2023

Investigation of Sneathia vaginalis and the Host Antibody Response to S. vaginalis Pore-Forming Toxin, CptA, in Vivo

Zion T. McCoy
Virginia Commonwealth University

INVESTIGATION OF *SNEATHIA VAGINALIS* AND THE HOST ANTIBODY RESPONSE TO *S. VAGINALIS* PORE-FORMING TOXIN, CPTA, IN VIVO

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

by

Zion McCoy
B.S., Howard University 2021

Major Director: Kimberly Jefferson, Ph.D.
Associate Professor, Department of Microbiology and Immunology
Virginia Commonwealth University
Richmond, Virginia

Virginia Commonwealth University
Richmond, Virginia
May 2023

Acknowledgements

Initially, I'd like to thank Dr. Kimberly Jefferson for being my mentor as I pursued my Masters' degree. Her patience, assistance, and direction has helped me tremendously and developed me as a student and scientist. Her constant motivation and encouragement allowed me to work through doubt and establish a foundation as an independent researcher. I am grateful for the two years I experienced under her mentorship and the knowledge I gained while completing my project in the Jefferson lab.

Secondly, I'd like to thank my graduate advisory committee members: Dr. Gregory Buck, Dr. Eun Lee, and Dr. Maria Teves. Their questions and advice allowed me to develop a relevant final project. I'm extremely appreciative of the attention to detail provided by my graduate advisory committee towards my project, helping me form conclusiveness and organization within my final project. I'd like to give a special thanks to Dr. Eun Lee for collaborating with Dr. Jefferson and I on the Sn35 mouse model. I would like to thank Dr. Myrna Serrano for providing data processing of the samples I used which was vital for the final project. I would like to thank Dr. David Edwards for assistance in choosing accurate statistical tests to analyze my data which was vital for determining significance.

Lastly, I would like to thank fellow members of the Jefferson lab, both past and present, that have kept me company for many days as we tackled our projects. I would also like to thank my lab mates for giving me very helpful advice throughout my research. I'm grateful to have had great lab mates that always made research in the Jefferson lab enjoyable and exciting.

Dedication

I would like to dedicate my final project to my mother, Angelene, and grandmother, Gwendolyn. I owe all my success to my mother whom I would not have made it this far without. She has constantly reminded me of how capable I am and how proud she is of me. She is my number one fan and has supported me in every way. My grandmother, who passed from metastatic breast cancer in 2011, was not able to witness me become who I am today. Still, she laid the foundation for my success, and I hope I am making her proud.

Table of Contents

Page

Acknowledgements ……2

Dedication ……..3

List of Figures…….6

List of Abbreviations…… 8

Chapters

1. Introduction……..……...……..14
    I. *S. vaginalis* in the vaginal microbiome……...……..14
    II. *S. vaginalis* strain Sn35……...…..18
    III. CptA toxin…….18
    IV. Bacterial vaginosis…….……21
    V. Preterm birth……..….22
    VI. Mouse models of pregnancy and PTB…….…...……...23
    VII. Introduction to Research and Objectives……...…25
2. Methods……..……...……....28
3. Analysis of Anti-CptA Antibodies in Vaginal Fluid……..……...…..…..42
    I. Host anti-CptA antibody relationship with *S. vaginalis*……43
        i. Qualitative analysis of host antibody presence……...…..….43
        ii. Qualitative analysis of Ig isotype presence……....…..45
        iii. Ig absence when *S. vaginalis* fails to decline……46
    II. Host antibody ability to neutralize CptA toxin…….……47
        i. Quantitative analysis of host antibody neutralizing activity…....47

ii. Qualitative analysis of host antibody neutralizing activity……...49

III. *S. vaginalis* and host antibody relationship with birth outcome…….....51

i. *S. vaginalis* abundance relative to birth outcome…….....51

ii. Host antibody response relative to birth outcome…….....52

IV. Longitudinal study from IgM positive samples……...……...53

4. Optimization of Mouse Model for *S. vaginalis* Colonization...……...……55

I. Preliminary five-day drip plate experiment……...……..55

II. Complications in uterine horn post inoculation with Sn35…….57

5. Discussion…….…….....……59

6. Future Directions…….…...……63

Literature Cited……...….....65

Vita……..…….67

List of Figures

Page

Figure 1: Illustration of the fetal membrane model……..…….16

Figure 2: *S. vaginalis* traverses chorionic membrane ……..……...……..16

Figure 3: *S. vaginalis* permeabilizes primary human amniotic epithelial cells ……...….17

Figure 4: *S. vaginalis* damages cells in human chorion……..……..17

Figure 5: Full length CptA and CptA protein fragments……...……...….……20

Figure 6: Cytotoxicity and hemolytic activity of CptA toxin ….…….……20

Figure 6A: Trypan blue staining of JEG-3 cells……20

Figure 6B: MTT assay on JEG-3 cells……...20

Figure 6C: Hemolysis assay on human RBCs……20

Figure 7: Mouse estrous cycle……....24

Figure 8: Longitudinal analysis of *S. vaginalis* during gestation……27

Figure 9: Longitudinal analysis of *S. vaginalis* during gestation in preterm and full term groups according to African American or European American ancestry……27

Figure 10: Indirect ELISA schematic……..33

Figure 10.1: Antigen……..……..33

Figure 10.2: Unlabeled primary antibody……....33

Figure 10.3: HRP-labeled secondary antibody……...…….....33

Figure 10.4: TMB substrate…….…33

Figure 11: Neutralization schematic…….……..….35

Table 1: Sample results from ELISAs and Neutralization Assays……..36

Figure 12: Qualitative analysis of host antibody presence relative to *S. vaginalis*……..…..44

Figure 13: Qualitative analysis of Ig isotype presence relative to *S. vaginalis*……..45

Figure 14: Ig absence when *S. vaginalis* fails to decline……46

Figure 15: Quantitative analysis of host antibody neutralizing activity…….…..…..48

Figure 16: Qualitative analysis of host antibody neutralizing activity……..….50

Figure 17: *S. vaginalis* abundance relative to birth outcome……..…51

Figure 18: Host antibody response in relation to birth outcome…….……...52

Figure 19: Longitudinal study from IgM positive samples……....53

Figure 19A: IgM positive- sample #6……53

Figure 19B: IgM positive- sample #65……..53

Figure 19C: IgM positive- sample #57……..53

Figure 20: Preliminary five-day drip plate experiment…….....56

Figure 21: Complications in uterine horn post inoculation with Sn35…….57

List of Abbreviations

°C ……degrees Celsius

µg ……microgram

µl ……microliter

µm ……micrometer

Amp ……ampicillin

BHI ……brain heart infusion

BL21 ……non-T7 expression *E. coli* strain

B-ME ……Beta-Mercaptoethanol

bp ……base pair

BV ……bacterial vaginosis

CFU ……colony forming units

Cipro ……ciprofloxacin

Cm ……chloramphenicol

COG ……Clusters of Orthologous Genes

CptA ……Cytopathogenic toxin, component A

CptB ……Cytopathogenic toxin, component B

DI ……deionized

E. coli ……*Escherichia coli*

ELISA……enzyme linked immunosorbent assay

FBS ……fetal bovine serum

GC …… ……guanine and cytosine

g ……gram

HAEC ……human amniotic epithelial cell

HD1……..high dose 1

HD2…….high dose 2

HIV……..Human immunodeficiency virus

HPV…….Human papilloma virus

HRP……..horseradish peroxidase

hr……..hour

Ig……..immunoglobulin

IPTG ……isopropyl β-D-1-thiogalactopyranoside

JEG-3 ……..human chorionic trophoblasts

kDa……..kilodalton

LB……Luria-Bertani

LD1……..low dose 1

LD2……..low dose 2

LefSe ……Linear discriminant analysis Effect Size

max…….maximum

Mbp…….Megabase pair

mg……..milligram

min…….minute

mL…….milliliter

M …….Molar

mM……..millimolar

MOMS-PI……Multi-Omic Microbiome Study, Pregnancy Initiative

MTT……3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

nm……..…….…….nanometer

nM……..nanomolar

NTerm ……amino-terminal, CptA 24-1349

OD…….optical density

PBS……phosphate-buffered saline

PBST ……phosphate-buffered saline solution with 0.1% Triton-X 100

pH …….power of hydrogen

PSI……pounds per square inch

PTB……..preterm birth

RAMS……..……Research Alliance for Microbiome Science

RBC…….red blood cells

RCF…….relative centrifugal force

rRNA …….ribosomal ribonucleic acid

RT…….room temperature

sec…….second

sBHI……supplemented brain heart infusion

Sn35…….Sn35 strain of *S. vaginalis*

STI……..sexually transmitted infection

S. vaginalis …….*Sneathia vaginalis*

TB…….term birth

TEM……..……..transmission electron microscopy

TMB…….3,3’,5,5’-Tetramethylbenzidine

Trx……..thioredoxin

UHD2 …….ultra high dose 2

VCU…….Virginia Commonwealth University

WHO ……..World Health Organization

Abstract

INVESTIGATION OF *SNEATHIA VAGINALIS* AND THE HOST ANTIBODY RESPONSE TO *S. VAGINALIS* PORE-FORMING TOXIN, CPTA, IN VIVO

By Zion T. McCoy
B.S., Howard University

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

Virginia Commonwealth University
2023

Major Director: Kimberly Jefferson, Ph.D.
Associate Professor, Department of Microbiology and Immunology

Sneathia vaginalis, formerly *Sneathia amnii*, is a gram-negative vaginal species that is associated with pregnancy complications including preterm birth. The only virulence factor of *S. vaginalis* that has been characterized is the cytopathogenic toxin, component A (CptA), a pore-forming toxin that lyses human red blood cells and human epithelial cells and plays a role in traversal of the bacteria through fetal membranes. The overarching goal of this work was to examine the host immune response to CptA. To investigate the immune response to CptA, levels of anti-CptA IgM, IgA, and IgG in human mid-vaginal swab samples obtained during pregnancy were quantified. All three antibody isotypes were detectable, suggesting that CptA is expressed in vivo and is recognized by the host immune defenses. Vaginal swab samples containing anti-CptA antibodies neutralized the hemolytic activity of CptA toxin in vitro. To extend these findings, an animal model is needed, so the second aim of this work was to begin to establish a

mouse model for *S. vaginalis* colonization. Conditions were optimized to maximize vaginal colonization of C57BL/6 mice.

Chapter 1

Introduction

I. *S. vaginalis* in the vaginal microbiome

Sneathia vaginalis, previously known as *Sneathia amnii*, is a gram-negative, non-motile facultative anaerobe that is a normally occurring bacteria in the vaginal microbiome. Through MOMS-PI, a study under the Vaginal Human Microbiome Project, samples were collected from 1,572 pregnancies with 992 pregnancies from clinics at VCU [1]. MOMS-PI Preterm Birth (PTB) study dataset was developed from a case-control study of 45 women who delivered spontaneously preterm and 90 case-matched women who delivered at term. Longitudinal vaginal swab samples were taken throughout gestation, and analysis of 16s rRNA encoding gene via deep-sequencing of these samples allowed bacteria present to be identified, even at low abundances. Out of four taxa in vaginal 16S rRNA profiles in the 6-to-24-week gestational age range that were associated with preterm birth, *S. vaginalis* had the highest LefSe score [1]. From 736 vaginal samples collected from a separate study at VCU [2], it was found that *Sneathia* species were present in 43.3% of samples with at least 0.1% abundance in total reads, confirming that the bacteria normally occur in the vaginal microbiome [3]. In 20.9% of samples, *S. vaginalis* abundance relative to other present bacterial taxa occurred between 1 and 10% [3]. Importantly, three samples contained more than half total reads that traced to *Sneathia* species suggesting that, in some cases, not only is *Sneathia* present, but the most abundant taxa present [3].

Within the genus *Sneathia*, there are three species that are believed to exist within the vaginal microbiome: *S. vaginalis, S. sanguinegens*, and a third putative species. In the vaginal

samples, both *S. vaginalis* and *S. sanguinegens* co-occurred in 70.1% of samples containing *Sneathia*; however, 76.3% of *Sneathia* reads traced to *S. vaginalis*. Though recent studies have begun to challenge this theory in women of African-American or Hispanic ancestry, it is believed that a lactobacillus-dominant vaginal microbiome is key to a healthy vaginal microbiome [4]. *Lactobacillus* species produce antimicrobial compounds including lactic acid, hydrogen peroxide, and bacteriocins in the normal vaginal microbiome. These compounds minimize the abundance of various anaerobic bacteria populations, like *Sneathia*, in the vaginal microbiome, as high biodiversity and low lactobacillus abundance in the vaginal microbiome is associated with BV, STI acquisition, and preterm birth.

S. vaginalis can ascend the female genital tract, and this phenomenon is associated with pathologic outcomes during pregnancy. *S. vaginalis* is associated with amnionitis and chorioamnionitis [5][6]. A fetal membrane model (figure 1) revealed that *S. vaginalis* damages and traverses chorionic membrane from maternal to fetal side (figure 2) in vitro. *S. vaginalis* has also been proven cytotoxic to epithelial cells from primary amniotic epithelial cells (figure 3) and primary chorion (figure 4) from healthy term birth via trypan blue stain and, TEM, respectively.

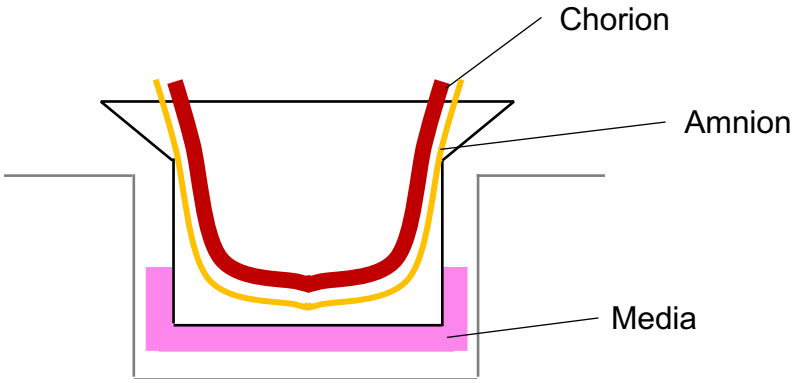
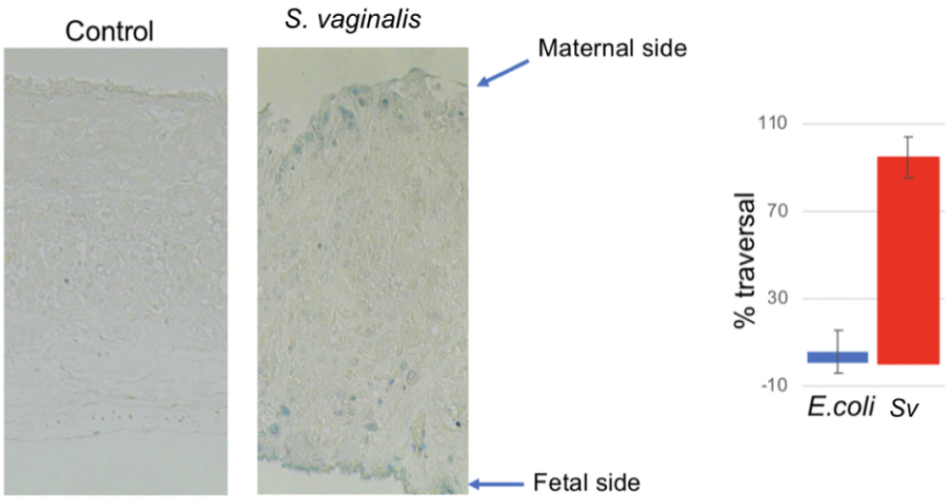
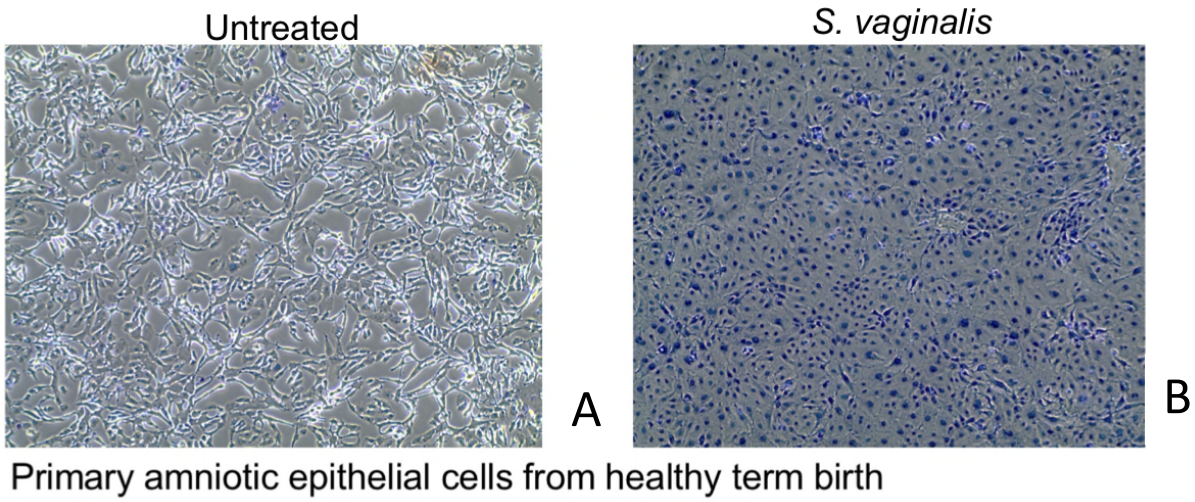


Figure 1. Illustration of the fetal membrane model.
Human chorionic membrane was used in a model to assess *S. vaginalis* ability to traverse human chorion.



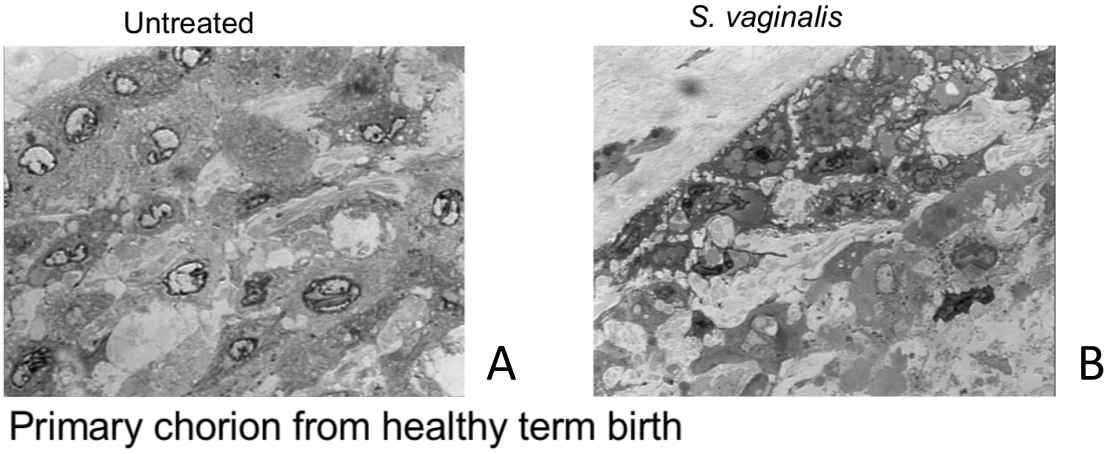
Adapted from Gentile et al. [7]

Figure 2. *S. vaginalis* traverses chorionic membrane.
Trypan blue staininng on chorionic membrane revealed that *S. vaginalis* traverses chorionnic membrane. Trypan blue is uptaken by cells that are permabilized by *S. vaginalis.*



Unpublished data from previous project in lab

Figure 3. *S. vaginalis* permeabilizes primary human amniotic epithelial cells (HAEC).
HAEC were extracted from placentas from healthy term births. They were cultured in vitro and produced monolayers in 6-well plates. Monolayers were either untreated or challenged with live S. vaginalis for 12 hours. Trypan blue staining of 2a) HAECs at 10x magnification or 2b) S. vaginalis treated HAECs at 10x magnification.



Unpublished data from previous project in lab

Figure 4. *S. vaginalis* damages cells in human chorion
Biopsies of chorion were taken from placentas from healthy term births and treated with live S. vaginalis. The membranes were fixed, embedded in paraffin sections, and visualized by tandem electron microscopy. 2a) Untreated primary human chorion under TEM or 2b) S. vaginalis treated primary human chorion under TEM

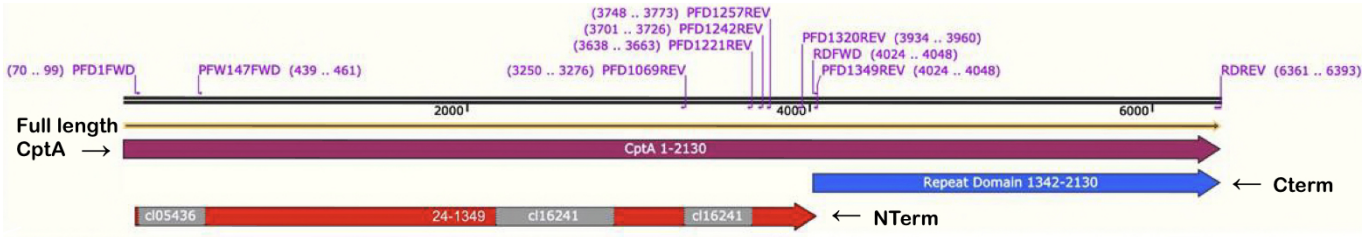
II. *S. vaginalis* strain Sn35

The Sn35 strain of *S. vaginalis* was isolated from vaginal fluid through the Vaginal Human Microbiome Project at Virginia Commonwealth University [3]. Sn35 was used throughout the project for this study of *S. vaginalis*. The genome is approximately 1.34 Mbp and has a GC content of ~ 28%, making it the smallest known genome in the Fusobacteriacae family. The *S. vaginalis* genome has a relatively high gene density at 968 genes per Mbp. There are 1,282 putative protein-coding genes in the genome. *S. vaginalis* can grow on or in porcine BHI with 10% FBS in anaerobic conditions at 37 °C. The bacteria is non-motile. Tandem electron microscopy revealed chains of 1-2 μm long bacilli with rounded ends that form long rods. *S. vaginalis* is resistant to antibiotics like tetracycline and ciprofloxacin. However, the species is sensitive to vancomycin to which most gram-negative bacteria are not.

III. Cytopathogenic toxin, component A (CptA toxin)

CptA toxin is encoded from the *cptA* gene within the *S. vaginalis* genome (figure 5). CptA is a cytotoxic exotoxin and the only virulence factor from *S. vaginalis* that has been characterized. NCBI Blastp reports 98.40% identity between CptA amino acid sequence in 52976 and Sn35 strains of *S. vaginalis*. This likeness in CptA protein amino acid sequences suggests that the immune system recognizes CptA produced by different strains of S. vaginalis similarly. The CptA protein is 1,881 amino acids with a molecular weight of 226 kDa. The neighboring gene, *cptB*, appears to be a Type Vb (two-partner secretion system) secretion apparatus. A pore-forming domain of the *cptA* gene, ranging from 24-1349 bp, is referred to as NTerm. NTerm fragment 24-1349 alone has the ability to permeabilize JEG-3 cells as illustrated by trypan blue

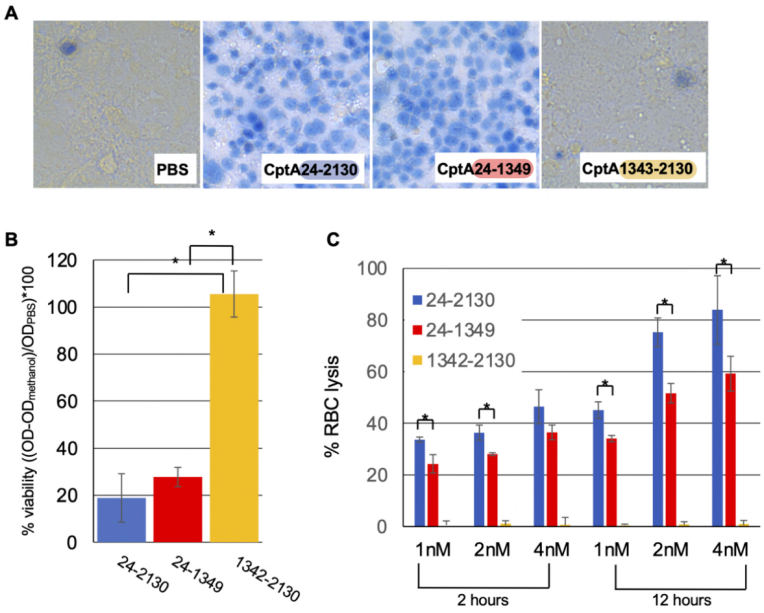
staining (figure 6a). NTerm is cytotoxic to JEG-3 cells via MTT assay (figure 6b) [8]. NTerm is also capable of being hemolytic to human red blood cells via hemolysis assay (figure 6c) [8]. There are multiple tags on the NTerm fragment: Trx tag, S-tag, and a histidine-6 tag. Thrombin can be used to remove these tags. The S-tag and histidine-6 tags have been used to purify the NTerm protein via affinity chromatography. NTerm contains two regions of antigenicity, identified by the model described [9].



Adapted from O'Brien et al. [8]

Figure 5. Full length CptA and CptA protein fragments.

Illustration of the cptA gene, the full length CptA protein (1-2130 amino acids), CptA protein fragment 1342-2130 identified as "CTerm" (1342-2130 amino acids), CptA protein fragment 24-1349 identified as "NTerm" (24-1349 amino acids). Primers used to amplify and clone the different portions of CptA are marked on the gene.



Adapted from O'Brien et al. [8]

Figure 6. Cytotoxicity and hemolytic activity of CptA toxin.

6a) JEG-3 cells were incubated with 2.5 nM of CptA 24-2130 (Full length CptA), CptA 24-1349 (NTerm), or CptA 1343-2130 (CTerm) for one hour then stained with trypan blue or 6b) assessed for cell viability via MTT assay. 6c) 1, 2, or 4 nM of CptA 24-2130, CptA 24-1349, or CptA 1343-2130 were incubated with washed human RBCs for 2 or 12 hours at 37°C via hemolysis assay and measured at OD_{405nm}.

IV. Bacterial vaginosis

Bacterial vaginosis is the most frequent vaginal infection in reproductive age women, and about $4.8 billion is spent annually on treatment of symptoms [10]. BV is described by a large decline or depletion of *Lactobacillus* species in the vaginal microbiome [10]. As stated, a healthy vaginal microbiome is associated with *Lactobacillus* predominance which appears to be especially true in women of European American ancestry [4][11]. However, the vaginal microbiomes of women of African [11] and Hispanic ancestry have higher Shannon diversity than women of European ancestry, and they are less likely to be dominated by *Lactobacillus* species [4]. Four common BV associated species include *Gardnerella vaginalis, Prevotella bivia*, *Atopobium vaginae,* and *Sneathia vaginalis* [12]. With the absence of the antimicrobial compounds produced by *Lactobacillus* like lactic acid, hydrogen peroxide, and bacteriocins, the presence of anaerobic bacteria greatly increases, potentially increasing individual's susceptibility to BV. BV diagnosis requires three of the following criteria, also including discharge: vaginal pH higher than 4.5, positive whiff test, and existence of clue cells on wet mount. Clinically, the Amsel criteria are often used to diagnose BV with at least three of four criteria required for positive diagnosis [13]. The Amsel criteria essentially consists of all criteria mentioned previously with the addition of a thin white or yellow homogeneous discharge. It has been proven that BV leads to a higher risk of STI acquisition and pregnancy complications like preterm birth [10][14][15][16].

V. Preterm birth

Live babies born before pregnancy reaches 37 weeks of gestation are considered preterm. According to WHO, there are subcategories of PTB based upon gestational age: extremely preterm (less than 28 weeks), very preterm (28 weeks to 32 weeks), and moderate to late preterm (32 to 37 weeks). Probability of survival of preterm babies increases as severity of PTB decreases. PTB is considered either spontaneous or medical induction. More than 1 in 10 babies are born preterm, and about 1 million children die annually due to complications stemming from PTB. Children that do survive PTB often experience lifelong challenges including but not limited to learning, visual, and hearing disabilities. There are drastically different survival rates for PTB depending on the economic status of the area where the birth occurred, making it a socioeconomic issue as well as a health issue. In the US, African American infants have the highest rate of PTB (14.2%), followed by American Indian/Alaska Native infants (11.6%), Hispanic American infants (9.8%), White American infants (9.2%), and Asian/Pacific Islander infants (8.8%).

There are various factors that result in PTB outcome of pregnancies including genetic, anatomic, infectious, and others. Furthermore, these factors may impact PTB outcome singularly or in combination. As a potential infectious factor, a relationship between *S. vaginalis* and PTB was confirmed by LefSe model [1]. Bacterial invasion of the normally sterile uterine cavity is the leading cause of early preterm birth. After intrauterine invasion, bacteria can invade further by traversing the chorion or chorion and amnion, leading to chorionitis or chorioamnionitis. Invasion of the amniotic sac can result in a maternal or fetal inflammatory response triggering preterm premature rupture of membranes or labor [17].

VI. Mouse models of pregnancy and PTB

The etiology of PTB is complex and may occur as a result of a combination of various factors including, but not limited to, genes, anatomy, and microbial infection. The use of the animal model provides an ideal environment to look at the role of specific factors, such as *S. vaginalis*, in PTB, by restricting genetic, anatomic, and infectious variability within the controlled system.

In female mice, the estrous cycle (figure 7) lasts 4 to 5 days on average [25]. The estrous cycle determines when mice ovulate, with estrus stage being the only stage in which mice can be impregnated. There are four stages in the estrous cycle: proestrus, estrus, metestrus, and diestrus [18][19]. Mouse estrous stage can be determined by the evident changes in mouse physiology and anatomy [20]. Stage of estrous can be determined using various methods such as evaluating vaginal cytology [19][21][18][22], visual observation of the external genitalia [23], biochemical analysis of urine [24], and measuring electrical impedance [25]. The four stages of estrous vary between 6 and 72 hr, depending on stage. In mice, the proestrus stage lasts less than 24 hr with mostly nucleated epithelial cells, estrus stage lasts between 12 and 48 hr with mostly cornified epithelial cells, metestrus stage lasts up to 24 hr with leukocytes and nucleated and cornified epithelial cells present, and diestrus stage lasts between 48 and 72 hr with mostly leukocytes [26]. Transitional stages are encountered in estrous cycle studies, and there are several strategies that can be used to determine these stages [26].

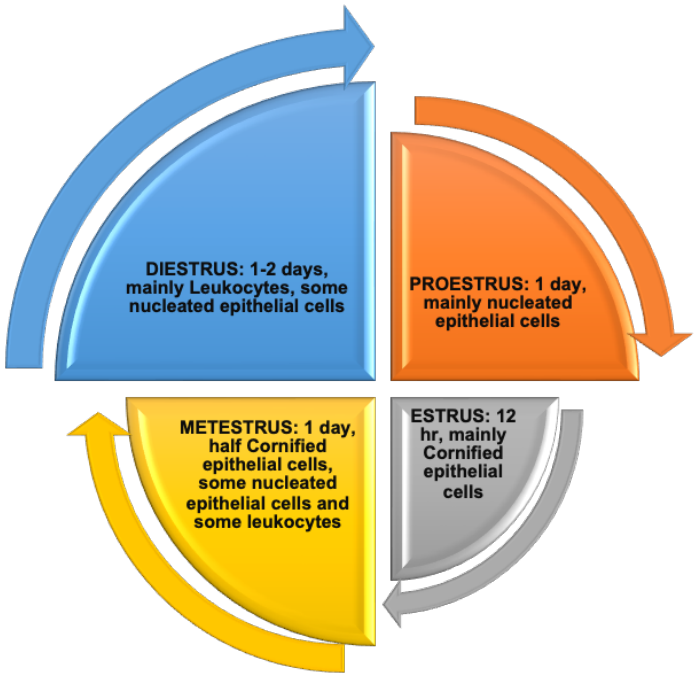


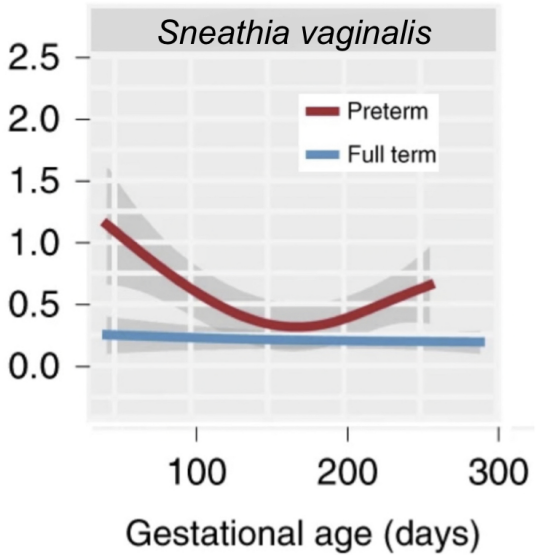
Figure 7. Mouse estrous cycle.
The mouse estrous cycle determines when mice can become pregnant. There are four stages of estrous with vaginal cytology changing as cycle progresses. Proestrus stage is the first stage of estrous, which lasts about 24 hours with mainly nucleated epithelial cells. The second stage of the estrous cycle is estrus, which lasts about 12 hours with mainly cornified epithelial cells. Metestrus stage is the third stage of the estrous cycle, which lasts about 24 hours with about half present cells being cornified epithelial cells, some nucleated epithelial cells, and some leukocytes. The fourth stage of the estrous cycle is the diestrus stage, which lasts 24 to 48 hours with mainly leukocytes and some nucleated epithelial cells.

VII. Introduction to Research and Objectives

In this study, experiments were designed to investigate CptA toxin produced by *Sneathia vaginalis* during vaginal colonization and the host antibody response mounted against the toxin. Samples from 93 participants were strategically selected from the MOMS-PI study to include samples that had term or preterm outcomes with high, low, or no *S. vaginalis* abundance at time of collection. 16S rRNA sequencing of longitudinal samples from participants of the MOMS-PI PTB study led to the hypothesis that *S. vaginalis* presence, especially during early pregnancy [1] (figure 8), has greater potential to result in preterm birth outcome. This hypothesis aligns with results from a study investigating the association between preterm delivery and bacterial vaginosis [27] as *Sneathia* genera is associated with the condition of bacterial vaginosis [28]. The longitudinal study revealed a decline followed by a slight increase in *S. vaginalis* before preterm birth (figure 8) suggesting that the host could be mounting an immune response against *S. vaginalis* as an effort to rid itself of the bacteria. Importantly, there is a difference in *S. vaginalis* abundance throughout pregnancy between individuals of African American or European American ancestry that gave birth preterm (figure 9) with the African American ancestry group resembling the trend of *S. vaginalis* seen in figure 8.

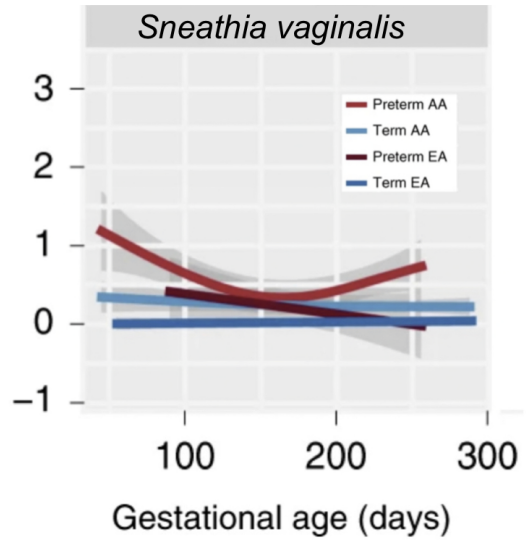
Via indirect ELISA, IgM, IgG, and IgA antibody isotypes in vaginal swab samples with affinity for the NTerm domain of CptA toxin were quantified. The neutralizing ability of anti-CptA host antibodies was also quantified via neutralization assays by incubating NTerm domain of CptA toxin and vaginal swab sample together then adding human RBCs and quantifying released hemoglobin via spectrophotometry. The relationships between *S. vaginalis* abundance and host antibody presence, host antibody presence or *S. vaginalis*

abundance and preterm birth, and host antibody presence and CptA toxin neutralization were statistically analyzed. Before experimentation, the potential relationship between host antibody presence and birth outcome was unclear. It was hypothesized that host antibody presence could signify clearing of infection resulting in TB outcomes or progression of infection resulting in PTB outcomes. Regarding host antibody activity, it was hypothesized that samples containing host antibodies would have greater neutralizing ability against the NTerm domain of CptA toxin than samples that were negative of host antibody to the toxin. To examine *S. vaginalis* pathogenicity in a controlled system, a mouse model of colonization was optimized.



Adapted from Fettweis et al. [1]

Figure 8. In pregnancies resulting in preterm birth, S. vaginalis relative abundance declines during pregnancy and then increases prior to birth.
The relative abundance values (y-axis) of S. vaginalis from longitudinal samples were plotted against gestational days at the time of sampling (x-axis) in preterm and full term groups.



Adapted from Fettweis et al. [1]

Figure 9. Longitudinal analysis of *S. vaginalis* during gestation in preterm and full term groups according to African American or European American ancestry
The relative abundance values (y-axis) of S. vaginalis from longitudinal samples were plotted against gestational days at the time of sampling (x-axis) in preterm and full term groups. Groups were separated based on self-reported ethnicity.

Chapter 2

Methods

Study design

Mid vaginal swab samples from the MOMS-PI PTB sub-study [1] were selected based on gestational age at delivery and relative abundance of *S. vaginalis*. All samples were previously subjected to 16S rRNA survey analysis. Samples tested consisted of 25 TB samples with no *S. vaginalis*, 25 TB samples with at least 0.01% *S. vaginalis* abundance relative to other bacterial taxa present, 22 PTB samples with no *S. vaginalis*, and 21 PTB samples with at least 0.01% *S. vaginalis* abundance relative to other bacterial taxa present. Indirect enzyme-linked immunosorbent assays and neutralization assays were performed to examine the presence and impact of host antibodies, IgG, IgM, or IgA, present in vaginal swab sample.

Sample collection

As part of the MOMS-PI study, vaginal samples were collected from participants at study enrollment, then longitudinally at each prenatal visit, at triage, at labor and delivery, and postpartum follow-up visits [1]. All swab samples were collected with BD BBL CultureSwab EZ swabs [1]. Participants were allowed to self-sample as self-sampled samples appeared to be equivalent to those collected by trained clinicians [1]. Vaginal swab samples were suspended in solution and stored at – 80 ^{O}C.

Maintenance of *S. vaginalis*

The Sn35 strain of *S. vaginalis* was described previously [3]. It is cultivated in porcine brain heart infusion (BHI) culture media supplemented with 5% fetal bovine serum at $37^{O}C$ under anaerobic conditions. In certain studies, solid and liquid media were supplemented with 5 µg ciprofloxacin /ml to prevent the growth of contaminants.

Plating of mouse vaginal swabs

Vaginal swabs from Sn35 mouse model were collected and immersed in 300 µl of PBS in a microcentrifuge tube. These tubes were vortexed to ensure thorough suspension of bacteria in PBS. From 300 µl bacteria suspension, ten-fold serial dilutions were made out to 1:10,000 bacteria suspension in PBS. Ten µl of non-diluted suspension, followed by dilutions, were placed on sBHI plates containing 5 µg ciprofloxacin /ml by the drop plate method. These plates were placed upside down to avoid contamination from condensation, in anaerobic conditions, at 37 °C and allowed to incubate for two days. After incubation, Sn35 CFU are counted.

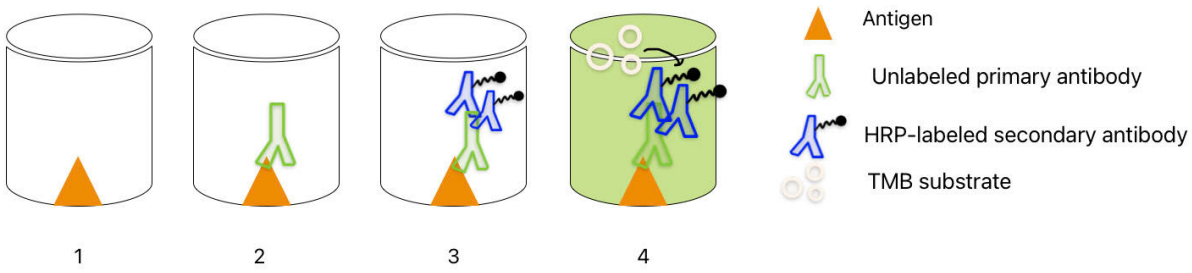
NTerm recombinant protein expression, extraction, and purification

For this study, the protein produced from the pore-forming domain of the *cptA* gene was used often as it displays the same antigenic properties as full length CptA protein. This fragment is referred to as NTerm. NTerm protein fragment of CptA toxin was expressed, extracted, and purified over 2 days [8]. On day 1, 50 ml of LB broth was made in a 250 ml baffled Erlenmeyer flask and 250 ml of LB broth was made in two 2L baffled Erlenmeyer flasks each. The three flasks were autoclaved for 30 mins at 121°C. Once the media was cooled, 50 µl of ampicillin and 50 µl of chloramphenicol were added to the 250 ml baffled Erlenmeyer flask and this flask was inoculated with *E. coli* BL21. This flask was put in the shaker and allowed to shake overnight at 37 °C. The next morning, 250 µl of ampicillin and 250 μl of chloramphenicol was added to each of the 2L baffled Erlenmeyer flasks, then both flasks were inoculated with 15 ml of the overnight culture. The two flasks were allowed to shake at 37 °C for one hr and 45 mins. After an hour and 45 mins, the shaker lid was opened and the heat was turned off. Then, the two flasks were allowed to shake for 30 mins at room temperature, RT. For protein induction, 1 mM IPTG was added to both flasks then they were allowed to shake at RT for 2 hr and 30 mins. When there were only 20 minutes left before the completion of incubation, two centrifuge bottles were prechilled at 2 °C, 6,800 rcf for 15 mins. After protein induction, both cultures in the 2L baffled flasks were poured into one of the prechilled centrifuge bottles and the bottle was filled with distilled water until the volume was over halfway the height of the bottle. The second centrifuge bottle was used as a blank to balance the centrifuge bottle containing the two cultures. The two bottles were spun in the centrifuge for 15 mins at 2 °C, 6,800 rcf. During centrifugation, a lysis buffer was made in a 50 ml centrifuge tube by combining 25 ml of complete Resin buffer [8], 1 Complete protease inhibitor tablet [8], 25 μl of Triton X-100, and 5 μl of B-ME. This was mixed

by end-over-end rotation then placed on ice to be used for resuspension of the centrifuge bottle pellet. Another lysis buffer was made by combining 50 ml of Complete Resin buffer A and 10 μl of B-ME. After centrifugation, as much media was removed from the centrifuge bottle as possible by pouring media out and tapping the rim of the bottle on a paper towel. The remaining pellet was resuspended in the lysis buffer that was put on ice, then one gram of lysozyme (14,388 g/mol) was added and this combination was put in a 50 ml centrifuge tube on ice. The rotor was set to prechill for 15 mins at 2 °C, 27,000 rcf. Using the French pressure cell, bacteria in the 50 ml centrifuge tube were lysed by passing the combination through the French pressure cell 3 times at 2000 PSI. This lysate was centrifuged for 15 mins at 2 °C, 27,000 rcf in the rotor. While spinning, a column was prepared for affinity chromatography. To do so, 500 μl of Complete resin [8] was put into a clean column and allowed to drain. Next, 10 ml of deionized water was added to the column and allowed to drain. Following, 10 ml of Complete buffer [8] was added to the column and allowed to drain. After centrifugation, the clarified lysate or supernatant, was transferred to a 50 ml centrifuge tube while making sure to avoid the pellet. The resin beads in the column were resuspended with 5 ml of the clarified lysate and the contents were transferred back to the 50 ml tube. The 50 ml tube was incubated on ice for 15 mins with occasional mixing by inversion. The lysate and beads were put back in the column and allowed to drain. The column was then washed four times with Complete Wash buffer containing B-ME. Then, the column was washed once with 150 μl of 10mM imidazole and Complete Wash buffer containing B-ME. Elution was done by adding Complete Wash buffer containing B-ME and 250 mM imidazole to the column, 250 μl at a time, and collecting the 250 μl in separate 1.5 mL microcentrifuge tubes. At least 8 fractions were collected. Lastly, the protein concentrations of the different fractions were determined by nanodrop.

ELISA protocol

To quantify the amount of host antibodies present that have binding affinity to CptA toxin, indirect enzyme-linked immunoassays, or ELISAs, were performed on all vaginal swab samples for IgG, IgA, and IgM. A 96-well polystyrene plate was coated overnight at 4 °C with 200 μl of NTerm domain CptA protein/PBS at consistent concentration of 1.86 mg/ml (figure 10.1). On the next day, the plate was inverted to remove the toxin and washed three times with PBST. Each wash during the ELISA protocol was completed by filling wells completely with PBST then inverting. After three washes, 200 μl of PBS with 5% skim milk were put on wells for 1 hr at 37 °C to block. After blocking, the plate was inverted to remove milky PBS then filled in column sets of 3 wells per row with 90 μl of PBST in for dilution of primary antibody of controls. In each row, 10 μl of undiluted vaginal swab sample were added to the first well and serially diluted in each column ten-fold to 1:1,000 as primary antibody (figure 10.2). For positive controls, undiluted or 1:1000 dilution of goat, anti-rabbit anti-CptA were added to a well and diluted out as previously stated. For a negative control, 10 µl of PBST were added to a well. After 1hr RT incubation, the plate was inverted and washed three times. After the third wash, the plate was "smacked" face-down against stacked paper towels to ensure the removal of primary antibody from wells. Then, 100 μl of 1:200 anti-human IgA, IgG, or IgM-HRP in PBST were added to all tested wells as secondary antibody conjugate (figure 10.3). After another hour of RT incubation, the plate was inverted and washed six times striking the plate as previously described after the last two washes to ensure removal of secondary antibody. Lastly, 50 μl of TMB substrate solution was put on tested wells for 15 min max. incubation. Then, 50 μl of 0.2 mM sulfuric acid were added to wells as STOP solution (figure 10.4) and immediately measured at OD450 nm via spectrophotometry.



Antigen: CptA fragment 24-1349

Unlabeled primary antibody: anti-CptA antibodies in vaginal swab sample

HRP-labeled secondary antibody: secondary anti-human IgM, IgA, or IgG- HRP

Figure 10. Indirect ELISA schematic
The ELISA is performed in a 96-well immune plate. To begin, 10.1) 96 well plate is coated with antigen. Then, vaginal swab sample is added to wells, where 10.2) unlabeled primary antibody binds to antigen if there is affinity. Next, 10.3) HRP-labeled secondary antibody is added to wells, where secondary antibody binds to primary antibody if there is affinity. Lastly, 10.4) TMB substrate (Promega corporation) is added to wells to form colored product after reaction with horseradish peroxidase, HRP, label.

Neutralization protocol

To analyze the ability of anti-CptA antibodies in vaginal swab samples to neutralize the toxic activity of CptA, hemolysis assays were performed by adding vaginal swab sample to NTerm fragment of CptA toxin and quantifying the hemolytic activity of NTerm (figure 11). Human RBCs were collected from the same source for consistency. In a 1.5 ml microcentrifuge tube, 500 µl of RBCs were added to 1 ml of PBS for washing of RBCs. To wash RBCs, a 1:2 dilution of RBCs in PBS was spun at 500 rcf for 7 mins and 30 seconds. After centrifugation, 1 ml of the supernatant was pipetted and discarded from the tube, completing one full wash, then 1 ml of PBS was added to the tube for another wash. The RBCs underwent three total washes with PBS to ensure lysed RBCs or free hemoglobin were removed before experimentation. After the third wash, washed RBCs were resuspended in 1 ml of PBS with the tube final volume totaling 1.5 ml. This tube was used as the RBC stock for experimentation. Individual vaginal swab samples were added to 1.5 ml microcentrifuge tubes at quantities of 5 µl. A consistent concentration of NTerm, 0.53 mg/ml, was added to each microcentrifuge tube at a quantity of 10 µl and allowed to incubate together for 15 mins at RT. After incubation, 100 µl of RBC stock were added to each tube and allowed to incubate for 2 hr in 37 °C water bath. After incubation, the tubes were gently shaken by hand then centrifuged for 7 mins and 30 secs at 500 rcf. After centrifugation, RBCs and free hemoglobin supernatant were separated. In a 96 well polystyrene plate, 90 µl of deionized water were added to wells that will be tested. Without disturbing the RBC pellet, 10 µl of free hemoglobin supernatant was placed in 90 µl of deionized water on 96 well polystyrene plate. The plate is then read at OD405 nm via spectrophotometry.

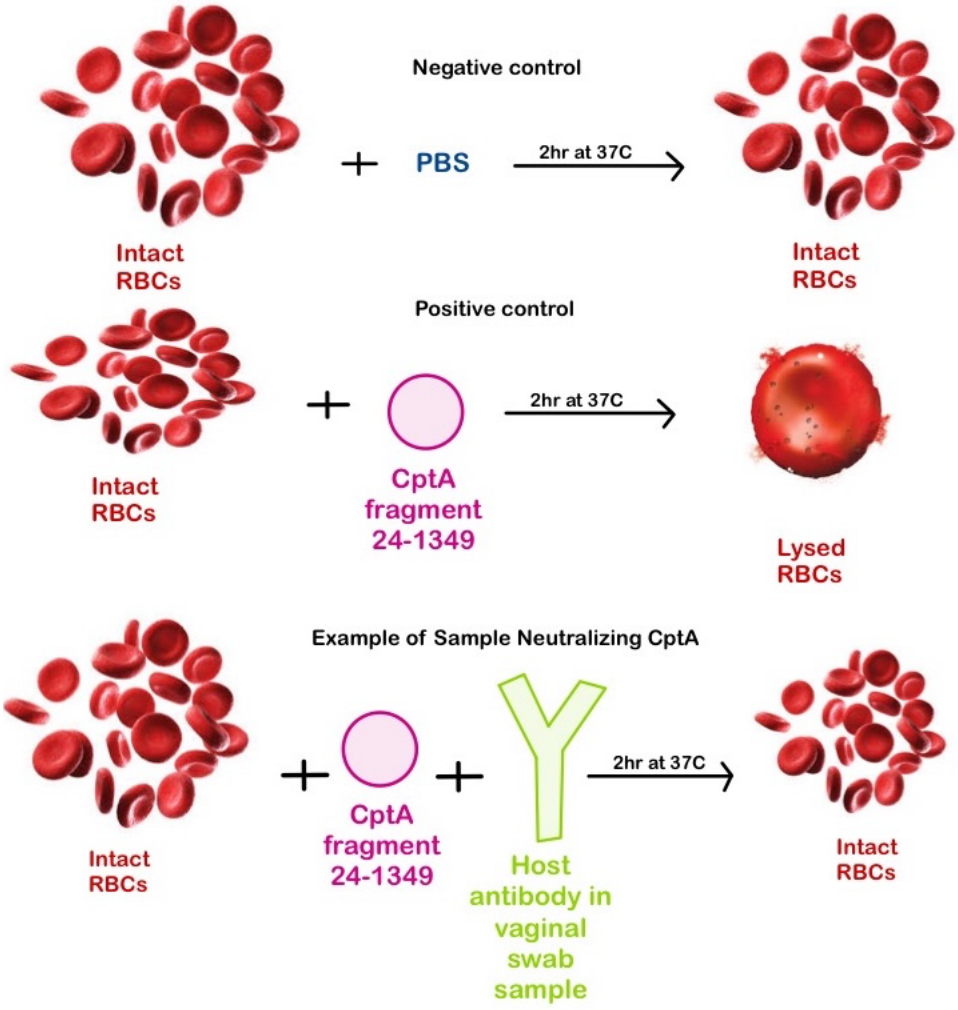


Figure 11. Neutralization assay schematic

Neutralization assays were performed in microcentrifuge tubes to determine if host anti-CptA antibodies in samples would be capable of neutralizing CptA toxin. Negative control (PBS), positive control (CptA fragment 24-1349 "NTerm"), or vaginal swab sample and CptA 24-1349 "NTerm" were incubated with human red blood cells for 2 hours at 37 °C to assess host anti-CptA antibodies ability to neutralize CptA toxin. Human RBCs should remain intact in the negative control and in samples that contain CptA neutralizing host antibodies. Human RBCs should be lysed in the positive control and in samples that do not contain CptA neutralizing host antibodies.

Sample results

Table 1. Sample results from ELISAs and Neutralization Assays

Sample ID	IgM	IgM	IgG	IgG	IgA	IgA	Percent hemolysis	Relative Sn abundance	Preterm/Term
51	0.0	0.0	0.115	0.054	0.017	0.051667		0.17%	T
45	0.0	0.0	0.206	No sample	0.06	0.054667		0.00%	P
37	0.0	0.0	0.04	-0.008	0.068	0.071667		0.98%	T
62	0.0	0.0	0.168	0.139	0.087	0.036667		0.02%	P
1	0.0	0.0	0.203	0.575	0.237	0.138667		0.00%	P
PBS	0.0	0.0							
23	0.0	0.0	0.151	0.078	0.1	0.019667	14.23%	0.00%	P
73	0.1	0.0	0.059	0.001	0.03	-0.04633	120.64%	0.00%	T
PBS	0.0	0.0							
59	0.0	0.0	0.007	-0.007	-0.00467	1.6		2.19%	P
26	0.0	0.0	0.133	0.102	0.2653	0.436667		0.00%	T
28	0.0	0.0	0.206	0.333	0.4713	0.449667	-0.07%	0.00%	P
13	0.0	0.0	0.154	0.023	0.0	0.0		0.00%	T
49	0.0	0.0	0.029	0.005	0.0	0.0		0.00%	P
44	0.0	0.0	0.133	0.031	0.0563	0.0		0.76%	P
9	0.0	0.0	0.163	0.09	0.1323	0.122667		0.00%	T
71	0.0	0.0	0.025	0.015	0.0	0.0		3.21%	P
52	0.0	0.0	0.247	0.123	0.0	0.0		3.54%	P

92	0.0	0.0	0.102	0.059	0.0	0.0		0.00%	P
PBS	0.0	0.0							
30	0.0	0.0	0.045	0.014	0.0	0.0		0.00%	T
7	0.0	0.0	0.108	0.078	0.0	0.0	61.76%	0.00%	P
64	0.0	0.0	-0.005	0.051	0.0	0.0	75.75%	2.87%	T
69	0.0	0.0	0.208	0.243	0.3543	0.299667		0.00%	T
66	0.0	0.0	0.012	0.087	0.0	0.0		12.85%	T
83	0.0	0.0	0.064	No sample	0.2	0.1		0.00%	T
4	0.0	0.0	0.014	0.236	0.0	0.0		0.00%	T
35	-0.1	0.0	0.208	0.774	0.1	0.283667	6.68%	0.00%	P
PBS	0.0								
anti-CptA	0.3	0.6							
anti-CptA 1/1000	0.0	0.0							
PBS	0.0	0.0							
24	0.0	0.0	0.196	0.187	0.04633	0.058667		0.19%	P
77	-0.1	0.1	0.235	0.153	0.2133	0.233667	2%	9.96%	P
10	0.0		0.049	-0.001	0.04	0.008		0.00%	P
61	0.0		0.094	0.016	0.05	0.007	50.41%	9.47%	T
86	0.0		0.098	0.053	0.067	0.036		0.00%	P
PBS	0.0								
88	0.0		0.333	0.413	0.2	0.1		0.00%	P
47	0.0		0.342	0.291	0.3	0.4		0.48%	P
PBS	0.0								

3	0.0		0.266	0.164	0.053	0.041		0.00%	T
85	0.0		0.006	-0.019	0.058	0.087		24.32%	P
58	0.0		0.089	0.027	0.054	0.029	43.14%	22.88%	P
63	0.0		0.137	0.101	0.005	-0.027		0.00%	T
17	0.0		0.093	0.02	0.067	0.011	77.83%	0.05%	T
25	0.0		0.12	0.008	0.106	0.035		0.29%	T
81	0.0		0.104	0.073	0.158	0.118		0.00%	T
32	0.0		0.306	0.237	0.075	0.005		0.00%	T
67	0.0		0.059	-0.009	0.032	0.001		2.33%	P
79	0.0		0.095	0.075	0.034	-0.006		0.00%	P
PBS	0.0								
14	0.0		0.049	-0.008	0.013			0.00%	T
57	0.3	0.1	0.062	0.026	0.118	0.17	-1.14%	19.17%	T
12	0.0		0.139	0.041	0.354	0.31		0.00%	P
2	0.0		0.238	0.57	0.107	0.028		0.00%	T
29	1.0	0.0	0.008	0.198	0.023	0.002	93.00%	0.00%	P
46	0.0		0.109	0.228	-0.002	0.041		0.27%	P
60	0.0		0.131	0.222	-0.003	0.032		2.87%	T
42	0.1	0.0	0.413	0.517	0.253	0.17	55.50%	3.34%	T
PBS	0.0								
antiCptA	1.1								
antiCptA 1/1000	0.0								
PBS	0.0								
38	0.0		0.207	0.005	0.007	0		0.03%	T

31	0.0		0.339	0.082	0.095	0.105		1.73%	T
11	0.0	0.0	0.297	0.252	0.213	0.174		0.00%	T
18	0.0	0.0	0.227	0.142	0.053	0.081		0.00%	T
54	0.0	0.0	0.06	0.024	0.0822	0.019		6.46%	T
PBS	0.0	0.0							
27	0.0	0.0	0.008	0.008	0.0832	0.037		0.00%	P
87	0.0	0.0	-0.013	0.038	0.0172	0.013		0.00%	T
PBS	0.0	0.0							
68	0.0	0.0	0.021	0.013	0.0112	0.018	68.82%	0.00%	P
80	0.0	0.0	0.051	0.031	0.0512	0.01		0.00%	T
48	0.0	0.0	0.144	0.097	0.2352	0.076		0.00%	T
53	0.1	0.0	0.237	0.115	0.3292	0.206	1.33%	0.02%	T
70	0.0	0.0	0.257	0.116	0.1702			0.04%	P
15	0.0	0.0	-0.009	0.001	0.0472	0.0415		0.72%	T
40	0.0	0.0	-0.012	0.257	0.2722	0.097		0.00%	T
8	0.0	0.0	0.335	0.065	0.0482	0.006		0.08%	T
72	0.0	0.0	0.128	0.171	0.7262	0.265		0.07%	P
55	0.0	0.0	0.151	0.094	0.1662	0.06		0.00%	P
PBS	0.0	0.0							
50	0.0	0.2	0.165	0.049	0.1422	0.065		3.82%	T
76	0.0	0.0	0.165	0.046	0.0472	0.0425		0.00%	P
36	0.0	0.0	-0.01	0.231	-0.022	-0.0055		0.14%	T
19	0.0	0.0	0.24	-0.005	0.01	0.0215		0.00%	T

65	0.4	0.3	0	0.176	0.1792	0.112	-0.11%	3.93%	T
41	0.0	0.0	0.121	0.081	0.0032	-0.005		0.00%	P
21	0.0	0.0	0.061	0.102	0.0122	0.007		1.69%	T
89	0.0	0.0	0.2	0.162	0.0082	-0.003		0.00%	T
PBS	0.0	0.0							
a-CptA UD	0.5	0.7							
a-CptA 1/1000	0.0	0.0							
PBS	0.0	0.0							
20	0.0	0.0	0.132	-0.007	0.0102	-0.003		0.01%	P
22	0.1	0.2	0.031	0.14	0.2062	0.112	-2.57%	7.39%	P
39	0.0	0.0	0.251	-0.028	0.0042	0.012	7.24%	1.35%	T
16	0.0	0.0	0.138	0.114	0.4362	0.277		0.87%	P
34	0.0	0.0	0.077	0.091	0.2102	0.12		0.73%	P
PBS	0.0	0.0							
84	0.0	0.1	0.02	0.07	0.0742	0.037		0.00%	P
90	0.0	0.0	0.011	0.063	0.057	0.011	55.18%	0.00%	P
PBS	0.0	0.0							
82	0.0	0.0	0.424	0.303	0.082	0.042	2.40%	2.50%	T
91	0.0	0.0	0.112	-0.032	0.093	0.072		0.00%	T
56	0.0	0.0	0.2	0.066	0.07	-0.036		0.00%	P
74	0.0	0.0	-0.005	0.037	0.108	0.119		0.01%	T
5	0.0	0.0	-0.023	0.01	0.074			0.01%	P
x	0.0	0.1	0.049	0.121	0.16			41.64%	P
6	0.4	0.3	0.374	0.28	0.075		0%	0.01%	T
75	0.0	0.0	0.07	0.164	0.402			0.00%	T

xx	0.0	0.0	-0.104	0.077	0.046			4.62%	T
PBS	0.0	0.0							
43	0.0	0.0	0.049	0.034	0.405			0.00%	T
93	0.0	0.0	-0.075	-0.031	0.077		7.92%	0.04%	P
PBS	0.0	0.0							
PBS	0.0	0.0							
a-CptA UD	1.0	0.7							
a-CptA 1/1000	0.0	0.0							

Chapter 3

Analysis of Anti-CptA Antibodies in Vaginal Fluid

Introduction

S. vaginalis commonly colonizes the vagina but it is not known whether it expresses the CptA toxin during colonization. In order to determine whether or not CptA is expressed during colonization and to investigate the mucosal immune response to S. vaginalis, swab samples were tested for anti-CptA IgM, IgG, or IgA via indirect ELISA, and host anti-CptA antibodies were tested for neutralizing ability of CptA toxin via neutralization assay. In this cohort, the relationships between host anti-CptA antibody and *S. vaginalis* abundance, host anti-CptA antibodies and neutralizing ability, and *S. vaginalis* abundance or host antibody response and PTB were statistically analyzed.

Interestingly, IgM positive samples resulted in TB. This could be due to IgM, associated with early infection, ability to neutralize. To investigate this idea, *S. vaginalis* abundance across longitudinal sampling of the IgM positive samples was determined to analyze the rise and decline of *S. vaginalis* through gestation to birth.

Results

I. Host anti-CptA antibody relationship with *S. vaginalis*

i. Qualitative analysis of host antibody presence

The relationship between host antibody presence and *S. vaginalis* abundance was examined (figure 12). Samples were considered Ig absent, Ig present (regardless of class), and isotype specific Ig present in relation to *S. vaginalis* abundance in samples. Samples were considered "Ig present" if ELISA OD ≥ 0.1 for any Ig isotype when tested. Samples were considered "Ig isotype present" if ELISA OD ≥ 0.1 for specific isotype. Any samples that failed to reach limit of detection, ELISA OD ≥ 0.1, for any Ig class were considered "Ig absent" (table 1). *S. vaginalis* abundance was quantitatively determined by 16S rRNA sequencing of vaginal swab samples upon collection. Kruskal-Wallis (unpaired t-test) test revealed no significant association between Ig presence and *S. vaginalis* abundance. In addition, when Ig presence/absence was compared to *S. vaginalis* presence/absence rather than abundance, Fisher's exact test revealed no significance.

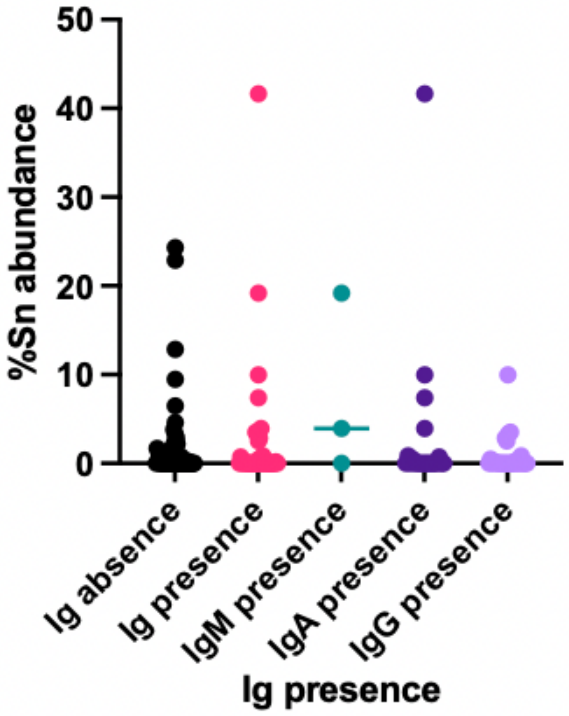


Figure 12. Qualitative analysis of host antibody presence relative to *S. vaginalis.*

The relationship between host antibody presence and *S. vaginalis* relative abundance at time of sample collection was analyzed. For this test, *S. vaginalis* abundance was analyzed quantitatively and host Ig presence was analyzed qualitatively. Kruskal-Wallis test revealed a p-value of 0.4506, not reaching significance.

ii. Qualitative analysis of Ig isotype presence

The relationship between host antibody presence specific to isotype and *S. vaginalis* abundance was examined (figure 13). Samples were considered if they were either Ig absent or had the Ig presence of only one isotype. Samples were considered "Ig isotype present" if ELISA OD ≥ 0.1 at least twice for specific Ig class. Furthermore, only samples that met "Ig presence" requirements for only one Ig class were selected. Any samples that failed to reach limit of detection , ELISA OD ≥ 0.1, at least twice for any Ig class were considered "Ig absent". The same Ig absent samples from the previous host antibody and *S. vaginalis* abundance analysis were used. *S. vaginalis* abundance was quantitatively determined by 16S rRNA sequencing of vaginal swab samples upon collection. Kruskal-Wallis test revealed no significance between Ig presence of singular Ig isotype and *S. vaginalis* abundance.

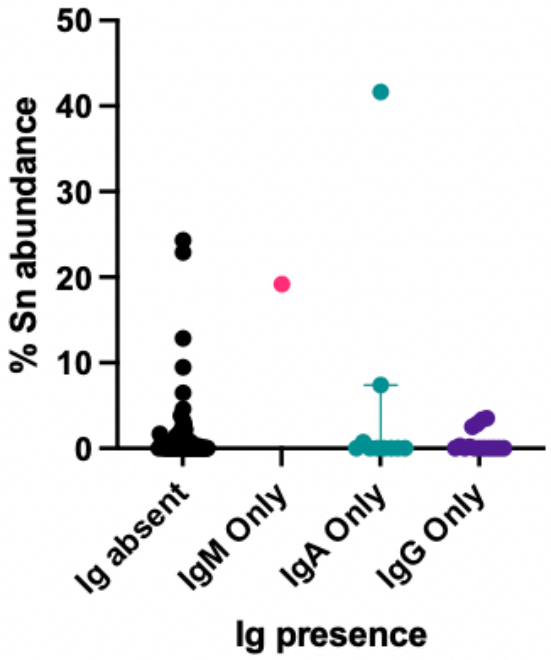


Figure 13. Qualitative analysis of Ig isotype presence relative to *S. vaginalis*
The relationship between host antibody presence of single isotype and *S. vaginalis* relative abundance at time of sample collection was analyzed. For this test, *S. vaginalis* abundance was analyzed quantitatively, and singular host Ig isotype presence was analyzed qualitatively. Kruskal-Wallis test revealed a p-value of 0.3856, not reaching significance.

iii. Ig absence when *S. vaginalis* fails to decline

After obtaining longitudinal *S. vaginalis* relative abundance for samples tested in this cohort, a trend relating to *S. vaginalis* abundance and Ig presence or absence. In most participant samples with Ig presence, according to ELISA results, there was a decline in *S. vaginalis* at the sample collection before the sample collection that underwent ELISA. However, participant samples that did not appear to experience a decrease in *S. vaginalis* abundance in the sample collection before the sample collection that underwent ELISA, have Ig absent ELISA results (figure 14).

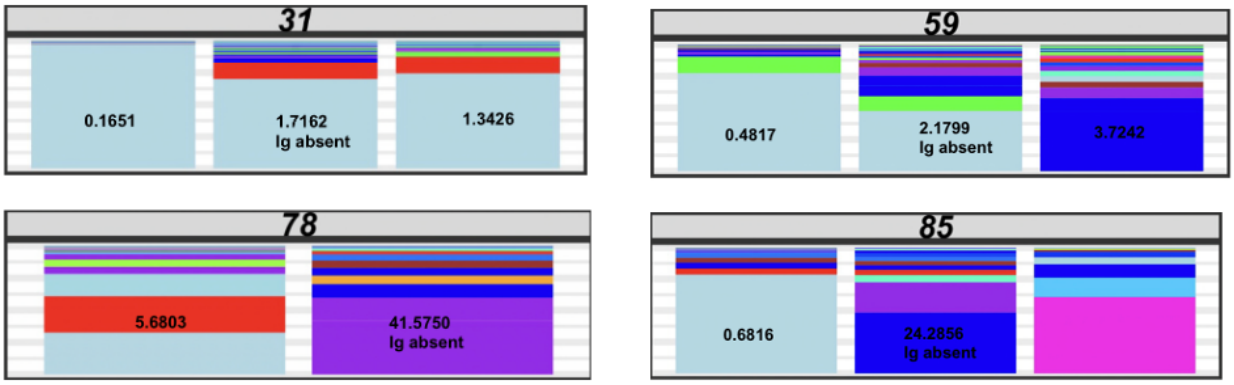


Figure 14. Negative Ig results in host samples when *S. vaginalis* fails to decline in the sample visit previous to the sample visit that is ELISA tested

Samples 31, 59, 78, and 85 were selected to display the lack of Ig presence when *S. vaginalis* does not decrease. In sample 31, *S. vaginalis* relative abundance increases from 0.1651 to 1.7162 with negative Ig results from ELISA. In sample 59, *S. vaginalis* relative abundance increases from 0.4817 to 2.1799 with negative Ig results from ELISA. In sample 78, *S. vaginalis* relative abundance increases from 5.6803 to 41.5750 with negative Ig results from ELISA. In sample 85, *S. vaginalis* relative abundance increases from 0.6816 to 24.2856 with negative Ig results from ELISA.

II. Host antibody ability to neutralize CptA toxin

i. Quantitative analysis of host antibody neutralizing activity

Assessment of the ability of host antibody to neutralize the pore-forming activity of CptA (figure 15). 22 samples were chosen from this cohort, 10 with Ig present and 12 without Ig presence as determined by ELISA results. was used for hemolysis assay. NTerm fragment CptA 24-1349, previously displayed to have hemolytic activity (figure 6c), was pretreated with 10 samples containing anti-CptA IgG, IgM, or IgA, and 12 samples below the limit of detection by ELISA. Following incubation of CptA24-1349 with the samples, the hemolytic activity relative to untreated toxin was determined. Of the 10 samples with detectable anti-CptA (any isotype), 9 reduced the hemolytic activity by more than 50%, while only 1 failed to neutralize to this level whereas out of the 12 samples without detectable antibody, only 4 neutralized to this level while 8 failed to neutralize (Fisher's exact test two-sided p-value- 0.0115). Spearman correlation determined that there was a significant association between the percent hemolysis and the quantitative value by ELISA for both IgM and IgA with two-sided p-values- 0.0467 and 0.0029 respectively at 95% confidence but there was not a significant association between reduction in hemolysis and pre-treatment with vaginal swab samples containing no detectable antibody.

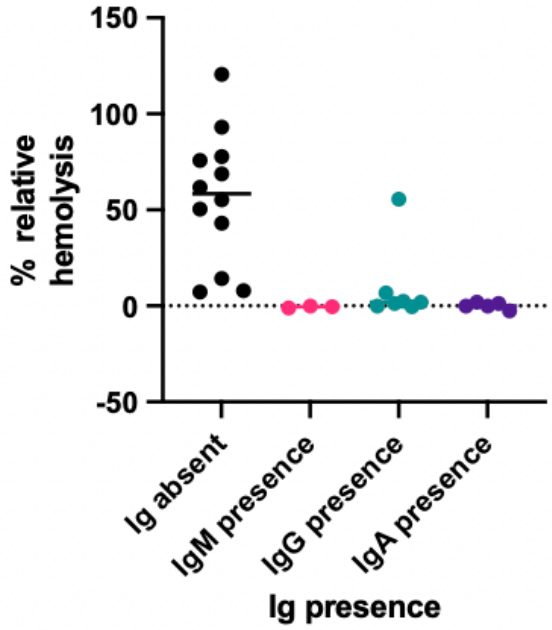


Figure 15. Quantitative analysis of host antibody neutralizing activity.
The ability of host anti-CptA antibodies to neutralize CptA was analyzed by quantitatively evaluating neutralizing activity, percent relative hemolysis, and qualitatively evaluating host antibody isotype presence. IgM and IgA positive samples neutralize CptA to nearly 0% relative hemolysis. IgG positive samples follow a similar trend with one outlier at around 55% relative hemolysis. Kruskal-Wallis test revealed a p-value of 0.0003, reaching significance.

ii. Qualitative analysis of host antibody neutralizing activity

A qualitative analysis of host antibody ability to neutralize CptA toxin was conducted using the same neutralization assay data as the quantitative analysis (figure 16). However, neutralization and Ig presence were considered "yes/no" categories for the vaginal swab samples. Samples that reached ELISA OD ≥ 0.1 at least twice when tested were considered Ig present while those that failed to meet limit of detection were considered Ig absent. Samples that resulted in $<$ 50% hemolysis relative to NTerm alone were considered to have neutralization ability while those that resulted in $\geq$ 50% hemolysis relative to NTerm alone were not. Of 10 samples considered to have Ig present, only one did not neutralize. 66.67% of samples with Ig absent were unable to neutralize. Fisher's exact test suggested that there is a significant relationship between sample Ig presence and sample ability to neutralize, two-sided p-value- 0.0115. This test supports the idea that host samples considered positive for antibody presence would have greater neutralizing ability against NTerm region of CptA toxin than those considered negative.

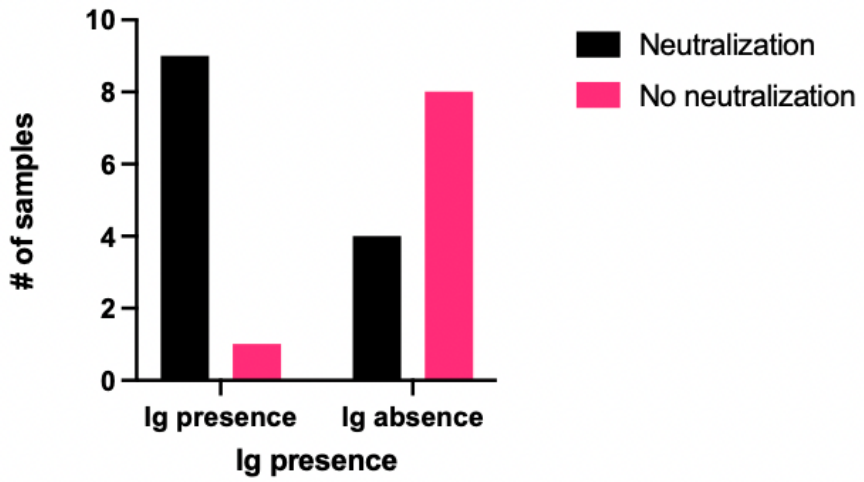


Figure 16. Qualitative analysis of host antibody neutralizing activity

The ability of host anti-CptA antibodies to neutralize CptA was analyzed by qualitatively evaluating neutralizing activity and qualitatively evaluating host antibody isotype presence. Out of 10 samples considered Ig present, 9 were considered able to neutralize CptA toxin. Fisher's exact test revealed a p-value of 0.0115, reaching significance.

III. *S. vaginalis* and host antibody relationship with birth outcome

i. *S. vaginalis* abundance relative to birth outcome

LefSe model confirmed a relationship between *S. vaginalis* abundance and risk of preterm birth [1], so the relationship was examined to see if significance would be met in this cohort (figure 17). *S. vaginalis* relative abundance was determined via 16S rRNA sequencing and compared to the birth outcome of the same participant. All participants were pregnant upon swab sample collection, and the pregnancy outcomes were categorized as term, ≥ 39 weeks, or preterm, < 32 weeks. Though the three samples with the highest *S. vaginalis* abundances had PTB outcome, a significant relationship between PTB and *S. vaginalis* abundance was not found in this cohort.

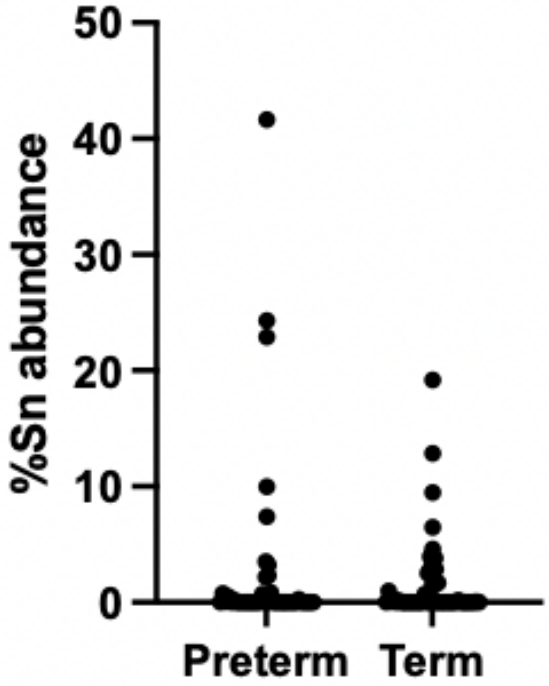


Figure 17. *S. vaginalis* abundance relative to birth outcome
The relationship between *S. vaginalis* abundance and preterm or term birth outcome was analyzed by quantitively considering *S. vaginalis* relative abundance at time of collection. Mann-Whitney test revealed a p-value of 0.8742, not reaching significance.

ii. Host antibody response relative to birth outcome

The relationship between PTB outcome and host antibody presence was analyzed qualitatively (figure 18). All participants were pregnant upon swab sample collection, and the pregnancy outcomes were categorized as term, ≥ 39 weeks, or preterm, < 32 weeks. Chi-square test for trend determined no significance between Ig presence and PTB outcome. However, it is notable that all samples, 3, positive for IgM resulted in TB outcome.

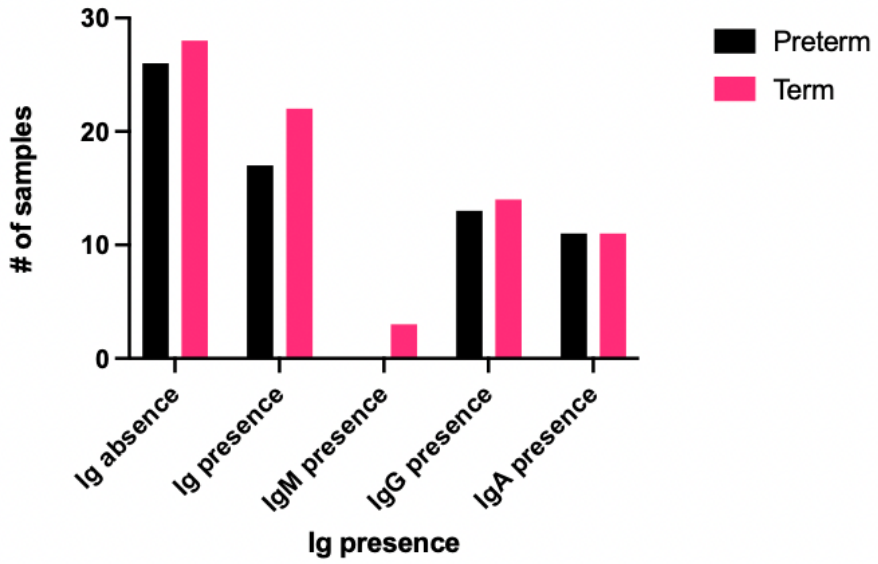


Figure 18. Host antibody response in relation to birth outcome.
The relationship between Ig presence and preterm or term birth outcome was analyzed by qualitatively considering Ig presence. Chi-square test for trend revealed a p-value of 0.9018, not reaching significance.

IV. Longitudinal study from IgM positive samples

The longitudinal samples collected from samples that tested positive for IgM were examined (figure 19). Each column bar present for samples represent sample collection. The column bars are in order, left to right, from earliest swabs collected to latest. Columns have relative *S. vaginalis* percentage or no *S. vaginalis* detected. Columns from samples apart of this study have IgM ELISA OD displayed. Figures 19a and 19b are IgM positive samples that appear to follow a similar trend. The samples appear to have very little, almost zero, *S. vaginalis* present relative to the other bacterial taxa present at collection in early pregnancy. Mid way through pregnancy, *S. vaginalis* presence is not detected. Towards the end of pregnancy, *S. vaginalis* relative abundance slightly increases. Figure 19c represents an IgM positive sample that does not follow the same trend as samples in figures 19a and 19b. In figure 17c, *S. vaginalis* relative abundance (19-27%) reaches a greater relative abundance than the other IgM positive samples (<0.1%).

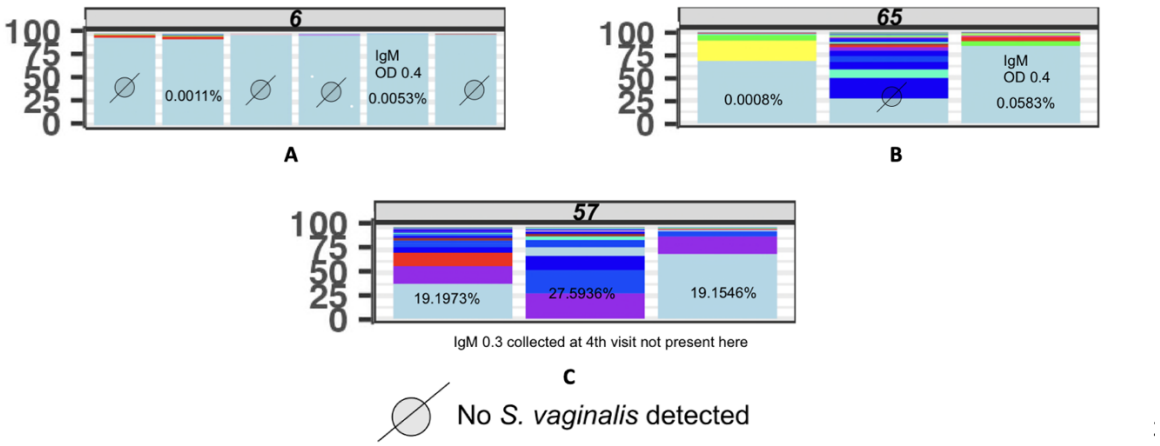


Figure 19. In most IgM positive samples, *S. vaginalis* relative abundance decreases to 0% in mid pregnancy The relative abundance of *S. vaginalis* was longitudinally observed for samples with consistent positive IgM results via ELISA. In samples 6 (19a) and 65 (19b), there is a phenomenon of nearly 0.001% relative abundance of *S. vaginalis* at early visits followed by a decline to 0% relative abundance in mid visits. In later visits, *S. vaginalis* relative abundance increases. This phenomenon is not present in sample 57 (19c). All three IgM positive samples resulted in TB outcome.

Discussion

For the first time, our results confirmed expression of CptA toxin in vivo and host immune response against CptA toxin in vivo. Results also confirmed a significant relationship between host antibody, specifically IgM and IgA, presence in sample and sample ability to neutralize CptA toxin. In two of three IgM positive samples, *S. vaginalis* presence appeared to be cleared during mid pregnancy with an increase in *S. vaginalis* abundance near birth. Notably, *S. vaginalis* relative abundance in the two samples never reached a percentage greater than 0.06%.

Chapter 4

Optimization of Mouse Model for *S. vaginalis* Colonization

Introduction

Mouse models are ideal for the study of *S. vaginalis* pathogenicity in vivo, and the study of the relationship between *S. vaginalis* and preterm birth. There are various factors that play a role in PTB outcome, including but not limited to, genetic, anatomic, and other factors. In mouse models, variability is lessened compared to human subjects allowing for more accurate analysis of *S. vaginalis* impact. The following research was conducted to optimize the mouse model for *S. vaginalis* colonization. The ultimate goal is to get *S. vaginalis* to last in mice as long as possible for study of pathogenicity and impact on PTB.

Interestingly, in a preliminary model, the mouse inoculated with the highest dose of Sn35 appeared to have uterine horn complications not recognized in mice inoculated with lower doses of Sn35.

Results

I. Preliminary five-day drip plate experiment

In Sn35 mouse model, one C57/BL6 mouse in either estrus or diestrus stage of estrous cycle 24 hours pre-inoculation were intravaginally inoculated with ~1,000,000 cfu Sn35 (low dose) or ~10,000,000 cfu Sn35 (high dose), and *S. vaginalis* presence in vaginal swabs was determined and quantified over a period of time (figure 20). Swabs were collected one-, two, and five-days post inoculation, diluted, and plated to allow quantification of *S. vaginalis* present in swab sample. The low dose inoculum did not result in Sn35 CFUs from any mice. High dose inoculum lasted five days in mice in estrus stage at time of inoculation with an increase in *S. vaginalis* presence from 8,400 cfu (day one) to 336,000 cfu (day two) followed by a decrease to

2,850 cfu (day five). High dose inoculum lasted one day in mice in diestrus stage at time of inoculation with *S. vaginalis* present at 132,000 cfu, which is greater than estrus day one *S. vaginalis* presence but less than estrus day two. *S. vaginalis* was verified via gram-stain.

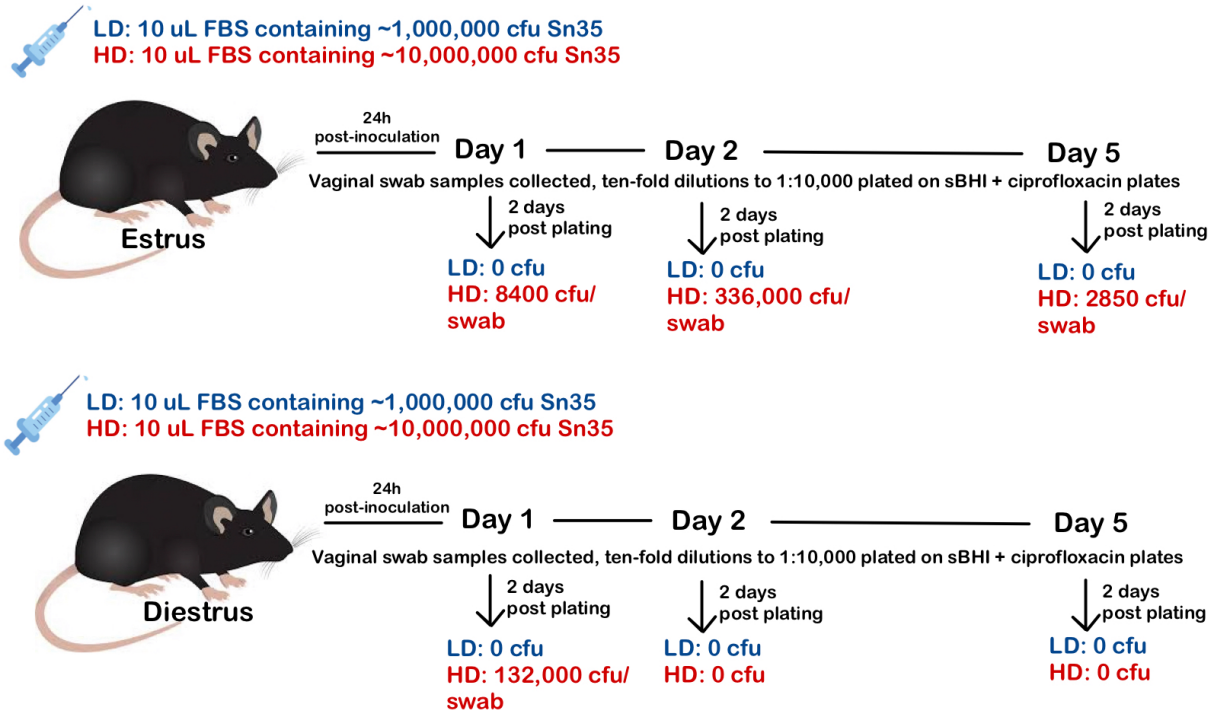


Figure 20. *S. vaginalis* can last for five days in mice inoculated with ~10,000,000 cfu Sn35 24 hours post estrus stage result of vaginal cytology examination

One out of two mice in each group, in diestrus or estrus stage of estrous cycle 24 hours pre-inoculation, were incubated with ~1,000,000 cfu Sn35 (HD) or ~10,000,000 cfu Sn35 (LD). Drop plates were made from dilutions of vaginal swab in PBS 1, 2, and 5 days post-inoculation, then Sn35 cfu was counted. In the estrus group, vaginal mouse swab from mouse inoculated with ~10,000,000 cfu Sn35 resulted in 8,400 cfu Sn35 on day 1 post-inoculation, 336,000 cfu Sn35 on day 2 post-inoculation, and 2,850 cfu Sn35 on day 5 post-inoculation. In the diestrus group, vaginal mouse swab from mouse inoculated with ~10,000,000 cfu Sn35 resulted in 132,000 cfu Sn35 on day 1 post-inoculation and 0 cfu Sn35 on days 2 and 5 post-inoculation. In either mouse inoculated with ~1,000,000 cfu Sn35 in estrus or diestrus groups, 0 cfu Sn35 were seen on plates from days 1, 2, or 5 post-inoculation.

II. Complications in uterine horn post inoculation with Sn35

In a preliminary experiment from the Sn35 mouse model, the uterine horns were removed from C57/BL6 mice intravaginally inoculated at estrus stage of estrous cycle with three doses of Sn35, ~37,500,000 cfu, ~3,750,000 cfu, or ~375,000 cfu, and examined (figure 21). Uterine horn from mouse inoculated with ~37,500,000 cfu appeared to have a blood clot. Uterine horns from mice inoculated with lower doses of *S. vaginalis* did not appear to have blood clots. In lab, blood clots were not previously observed in mice uterine horns.

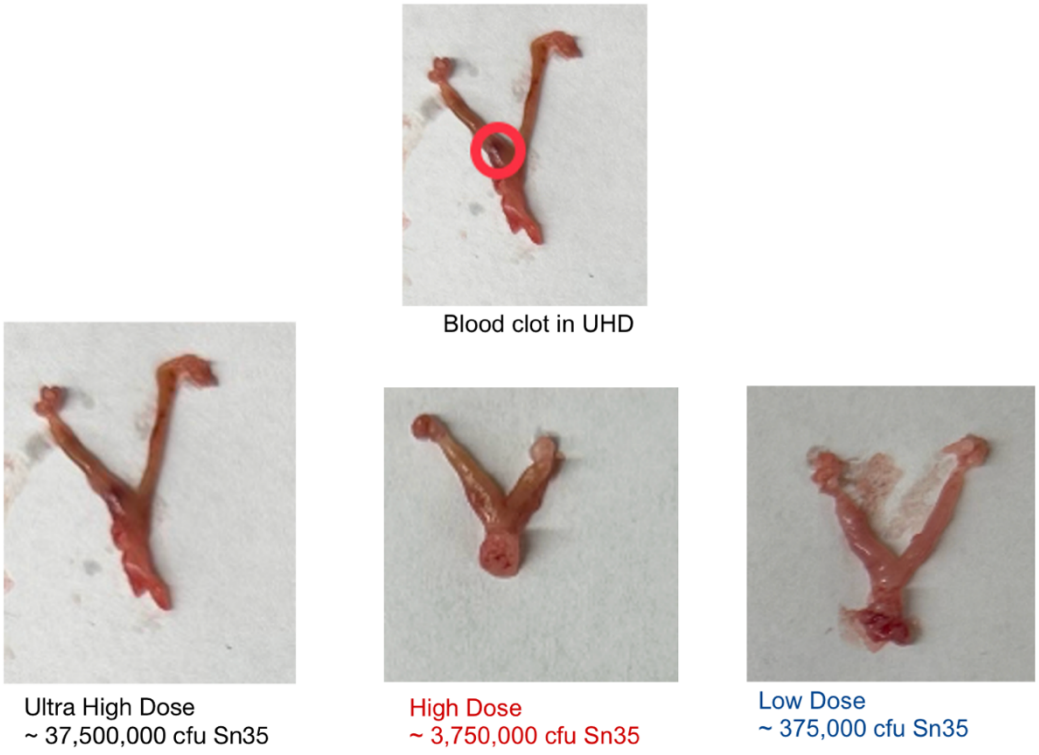


Figure 21. Blood clot in uterine horn post-inoculation with ~37,500,000 cfu Sn35
Three mice in estrus stage at inoculation were inoculated with ~375,000, ~3,750,000, or ~37,500,000 cfu Sn35. Only the mouse inoculated with ~37,500,000 cfu Sn35 appeared to contain a blood clot.

Discussion

From results, our *S. vaginalis* can remain present in mouse vaginal microbiome for at least 5 days. Stage of estrous cycle at time of inoculation appears to impact *S. vaginalis* survival in vivo as mice in estrus stage maintained *S. vaginalis* longer than mice in diestrus, even though both sets of mice were inoculated with the same inoculum, ~10,000,000 cfu Sn35. Only at the highest dose, ~37,5000,000 cfu Sn35, a blood clot was present in mouse uterine horn suggesting that abundant S. vaginalis colonization can cause complications in reproductive system.

Chapter 5

Discussion

Our data implies that vaginally residing *Sneathia* becomes pathogenic following its ascension into the upper urogenital tract, amniotic fluid, placenta, and fetal membrane. There is evidence of *Sneathia's* aid in pathogenesis of bacterial vaginosis, chorioamnionitis, preterm prelabor rupture of membranes, spontaneous preterm labor, stillbirth, maternal and neonatal sepsis [28], making it an important pathogen in the world of reproductive age women's health, especially during pregnancy. Our cohort consisted of 93 pregnant women. Vaginal swab samples were taken longitudinally until birth, which was either term, 50 participants, or preterm, 43 participants. The samples underwent 16s rRNA sequencing to determine *S. vaginalis* abundance relative to other bacteria present at collection [1].

In this study, the host antibody response to the pore-forming toxin produced by *S. vaginalis* during pregnancy was examined. ELISAs were performed for quantitative representation of host antibody, IgM, IgA, or IgG, in each sample. Samples were selected, 22 of 93, to undergo neutralization assays to assess host antibody neutralizing ability against NTerm protein fragment of the CptA toxin. Experiments were repeated various times to ensure replicable results. Before experimentation, it was assumed that *S. vaginalis* abundance would cause a host antibody response if CptA is being produced in vivo. However, there was still curiosity of the relationship between host antibody response and birth outcome. It was believed that host antibody response could signify a clearing of infection which could result in TB, or an antibody response could signify a severity of infection which could result in PTB. Three isotypes of immunoglobulins were tested for affinity to CptA toxin via indirect ELISA: IgM, IgA, and IgG. These isotypes were chosen due to IgM association with primary and early infection, IgA

association with mucosal membranes, and IgG being the most prevalent isotype in vaginal fluid [29]. IgM association with early colonization resulted in the idea that samples with IgM presence would result in TB outcome due to too little *S. vaginalis* abundance in host vaginal microbiome to impact birth outcome.

It was hypothesized that samples containing host antibodies would have greater neutralizing ability against the NTerm domain of CptA toxin than samples that were considered negative of host antibody to the toxin. After statistical analyzation of the data collected, tests suggested that there was a significant relationship between sample host antibody presence, specifically IgM and IgA, and sample neutralizing ability. Tests suggested that there was no statistical significance between host antibody presence, regardless of Ig isotype, and *S. vaginalis* abundance. The relationship between *S. vaginalis* abundance and host antibody presence was examined and there was no significance between the two. When experimentation first began, there was limited information known about the samples. Originally, the *S. vaginalis* abundance at time of sample collection and birth outcome of each pregnant individual were the only known variables. For this reason, samples were chosen on either end of extremity to portray the difference in host antibody response when host has high or low *S. vaginalis* abundance and PTB outcome. For a control group, samples were also chosen from host who had high or low *S. vaginalis* abundance with TB outcome. As a result of this intentional selection of samples, the samples selected for testing could have created an artificial bias, making it an inappropriate group for statistical analyzation of the relationship between *S. vaginalis* abundance and PTB. The size of the cohort could have a role in the outcome of results.

For the first time, this paper proves that CptA is produced in vivo by *S. vaginalis,* CptA provokes a host immune response, and host antibody can neutralize CptA. This is an important

revelation since CptA toxin is currently the only known virulence of *S. vaginalis*. Further investigation and study of *S. vaginalis,* especially pathogenic characteristics, is critical. *S. vaginalis* has been associated with vaginal disease and serious pregnancy complications. These associations suggest *S. vaginalis* enhances the chance of disease manifestation or severity of disease. Understanding *S. vaginalis* pathogenicity is necessary to decrease or eliminate *S. vaginalis* aid in bacterial vaginosis, amnionitis, preterm premature rupture of membranes, preterm birth, and other extreme pregnancy complications.

Currently, *S. vaginalis* has lasted in C57/BL6 mice for 5 days with an intravaginal inoculum of 10 µl FBS containing approximately 10,000,000 cfu Sn35 (HD1), with an increase in *S. vaginalis* presence in vaginal swab on day 2 compared to day 1 post-inoculation and decline by day 5. However, survival of Sn35 was not noted in mice inoculated with 10 µl FBS containing approximately 1,000,000 cfu Sn35 (LD1), suggesting that this inoculum may consist of too little *S. vaginalis* for survival. Interestingly, difference in stage of estrous cycle appear to impact *S. vaginalis* survival. This may be due to the difference in mouse vaginal cytology at different points in estrous stage and *S. vaginalis* ability to be pathogenic against different cell types. When inoculated with HD1, mice in estrus stage of estrous cycle at time of inoculation resulted in Sn35 cfu until plating day 5 post-inoculation whereas mice in diestrus stage resulted in Sn35 cfu only on day 1 post-inoculation. In another preliminary model, estrus stage mice were inoculated with one of three doses of Sn35: ultra-high dose containing approximately 37,500,000 cfu Sn35 in 10 μl FBS (UHD2), high dose containing approximately 3,750,000 cfu Sn35 in 10 μl FBS (HD2), and low dose containing approximately 375,000 cfu Sn35 in 10 μl FBS (LD2). At 6 days post-inoculation, uterine horns were collected for physical observation. Interestingly, a blood clot was noticed in the uterine horn of a mouse inoculated with UHD2. Blood clots in mice

uterine horns are not known to be common, nor has this been a common occurrence in lab. This leads to the belief that this blood clot was formed due to injury in the uterine horn resulting from the high dosage of S. vaginalis in the UHD2 inoculum, supporting the idea that S. vaginalis can be pathogenic in vivo.

Chapter 6

Future Directions

In the near future, there is interest in observing host antibody presence and neutralizing ability of anti-CptA IgG, IgM, and IgA antibodies in other samples collected longitudinally from participants of this study. As of now, it is understood that *S. vaginalis* produces CptA in vivo and a host immune response is mounted against the toxin. It is also understood that *S. vaginalis* abundance, relative to other bacterial taxa present, varies throughout gestation in pregnancies that result in preterm birth (figure 8). There is a chance that *S. vaginalis* abundance or stage of colonization could have an impact on the presence or abundance of immunoglobulin isotypes, as it is known that different isotypes appear at different points in the adaptive immune response according to point of infection. Potentially, samples taken from timepoints in gestation when *S. vaginalis* relative abundance begins to decline may contain host antibodies with greater neutralizing ability than antibodies collected from other timepoints throughout gestation. The relationship between host immune response to CptA and PTB should be further investigated in a larger case-controlled study. Though samples selected originated from a case-controlled study, this study was not case-controlled as samples were chosen with the goal of determining whether CptA toxin is expressed during colonization.

There is great interest in investigating *S. vaginalis* pathogenesis in the female genital tract and early colonization impact on pregnancy in a controlled system. Future plans include using a mouse model for investigation. Mice will be genetically identical, and it will be certain that mice were not pre-exposed to *S. vaginalis*. This will reduce the variability of results and outcomes during experimentation and allow *S. vaginalis* to be reliably studied. Mice will be intravaginally inoculated with *S. vaginalis,* and *S. vaginalis* impact on the female genital tract and host immune

response will be observed. In another model, mice will be intravaginally inoculated with *S. vaginalis* 12 hr pre- or post- impregnating. More specifically, there is a plan to knockout the *cptA* gene in *S. vaginalis* genome and inoculate a group of mice with this genetically modified strain and a group with WT Sn35 strain 12 hr pre- or -post impregnating and compare differences with control group throughout pregnancy and pregnancy outcome. This would determine CptA toxin impact on *S. vaginalis* virulence as CptA toxin is the only known virulence factor of *S. vaginalis*. Various preliminary mouse model experiments have been completed in attempt to optimize protocol and procedures before larger experimentation. Importantly, strives are made to keep Sn35 alive in mice as long as possible or at least 20 days, being that this is the average gestation of mice. There are also attempts to develop an inoculum containing an optimal amount of Sn35 that will allow *S. vaginalis* to last and persist in the mouse models. Once this inoculum is developed, there is a plan to freeze a large stock and use this inoculum for consistency throughout experimentation and quantitative measure of Sn35 inoculated in mice. Future directions will lead to a better understanding of *S. vaginalis* pathogenesis and CptA toxin activity in vivo and the host antibody response to CptA toxin produced in vivo.

Literature Cited

[1] J. M. Fettweis *et al.*, "The vaginal microbiome and preterm birth," *Nat. Med.*, vol. 25, no. 6, Art. no. 6, Jun. 2019, doi: 10.1038/s41591-019-0450-2.

[2] J. Fettweis *et al.*, "The Vaginal Microbiome: Disease, Genetics and the Environment," *Nat. Preced.*, pp. 1–1, Nov. 2010, doi: 10.1038/npre.2010.5150.1.

[3] M. D. Harwich *et al.*, "Genomic sequence analysis and characterization of Sneathia amnii sp. nov," *BMC Genomics*, vol. 13, no. 8, p. S4, Dec. 2012, doi: 10.1186/1471-2164-13-S8-S4.

[4] J. Ravel *et al.*, "Vaginal microbiome of reproductive-age women," *Proc. Natl. Acad. Sci.*, vol. 108, no. supplement_1, pp. 4680–4687, Mar. 2011, doi: 10.1073/pnas.1002611107.

[5] L. Gruwier, A. Sprenkels, S. Hulsbosch, A. Vankeerberghen, and R. Cartuyvels, "Sneathia amnii bacteraemia and chorioamnionitis leading to second trimester abortion: a case report," *Access Microbiol.*, vol. 3, no. 12, p. 000290, Dec. 2021, doi: 10.1099/acmi.0.000290.

[6] P. Vitorino *et al.*, "Sneathia amnii and Maternal Chorioamnionitis and Stillbirth, Mozambique," *Emerg. Infect. Dis.*, vol. 25, no. 8, pp. 1614–1616, Aug. 2019, doi: 10.3201/eid2508.190526.

[7] G. L. Gentile *et al.*, "Identification of a Cytopathogenic Toxin from Sneathia amnii," *J. Bacteriol.*, vol. 202, no. 13, pp. e00162-20, Jun. 2020, doi: 10.1128/JB.00162-20.

[8] "C. O'Brien, J. Raskin, et. al.,"Identification of the pore-forming and binding domains of the Sneathia vaginalis cytopathogenic toxin A." PLoSOne (accessed Apr. 04, 2023).

[9] A. s. Kolaskar and P. C. Tongaonkar, "A semi-empirical method for prediction of antigenic determinants on protein antigens," *FEBS Lett.*, vol. 276, no. 1–2, pp. 172–174, 1990, doi: 10.1016/0014-5793(90)80535-Q.

[10] X. Chen, Y. Lu, T. Chen, and R. Li, "The Female Vaginal Microbiome in Health and Bacterial Vaginosis," *Front. Cell. Infect. Microbiol.*, vol. 11, p. 631972, Apr. 2021, doi: 10.3389/fcimb.2021.631972.

[11] J. M. Fettweis *et al.*, "Differences in vaginal microbiome in African American women versus women of European ancestry," *Microbiol. Read. Engl.*, vol. 160, no. Pt 10, pp. 2272–2282, Oct. 2014, doi: 10.1099/mic.0.081034-0.

[12] P. Łaniewski and M. M. Herbst-Kralovetz, "Bacterial vaginosis and health-associated bacteria modulate the immunometabolic landscape in 3D model of human cervix," *Npj Biofilms Microbiomes*, vol. 7, no. 1, Art. no. 1, Dec. 2021, doi: 10.1038/s41522-021-00259-8.

[13] H. Verstraelen and R. Verhelst, "Bacterial vaginosis: an update on diagnosis and treatment," *Expert Rev. Anti Infect. Ther.*, vol. 7, no. 9, pp. 1109–1124, Nov. 2009, doi: 10.1586/eri.09.87.

[14] R. Russo, E. Karadja, and F. De Seta, "Evidence-based mixture containing Lactobacillus strains and lactoferrin to prevent recurrent bacterial vaginosis: a double blind, placebo controlled, randomised clinical trial," *Benef. Microbes*, vol. 10, no. 1, pp. 19–26, Feb. 2019, doi: 10.3920/BM2018.0075.

[15] J. Deese, S. Pradhan, H. Goetz, and C. Morrison, “Contraceptive use and the risk of sexually transmitted infection: systematic review and current perspectives,” *Open Access J. Contracept.*, vol. 9, pp. 91–112, Nov. 2018, doi: 10.2147/OAJC.S135439.
[16] A. Javed, F. Parvaiz, and S. Manzoor, “Bacterial vaginosis: An insight into the prevalence, alternative treatments regimen and it’s associated resistance patterns,” *Microb. Pathog.*, vol. 127, pp. 21–30, Feb. 2019, doi: 10.1016/j.micpath.2018.11.046.
[17] “The Bacterial Etiology of Preterm Birth | Elsevier Enhanced Reader.” https://reader.elsevier.com/reader/sd/pii/B9780123943811000015?token=D90E8EE81D9EF0483EE3205443078C19681D5CD222489039212AE21E7F8EADF02DF5FF298129668CAF588FE655A6F65A&originRegion=us-east-1&originCreation=20230425155154 (accessed Apr. 25, 2023).
[18] *Biology of the laboratory mouse*. in Biology of the laboratory mouse. Oxford, England: Blakiston, 1941, pp. ix, 497.
[19] *The American Journal of Anatomy*. For the Wistar Institute by Alan R. Liss, Incorporated, 1922.
[20] S. L. Byers, M. V. Wiles, S. L. Dunn, and R. A. Taft, “Mouse Estrous Cycle Identification Tool and Images,” *PLoS ONE*, vol. 7, no. 4, p. e35538, Apr. 2012, doi: 10.1371/journal.pone.0035538.
[21] J. F. Nelson, L. S. Felicio, P. K. Randall, C. Sims, and C. E. Finch, “A Longitudinal Study of Estrous Cyclicity in Aging C57BL/6J Mice: I. Cycle Frequency, Length and Vaginal Cytology1,” *Biol. Reprod.*, vol. 27, no. 2, pp. 327–339, Sep. 1982, doi: 10.1095/biolreprod27.2.327.
[22] C. Caligioni, “Assessing Reproductive Status/Stages in Mice,” *Curr. Protoc. Neurosci. Editor. Board Jacqueline N Crawley Al*, vol. APPENDIX, p. Appendix-4I, Jul. 2009, doi: 10.1002/0471142301.nsa04is48.
[23] A. K. Champlin, D. L. Dorr, and A. H. Gates, “Determining the Stage of the Estrous Cycle in the Mouse by the Appearance of the Vagina,” *Biol. Reprod.*, vol. 8, no. 4, pp. 491–494, May 1973, doi: 10.1093/biolreprod/8.4.491.
[24] S. Achiraman *et al.*, “Biochemical Analysis of Female Mice Urine with Reference to Endocrine Function: A Key Tool for Estrus Detection,” *Zoolog. Sci.*, vol. 28, no. 8, pp. 600–605, Aug. 2011, doi: 10.2108/zsj.28.600.
[25] S. D. Ramos, J. M. Lee, and J. D. Peuler, “An inexpensive meter to measure differences in electrical resistance in the rat vagina during the ovarian cycle,” *J. Appl. Physiol. Bethesda Md 1985*, vol. 91, no. 2, pp. 667–670, Aug. 2001, doi: 10.1152/jappl.2001.91.2.667.
[26] M. C. Cora, L. Kooistra, and G. Travlos, “Vaginal Cytology of the Laboratory Rat and Mouse: Review and Criteria for the Staging of the Estrous Cycle Using Stained Vaginal Smears,” *Toxicol. Pathol.*, vol. 43, no. 6, pp. 776–793, Aug. 2015, doi: 10.1177/0192623315570339.
[27] M. Shimaoka *et al.*, “Association between preterm delivery and bacterial vaginosis with or without treatment,” *Sci. Rep.*, vol. 9, p. 509, Jan. 2019, doi: 10.1038/s41598-018-36964-2.
[28] K. R. Theis *et al.*, “Sneathia: an emerging pathogen in female reproductive disease and adverse perinatal outcomes,” *Crit. Rev. Microbiol.*, vol. 47, no. 4, p. 517, Aug. 2021, doi: 10.1080/1040841X.2021.1905606.

[29] J. P. Bouvet, L. Bélec, R. Pirès, and J. Pillot, “Immunoglobulin G antibodies in human vaginal secretions after parenteral vaccination.,” *Infect. Immun.*, vol. 62, no. 9, pp. 3957–3961, Sep. 1994.

VITA

Zion McCoy was born on August 29th in Laurel, Maryland. Her mother decided to relocate to her hometown, Portsmouth, Virginia, when Zion was two years old. She graduated from Nansemond River High School in Suffolk, Virginia in spring 2017. In spring 2021, Zion received her Bachelor of Science in Biology from Howard University. Zion began the Master of Science program at Virginia Commonwealth University School of Medicine Department of Microbiology and Immunology in fall 2021.

Publications:

C. O'Brien, J. Raskin, et. al.,"Identification of the pore-forming and binding domains of the Sneathia vaginalis cytopathogenic toxin A.”

Abstracts and Presentations:

VCU Institute for Women’s Health 16th Annual Women’s Health Research Day, April 2022. Antibody response to the pore-forming toxin from *Sneathia vaginalis* during pregnancy. Poster.