed, the pathogenic strains of *E.coli* are not blocking the formation of the membrane attack complex (MAC), or the activation of the complement system. This is supported by both the immunofluorescent experiment and the ELISA experiment. The ELISA results showed that there was some C5b-9 terminal protein complex left in the serum after incubation with the pathogenic APEC O2 (+/+) strain, as well as the mutant APEC O2Δ*iss* (-/+) strain, which means that the MAC complex is being formed and is therefore at least partially binding to the outer cell wall of

the pathogenic strains. The ELISA results support that *iss* and *bor* do not prevent MAC from forming, since there is not a significance difference between each strain at 15 minutes or 60 minutes. The difference in MAC formation is not statistically significant at 15 minutes or 60 minutes with APEC O2 (+/+), APEC O2Δ*iss* (-/+), or DH5α, so the time difference does not overly produce more MAC. This suggests that APEC strains do have some insertion of MAC so excess MAC is not depleted from serum, and the bacteria somehow prevent lysis by the MAC complex. The gene *bor* is different in that it has significantly more C5b-9 terminal protein activated and remaining at 60 minutes than at 15 minutes. The immunofluorescent (IF) experiment confirms the formation of the C5b-9 terminal protein complex for all the strains used. The APEC O2 (+/+) that underwent the primary and secondary antibody incubation and staining for the IF experiment supports the hypothesis that the MAC protein forms, but is somehow being prevented from lysing the cell wall.

In conclusion, the first hypothesis was rejected. There is something other than the protein Bor assisting the protein Iss in complete serum resistance. The second hypothesis was also rejected. Bor does not assist Iss in surface exclusion tactics to prevent MAC from properly binding.

There were several possible sources of error. One source of error could have occurred during heat inactivation of serum phase of experimentation. The hot water bath used was old, and may not have been working as efficiently as expected, causing the serum to not be completely inactivated. The bacterial strains themselves were pre made, which was convenient, however, there was not an easy way to confirm their genotypes, and they had to be assumed.

Future work would involve knocking out the gene *traT* to see if its protein product is assisting the protein Iss in serum resistance and inhibiting MAC cell wall binding. The glow intensity can also be measured for the immunofluorescence in the future, or an SEM conducted in order to get a clearer picture of what is occurring with the bacteria. Finally, a measurement can be conducted on the membrane fluidity to see how it is affected by these genes.

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VITA

Cindy Botero

Education

M.S. Biology, 2012, Department of Biology, Sam Houston State University, Huntsville, TX
Major Advisor: Dr. Aaron Lynne
Thesis: Visualizing the Interactions Between Serum Proteins of the Complement System and Outer Membrane Proteins in Avian Pathogenic *Escherichia coli.*

B.S. Biology, 2011, Department of Biology, Sam Houston State University, Huntsville, TX

Current and Recent Employment

Veterinary Technician, Trinity Pet Clinic, Trinity TX. December 2011- August 2012.
Veterinarians, Dr. Gerry Reece and Dr. Betty Drummond
Ran centrifuge to separate plasma and serum for testing, and the vet test and laser site for blood analysis for diagnosis. Identification of internal parasites in blood and fecal matter. Set up samples of potential skin infections in algae bottles for later identification. Proper restraint of animals and vaccine administration. FIV/FEV testing in cats, heartworm testing in dogs.

Teaching Experience

Botany lab (BIO 1111). Undergraduate Freshman Level Course.
Taught Fall 2012-Spring 2016. Sam Houston State University. Department of Biology. Chair, Dr. Todd Primm. Lab Coordinator, Mrs. Rose.

Instruct students on the proper usage and parts of a microscope, hypothesis formulation, review the external and internal morphology of the plant body, review the growth regions, and types of roots, examine the citric acid cycle reactions in bean seeds, investigate the different factors that affect catalase activity, investigate the light reactions of photosynthesis, review the mechanism of meiosis

General Microbiology lab (BIO 3470). Undergraduate Junior Level Course.
Taught Spring 2013. Sam Houston State University. Department of Biology.
Chair, Dr. Todd Primm. Lab Coordinator, Dr. Todd Primm.

Guide students in the conduction of a semester long metaproject experiment involving the isolation and identification of unknown bacteria from retail meats. Instructed students on proper aseptic techniques, the use of serial dilutions and the use of the pipette. Assisted students in gaining practical knowledge over microbiology techniques such as Gram stain, capsule stain, acid fast stain, endospore stain, antimicrobial testing, and conduction of a plaque assay. Demonstrated the use and differences between different types of growth media, effects of radiation, and influences of the environment on the growth of microbes.

Nursing Microbiology lab (BIO 2420). Undergraduate Junior Level Course. Taught Fall 2013- Fall 2015. Sam Houston State University. Department of Biology. Chair, Dr. Todd Primm. Lab Coordinator, Dr. Todd Primm.

Guide students in the conduction of a semester long metaproject experiment involving the isolation and identification of unknown bacteria from the skin of a Western mosquitofish. Instructed students on proper aseptic techniques, the use of serial dilutions and the use of the pipette. Assisted students in gaining practical knowledge over microbiology techniques such as Gram stain, capsule stain, acid fast stain, endospore stain, antimicrobial testing, DNA extraction and PCR amplification. Demonstrated the use and differences between different types of growth media and influences of the environment on the growth of microbes.

Undergraduate Experience

General Ecology (BIO 3409). Fall 2011. Dr. Chad Hargrave

Designed and completed an experiment over the course of the semester to determine the amount of fish species overlap in Harmon Creek, Texas using mosquito fish, blacktail shiner fish, and bull minnows

General Entomology (BIO 431). Spring 2011. Dr. Sibyl Bucheli

Captured, preserved, and properly pinned two different families from 50 different orders of insects learned during the class.

Introductory Biochemistry (BIO 348). Fall 2010. Dr. Ilona Petrikovics

Prepared biological Tris buffers, practiced gel filtration chromatography to separate proteins, casted a polyacrylamide gel for protein separation via gel electrophoresis, ran an SDS-PAGE, isolated mitochondria from yeast, preformed the Cytochrome c Oxidase Assay, extracted DNA from *Vibrio fischeri* clones and inserted it into a plasmid which was then transferred into *Escherichia coli* clones using PCR in order to get the *E. coli* to express the Lux Operon genes

Organic Chemistry I and II (CHEM 218, 219). Dr. Rick White

Extraction techniques, recrystallization, distillation, dehydration of an alcohol to form an alkene, nucleophilic substitution reactions, practice in reading IR and NMR spectroscopy, synthesis and purification of aspirin, electrophilic aromatic substitution reaction

General Microbiology (BIO 347). Dr. Aaron Lynne

Completed a semester long project over the identification of a coliform bacteria found in retail turkey meat by determining the microbe's metabolism, testing for the presence of nitrite, protease production, catalase production, presence of cytochrome oxidase, presence of H2S, gram staining, the effects of oxygen, osmotic pressure, and temperature on growth, IMViC series testing

Introductory Genetics (BIO 345). Dr. Madhusudan Choudhary

Covered the physical bases of inheritance and principles of heredity and variation. Studied Mendelian genetics, cytogenetics, molecular basis of genetics, gene expression and regulation, and DNA technologies. Practical application in the lab using *Drosophilia melanogaster*

Graduate Experience

Molecular Biology (BIO 4480W). Fall 2012. Dr. Aaron Lynne

Used α-amylase to predict enzyme activity levels by comparing it to a Maltose Standard Curve. Determined the optimum temperature and pH for enzyme activities to find the probable source of unknown α-amylase enzyme sources. Used SDS-PAGE to analyze the protein composition of several α-amylase containing samples. Learned the procedures involved with Western blotting, DNA extraction, isolation and cloning, PCR amplification, restriction mapping, and construction of an agarose gel for electrophoresis.

Model Systems in Biology (BIO 5394). Fall 2012. Dr. Todd Primm

Explored a variety of biological topics and concepts in a journal club setting, gained factual knowledge over aspects of the immune system, and acquired practice in presenting information to a group of people in a concise and intellectually stimulating manner. Practice in creating a proposal.

Cell Structure and Function (BIO 5364). Spring 2013. Dr. Joni Seeling

Overview of signal transduction, including growth factors, receptors, intracellular signaling molecules, specific signaling pathways, and pathways in development and medicine.

Virology (BIO 5378). Spring 2013. Dr. Aaron Lynne

Studied viruses that infect plants, animals, and bacteria. Areas of study included the chemical and structural properties of viruses, virus-host relations, and infection and growth phenomena, including interference and regulation. Also studied the roles of viruses as agents of disease and malignancy, and as gene vectors in a natural setting, but also as tools in biotechnology and gene therapy. Preparation of a small grant proposal on a research topic related to virology, and the presentation of a research article during ASM Journal Club.

Advanced Genetics (BIO 5364). Fall 2013. Dr. Madhusudan Choudhary

This course covered advanced topics related to prokaryotic and eukaryotic gene structure and regulation, with special emphasis on developing the activity to solve problems using genetic ideas and methods, and to understand the nature and the reliability of genetic inferences, and to apply genetic reasoning to genetic and forensic research. A research proposal was designed detailing the introduction of novel proteins that contribute to UV resistance in *Deinococcus radiodurans* into the germ line of *Drosophila melanogaster.*

The Biology of Aging (BIO 5364). Fall 2013. Dr. James Harper

Studied the current state of biogerontological research, hitting upon a number of the major topics of interest using the primary literature as the sole source of lecture material. Discussed the selected papers pertinent to a weeks's topic including subjects over progeria, caloric restriction, oxidative stress, DNA damage response, telomeres, sirloins, TOR, rapamycin, resveratrol, and comparative biology. At the end of a semester, a grant proposal was written over the klotho gene and its effects on longevity in mice.

Immunology (BIO 4350). Spring 2014. Dr. Todd Primm

This course covered the major terns and concepts of immunology, and provided a deeper understanding of the major components in immunity and how they function. A working understanding of how the immune system interacts with

pathogens and cancerous cells was provided, and allowed for the application of this understudying of immunology to research and clinical problems. Also covered were immunogenetics, cancer, and autoimmunity.

Molecular Evolution (BIO 5371). Spring 2014. Dr. Madhusudan Choudhary

This course covered the modern views of the origin of life, as well as the evolution of molecular structures such as RNA, the genetic code, introns, and tRNA. A term paper covering the evolution of double stranded DNA viruses was written for the end of the semester.

Practical Development of Natural History Collections (BIO 5394). Summer 2015. Dr. Jeffrey Wozniak

This course discussed the history, cultures, landscapes, and biota of the region. Different archaeological sites, rock art, museums, national parks and world heritage sites were visited. There was interaction with local scientists and guides, as well as with trained faculty. Work was done with local schools and with fixing water sources for elephants in national parks.

Natural History of Southern Africa- Methods in Field Biodiversity (BIO 5394). Summer 2015. Dr. Monte Thies

This course was a study in Zimbabwe that focused on the natural history of the region. The modern biota were studied, including mammals, reptiles, birds, and insects, as well as the evolutionary history of the region. A large portion of the time was spent with hands-on experiential learning. This course involved individual as well as group projects. A survey was completed over the biodiversity of arthropods found on the mountain fig tree, *Fiucs glumosa.*

Field Experience

September 2, 2012: Went to the SHSU center for Biology Field Studies to collect Mompha moths and miscellaneous insects with Dr. Sibyl Bucheli and Mo Sisson using the sheet and mercury vapor light method.

October 18-20, 2012: Attended the American Society of Microbiology Branch Fall Meeting at Baylor University in Waco, TX.

February 23, 2013: BSGSO 1 mile Trash Pickup off Fish Hatchery Road, Huntsville, TX

October 31- November 2, 2013: Attended the American Society of Microbiology Regional Fall Meeting in New Orleans, LA.

September 13, 2014: Took over the inactive fish tanks left over after the leaving of previous professors in the Biology Department. Tanks that previously held fish were prepared for public displays including micro farming and composting. Two tanks were set up to hold compost worms and aquaponics.

November 6-8, 2014: Presented a poster at the American Society of Microbiology Texas branch Fall meeting in Houston, TX.

November 29, 2014. Attended the Sam Houston State University tailgating event for the

Department of Biological Sciences.

January 22, 2015. Planted herb seeds into various containers in order to begin the herb garden for the B.U.G.S. organization.

February 12, 2015: Cleaned out the department greenhouse building and transplanted sprouted plants to larger pots to continue the herb garden for B.U.G.S.

May 25, 2015. Organized a Date for the Cause event where volunteers were auctioned off on dates in order to raise money for school supplies for underprivileged children in Zimbabwe, Africa. $1500 total were raised in the event.

June 4-5, 2015. Helped with identifying and sorting insect collections in the entomology department of the Museum of Zimbabwe

June 4, 2015. Hiked through the Matopos region to observe and identify the local wildlife.

Honors, Scholarships, and Awards

National Science and Mathematics Access to Retain Talent (SMART). 2010. $2500
College of Sciences Special Graduate Scholarship. Spring 2013. $1500
College of Sciences Special Graduate Scholarship. Fall 2013. $1500
Texas Public Education Grant Resident (TPEGR). Fall 2013. $3000
Sam Houston State University Teaching Assistant of the Year Nomination. Fall 2013.
The 2014 Department of Biological Sciences Graduate Teaching Award. Spring 2014.
Graduate Bearkat Grant. Fall 2014. $2303
Mathematics Peers II. Fall 2014. $7250
Graduate Bearkat Grant. Spring 2015. $1151
College of Sciences Special Graduate Scholarship. Spring 2015. $1500
Summer Graduate Bearkat Grant. Summer 2015. $1800
Mathematics Peers II. Fall 2015. $7250
College of Sciences Special Graduate Scholarship. Spring 2015. $1500
Texas Public Education Grant Resident (TPEGR). Spring 2016. $1250

Memberships

Secretary of the Biological Sciences Graduate Student Organization (BSGSO). I am in charge of taking down the minutes of each meeting, and ensuring that this information is used to remind officers of plans that were agreed upon in the meetings.

Secretary of the Better Understanding of Global Sustainability (BUGS) organization. I am in charge of taking down the minutes of each meeting, reminding the president of important dates, scheduling future meetings, and emailing important information to all the members.