DEVELOPMENT OF LOW-COST, MICRO-VOLUME ANTIMICROBIAL RESISTANCE ASSAYS FOR PATHOGENIC BACTERIA

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Taylor Mae Oeschger

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Taylor Mae Oeschger, Ph.D.

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A lack of diagnostics and growing antibiotic resistance worldwide threatens to make once treatable disease deadly again. The discovery of antibiotics drastically reduced the mortality of bacterial infectious diseases; however, persistence is still rampant in low- and middle-income countries. Even when infections are properly diagnosed and treatments are available, resistance genes are spreading and leading to more treatment failures. Paper-based diagnostics require no cold chain or power supply and are magnitudes cheaper than standard nucleic acid amplification techniques. They are also easier to perform and run faster than typical cell culture-based diagnostics. Thus, paper diagnostics provide global solutions to curbing bacterial diseases.

In this work, I describe efforts to develop low cost, accessible technologies for identifying the presence and the antimicrobial susceptibility profile of various pathogenic bacteria on the World Health Organizations top threat list. Specifically, I focus on Carbapenem-resistant Enterobacterales (CRE), a group of bacteria commonly responsible for hospital acquired infections, and *Neisseria gonorrhoeae*, the causative agent of the sexually transmitted infection gonorrhea.

First, I describe a paper-based chip made of chromatography paper and wax that supports the growth of CRE in the presence of three different clinically relevant antibiotics at three concentrations each. For the low cost of $0.77, this technology can enable personalized antibiotics prescriptions by giving personalized susceptibility information to prescribing clinicians. Next, I describe the evaluation of various colorimetric dyes capable of indicating viability of *N. gonorrhoeae*. By focusing on dyes that can be seen with the naked eye, we aim to reduce the need for complex equipment and reduce the level of training required for end users. Thirdly, I developed a lateral flow assay to detect the presence of *Neisseria gonorrhoeae* quickly and accurately in a sample and concentrate and isolate whole, live bacteria that can be used in downstream susceptibility testing. Finally, I combine these technologies to develop a paper chip for antibiotic susceptibility testing of *N. gonorrhoeae*.

BIOGRAPHICAL SKETCH

Taylor Oeschger grew up in Sacramento, California and graduated from Sacramento Country Day High School in 2013. She received a Bachelor of Science in Chemical Engineering and a Bachelor of Science in Biological Engineering from Montana State University in Bozeman, Montana in 2017. She matriculated to Cornell University in August 2017 in pursuit of a Doctorate of Philosophy in Biomedical Engineering. Under the guidance of Professor David Erickson, she developed point of care diagnostics for use in low resource settings. Utilizing her background in microbiology, she designed paper diagnostic chips and lateral flow assays for important antimicrobial resistant pathogens including Carbapenem-resistant Enterobacterales and *Neisseria gonorrhoeae*. In 2020, Taylor was awarded a Master of Science degree and will complete her doctoral degree in April 2022.

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CHAPTER ONE

INTRODUCTION

1.1 Overview

In this dissertation I will describe the development and testing of multiple point of care diagnostics with the goal of improving access to antimicrobial susceptibility testing. These paper-based lab-on-a-chip devices are intended to be used globally, both as a replacement for first world microbiology labs and in low- and middle-income countries where access to cold chain and electricity may be limited. The end goal is to increase access to antibiotic susceptibility testing broadly so that personalized prescriptions are possible worldwide.

In 2019, antimicrobial resistance (AMR) directly contributed to 1.27 million deaths putting it above other infectious diseases such as HIV and malaria[1]. While AMR is sometimes considered a first-world problem, the greatest burden of AMR exists in low-income countries like sub-Saharan Africa and south Asia. This is because while high income countries have greater antibiotic availability, low- and middle-income countries have higher incidence of infections which spread more readily, fewer options for narrow spectrum antibiotics, an abundance of counterfeit medications, and a broad lack of oversight and regulation, all which drive resistance[1]. In the United States specifically, the Centers for Disease Control estimates there are 2.8 million infections that are resistant to antibiotics, which case and 35,000 deaths every year[2]. The six most common AMR pathogens that may cause death are *Escherichia coli*, followed by *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*, all of which were studied in this research[1]. It is estimated that

by the year 2050 more than 10 million deaths will be attributable to AMR worldwide, with nearly 5 million of these cases occurring in Asia and more than 4 million in Africa[3]. Methods to slow the spread of AMR include careful use of antibiotics in medicine and agriculture, increased surveillance of resistant threats, antibiotic stewardship programs, and individualized prescriptions for infections. Most of these methods will require lower cost and higher throughput methods for antibiotic profiling to monitor and treat AMR infections.

There are two main methods for antibiotic susceptibility testing (AST): genotypic, where one evaluates the presence of specific genes required for resistance, and phenotypic, where one evaluates the overall presentation of antibiotic resistance. In this case, I chose to focus on phenotypic methods because they are 1) more clinically relevant, 2) can report on resistance caused by multiple genes simultaneously and 3) do not require specific resistance genes to be known and sequenced. This is especially important for an organism such as *N. gonorrhoeae* which is known to have 20 or more antibiotic resistance genes, which it readily acquired through mutation and horizontal gene transfer with commensal microbiota[4].

The current gold standard for AST varies by organism but the most general is automated systems like the VITEX 2 by BioMerieux or the MicroScan Walkaway by Beckman Coulter which identify pathogens and provide phenotypic AST profiles. These machines cost tens of thousands of dollars in initial costs and weigh hundreds of pounds each, making them inaccessible to low resource settings. Each test requires a disposable test card containing reagents that costs $5-15 per test depending on the culture type and source. Other lower resource culture-based AST requires a large number of liquid media

tubes or agar plates with 10 or more antibiotic concentrations, each which is inoculated with the bacterium. This method can be interpreted by eye but requires extensive user input to prepare and 18 hours or more for results. Instead, a patient specific solution without startup costs that can be performed by untrained users could drastically increase access to AST globally.

Paper microfluidics is a field that dates back as far as the first century AD with the first reported use of chromatography paper to separate particles on the basis of size[5]. Dipstick type tests and lateral flow assays were popularized in the 1950s and 1960s which rely on capillary action to carry antigens and reagents across the paper test[5,6]. More recently, advanced technologies combining paper with paraffin wax or other hydrophobic barriers have created more complex paper microfluidics capable of complex fluid handling in two and three dimensions[7]. Overall, paper microfluidics has significantly improved the diagnostics field and can impact AST as well.

1.2 Paper Based Testing for Triple Antibiotic Susceptibility of Non-Fastidious Carbapenem-resistant Enterobacterales (CRE)

Carbapenems were previously considered the last resort antibiotic for many gram-negative bacterial infections, however their widespread use has given rise to a group of carbapenem-resistant bacteria collectively referred to as Carbapenem-resistant Enterobacterales (CRE)[8]. This group includes *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Klebsiella aerogenes*, *Citrobacter freundii*, and *Citrobacter koseri* among others[9]. Instead of causing one specific disease, CREs can cause a variety of infections including pneumonia, skin infections, urinary tract infections, and sepsis[10]. Risk factors for developing CRE infections include hospital stays, intensive care unit

visits, residence in a long term care facility, use of mechanical ventilation, recent organ or stem-cell transplants, and previous exposure to antimicrobial therapies[11,12]. Some studies estimate that mortality of CRE infections can be as high as 26-44%, significantly higher than carbapenem susceptible Enterobacteriaceae infections[13,14]. In addition to improved sterilization of medical equipment, better hygiene in hospitals and long-term care facilities, and the development of novel antibiotics to treat CRE, further resistance can be delayed through personalized susceptibility profiling on a patient-by-patient basis.

To address this, Chapter 2 of this thesis details the Bac-PAC: **Bac**terial **P**aper **A**ntibiotic Susceptibly Testing **C**hip which provides a colored readout of the antibiotic profile for a patient specific strain of CRE using a small paper chip and a low power rechargeable coffee mug. This paper-based assay provides semi-quantitative categorization of "susceptible", "intermediate", or "resistant" for 3 antibiotics on a single assay, thus reducing the need for repeated testing if the antibiotic of interest comes up resistant. This diagnostic tool that costs an average of $0.81 could be the first step in enabling personalized antibiotic prescriptions for a variety of bacterial diseases worldwide.

1.3 Development of Point of Care Assays for Diagnosis and Antibiotic Susceptibility Testing of *Neisseria gonorrhoeae*

Neisseria gonorrhoeae, the causative agent of the sexually transmitted disease gonorrhea, is one of the top antibiotic resistant threats of our time. In 2016, there were an estimated 87 million cases of gonorrhea globally, corresponding to 0.9% of all women and 0.7% of all men[15]. While gonorrhea is prevalent worldwide, developing countries bear 80-90% of the disease burden[16]. While well-resourced countries have switched from

laboratory based testing to nucleic acid amplification testing to diagnose gonorrhea, low- and middle-income countries still rely on syndromic based diagnosis which has led to both overtreatment and missed treatments[17].

In addition to being difficult to diagnose, gonorrhea is becoming harder to treat due to AMR. *N. gonorrhoeae* has a unique ability to rapidly acquire resistance genes through spontaneous mutation and horizontal gene transfer and its extremely fastidious nature have made it a challenge for researchers[4]. Experts agree that a low cost, easy to use test for *N. gonorrhoeae* would facilitate antimicrobial stewardship and extend the usage life of classic and emerging antimicrobial therapies[18]. Modeling studies suggest that the use of a theoretical point of care AST for gonorrhea in even 20-50% of cases could delay the onset of multidrug resistant gonorrhea by decades and reduce the use of last line therapy by 66%[19,20]. In the United States, approximately half of all gonorrhea infections are resistant to antibiotics resulting in $133 million in annual direct costs[21].

According to an expert consensus published in 2020, an ideal point of care test to diagnose gonorrhea should cost less than $1USD, provide results within 30 minutes, require no external power and have a sensitivity of 80% or higher[22]. Meanwhile, an applicable point of care test to monitor AMR should be usable at a district hospital level, cost $15-25 per test, give results in 1 hour, and may rely on rechargeable or battery-operated equipment[22]. The consensus notes that these are lofty, potentially unattainable goals and that any technology meeting the majority of these requirements would be a significant contribution to medicine.

Gonorrhea has been a difficult organism to design diagnostics for because of its fastidious nature. While it readily grows and spread through the human population, it

does not survive well in the lab, in liquid medium, without 3-5% CO_2, or outside of the ideal 35-37°C temperature range. In addition, it is highly sensitive to many of the typical colored dyes used in microbiology. Thus, as seen in Chapter 3, prior to the development of a paper assay, I had to explore specific dyes that would indicate gonococcal growth without inhibiting growth and confounding results. In Chapter 4, a traditional lateral flow assay is combined with the Bac-PAC technology from Chapter 1 to create a complete paper system for personalized diagnosis and treatment of gonorrhea infections.

CHAPTER TWO

MULTIPLEXED PAPER-BASED ASSAY FOR PERSONALIZED ANTIMICROBIAL SUSCEPTIBILTY PROFILING OF CARBAPENEM-RESISTANT ENTEROBACTERALES PERFORMED IN A RECHARGABLE COFFEE MUG

2.1 Abstract

The increasing prevalence of antibiotic resistance threatens to make currently treatable bacterial diseases deadly again. As drug resistance rises, antibiotic susceptibility testing needs to adapt to allow for widespread, individualized testing. Paper-based diagnostics offer low-cost, disposable alternatives to traditional time consuming and costly in-house methods. Here, we describe a paper-based microfluidic device, called the Bac-PAC, capable of categorizing the antibiotic susceptibly of individual strains of Carbapenem-resistant Enterobacterales. Each chip provides a colored readout with actionable susceptibility classification of three antibiotics, thus maximizing the chances of identifying a viable therapy. We verified the technology on thirty bacterial strains with two dyes using six clinically relevant antibiotics. We demonstrated that the dried tests are stable for one month, can work with human bodily fluids such as urine, and can be incubated in a rechargeable coffee mug that reduces the need for external infrastructure.

2.2 Introduction

Antimicrobial resistance (AMR) is immediate pressing global threat. In 2016, AMR caused 700,000 deaths worldwide, and models predict an increase of up to 10 million deaths per year by 2050, resulting in 100 trillion USD lost in global production1. Carbapenem-resistant Enterobacterales (CRE) is a highly antibiotic resistant group of

organisms dubbed the “nightmare bacteria” because of their estimated annual deaths, prevalence in hospitals, and rapid transfer of antibiotic resistance genes2. Many experts in AMR broadly recommended that antimicrobial susceptibility testing (AST) be performed on an individual basis so that personalized treatments can be prescribed3,4. This would prevent the misuse of last-line antibiotics and delaying the onset of full resistance.

The gold standard method of AST is broth serial dilution, where solutions of antibiotics diluted two-fold are prepared, spiked with bacteria, and the minimum inhibitory concentration is obtained5 and classified based on breakpoints as determined by the Clinical and Laboratory Standards Institute (CLSI)6. While this produces reliable, reproducible, quantitative results, this method is also limited by its tedious preparation, high cost, and laboratory requirments5. It is currently impractical to preform broth serial dilutions for multiple antibiotics for several patients without the aid of automated equipment. Other methods for AST may include automated devices, premade 96-well plates, E-test gradient strip diffusion, or agar disk diffusion. While these technologies are logistically simpler than broth dilution, they may be more expensive, bulkier, or require refrigerated agar plates making them unamenable to low resource settings. When none of these methods are available, clinicians may prescribe patients an antibiotic treatment based on infection source or current regional resistance trends7,8.

The consensus Review on Antimicrobial Resistance concluded that “the solution to the problem (of AMR) must work for the world and benefit as many people as possible, not one country or one group of countries,” and thus “the solutions should be cost-effective, affordable and support economic development”1. This is especially important

for low- and middle-income countries that lack the resources or infrastructure necessary to perform drug susceptibility testing9,10. It is therefore necessary to develop a low-cost point-of-care diagnostic to accurately predict effective antibiotics for treating a patient based on their individual infection resistance profile. Point-of-care diagnostic devices should be affordable, portable, robust, designed to work in a wide variety of settings, use small sample volumes, and have a quick turnaround time11–13. Paper microfluidics specifically offer several additional benefits: they are disposable, require no power supply, and are easy to scale for mass manufacturing11,14,15. Such a test could provide a global picture of emerging AMR, guide public health and policy, and improve individual patient outcomes.

Here we introduce the Bac-PAC: Bacterial Paper Antibiotic Susceptibly Testing Chip. The Bac-PAC is a paper microfluidic chip capable of giving colored readouts visible to the naked eye corresponding to the susceptibly of a patient-specific bacterial strain. This work improves on our previous publication by Wang and Erickson16 through 1) multiplexing to include three antibiotics while maintaining semi-quantitative readouts, 2) exploration of additional colorimetric dyes that demonstrated better performance with some antibiotics, 3) proof of concept validation with 30 bacterial strains from 12 different species contained in the “Enterobacterales Carbapenem Breakpoint" panel17 (Figure A.1, 2.2), 4) implementation of packaging to store the device at different temperatures for extended periods of time, and 5) the incorporation of a low-cost rechargeable SmartMug in place of a commercial incubator. Altogether, the Bac-PAC assay described herein demonstrates the first multiplexed, colored AMR assay on paper that is optimized for clinically actionable information in low resource settings.

2.3 Methods

2.3.1 Paper microfluidic printing

A circular based paper microfluidic was designed in Adobe Illustrator 25.3.1 and printed on Whatman Grade 1 paper using a Xerox ColorQube 8570 wax printer. Wax was melted through the paper on a hot plate at 100°C for ~30 seconds until the wax visibly melted through the paper. The back side of the chips was sealed with clear packing tape. Chips were stored in petri dishes at room temperature until use. The overall size of the device is 52 x 52mm with 1 x 8mm channels, 5 x 7mm wells, and a 15mm diameter central loading zone.

2.3.2 Bacterial strains

Test strains were obtained from the Centers for Disease Control (CDC) and Food and Drug Administration (FDA) Antibiotic Resistance Isolate Bank[23]. We utilized 30 strains from the "*Enterobacterales* Carbapenem Breakpoint" panel containing 12 different species of Enterobacteriaceae with varying levels of resistance to ciprofloxacin, meropenem, gentamicin, cefazolin, tobramycin, and ampicillin (Figure A.2, 9-11). Species included: *Escherichia coli, Enterobacter cloacae, Klebsiella pneumoniae, Klebsiella aerogenes, Citrobacter freundii, Citrobacter koseri, Providencia stuartii, Serratia marcescens, Klebsiella oxytoca, Proteus mirabilis, Shigella sonnei,* and *Salmonella Typhimurium.* Gold standard minimum inhibitory concentrations were obtained by the CDC/FDA Antibiotic Resistance Isolate Bank through broth microdilution[23]. Bacteria from freezer stocks were grown for 20-24 hours on Mueller Hinton II agar and re-plated onto fresh BBL Mueller Hinton II agar and grown 20-24 hours prior to testing. Bacterial strains were adjusting to a cell density of ~1 x 10^8 using

the optical density at 625nm and diluted 1 to 10 in BBL Mueller Hinton II media prior to testing.

2.3.3 Antibiotics

Gold standard antibiotic susceptible categories were assigned by the CDC/FDA Antibiotic Resistance Isolate Bank[23] based on Clinical Laboratory Standard Institute's M100 Performance Standards for Antimicrobial Susceptibility Testing[24]. Certified Pharmaceutical reference or secondary standards were used for all six antibiotics tested.

2.3.4 Microfluidic device loading

Antibiotic stock solutions were diluted in 2mg/mL XTT with 5μg/mL phenazine methosulfate or pure PrestoBlue Cell Viability dye. The highest working concentration of antibiotic dye solution was diluted twice either 1 to 2, 1 to 4, or 1 to 6 depending on the trial requirements. 3μL of dye-only solution or antibiotic dilution were loaded on to the pre-printed and melted microfluidic chip starting at the 3 o'clock position and continuing from lowest to highest antibiotic concentration moving counterclockwise (Figure A.2). Dye and antibiotic solutions immobilized in the wells by drying at room temperature prior to testing. Multiplexed chips contained either ciprofloxacin, meropenem, and gentamicin or cefazolin, tobramycin, and ampicillin. 90μL of diluted bacteria solution was placed in the center well of the chip and allowed to diffuse outward, filling all 10 outer wells. 30μL of sterile deionized water was placed in each corner of the chip to increase humidity. The chip was sealed between two half pieces of sterile ELISA sealing film by applying pressure with a finger around all the edges, labelled with sample name and antibiotics, and incubated overnight incubation at 37°C.

2.3.5 Microfluidic device storage

For one-week and one-month storage trials, chips were prepared with the dye and antibiotic solutions as above. After fully drying at room temperature, chips were enclosed in an opaque heat-seal packaging with a single desiccator packet. Chips were then stored at either 4°C or room temperature for the appropriate duration before use.

2.3.6 Smart Mug Incubator

A VSITOO brand S3 Pro Smart Mug Warmer with Double Vacuum Insulation was purchased online. The mug was loaded with approximately 1 inch of water before adding Bac-PACs to prevent the mug automatic shutoff feature. Bac-PACs were prepared as usual and loaded vertically into the mug and the lid was sealed. Bac-PACs were removed at regular time intervals to check for growth and accuracy.

2.3.7 Data analysis

Data was analyzed using GraphPad Prism 9 and Microsoft Excel 2016. For each antibiotic, the number of wells that did undergo a color change were recorded, where zero or one wells corresponded to a susceptible sample, two wells corresponded to an intermediate sample, and three wells corresponded to a resistant sample. Chips that did not undergo a color change in the dye-only positive control well were marked as incomplete. Percent accuracy was calculated as the number of correct susceptibility categorizations out of the total number of categorizations, once incomplete tests were removed. Percent “minor” discrepancies were calculated as the number of categorizations that were one category off (i.e., susceptible sample reading as intermediate) divided by the total number of categorizations, once incomplete tests were removed. Percent “major”

discrepancies were calculated as the number of false-resistant results (i.e., susceptible sample reading as resistant) divided by the total number of categorizations, once incomplete tests were removed. Percent "very major" discrepancies were calculated as the number of false-susceptible results (i.e., resistant sample reading as susceptible) divided by the total number of categorizations, once incomplete tests were removed.

2.4 Results

Development of the Bac-PAC. The Bac-PAC is a wax printed paper-microfluidic, based on the original design described by Wang and Erickson[25], where bacteria are placed in the center and diffuse radially into ten wells containing three antibiotics of interest at three concentrations each, plus one positive control with no antibiotics. Antibiotic concentrations increase in a counterclockwise direction. After incubation, the wells change color to indicate bacterial replication and a failure of the antibiotic to inhibit the infection. Zero or one wells changing indicates a susceptible sample, while two wells and three wells changing implies an intermediate and resistant sample, respectively. Simply put, less color change indicates more susceptible bacteria.

Prior to incubation, water is added to each of the four corners and the entire chip is sealed in a transparent film. This increases humidity, encourages bacterial growth, prevents contamination, and contains all biohazards. The multiplexed design includes three antibiotics on a single chip, thus producing more clinically actionable information from a single test compared to the previous system[25]. We compared resazurin-based PrestoBlue dye (Figure 2.1a) with the tetrazolium dye XTT (Figure 2.1b). PrestoBlue is a redox dye that starts as blue colored resazurin and is reduced to resorufin, a pink product, or further reduced to dihydroresorufin, a colorless product[26,27]. In contrast, XTT in

solution starts colorless and is reduced to a vibrant orange, especially in the presence of additional electron acceptors such as phenazine methosulfate.

Antibiotics were selected using the CLSI M100 Performance Standards for Antimicrobial Susceptibility testing[24]. All four Group A antibiotic agents considered to be appropriate for inclusion in routine, primary testing panel were used. These included ampicillin, cefazolin, gentamicin, and tobramycin. Additionally, we chose two Group B antibiotics which are recommended to be included in testing if failure to agents in Group A is observed. The Group B agents selected were meropenem and ciprofloxacin due to their unique antibiotic classes compared to those in Group A.

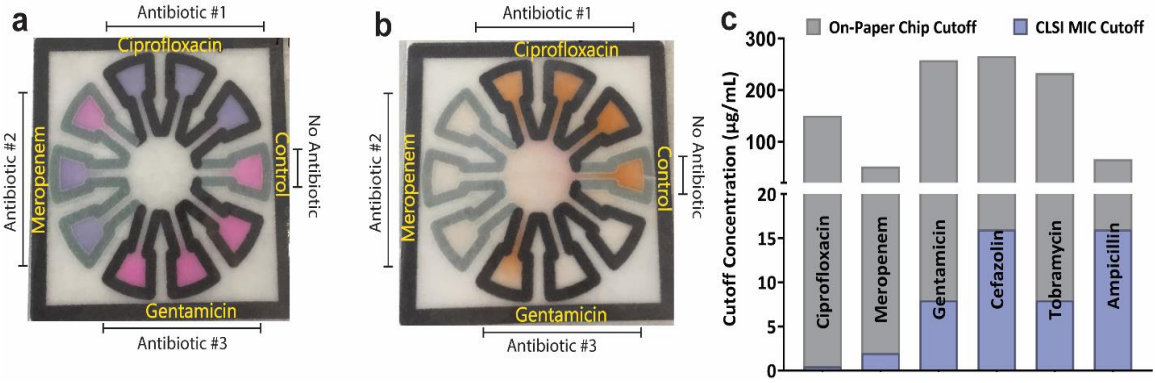

Figure 2.1 Bac-PAC design. **a)** Example test using PrestoBlue dye displaying ciprofloxacin susceptible, meropenem susceptible, and gentamicin resistant results**. b)** Example test using XTT dye displaying ciprofloxacin resistant, meropenem susceptible, and gentamicin susceptible results**. c)** Concentrations used on paper chip (top, grey) compared to CLSI MIC liquid culture cut-offs (bottom, blue).

We found that the concentration of antibiotics used on the Bac-PAC needed to be much higher than that used in traditional liquid MIC testing. Therefore, the concentration of each antibiotic on paper corresponding to susceptible, intermediate, and resistant cutoffs had to be identified experimentally. For each antibiotic, at least one strain from the panel was selected that contained susceptible, intermediate, and resistant gold

standards and the antibiotic concentration were adjusted, or "tuned", on paper until the categorization aligned. Overall, the intermediate cut-off level varied between 50 and 250 µg/mL, an average of 75-fold higher than the CLSI MIC breakpoints of 0.5 to 16 µg/mL[24] (Figure 1c). Gentamicin and cefazolin had an intermediate cut-off of 250 µg/mL, tobramycin 225 µg/mL, ciprofloxacin 150 µg/mL, and ampicillin and meropenem 50 µg/mL.

This magnitude difference in antibiotic concentrations warrants further investigation. Agar disk used for disk diffusion antibiotic susceptibility testing are loaded with high concentrations of antibiotics because the antibiotics must diffuse into the agar before contacting the bacteria. However, here the antibiotics should not be diffusing, and contact occurs at the site where antibiotics were dried. We suspect that higher concentrations of antibiotics are needed because of the higher bacterial inoculums needed to obtain growth and colored readouts on the paper format. Additionally, some antibiotics may be leaking to the center of the chip. The effectiveness of the antibiotics could also be decreased by the drying process or interaction with the chromatography paper itself. Despite possible antibiotic diffusion across the chip, antibiotics are not contaminating adjacent wells as can be seen from positive controls which are located directly besides the highest concentration of one of the antibiotics.

For analysis of results, discrepancies were separated into minor (a false resistant or susceptible result for an intermediate isolate or a false intermediate result), major (false resistant), and very major (false susceptible) in accordance with CLSI[24] and the FDA[28] guidelines. False resistant major discrepancies could cause a viable treatment option to be missed; however, this is preferable to a very major false susceptible in which the wrong

treatment might be prescribed. The overall aim was to reduce all discrepancies, with an emphasis on very major discrepancies.

Four-fold dilution is more ideal than two- or six- fold. In designing the Bac-PAC, we sought to maximize categorical agreement and to minimize major and very major discrepancies while also spanning the widest range of antibiotic concentrations possible. Gold standard broth microdilution testing involves adding bacteria to broth containing antibiotics that are diluted two-fold until growth of the bacteria ceases (Figure 2.2a). These tests utilize eight to ten different antibiotic concentrations in a single test; however, for the Bac-PAC this was limited to three concentrations to minimize the device footprint. By exploring four-fold and six-fold dilutions, we could span a wider range of concentrations on a single chip. For example, a two-fold dilution chip could span 75 to 300 μg/mL while a six-fold dilution chip could span 25 to 900 μg/mL (Figure A.3).

Prior to testing, dyes and antibiotics were mixed and immobilized on the chip by drying. No color change was seen in the center of the chip implying that the dye stayed well confined in the wells. As mentioned previously, the antibiotics may flow back into the center of the chip, but they do not impact adjacent wells. If antibiotics were influencing adjacent wells, the positive control would disappear, especially in the case of high susceptible samples, and the test would be invalidated. Therefore, since no mixing of antibiotics was observed, the antibiotic configurations can easily be changed based on clinical needs, such as local resistance trends or antibiotic availability.

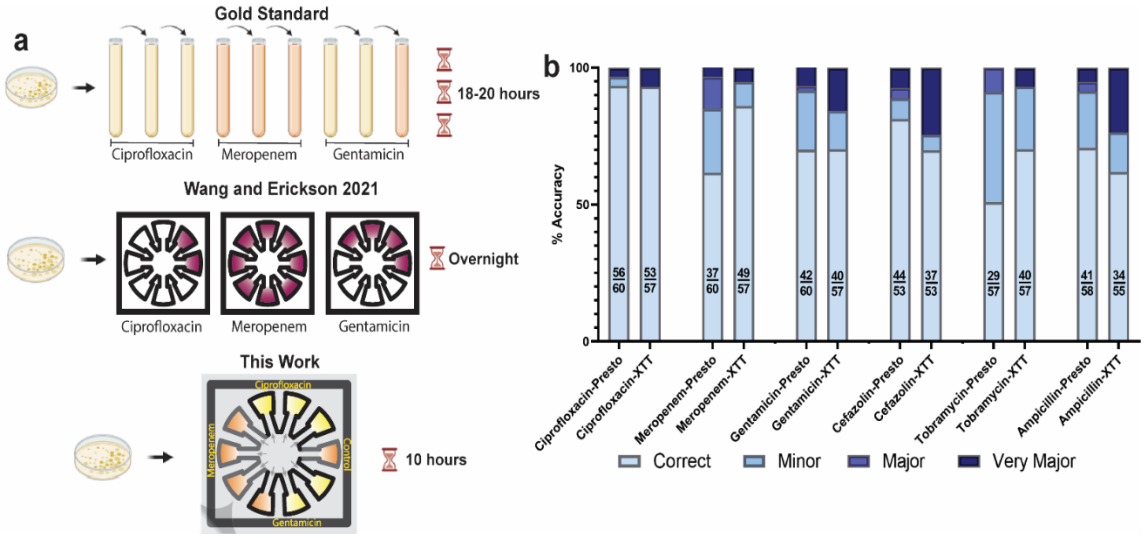

Figure 2.2 Bac-PAC compared to other technologies **a)** Comparison of time and equipment of broth dilution (top), previous work (middle) and this work (bottom) **b)** Bac-PAC four-fold dilutions results. Fractions represent number of correct samples over number of total samples

Ciprofloxacin, meropenem, and gentamicin two-fold and four-fold dilutions had an average categorical agreement of 79% compared to 77% for six-step dilutions (Figure 2.2b, Figure A.4). Cefazolin, tobramycin, and ampicillin had average accuracies of 68%, 78%, and 67% for two-fold, four-fold, and six-fold dilutions respectively (Figure 2.2b). Therefore, we used four-fold dilutions in all future testing. Out of all the antibiotics, ciprofloxacin performed the best with over 92% accuracy in every dilution and dye tested. For context, the World Health Organization (WHO) is aiming for 90% accuracy with less than 5% major discrepancies for an ideal antibacterial susceptibility test[29].

XTT displays higher overall accuracy but with higher proportions of very major errors compared to PrestoBlue. We compared XTT and PrestoBlue side by side to identify any major differences in accuracies between the two dyes for each antibiotic. Meropenem performed significantly better when paired with XTT compared to PrestoBlue: Meropenem-Presto displayed only a 50% accuracy with 23% major discrepancies compared to 83% accuracy with no major discrepancies for Meropenem-XTT. We suspect this is due to a chemical interaction between meropenem and the resazurin component of PrestoBlue dye. Cefazolin, tobramycin, and ampicillin displayed significantly higher very major discrepancies rates using XTT dye. In all dilution cases, gentamicin displayed similar accuracies between PrestoBlue and XTT but had fewer very major discrepancies using PrestoBlue. Overall, XTT had better accuracy in general but at the expense of higher very major errors rates, which could lead to misprescribing of antibiotic therapies.

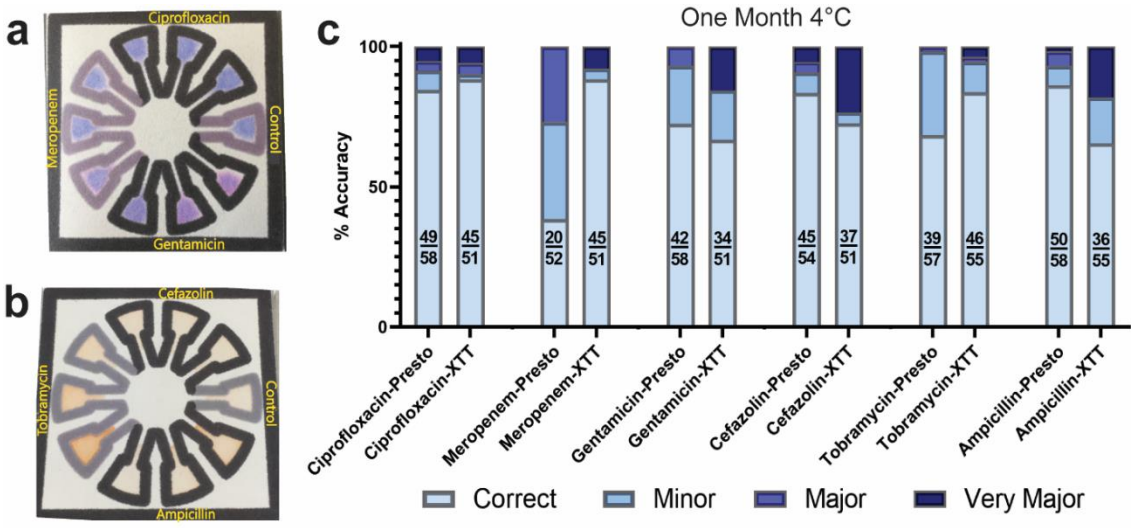

Figure 2.3 Shelf-life testing for 1 week and 1 month. **a)** PrestoBlue with ciprofloxacin, meropenem, and gentamicin after 1 month at room temperature but before any bacterial loading. **b)** XTT with cefazolin, tobramycin, and ampicillin after 1 month at room temperature but before any bacterial loading. **c)** Ciprofloxacin, meropenem, gentamicin, cefazolin, tobramycin, and ampicillin results after 1 month of storage at 4°C Fractions represent number of correct samples over number of total samples.

Bac-PACs are shelf-life stable for 1 week at room temperature and 1 month under refrigeration. For clinical use, diagnostic assays are manufactured, transported, and stored for some amount of time prior to use. Therefore, the Bac-PAC was evaluated for storage temperature and shelf-life. The paper chips were prepared as in all previous experiments, dried at ambient air for approximately ten minutes, and placed in heat seal packaging with a single desiccator packet. Half of the chips were stored at room temperature (approximately 20°C) while the other half were stored in the fridge (approximately 4°C).

Chips stored at room temperature for one month displayed some color change even without bacteria. PrestoBlue appeared to be converted by the highest concentrations of meropenem and gentamicin (Figure 2.3a) while XTT was readily converted by high levels of tobramycin and ampicillin (Figure 2.3b). When bacterial samples were added, this effect increased the discrepancy rate (Figure A.5 and A.6). This effect is unsurprising given that XTT and ampicillin are typically stored at -20°C while PrestoBlue, meropenem, gentamicin, cefazolin, and tobramycin are stored at 4°C.

Chips stored in the fridge at 4°C fared much better and retained higher accuracies after one month when compared to those at room temperature. In all cases except for meropenem, the choice of dye made little difference in the overall accuracy of the test. With meropenem, however, use of PrestoBlue lead to a very high minor and major discrepancy rate compared to XTT after one month of storage in the fridge (Figure 2.3c). This is likely caused by high levels of meropenem converting PrestoBlue over time, resulting in false positives. This theory is further supported by the lack of very major discrepancies. The best antibiotic dye pairing results after one month of storage at 4°C were Ciprofloxacin-XTT, Meropenem-XTT, and Gentamicin-Presto displaying 88%,

88%, and 72% accuracy, respectively, and Cefazolin-Presto, Tobramycin-XTT, and Ampicillin-Presto displaying 83%, 84%, and 86% accuracy, respectively. In the case of Meropenem-XTT, Ciprofloxacin-Presto, and Ciprofloxacin-XTT, storage at room temperature instead of refrigerated made little difference in the performance of the test (Figure 6). Therefore, most antibiotic-dye combinations on Bac-PAC are stable for at least one month when stored at 4°C, and some pairings could be stored at room temperature for shorter time periods, such as in resource-poor areas without reliable access to electricity.

2.4.1 Spiked human urine on the Bac-PAC

Previously described testing was all completed with pure bacterial isolates, however, direct from patient sampling would be preferable. CRE infections can occur in multiple locations including the blood stream, cerebrospinal fluid, and urinary tract[30–32]. Here we tested the preliminary potential of a direct urine inoculation using spiked sterile filtered, de-identified pooled human urine with eight different bacteria from our panel. Bacteria were initially spiked to a concentration of 10^8 CFU/mL in pure urine, as determined by optical density. Since urinary tract infections typically present in

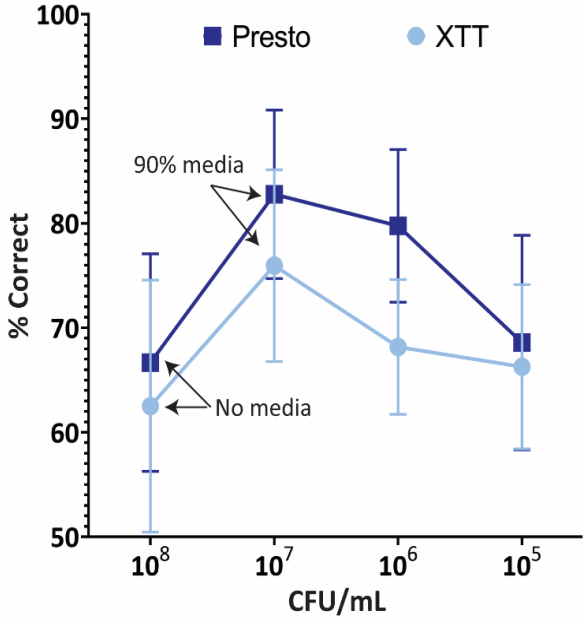

Figure 2.4 Spiked Human Urine Testing. 10^8 CFU/mL spiked into sterile pooled human urine then diluted 1:10, 1:100, and 1:1000 to obtain 10^7, 10^6, and 10^5 CFU/mL respectively. Tested on Bac-PACs with PrestoBlue and XTT. % Correct represents the average of all 6 antibiotics and error bars show standard error of the mean.

concentrations ranging from 10^5-10^8 CFU/mL[33], we diluted the sample 1/10, 1/100, and 1/1000 in media to cover this clinical range (Figure 2.4, Figure A.7). For every antibiotic and dye, correct susceptibility categorization was worse for 10^8 CFU/mL in pure urine compared to 10^7 CFU/mL in 10% urine and 90% media (Figure 2.5). This implies that media is an important component of the test system and that bacteria do not properly replicate in urine alone. However, this could be an effect of spiking with non-urinary tract adapted bacteria, and thus this result should be confirmed in clinical trials.

Rechargeable SmartMug can be used for low-cost incubation. While our results thus far are promising, they still rely on a large, water jacketed, energy intensive standing incubator which is not conducive to the point-of-care. Therefore, we substituted the 400lb incubator for a 2.5oz Smart Mug Warmer (Figure 2.5a). The mug was capable of maintaining a constant 35°C temperature and holding more than 30 Bac-PAC assays at a time. A significant change in assay accuracy was not seen when using the mug compared to the jacketed incubator as the heat source. Additionally, when fully charged, the mug could maintain a 34-35°C temperature for 10-12 hours, which was approximately the same time required for the PrestoBlue assays to reach their peak accuracies (Figure 2.5b).

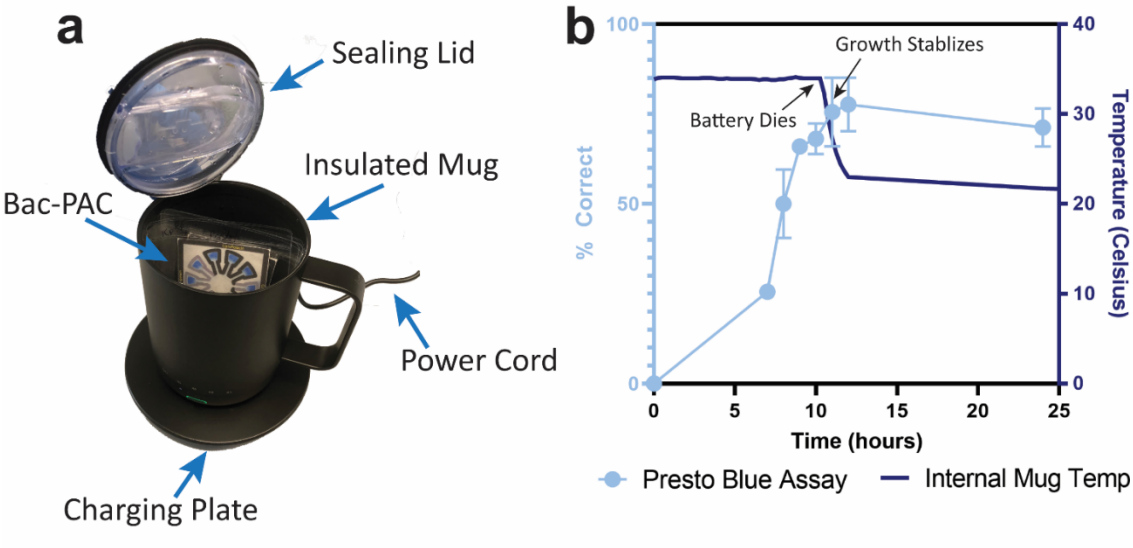

Figure 2.5 SmartMug Incubator. **a)** Picture of the Smart Mug rechargeable incubator system **b)** Accuracy of Bac-PACs in Smart Mug and temperature over 24 hours when on battery power. Error bars show standard error of the mean.

2.5 Conclusions

The need for individualized AMR testing worldwide is expected to increase over the next decade. Traditional phenotypic culture methods for AMR, such as broth dilution and Kirby-Bauer disk diffusion, are costly, time consuming, and require trained personnel, making them inaccessible in low- and middle- income countries that will be hardest hit by drug resistant epidemics. Instead, the improved Bac-PAC system brings us closer to clinically actionable information at the point-of-care by being low-cost, disposable, easy to use, and portable.

While many studies have compared PrestoBlue and XTT in liquid culture[27], relatively little is known about their applicability to paper diagnostics. While the XTT cell viability assay is commonly used in microbiology, it is typically suspended in liquid and read by spectrophotometry. PrestoBlue has been more commonly utilized for paper

microfluidics[34]; however, our side-by-side comparison demonstrates that XTT may give more accurate antibiotic categorization under certain conditions. The use of six different antibiotics, all with accuracies of at least 75% with proper dye pairing, demonstrates the flexibility of this platform to be expanded to countless other antibiotics. This is on par with the E-test and agar dilution methods which display 90% and 79% agreeance respectively with broth microdilution[35]. It is also approaching the 90% accuracy level sought by WHO[29]. While many novel technologies are tested with only a handful species or strains, the utilization here of 30 bacterial strains representing 12 different bacterial species demonstrates the potential for this technology to be expanded to other urgent antibiotic resistant threats such as *Neisseria gonorrhoeae* and *Candida auris*[2].

Other paper-based devices have been developed to diagnose bacterial infections[36–40], detect viral infections[41], and concentrate infectious DNA[42], but only a few paper platforms perform some kind of susceptibility testing. Deiss *et al*'s.[43] portable paper chip replicates the Kirby-Bauer disk diffusion method using PrestoBlue, but the readout requires measuring the zone of inhibition around each disk and comparing the results to published CLSI standards. Each chip also only provides the resistance profile for two antibiotics. Meanwhile, Michael *et al.*[44] designed a fidget spinner like device capable of concentrating urinary pathogens and estimating their resistance using another visible dye, WST-8. While their AST readout takes approximately 2 hours, it requires off-chip antibiotic exposure, multiple loading steps, separate devices for each antibiotic of interest, and has only been tested on *E. coli*. Meanwhile, the Bac-PAC is simpler to use with only one loading step and provides information on three antibiotics per device.

The Bac-PAC meets many of the priorities and goals for the development of accessible technologies for AMR outlined by the WHO[29]. For example, we calculated the disposable cost per chip as being $0.77 for XTT chips and $0.85 for the Presto Blue chips, well below the WHO recommended cost of $10-15 per assay[29] (Figure A.8). Additionally, this assay can be used by an untrained user with a few hours of training, again below the WHO recommended user of a trained laboratory personal with 2 days of training. Not only is this test low-cost and easy to use, but also stable over one month when stored protected from light at 4°C and for at least one week at 20°C. Literature suggests that storage under nitrogen vacuum may extend this shelf life even further[45]. Finally, we drastically reduced capital costs by replacing the typical water jacketed incubator with a rechargeable, low power coffee mug that is capable of maintaining the required 35°C for at least 10 hours on battery or indefinitely using a typical outlet. This approach meets the recommended < 25kg weight, > 8-hour battery life backup, and instrument costs of less than $10,000 also listed by WHO[29]. In the future, it may be feasible to replace the SmartMug incubator with a low-cost resistive microheater, further reducing cost and weight[46].

Although this work is a promising proof of concept for a cheap, dependable, and accessible antibiotic susceptibility test, some limitations of the study should be noted. First, the panel of bacteria utilized in testing contained a higher portion of resistant samples than may be found in the general population. Additionally, urine samples were sterilized to remove other trace bacteria before CRE samples were added. Therefore, it is unknown how the presence of such trace bacteria may impact test results To eliminate these limitations, further studies should focus on clinical trials of real samples from a

diverse patient population. Additionally, *Providencia stuartii* reads as susceptible to aminoglycosides in antibiotic susceptibility testing but is intrinsically resistant, and *Shigella sonnei* and *Salmonella Typhimurium* are not effectively treated with cephalosporins, such as cefazolin. Therefore, this test may need to be paired with a rapid bacterial identification strategy to eliminate these specific treatment options that are species dependent. In the future, we hope to incorporate direct from patient inoculation and rapid species identification in order to fully meet the WHO AMR criteria.

CHAPTER THREE

VISIBLE COLORIMETRIC GROWTH INDICATORS OF *NEISSERIA GONORRHOEAE* FOR LOW-COST DIAGNOSTIC APPLICATIONS[*]

3.1 Abstract

N. gonorrhoeae is one of the most pressing antibiotic resistant threats of our time and low-cost diagnostics that can easily identify antibiotic resistance are desperately needed. However, *N. gonorrhoeae* responds so uniquely to growth conditions that it cannot be assumed gonorrhea will respond to common microbiological methods used for other pathogenic organisms. In this paper, we explore visual colorimetric indicators of *N. gonorrhoeae* growth that can be seen without a microscope or spectrophotometer. We evaluate growth media, pH indicators, resazurin-based dyes, and tetrazolium-based dyes for their use in simple colorimetric system. Overall, we identified Graver Wade media as the best at supporting robust gonococcal growth while also providing the least background when analyzing results of colorimetric tests. XTT, a tetrazolium-based dye, proved to show to brightest color change over time and not negatively impact the natural growth of *N. gonorrhoeae*. However, other dyes including PrestoBlue, MTT, and NBT are less expensive than XTT and work well when added after bacterial growth has already occurred. By identifying the specific use cases of these dyes, this research lays the groundwork for future development of a color-based antibiotic susceptibility low-cost test for *N. gonorrhoeae*.

[*] Adapted with permission from Oeschger and Erickson. PloS ONE, 2021.

3.2 Introduction

The rapidly developing multi-drug resistance of *Neisseria gonorrhoeae* has been identified as a major public health threat by the World Health Organization[47], the National Institute of Health[48], and the Center for Disease Control[49]. For *N. gonorrhoeae*, phenotypic antibiotic susceptibility testing remains standard due largely to the cost of genotypic testing and the vast array of genes involved in resistance[18,50]. Point-of-care (POC) diagnostics for identification of *N. gonorrhoeae* infections have increased the number of patients treated compared to symptomatic management alone[51,52], however, increased treatment without careful consideration of specific antibiotics used may drive further antibiotic resistance in this organism[53]. Therefore, many experts have called for the creation of a POC test that enables gonococcal antimicrobial resistance monitoring worldwide [54–57] . Modeling studies have demonstrated that an AMR POC test for gonorrhea could drastically delay the onset of untreatable gonorrhea by 10 years or more[19,20,58–60].

N. gonorrhoeae is a notoriously fastidious, obligate human pathogen with limited clinically relevant models that does not grow well in standard liquid medias[61]. Previous research has shown that even the widely used, non-toxic resazurin dye inhibits *N. gonorrhoeae* growth so significantly that it could be used a treatment option[62]. Therefore, it is important for growth media and colorimetric indicator combinations to be specifically tested on this unique organism. The ideal colorimetric indicator system should be: 1) visible to the naked eye, 2) low cost, 3) not inhibit the natural growth of bacteria, and 4) display minimal strain-to-strain variability. Herein, we evaluate three

categories of dyes: pH indicators, resazurin indicators, and tetrazolium salts. These dyes were selected for their applicability to suspended cell cultures, ease of use, and low cost.

pH indicators are one of the oldest colorimetric indicators and are often included in commercial medias, but until now their usefulness has not been evaluated for studying *N. gonorrhoeae*. Meanwhile, resazurin is a redox dye that is reduced by bacteria to form a differently-colored product[63]. While many forms and protocols for resazurin exist, we selected the commercially modified PrestoBlue which advertises faster reduction times compared to resazurin salt. Similarly, tetrazolium salts have many forms and multiple generations, each with unique chemical properties. From commonly used tetrazolium-based dyes, we selected two first-generation salts, MTT and NBT, which form insoluble formazan and one second-generation salt, XTT, which forms a soluble formazan, all reactions that produce a visual color change. We used the Clinical and Laboratory Standard Institute's recommend clinical control strain *N. gonorrhoeae* ATCC 49226[64,65] and the WHO Neisseria Reference panel from the Center for Disease Control and Food & Drug Administration's Antibiotic Isolate Bank[9,66] to test the various dye and media pairs.

3.3 Materials and Methods

N. gonorrhoeae ATCC 49226 and the WHO *N. gonorrhoeae* Reference panel[9,66] (CDC & FDA Antibiotic Resistance Isolate Bank. Atlanta, GA). All strains were stored at -80˚C and revived and replated on prewarmed Chocolate Agar (Hardy Diagnostics, USA) at 37˚C and 5% CO_2 prior to use in all experiments. Graver Wade media was made following standard procedures by Graver and Wade[67] and Fastidious Broth was purchased from Hardy Diagnostics (USA). Bacteria were adjusted to an optical density of 0.1 and diluted 1/20 resulting in ~$5x10^6$ cells/mL starting concentration.

PrestoBlue Cell Viability Dye was purchased from ThermoFisher Scientific (USA), stored at 4˚C, and diluted to a working concentration of 10% v/v as per manufacturer instructions. MTT and NBT were stored at 4˚C, and 1mg/mL stock concentrations were made prior to each experiment. XTT was stored at -20˚C, and 1mg/mL stock concentrations were made prior to each experiment. MTT, NBT, and XTT were all sterile filtered using a 0.2µm filter and diluted 1/10 for a working concentration of 0.1mg/mL.

3.4 Results

3.4.1 Media Selection

N. gonorrhoeae struggles to grow in liquid media since it is a bacterium of mucosal surfaces and requires CO_2 to initiate growth. Therefore, higher inoculating concentrations are required [68]. Since there is no clinically defined standard liquid media, we compared the two most robust liquid gonococcal growth medias used in previous studies: Fastidious Broth and Graver Wade Media[67]. Over 48 hours of growth, both medias were able to support growth of *N. gonorrhoeae* strain ATCC 49226 as measured by spectrophotometry and results were averaged across 3 replicates (Figure 3.1a). The difference between the two medias was not statistically significant ($p = 0.402$). Both medias have previously been validated in a variety of *N. gonorrhoeae* strains[67,69,70] but these medias have never been specifically examined in the context of colorimetric growth indication. While the two medias offered similar growth support, Graver Wade media is clear and colorless (Figure 3.1b) compared to the dark yellow appearance of Fastidious Broth (Figure 3.1c). However, Fastidious Broth is commercially available in a ready-to-use format, whereas Graver Wade must be measured, mixed, and sterilized by the user. Therefore, the designer of point-of-care technology relying on liquid culture of *N.*

gonorrhoeae must prioritize either media color or ease of media acquisition when selecting between these options. For our studies, we chose to use Graver Wade in future experimentation so we could visualize the true color of the dyes.**pH Indicators**

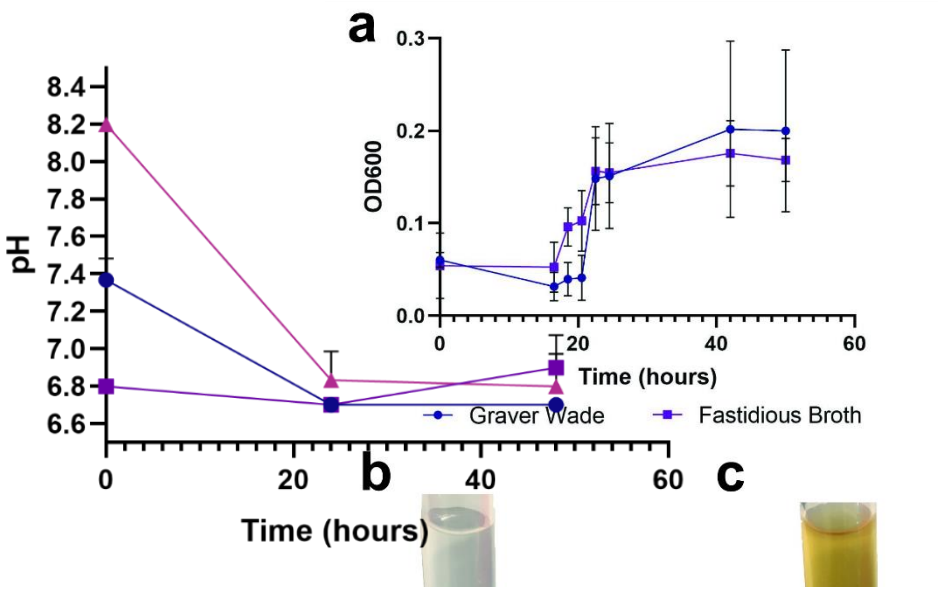

Figure 3.2 pH During Culture pH over 48-hour Cultures of *N. gonorrhoeae* ATCC 49226

Figure 3.1 Liquid Growth Medias. **a)** Growth curve of *N. gonorrhoeae* ATCC 49226 in Graver Wade (circles) and Fastidious Broth (squares). **b)** 24-hour culture tube of Graver Wade and **c)** 24-hour culture tube of Fastidious Broth

pH indicators medias are often added to commercial agar or liquid media as an indicator of growth or metabolic function. When bacteria are grown in weakly buffered solutions, they release acidic waste products during growth and replication that change the pH of the solution. Thus, a pH indicator that changes color between the starting pH and the final pH of the media can be used to signal that acidic products have been release by viable bacteria[71]. pH indicators have been used in food and environmental monitoring for this purpose[71–73]. For gonorrhea specifically, pH indicators have been shown to detect the presence of penicillinase release by some *N. gonorrhoeae* strains[74], but the use of pH indicators as a

broader viability marker has never been explored. Like many microorganisms, *N. gonorrhoeae* prefers a near neutral pH, specifically a pH of approximately 6.8. For this reason, Graver Wade media is adjusted to a pH of 6.8 prior to inoculation to support ideal growth, however, we found that *N. gonorrhoeae* will grow at more basic pH, up to at least 8.2, and release acidic products that reduce the pH back to the preferred 6.8 (Figure 3.2). While it is theoretically possible to use a pH indicator that changes color between 8.2 and 6.8, those typically used in microbiology such as bromothymol blue, methyl red, and phenol red are not sensitive enough in this range to detect low levels of *N. gonorrhoeae* growth needed for clinical diagnostics. Additionally, adjusting the pH away from ideal growth could inhibit initial growth and therefore impact clinical results. Therefore, pH indicators are not an ideal choice as a POC gonococcal growth indicator.

3.4.3 Viability Dyes

PrestoBlue (ThermoFischer Scientific, USA) is a proprietary, ready to use, lysis-free, resazurin-based cell viability dye. The purple-colored resazurin (Figure 3.3a) is reduced by metabolic products of bacterial cells to produce pink-colored resorufin (Figure 3.33b and 3.3c), which can be further reduced to hydroresorufin, which is clear and colorless[75]. This color change is fluorescent at a excitation wavelength of 530-570nm with emissions at 585-590nm wavelength[76], but changes are also easily seen by eye[77], making it applicable to a variety of POC technologies[78–80]. Resazurin is typically diluted to a working concentration of ~0.1mg/mL[81], but for PrestoBlue, the manufacturer recommends a standard 10% v/v dilution of the stock solution to obtain a working concentration. PrestoBlue is pH buffered and is reduced in as little as 10 minutes, significantly shorter than resazurin alone[82].

When *N. gonorrhoeae* is incubated for 24 hours with 10% v/v PrestoBlue less color change is seen (Figure 3.3b) compared to adding 10% v/v at hour 23 and allowing an additional hour for bacterial growth and dye reduction (Figure 3.3c). As these cultures are otherwise identical, this implies that PrestoBlue is inhibiting the metabolism of *N. gonorrhoeae*, an unfortunate side effect of many viability dyes. Indeed, this is confirmed by Schmitt *et al*.[62] who determined that pure resazurin exhibits antimicrobial activity against a broad range of *N. gonorrhoeae* strains, even at only 2.8µg/mL. Even the reduced form, resorfurin pentyl ether, inhibited *N. gonorrhoeae* growth, although with a higher minimum inhibitory concentration[62]. This result is surprising since resazurin is generally considered to be non-toxic[82], however, *N. gonorrhoeae* and *Francisella tularensis* both possess unique lipoprotein sorting machinery which resazurin may block [62,83]. Regardless, resazurin-based assays for *N. gonorrhoeae* have been developed, with resazurin added at the end of growth [81] and no variation was found between the colors of

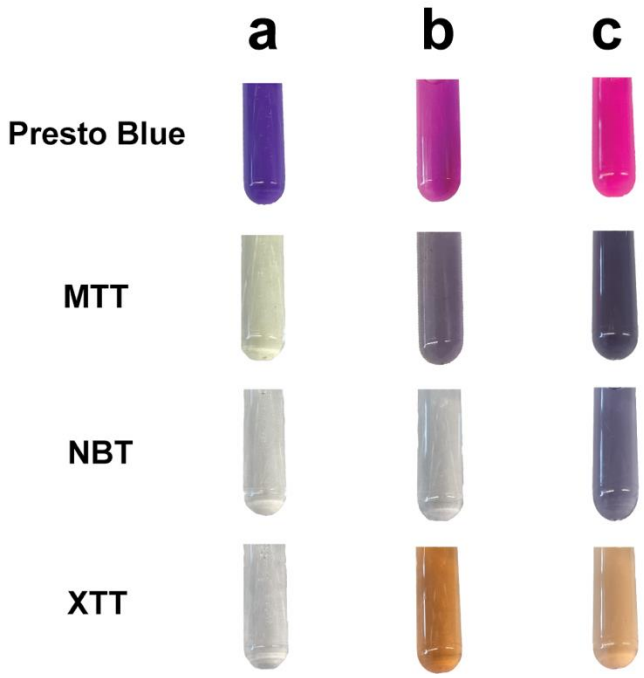

Figure 3.3 PrestoBlue, MTT, NBT, XTT during and after culture *N. gonorrhoeae* ATCC Clinical Control Strain 49226 in 1/10 PrestoBlue, 0.1mg/mL MTT, 0.1mg/mL NBT, and 0.1mg/mL XTT: **a)** At time 0, **b)** At time 24hr with dye added at time 0, and **c)** At time 24hr with dye added at 23hr

the dye post growth (Figure B.1). The exact concentration of pure resazurin in PrestoBlue is unknown, however, it still appears to display reliable color change in the presence of a 24-hour gonococcal culture, implying that the toxic effects of PrestoBlue may be less than that of resazurin alone, although further experimentation is needed to confirm this.

MTT, NBT, and XTT are tetrazolium salts that are reduced to visibly colorful formazans by cells during growth. MTT and NBT are first-generation tetrazolium salts that are pulled across the cell membrane due to their net positive charge and form insoluble formazans inside bacteria[84]. XTT on the other hand is a second-generation tetrazolium salt with a negative charge that requires an electron acceptor to be completely reduced to form a soluble formazan[85]. Most research on tetrazolium salts has been primarily performed on mammalian cultures [86–88], but they are broadly applicable for

bacterial cultures as well[89–91]. Here we evaluated MTT, NBT, and XTT for their use in identifying *N. gonorrhoeae* growth rapidly and consistently.

MTT at a working concentration of 0.1mg/mL appears pale yellow before growth (Figure 3.3a). When added at the beginning of 24 hours of *N. gonorrhoeae* growth, it turns a grey purple (Figure 3.3b). This color is more distinct when the dye is added after 23 hours of growth and incubated for an additional hour (Figure 3.3c). This implies that MTT has some inhibitory effect on the *N. gonorrhoeae* growth, which is not surprising since some tetrazolium dyes display microbial toxicity[92]. This inhibitory effect is especially apparent for *N. gonorrhoeae* strain WHO X (Figure B.2).

Like MTT, NBT at a working concentration of 0.1mg/mL appears slightly yellow before growth (Figure 3.3a) and turns to a grey purple after growth. However, NBT inhibits *N. gonorrhoeae* growth much more than MTT, resulting in no noticeable color change when dye and bacteria are incubated together (Figure 3.3b). This dye only works reliably as a gonorrhea indicator when added 1 hour before the end of growth (Figure 3.3C). It also has the least color change when compared to the other dyes studied and did not display strong color change at all in *N. gonorrhoeae* strains WHO G, U, V, W, or Y (Figure B.3).

XTT was by far the best tetrazolium dye tested. At a working concentration of 0.1mg/mL, it appeared clear and colorless before growth (Figure 3.3a) and turned a vibrant orange after growth. It did not appear to inhibit *N. gonorrhoeae* growth, in fact, the color was more vibrant when added at the beginning of the 24-hour growth phase (Figure 3.3b) rather than during the last hour of incubation (Figure 3.3c) implying XTT may take longer to be reduced than MTT and NBT. XTT was the only dye tested that did

not significantly inhibit natural gonococcal growth, making it ideal for clinical diagnostics that aim to minimize sample handling and processes steps since the dye can be pre-loaded with the sample. However, XTT did exhibit some strain variation with poor color change being seen in *N. gonorrhoeae* strains WHO U, X, and Z (Figure B.4).

3.5 Discussion

The optimal cell viability media and dye pair for *N. gonorrhoeae* point-of-care detection should be: 1) highly visible to the naked eye, 2) low cost, 3) not inhibit the natural growth, and 4) display minimal strain-to-strain variability. Graver Wade media displayed equivalent growth to Fastidious Broth and was clear and colorless, therefore we selected it for further experimentation with various dyes. *N. gonorrhoeae* was able to grow at a pH ranging from 6.8-8.2 but preferred 6.8, since this is considered the cutoff for most pH indicators, the sensitivity would be lacking. Next, we tested resazurin in the form of PrestoBlue and found the color changed dramatically when added after growth and is cost effective (Table 3.1). However, consistent with research by Schmitt *et al.* [62], we found that resazurin was toxic to *N. gonorrhoeae* over time, resulting in poor color change if added prior to growth. MTT was the cheapest option evaluated and displayed robust color change regardless of the growth stage in which it was added but it also showed signs of inhibiting growth. NBT seemed to be the most toxic, apparent by the complete lack of color change when co-incubated for 24 hours. Overall, XTT was the least toxic but the most expensive (Table 3.1).

Table 3.1 Dye Cost Comparison of PrestoBlue, MTT, NBT, and XTT based on listed working concentrations and prices from ThermoFischer Scientific.

Indicator	Concentration	Cost for 1mL Culture
Presto Blue	10% v/v	$0.06
MTT	0.1mg/mL	$0.01
NBT	0.1mg/mL	$0.02
XTT	0.1mg/mL	$0.19

Every strain responded slightly differently to each dye (Figure B.1-B.4) which has also been seen with other organisms[93]. Therefore, it is important to test any colorimetric diagnostic on a wide variety of *N. gonorrhoeae* strains to ensure accurate results. Our study was constrained to only a single concentration of each dye (1/10 PrestoBlue and 0.1mg/mL MTT, NBT, and XTT) to allow for a comparison of relative toxicity. Further research is required to determine optimum concentrations for each dye that balances strong color change with minimum bacterial growth inhibition. Additionally, MTT, NBT, and XTT were dissolved in water to minimize confounding factors that could limit *N. gonorrhoeae* growth, but PBS, DMSO, or another solvent may improve their functionality[94,95]. Overall, these resazurin and tetrazolium-based dyes have potential for simple, low-cost colorimetric readouts of *N. gonorrhoeae* viability that could enable a desperately needed point-of-care test for antibiotic susceptibility.

CHAPTER FOUR

DEVELOPMENT OF LATERAL FLOW AND PAPER MICROFLUIDIC ASSAYS FOR DETECTION AND ANTIBIOTIC SUSCEPTIBILITY TESTING OF WHOLE CELL *NEISSERIA GONORRHOEAE*

4.1 Abstract

Neisseria gonorrhoeae, the causative agent of gonorrhea, is an urgent worldwide threat and rapidly expanding antibiotic resistance is threating to make infections untreatable. Low-cost testing for diagnosis and antibiotic susceptibility are desperately needed to combat this obligate human pathogen. Accessible antimicrobial resistance testing is needed to gather data on the full spread of this resistance and to enable personalized prescriptions to avoid treatment failures. Here-in we develop a two-step paper microfluidic platform that identifies *N. gonorrhoeae* in a patient sample using a lateral flow assay and then performs antibiotic susceptibility on a wax printed chip, both at the point of care. The lateral flow assay utilizes whole cell bacteria without pretreatment, allowing viable cell recovery after testing. These cells are then exposed to antibiotics and dye using a single bacterial loading step on the wax printed NG Bac-PAC, providing bright, orange-colored readouts to indicate high levels of resistance. This colorimetric, paper-based, reader free system costs less than $2 per test can improve upon syndromic management currently employed in low resource setting.

4.2 Introduction

Neisseria gonorrhoeae (NG) is the obligate human pathogen responsible for the sexually transmitted infection gonorrhea. The World Health Organization estimates there were 82 million new gonorrhea infections in 2020[96]. Unresolved gonococcal infection are

a leading cause of infertility, ectopic pregnancy pelvic inflammatory disease, and can increase the risk of acquiring HIV[96,97]. It is estimated that 80-90% of STI infections, including 70 million cases of gonorrhea, occur in the developing world[16]. Because of these factors, experts have called for increased focused on affordable and reliable point of care technologies for detection of sexually transmitted infections, especially in low- and middle-income countries[98].

Many low-income settings rely on syndrome-based diagnostics, identifying patients based on non-specific symptoms such a pelvic or abdominal pain and vaginal or urethral discharge[99]. Even once properly diagnosed, treatment of *N. gonorrhoeae* infections is becoming increasingly challenging due to widespread antimicrobial resistance (AMR) of clinically relevant antibiotics. Gonorrhea has developed resistance to every previously recommended antibiotic monotherapy including sulphonamides, penicillins, cephalosporins, tetracyclines, macrolides, and fluoroquinolones within a few decades of their first clinical recommendations[100]. Recently, the first cases of multidrug resistant gonorrhea with decreased susceptibility to ceftriaxone, the last remaining monotherapy, were detected in Thailand[101]. It is estimated that half of all gonorrhea infections in the United States display resistance resulting in an annual direct cost $133 million dollars[21]. Modeling studies suggest that the use of a theoretical point of care (POC) antibiotic susceptibility test (AST) for gonorrhea in even 20-50% of cases could delay the onset of multidrug resistant gonorrhea by decades and reduce the use of the last line therapy by 66%[19,20].

Much of the recent innovation around point of care diagnostics for *N. gonorrhoeae* have focused on molecular based detection. Cephid's XPert NG/GC PCR based cartridge

system was shown to significantly reduce turnaround time and the unnecessary use of antibiotics in emergency healthcare settings[102]. Horst *et al.*[40] developed a nucleic acid based paperfluidic platform to detect gonorrhea in 80 minutes. Additionally, the SpeeDx Resistance Plus GC molecular diagnostics has been given emergency use authorization to detect ciprofloxacin resistance in *N. gonorrhoeae* [103]. These tests all look for specific genes to identify *N. gonorrhoeae* species or ciprofloxacin resistance. However, since ciprofloxacin resistance is already greater than 90% in Southeast Asian countries[104], such a test may have limited use, and future AMR NG tests will need to cover multiple antibiotics to increase the likelihood of identifying susceptibility in a patient's sample. While extremely rapid and accurate, nucleic acid based diagnostics are more sensitive but less specific than phenotypic culture methods and are limited to known resistance genes[105]. This is especially important for *N. gonorrhoeae* which is known to have 20 or more resistance genes which it readily acquires through mutation and horizontal gene transfer with commensal microbiota[4]. Genotypic tests are also unable to provide minimum inhibitory concentrations and can over or underrepresent true resistant or susceptible phenotypes[106].

Herein we describe a two-part paperfluidic system using a traditional lateral flow assay (LFA) and a wax printed microfluidic to perform detection, isolation, and antibiotic susceptibility testing for *N. gonorrhoeae* at the point of care. The lateral flow assay utilizes whole, live *N. gonorrhoeae* cells, providing a colored readout to indicate infection and simultaneously isolating *N. gonorrhoeae* from other contaminating bacteria. Then we modified the previously described Bac-PAC (**Bac**terial **P**aper **A**ntibiotic Susceptibly Testing at Point-of-**C**are) technology[25,107] which gives a colored readout

corresponding to cell viability in the presence of antibiotics. The lateral flow tests provide an easy to interpret positive or negative readout. Live, purified bacteria can be recovered from the positive test line from the LFA and transferred to the NG Bac-PAC system which provides a personalized semi-quantitative antibiotic resistance profile.

4.3 Materials and Methods

4.3.1 Lateral Flow Assay Design and Reagents

LFAs were assembled using Hi-Flow Plus 180 backed membrane cards (Millipore Sigma, USA) with a 10mm Glass Fiber Diagnostic Pad (Millipore Sigma, USA) and two 20mm Cellulose Fiber Sample Pads (Millipore Sigma, USA) as the sample pad and waste pad. Control line antibodies were goat anti-rabbit IgG H+L secondary antibody (Invitrogen, USA) depending on antibody used for conjugation. Antibodies evaluated for the test line include PA1-7233 (ThermoFisher, USA) and ab19962 (AbCam, USA), GC12-323.6 (ThermoFisher, USA), GC12-351.4 (ThermoFisher, USA), GC12-316.4 (ThermoFisher, USA), and MOMP 386/418 (ThermoFisher, USA). Running buffer consisted of 1X Borate Buffer with 1.5% Tween-20, 1% bovine serum albumin, and 0.02% sodium azide. Conjugate release buffer consisted of 20mM borate in 1X PBS, 1% bovine serum albumin, 0.025% Tween-20, 0.02% sodium azide, 150mM sodium chloride, and 10% sucrose. Test and control lines were printed 3mm apart using an Automated Lateral Flow Reagent Dispenser (ClaremontBio) and a Legato 200 Dual Syringe Infusion Pump (KD Scientific) at a rate of 6.7 mL/min.

Standard Gold 20nM Nanoparticles 1OD (CytoDiagnsotics) were conjugated with anti-*N. gonorrhoeae* antibodies ab19962 (AbCam, USA) at a concentration of 0.5mg/mL at pH 8.7 overnight. Nanoparticles were concentrated to 20OD and stored in 1X TBS

with 1% bovine serum albumin until use. 2.5µL of conjugate release buffer was applied to the glass fiber conjugated pad and dried for 20 minutes before applying 2µL of 20OD conjugated nanoparticles. Strips were dried for 2 hours before use. For testing, 40uL of sample was applied to the sample pad immediately followed by 70uL of running buffer. Results were read and imaged between 30 and 60 minutes.

4.3.2 Test Line Analysis

LFAs were imaged after 30 minutes but before 1 hour using the TIDBIT system described by Lu et al., 2017. Test to control line ratios (T/C) were calculated according to methods described by Wang et al., 2019. Briefly, images taken of the LFA by the TIDBIT reader were loaded into MATLAB and white balanced to the strip background. The code located the test and control lines, calculated the red pixel intensity, and normalized the data against the background. If no test line could be found, the intensity value was assumed to be zero. The T/C ratio was obtained by dividing the test line red pixel intensity by the control line red pixel intensity.

4.3.3 Bacterial Strains and Recovery from LFA

Glycerol freezer stocks of *N. gonorrhoeae* clinical control strain American Type Culture Collection (ATCC) 49226 and "WHO *Neisseria gonorrhoeae*" panel from the Center for Disease Control and Food and Drug Administration Antibiotic Resistant Isolate Bank (CDC/FDA AR Isolate Bank) [9] were obtained and kept at -80°C until use. Samples were thawed and streak on Chocolate Agar plates (Hardy Diagnostics, USA) and grown 18-24 hours prior to use on LFAs or Bac-PACs. Suspended cell concentrations in terms of CFU/mL were determined using an optical density at 625nM.

E. coli ATTC 25922 and CDC/FDA AR Isolate Bank strains AR001, AR006, and AR0011 from the Carbapenem Resistant Enterobacterales panel [9] were obtained as glycerol freezer stocks and kept at -80°C until use. Strains were grown on Mueller Hinton II Agar (Hardy Diagnostics, USA) for 18-24 hours prior to use on LFAs. Lyophilized *N. meningitidis* strains 13077 serogroup A, 13090 serogroup B, and 13102 serogroup C were obtained from ATCC, suspended in BBL Mueller Hinton Medium II (BD Biosciences, USA), and stored as glycerol stocks at -80°C until use. Samples were thawed and streaked on Chocolate Agar plates (Hardy Diagnostics, USA) and grown 18-24 hours prior to use on LFAs. *L. gasseri* was obtained from the lab of Illana Brito and grown on De Man, Rogosa and Sharpe (MRS) agar plates for 24 hours prior to use on LFAs.

Live bacteria were recovered from a used LFA by either cutting the test line out with scissors or scrapping the nitrocellulose backing. The test line was then suspended in 110µL of pre-warmed Graver Wade media and incubated at 37°C for 2 hours. Graver Wade media was prepared fresh weekly according to the recipe defined by Wade and Graver, 2007. After pre-incubation 100µL was plated on pre-warmed chocolate agar plates. Cells were grown for 24 hours before counting or use in further experiments.

4.3.4 NG Bac-PAC Design

Bac-PAC assays were made as described by Oeschger et al., 2022.. Briefly, a radial based design was printed using Xerox ColorQube 8570 wax on Whatman Grade 1 paper. Wax was melted into the paper and the back of the chip was sealed with packing tape. The total chip size is 52 x 52mm with 1 x 8mm channels, and 7 x 5mm wells and a 15mm diameter central loading zone. 25 mg of agar dissolved in 20mL of Graver Wade media and heated to 95°C. When the mixture cooled to 50°C, antibiotics stocks were added.

Each well was loaded with 8uL of antibiotic/agar solution and allowed to dry overnight before testing. Testing was performed with 10^8 CFU/mL solution of bacteria suspended in Graver Wade media and sealed with ELISA sterile sealing film. WST-8 diluted 1 to 5 (ThermoFisher Scientific, USA) was added through a center hole after 1 hour of incubation. Final incubation was run overnight for ~12 hours before results were interpreted.

4.3.5 Commercial NG LFA

Commercially available gonorrhea rapid test kits were obtained directly from the manufacturer. Kits included lateral flow assay cassettes, Extraction Reagent 1 (0.15M NaOH), Extraction Reagent 2 (0.2M HCl), sterile swabs, extraction tubes, and dropper tips. Bacteria samples were prepared in Graver Wade media at concentrations between $5x10^9$ and 10^4 CFU/mL. Sterile swabs were dipped in the bacteria solutions and suspended in 5 drops Extraction Reagent 1 according to manufacturer's instructions. After 2 minutes, 4 drops of Extraction Reagent 2 were added, and the swab was soaked for an additional 1 minute. The swab was compressed to release trapped liquid and 3 drops were applied to the cassette. The assay was run for at least 10 minutes but not longer than 30 minutes, as per manufacturer's instructions. Images, analysis, and bacterial recovery were performed as stated above.

4.4 Results

4.4.1 A Whole-Cell Lateral Flow Assay to Determine Presence of *Neisseria gonorrhoeae*

While most bacterial LFAs include some combination of lysing and heating steps to kill and break apart the bacteria, we designed a sandwich style LFA to work with whole live cells so that bacterium maintain viability for downstream testing. Figure 4.1a shows a schematic of the LFA design which includes a cellulose sample pad, glass fiber conjugate pad, nitrocellulose membrane, test and control antibody lines, and a cellulose waste pad. Six commercially available anti-*Neisseria gonorrhoeae* antibodies, including four monoclonal and two polyclonal, were tested both as test lines and conjugated to nanoparticles. We found that a combination of two polyclonal antibodies, ab19962 and PA1-7233 at concentrations of 0.4 and 0.3 mg/mL paired with 20nm standard gold nanoparticles conjugated with ab19962 gave optimum test line signal and minimal non-specific binding. The control line consisted of 0.1mg/mL anti-rabbit IgG. We found no benefit to increasing test line concentrations to 0.5 mg/mL of ab19962 and 0.4 mg/mL of PA1-7233 did not improve the limit of detection and increased the background signal detected in the negative control (Figure C.1).

Literature suggested that the clinically relevant concentrations of gonorrhea in urine catch sample during symptomatic infection is 7 x10^3 to 9x10^8 CFU/mL[110]. The LFA was validated with *N. gonorrhoeae* ATCC 49926 at concentrations from 10^4 -5x10^9 CFU/mL. We suspend pure colonies of *N. gonorrhoeae* clinical control strain ATCC 49226 in running buffer and applied 40uL to each test strip followed by 70uL of running buffer. Test strips were allowed to run for 30 minutes, but no longer than 1 hour to prevent false positive signals. Negative controls consisted of running buffer only containing no bacteria. We quantified the intensity of the test lines and normalized them to the intensity of the control lines, forming a T/C ratio. We found the visible limit of detection to be 10^6 CFU/mL which aligned with a T/C ratio of 0.1 (Figure 4.1b, c). Using this cutoff ratio, we were correctly able to identify samples spiked with 10^6 CFU/mL or higher with 92% accuracy.

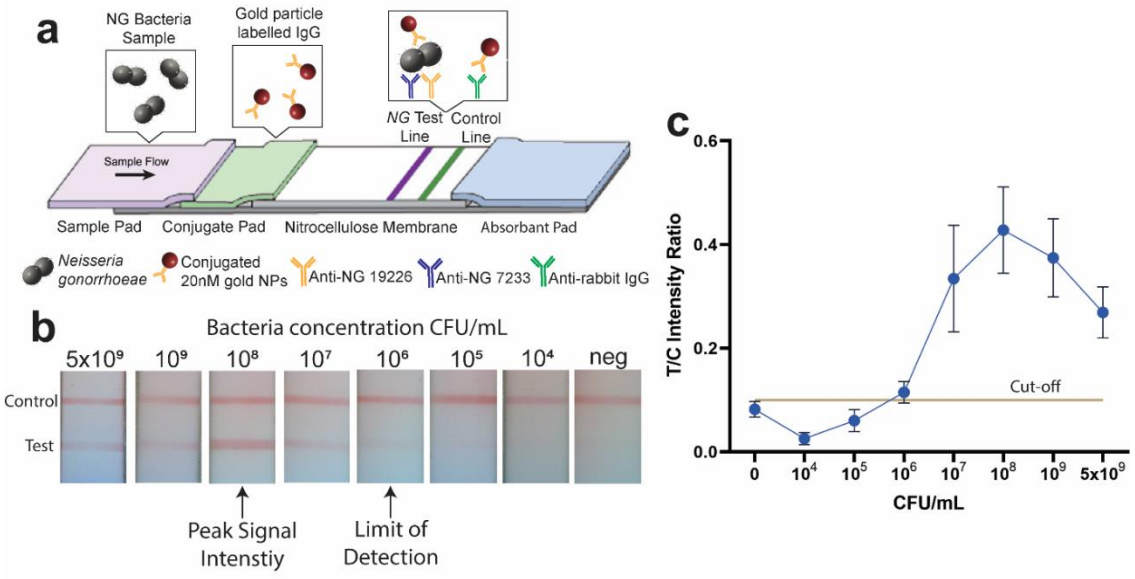

Figure 4.1 NG Lateral Flow Assay**. a)** Schematic of sandwich lateral flow assay with components labelled. **b)** Photographs of LFA run for 30 minutes with NG ATCC at 5 x 10^9 – 10^4 CFU/mL with a limit of detection at ~10^6 CFU/mL. Negative control contains no bacteria and shows no test line. **c)** Ratio of test line intensity to control line intensity plot versus *N. gonorrhoeae* ATCC 49226 bacteria concentration in CFU/mL. n = 8. Errors bars represent SEM.

When the T/C ratio was plotted versus bacteria concentration, an obvious hook effect can be seen after the peak signal at 10^8 CFU/mL (Figure 4.1c). The hook effect is a common occurrence with LFAs and occurs when the antigen concentration is significantly higher than the nanoparticle concentration such that the test line becomes blocked with unlabeled antigen[111]. While we could increase the concentration of nanoparticles on the conjugate pad to potentially expand the testing range higher than 5 x 10^9 CFU/mL there would be no additional clinical gain.

4.4.2 Specificity of LFA to Urinary Pathogens and Vaginal Microbiota

Multiple sampling sites could be considered for use with our LFA including male and female urethral swabs, male urine catch samples, female vaginal swabs, and pharyngeal swabs. In the case of urethral or urine samples, one should consider the possibility of comorbidities such as urinary tract infections. Thus, we examined the specificity of our LFA with three of the most common urinary pathogens *Escherichia coli*, *Proteus mirabilis*, and *Klebsiella pneumoniae*. *E. coli* is the most common pathogen identified up to 75% of urinary tract infections and forms reservoirs in the vagina[112,113]. We found no cross reactivity of the assay with the four strains of *E. coli* tested (Figure C.3). *P. mirabilis* is another common urinary pathogen in the same family as *E. coli* that is known to transmit from human to human [114]. We found minimal cross reactivity of our LFA with *P. mirabilis* with very faint false positives at 10^8 CFU/mL. We also tested 3 strains of *K. pneumoniae* and found no cross reactivity.

While the urethra is generally a sterile anatomical site, the human vagina on the other hand is known to be naturally inhabited by an array of commensal bacteria. While bacterial species and concentrations can vary widely from person to person, Lactobacillus

are known to be the predominate microflora [115]. Thus, we tested our LFA with *L. gasseri* for cross reactivity and found very faint false positives 10^8 CFU/mL. Overall, using the visually determined T/C cutoff of 0.1, we have a specificity of 86%.

4.4.3 Cross Reactivity of Neisseria species

N. gonorrhoeae shares significant genetic similarity with other human Neisseria species, thus we suspected that our polyclonal antibodies may cross react. Within the Neisseria species two pathogens, *N. gonorrhoeae* and *N. meningitides,* and at least 10 commensal Neisseria species have been identified [116]. *N. lactamica* is the closest relative of *N. gonorrhoeae* and *N. meningitides*. Other commensal species include *N. cinerea*, *N. mucosa*, and *N. elongate* all of which are commonly found on mucosal surface of the nasal pharynx (Figure 4.2a). *N. meningitides* and *N. gonorrhoeae* may occasionally co-located within the oropharynx, rectum, or urethra [117], thus, we sought to challenge our LFA with *N. meningitides*. *N. meningitides* showed crossed reactivity for all three serogroups tested (Figure 4.2b). This indicates that our LFA may not be suited for discriminating between *N. gonorrhoeae* and *N. meningitides* infections but rather to identify the presence of a Neisseria species broadly. Therefore, without changing the antibodies used on the test line, we cannot recommend the use of this LFA for pharyngeal sites where other *Neisseria* species may be present.

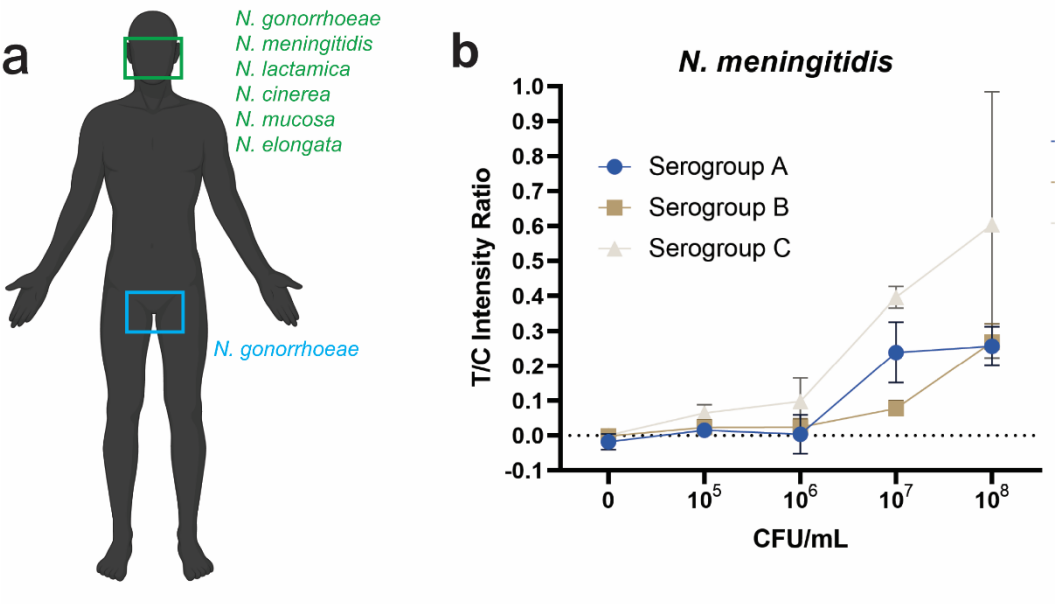

Figure 4.2 Neisseria species testing **a)** Sampling sites for *Neisseria* species **b)** Specificity of the lateral flow assay when challenged with the closely *related N. meningitidis* n =2. Error bars show SEM.

4.4.4 Recovery of Viable Cells from LFA

One of the main limitations of phenotypic AST is the need for prior isolation of pure bacteria from patient samples. For gonorrhea, this process often requires growing samples for 20-24 hours on chocolate agar plates in a certified laboratory. We aimed to use an LFA as a means to isolate and concentrate bacteria directly from patient urine or swab samples in hopes of circumventing this common problem. Many LFAs preprocess whole bacterial samples prior to flow by heating the cells to 100°C for 1 hour or exposing them to lysing buffers. These pretreatments break cells into smaller pieces to increase assay sensitivity but also kill the cells so they cannot be reused. In the LFA described here, *N. gonorrhoeae* cells are not lysed prior to or during assay flow, so that live cells could be recovered for further testing.

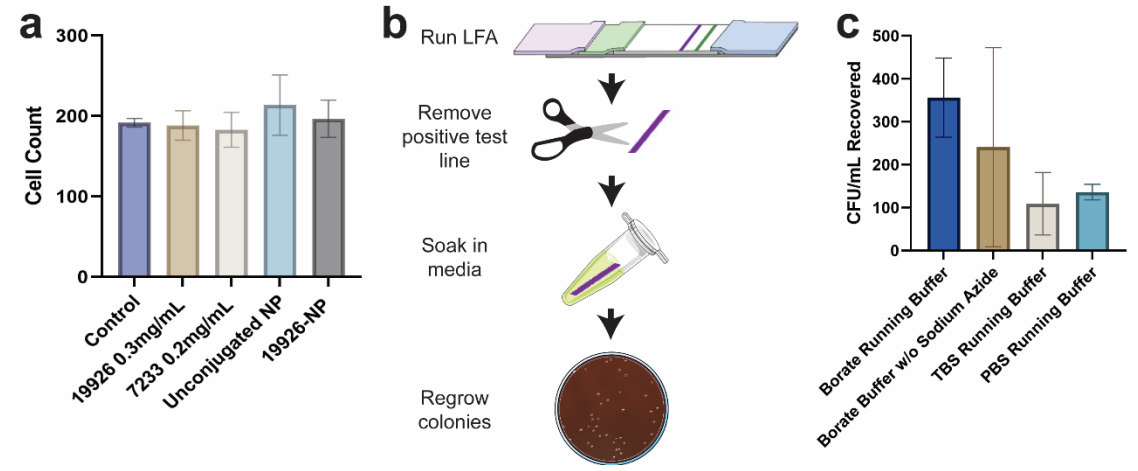

Figure 4.3 Whole Cell Recovery **a)** Bar graph showing CFU/mL growth after 24 hours of N. gonorrhoeae ATCC 49926 after exposure to components of lateral flow assay. Test groups were not significantly different than control as determined by paired t-tests with $p < 0.05$. **b)** Schematic of recovery workflow involving run the LFA, removing the test line through cutting or scrapping, soaking Graver Wade media, and plating on chocolate agar to obtain colonies **c)** Cells recovered from test lines of LFA assays run with 108 CFU/mL. n = 2

First, we ensured that the binding of antibodies and nanoparticles to the bacterial cells would not limit its ability to be regrown later. In Graver Wade liquid cultures, *N. gonorrhoeae* ATCC 49926 cells were exposed to antibodies, unconjugated nanoparticles, and conjugated nanoparticles at the same concentrations used in the LFA and then plated on chocolate agar and grown for 24 hours. The colony forming units were counted and compared to a control sample of *N. gonorrhoeae* in Graver Wade media. No significant difference in growth was seen between any of the samples and the control, as determined by paired t-tests ($p < 0.05$) (Figure 4.3a). Thus, it was hypothesized that *N. gonorrhoeae* could replicate even while the antibodies from the IgG test line or the nanoparticles were still attached.

We suspended 10^8 CFU/mL of *N. gonorrhoeae* ATCC in Graver Wade media and ran LFAs with 40uL of sample followed by four different running buffers: original borate running buffer, borate running buffer without sodium azide, TBS running buffer, or PBS running buffer. The resulting positive test line from the nitrocellulose membrane was

scraped using flat edged tweezer, suspended in 110uL of Graver Wade media, incubated for 2 hours, and was spread on a chocolate agar plate and grown for 20-24 hours (Figure 4.3b). Cells were counted to determine recovery efficiency in terms of CFU/mL. We were able to obtain ~226 individual viable colonies per mL across all buffers with the original borate running buffer performing the best with 356 CFU/mL recovered (Figure 4.3c). Additionally, we tried increasing the pH to 8.6, decreasing the pH to 4.0, and adding IdeZ IgG protease to aid in the removal of gonococcal cells from the nitrocellulose membrane. All these methods seemed to kill the bacteria and reduced recovery to zero cells. This recovered cell density is significantly lower than anticipated, and far too low for immediately susceptibility testing. It is possible that this LFA concentration method may still hold promise for other bacteria such as *E. coli* that are less fragile than *N. gonorrhoeae*.

4.4.5 Antibiotic Susceptibility Testing on the NG Bac-PAC

Wang and Erickson[25] and Oeschger *et al.*[107] previously described the Bac-PAC, a paper microfluidic for point of care ABR testing. Here, we modified this device for *N. gonorrhoeae* by changing the antibiotics, colorimetric dye, and incorporating agar to create the NG Bac-PAC. A 4uL aliquot of 2mg/mL agar was heated and added to each well of the Bac-PAC to support *N. gonorrhoeae* growth. Without the addition of the agar, less growth and conversion of the dyes we seen (Figure 4.4a). We suspect that the agar encouraged gonorrhea growth because it mimics the mucosal membrane environment where *N. gonorrhoeae* is naturally found more than the paper substrate alone.

The PrestoBlue dye used in previous version of the Bac-PAC is toxic to *N. gonorrhoeae* [62,118], thus could not be used. XTT and Graver Wade media were previously

found to be a good media and dye combination for point of care testing[118]. For susceptibility testing, we selected ciprofloxacin, azithromycin, and ceftriaxone as antibiotics to test based on their clinical use for gonorrhea and demonstration of resistance globally. Antibiotics were embedded in the agar to prevent them from flowing back into the center of the chip or into adjacent wells. As with previous versions of the Bac-PAC, we found that higher concentrations of antibiotics were needed to provide accurate results. We suspect that this is because of the addition of bacteria, antibiotics, and dye at the same time through a single loading step rather than allowing the bacteria to incubate with the antibiotics before adding the dye later. Figure 4.4b demonstrates possible readouts from the NG Bac-PAC where in more color change indicates higher resistance. A positive control containing no antibiotics is located at the right most 3 o'clock position. If this well does not change color, the test should be considered invalid because bacterial growth did not occur at all. To our knowledge, the NG Bac-PAC demonstrates the first time that *N. gonorrhoeae* has been grown on a paper substrate.

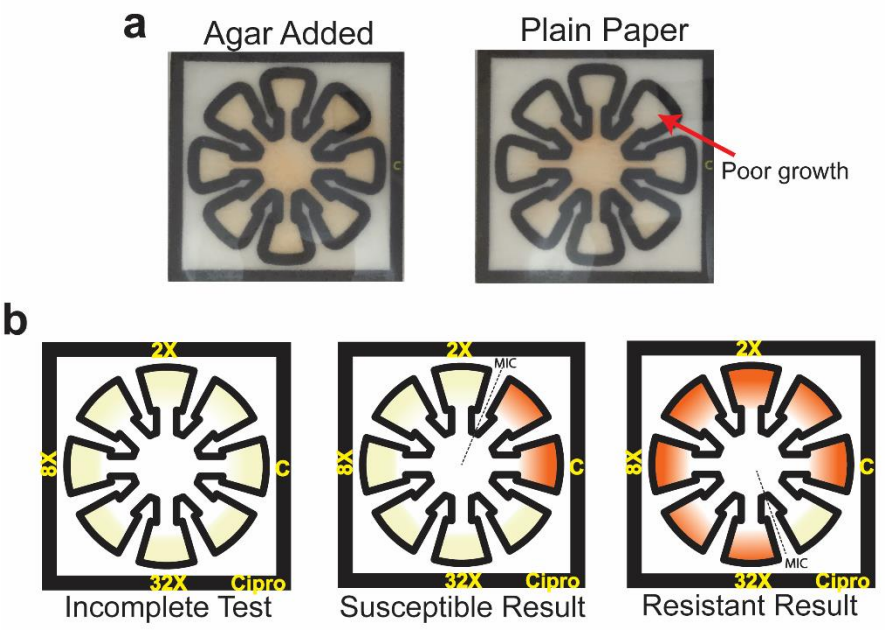

Figure 4.4 NG Bac-PAC **a)** Growth and color change of N. gonorrhoeae ATCC 49226 on agar and paper substrate and plain paper substrate **b)** Rendering of NG Bac-PAC testing for ciprofloxacin resistance displaying incomplete test, susceptible results, and highly resistant results

4.5 Discussion

Herein, we have described the development of paper-based assays for the diagnosis and antibiotic susceptibility testing of gonorrhea in low resource settings. Our NG LFA can detect 10^6 CFU/mL with high accuracy and specificity while the NG Bac-PAC offers a disposable and accessible solution to personalized susceptibility testing. The use of both assays together can replace traditional culture based clinical workflows for *N. gonorrhoeae* and improve access globally. Compared with the standard method of isolating gonorrhea on chocolate agar, identifying with gram stain, and performing agar dilution to determining antibiotic susceptibility, our method is both easier and cheaper. By capturing live bacteria directly from patients, we can eliminate the overnight culture on chocolate agar and by performing AST on a paper chip we drastically reduce cost and user input steps.

In 2020, an expert working group assembled by the World Health Organization developed a target product profile for *N. gonorrhoeae* diagnostics[22]. They determined there was a significant need for the development of diagnostics to detect gonorrhea and its antimicrobial resistance. They determined that specificity of >80% was acceptable for replacing syndromic management and screening the highest risk populations. Identification tests should be designed for health posts or district hospitals and be a standalone non-instrumented disposable device. Additionally, an ideal test would take less than 30 minutes and cost less than $3 USD. The most ideal susceptibility tests were expected test 1-2 antibiotic markers while costing less than $25 USD and run in 60 minutes at a district hospital level. Our whole cell NG LFA meets all of the recommended targets while the NG Bac-PAC misses the 1-hour time to result cutoff, it is 25 times cheaper than expected.

Currently there are no United States Food and Drug Administration cleared gonorrhea LFA tests on the market, however, Conformitè Europëenne (CE) marketed kits from Europe are available. When the sensitivity of our LFA was experimentally compared to a commercially available rapid test kit, we found our assay to demonstrate a 100-fold lower limit of detection with fewer sample handling steps when run on identical samples (Figure C.5).

This study has limitations that should be addressed in future work. One limitation of this assay is the relatively high limit of detection at 10^6 CFU/mL. It is possible that preconcentration by separating the test line binding and nanoparticle binding into separate steps could improve the LFA limits of detection by 10-100-fold (Zhang et al., 2020). Additionally, there is need for clinical validation and assessment of effectiveness

on clinical specimens, especially from asymptomatic individuals. Additionally, our LFA cannot discern between Neisseria species, so care should be taken when sampling sites that may be co-infected with N. meningitides, such as the pharynx.

CHAPTER FIVE

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Improvements to Paper-Based Diagnostics for Antibiotic Susceptibility Testing

With antibiotic resistance quickly becoming one of the most pressing health threats of our generation, new diagnostics are needed to help determine the scope of the problem and to address said problem directly. The **Bac**terial **P**aper-based **A**ntibiotic Susceptibility **C**hip or Bac-PAC described in Chapter 2 offers an alternative to traditional culture-based method of AST. The wax printed chip offers multiplex testing of three antibiotics at three concentrations with a single bacterial loading step. The chip is self-contained in sealing film both to protect the use and to prevent evaporation during use. The readout is a color change that is visible to the naked eye, removing the need for a separate reader. Results are available in ~12 hours and the overall cost per test is less than $1. The chip requires a simple heating source to maintain 37°C during the testing time, however, this is easily obtained with something like a rechargeable coffee mug, as demonstrated.

The Bac-PAC enables more clinics and laboratories to start AST testing programs without high capital investments. In low- and middle-income countries, data on the scope of AST is limited because of the fundamental lack of testing. This test with its simple use case, low cost, and low power requirements could change that. By reducing barriers to testing in higher income countries, we can also enable personalized prescriptions for antibiotics based on individual patient strains. Doing so now could help delay the onset of multi-drug resistance and untreatable pathogens, giving us more time to discover new antibiotic therapies. Once multi-drug resistance is widespread, personalized testing will

be required to avoid frequent treatment failures. In the future, methods for direct patient sampling from urine, blood, or spinal fluid should be evaluated.

5.2 Developing Color-Based Gonorrhea Diagnostics

Chapters 3 and 4 of this text focus on *N. gonorrhoeae,* the pathogen responsible for the sexually transmitted infection gonorrhea. Experts have increasing called for low-cost diagnostics for gonorrhea because of its widespread dissemination and increasing resistance. Chapter 3 details the interaction of a variety of commercially available dyes for determining cell viability with *N. gonorrhoeae*. Previously, it was known that this fastidious bacterium was easily harmed by common colorimetric viability dyes such as resazurin, but a comprehensive study had not been performed to determine what dyes would work instead. The work presented in Chapter 3 allows researchers moving forward to easily select dye and media pairs to design point of care assays. Overall, we found the clear colorless Graver Wade media paired with XTT or similar tetrazolium dye to be the most effective for single loading step paper microfluidics.

In Chapter 4, we develop two point of care assays: one for the diagnosis and one for the antibiotic susceptibly profiling of *N. gonorrhoeae*. The first is a lateral flow assay (LFA), one of the most well-known point-of-care technologies that has fundamentally changed clinical treatments for diseases like COVID-19. Our LFA is unique in the sense that it requires no preprocessing such as heating or lysing of the bacterial cells. The assay runs with whole, live bacteria so the cells can be recovered for further AST. The easy to read one line for negative result, two lines for positive result is intuitive even for untrained users, making this a possible at home screening tool. Although a reader system was used in our paper to obtain quantitative results, this is not necessary for, nor does it

change, clinical outcome. The assay runs completely in under 30 minutes, but results are stable for up to 1 hour. This test could replace or supplement syndromic management that is current practice in low- and middle-income countries. Further evaluation is necessary to determine the clinical sample types (i.e., urine, urethral swab, or vaginal swab) this assay could work with. Additionally, it is currently unknown how this test would fare against asymptomatic gonorrhea infections which are known to often have lower bacterial concentrations.

For the second part of Chapter 4, we modified the Bac-PAC system from Chapter 2 to work for *N. gonorrhoeae*. To do this we introduced agar to the paper to simulate natural mucus membranes and encourage growth of this fastidious organism. We also switch the colorimetric dye to WST-8, a tetrazolium salt like XTT identified in Chapter 3, because of its faster conversion time. In the future, clinical trials as well as long term shelf-life stability test should be performed. This NG Bac-PAC system could enable personalized treatment of gonorrhea infections, which will be increasingly important as antibiotic resistance continues to develop to the last line antibiotic.

APPENDICES

APPENDIX A: CHAPTER 2 SUPPLEMENTS

Table A.1 Bacterial Panel with gold standard MIC values from "Enterobacterales Carbapenem Breakpoint" from CDC/FDA Antibiotic Resistance Isolate Bank

AR Bank #	Organism	Ciprofloxacin MIC	Meropenem MIC	Gentamicin MIC	Cefazoline MIC	Tobramycin MIC	Ampicilin MIC
#0001	*E. coli*	> 8 R	4 R	4 S	>8 R	>16 R	>32 R
#0002	*E. cloacae*	> 8 R	> 8 R	8 I	>8 R	16 R	>32 R
#0003	*K. pneumoniae*	> 8 R	> 8 R	4 S	>8 R	16 R	>32 R
#0004	*K. pneumoniae*	> 8 R	> 8 R	1 S	>8 R	16 R	>32 R
#0005	*K. pneumoniae*	> 8 R	> 8 R	2 S	>8 R	>16 R	>32 R
#0006	*E. coli*	> 8 R	8 R	> 16 R	>8 R	>16 R	>32 R
#0007	*K. aerogenes*	> 8 R	0.25 S	0.5 S	>8 R	<=0.5 S	>32 R
#0008	*E. cloacae*	> 8 R	2 I	<= 0.25 S	>8 R	<=0.5 S	>32 R
#0009	*K. aerogenes*	<= 0.25 S	2 I	0.5 S	>8 R	1 S	>32 R
#0010	*K. pneumoniae*	<= 0.25 S	1 S	2 S	>8 R	4 S	>32 R
#0011	*E. coli*	> 8 R	<= 0.12 S	> 16 R	>8 R	>16 R	>32 R
#0012	*K. pneumoniae*	> 8 R	0.25 S	16 R	>8 R	>16 R	>32 R
#0013	*E. coli*	<= 0.25 S	<= 0.12 S	> 16 R	>8 R	8 I	>32 R
#0014	*E. coli*	> 8 R	<= 0.12 S	> 16 R	>8 R	>16 R	>32 R
#0015	*E. coli*	> 8 R	<= 0.12 S	> 16 R	>8 R	>16 R	>32 R
#0016	*K. pneumoniae*	<= 0.25 S	<= 0.25 S	<= 0.25 S	4 I	<=0.5 S	>32 R
#0018	*K. aerogenes*	<= 0.25 S	<= 0.12 S	0.5 S	>8 R	<=0.5 S	>32 R
#0019	*E. coli*	> 8 R	<= 0.12 S	<= 0.25 S	8 R	<=0.5 S	>32 R
#0020	*E. coli*	>8 R	<= 0.12 S	2 S	>8 R	2 S	>32 R
#0021	*C. freundii*	<= 0.25 S	<= 0.12 S	0.5 S	>8 R	<=0.5 S	32 R
#0022	*C. freundii*	<= 0.25 S	<= 0.12 S	<= 0.25 S	>8 R	<=0.5 S	>32 R
#0023	*C. freundii*	<= 0.25 S	<= 0.12 S	0.5 S	>8 R	0.5 S	32 R
#0024	*C. koseri*	<= 0.25 S	<= 0.12 S	0.5 S	2 S	0.5 S	>32 R
#0025	*C. koseri*	<= 0.25 S	<= 0.12 S	> 16 R	8 R	4 S	>32 R
#0026	*Providencia stuartii*	<= 0.25 S	<= 0.12 S	0.5 S (R on web)	>8 R	<=0.25 S (R on web)	>32 R
#0027	*Serratia marcescens*	1 R	<= 0.12 S	<= 0.25 S	>8 R	0.5 S	>32 R
#0028	*Klebsiella oxytoca*	<= 0.25 S	<= 0.12 S	0.5 S	>8 R	0.5 S	32 R
#0029	*Proteus mirabilis*	<= 0.25 S	0.5 S	1 S	8 R	1 S	<=1 S
#0030	*Shigella sonnei*	<= 0.25 S	<= 0.12 S	1 S	/	1 S	>32 R
#0031	*Salmonella Typhimurium*	0.25 S	<= 0.12 S	0.5 S	/	1 S	<=1 S

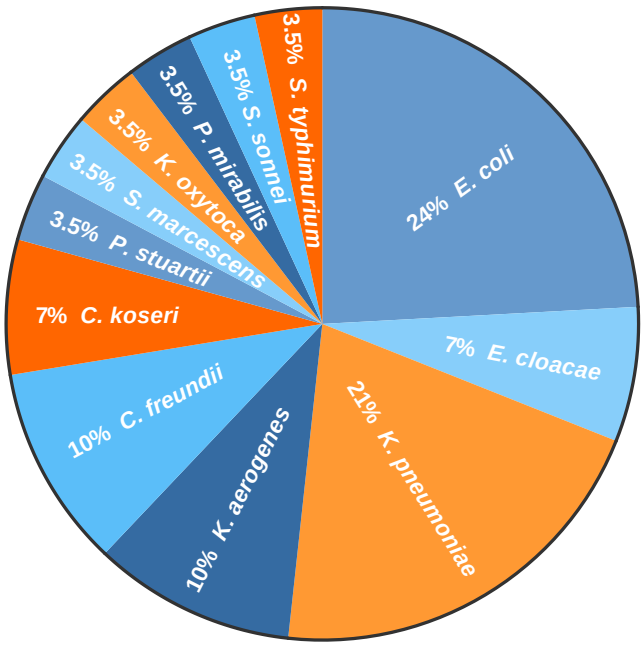

Figure A.1 Bacterial species included in this study from the CDC/FDA AR Isolate Bank Panel "Enterobacterales Carbapenem Breakpoint"

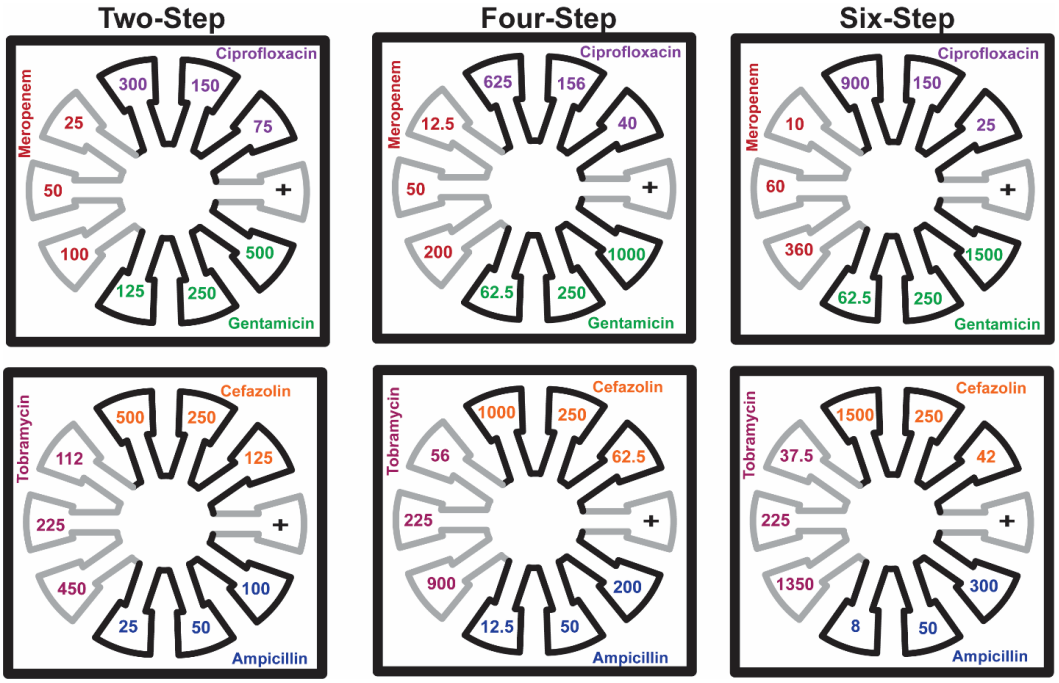

Figure A.2 Concentrations used in 2-step, 4-step, and 6-step experiments shown on the chips in the order they were applied and dried

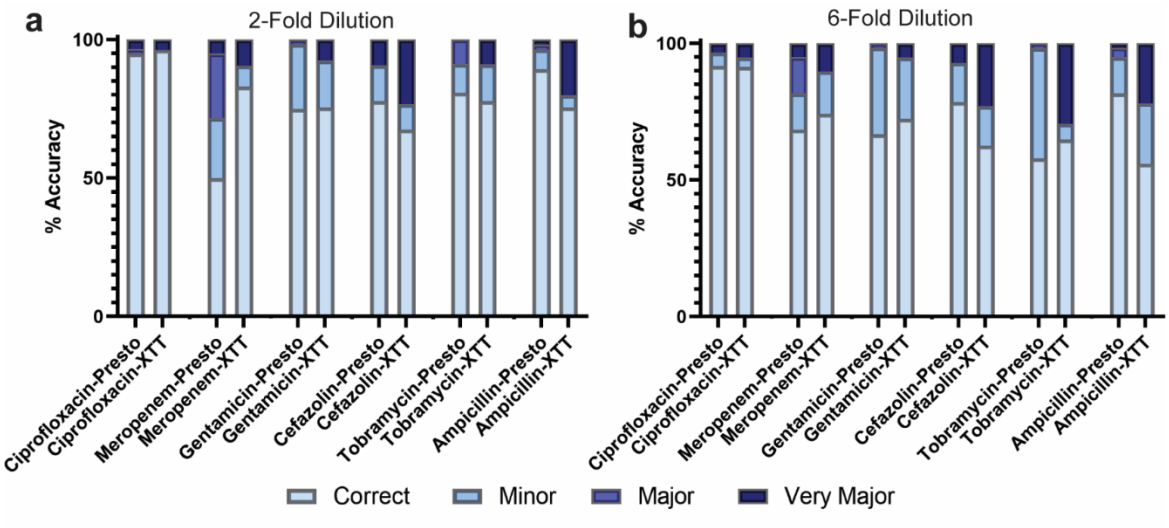

Figure A.3 Results from diluting antibiotics a) 2-fold and b) 6-fold

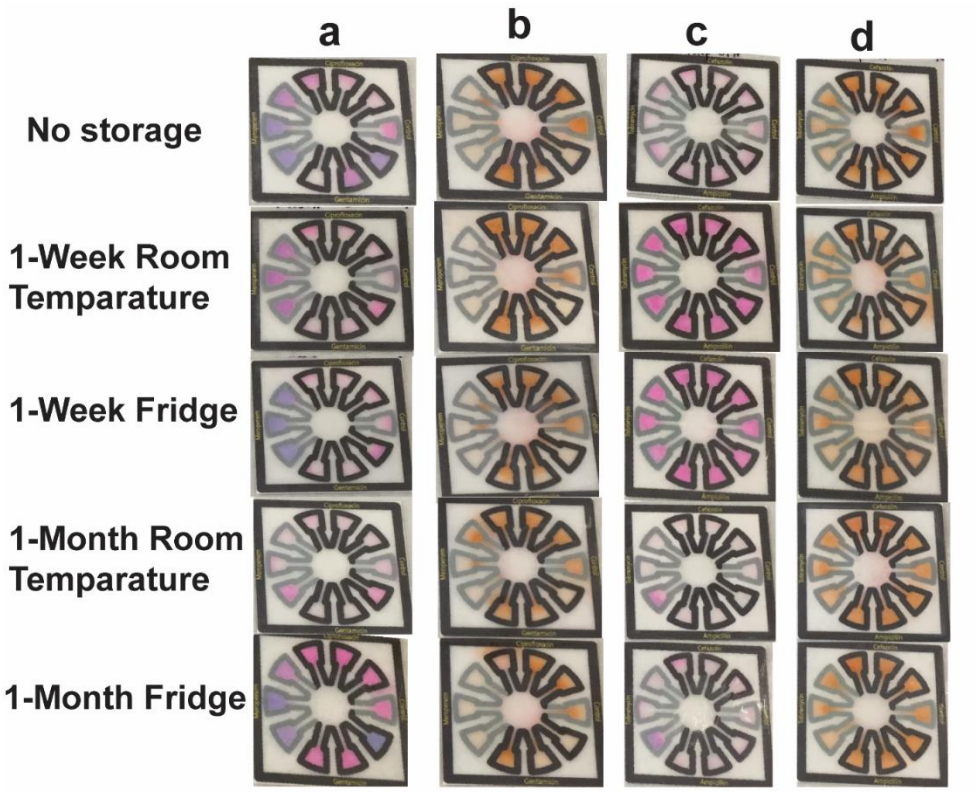

Figure A.4 Example results of *E. coli* Sample AR#0011 with gold standard result susceptible to meropenem and resistant to ciprofloxacin, gentamicin, cefazolin, tobramycin, and ampicillin **a)** Ciprofloxacin, meropenem, and gentamicin with PrestoBlue **b)** Ciprofloxacin, meropenem, and gentamicin with XTT **c)** Cefazolin, tobramycin, and ampicillin with PrestoBlue **d)** Cefazolin, tobramycin, and ampicillin with PrestoBlue

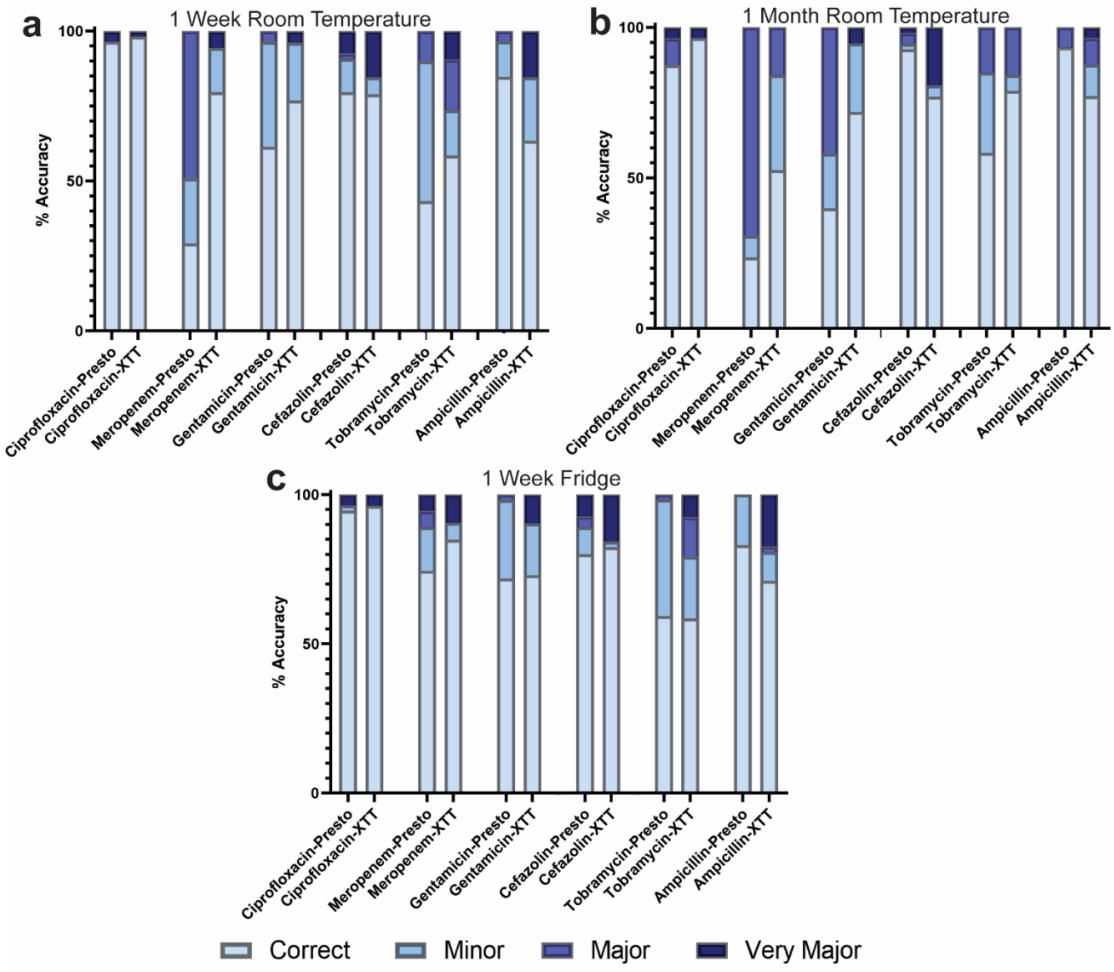

Figure A.5 Additional Shelf-Life Storage Results of CRE BacPAC **a)** 1 week stored at room temperature **b)** one month stored at room temperature **c)** one week stored in the fridge

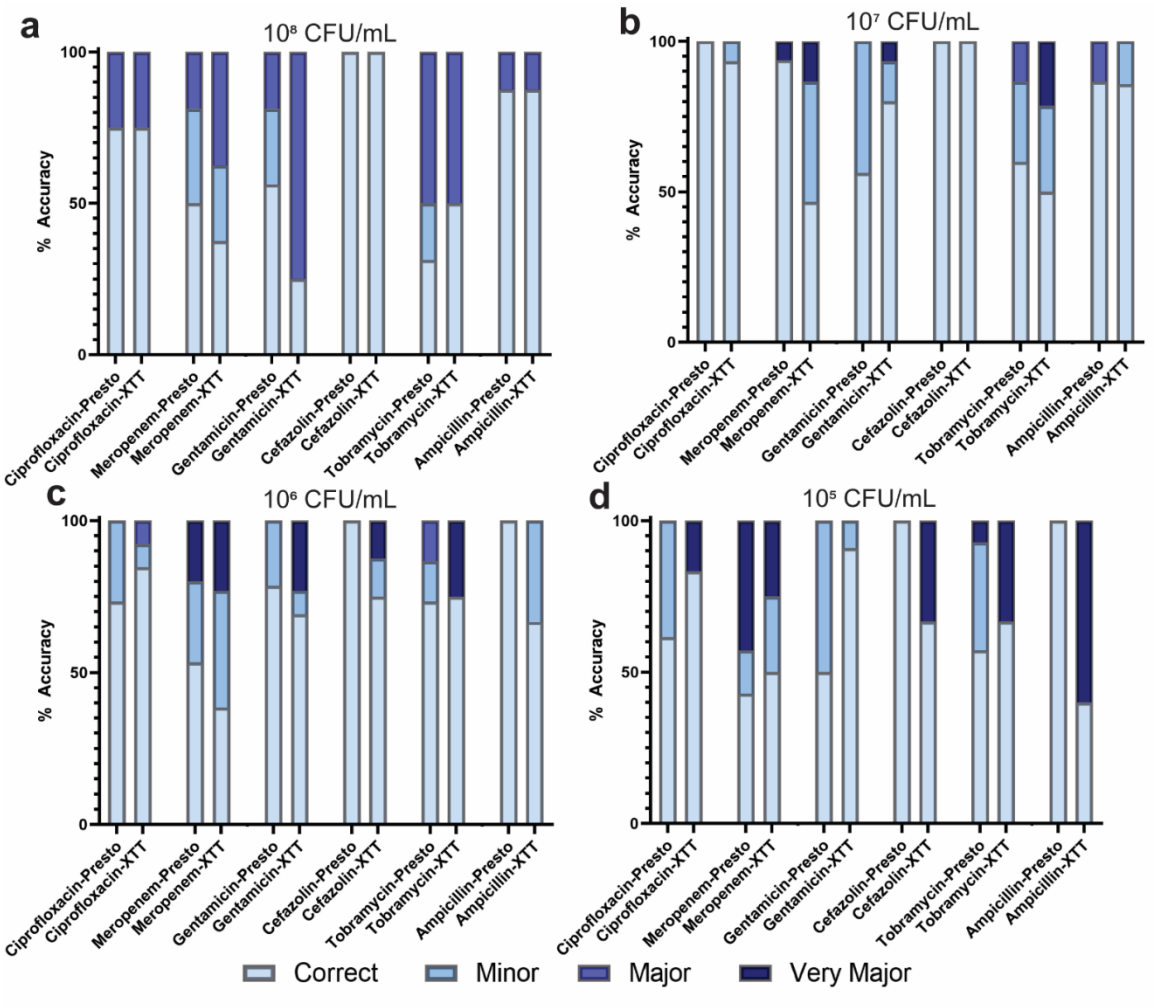

Figure A.6 Spiked Human Urine Testing at 10^8 - 10^5 CFU/mL. **a)** 10^8 CFU/mL in pure urine **b)** 10^7 CFU/mL 10% urine 90% media **c)** 10^6 CFU/mL 1% urine 99% media **d)** 10^5 CFU/mL 0.1% urine 99.9% media

Table A.2 Costs per chip including all disposables such as paper, sealing film, and pipette tips sourced from ThermoFischer Scientific and VWR

Item	**Cost per unit**	**Unit/chip**	**Chip Cost**
Grade 1 Chromatography Paper	$370/ 400 pages	1/12 page	$0.08
Wax Color Cubes	$72/100 pages	1/12 page	$0.06
PrestoBlue	$555/100mL	0.030mL	$0.17
XTT	$157/50mL	0.030mL	$0.09
PMS	$28/500mg	0.05mg	< $0.01
Ciprofloxacin	$74/1g	820 µg/mL	< $0.01
Meropenem	$203/500mg	260 µg/mL	< $0.01
Gentamicin	$76/1g	1300 µg/mL	< $0.01
Cefazolin	$163/500mg	1300 µg/mL	< $0.01
Tobramycin	$88/1g	1200 µg/mL	< $0.01
Ampicillin	$152/500mg	260 µg/mL	< $0.01
Mueller Hinton II Media	$62/500mL	0.090mL	$0.01
Packing tape	$6/40 yards	0.05yards	$0.01
Sealing Films	$43/100	1	$0.43
100uL pipette tips	$40/960 tips	2	$0.08
Total	----	----	$0.77-$0.85

Table A.3 Data in table form for two-fold, four-fold, and six-fold dilutions of CRE BacPAC

2-Fold Dilution										
Antibiotic	**Dye**	**Total Samples**	**Correct**	**Minor**	**Major**	**Very Major**	**% Correct**	**% Minor**	**% Major**	**% Very Major**
Ciprofloxacin	Presto	60	57	0	1	2	95.00	0.00	1.67	3.33
Meropenem	Presto	60	30	13	14	3	50.00	21.67	23.33	5.00
Gentamicin	Presto	60	45	14	1	0	75.00	23.33	1.67	0.00
Ciprofloxacin	XTT	53	51	0	0	2	96.23	0.00	0.00	3.77
Meropenem	XTT	53	44	4	0	5	83.02	7.55	0.00	9.43
Gentamicin	XTT	53	40	9	0	4	75.47	16.98	0.00	7.55
Cefazolin	Presto	54	42	7	0	5	77.78	12.96	0.00	9.26
Tobramycin	Presto	57	46	6	5	0	80.70	10.53	8.77	0.00
Ampicillin	Presto	56	50	4	1	1	89.29	7.14	1.79	1.79
Cefazolin	XTT	43	29	4	0	10	67.44	9.30	0.00	23.26
Tobramycin	XTT	45	35	6	0	4	77.78	13.33	0.00	8.89
Ampicillin	XTT	45	34	2	0	9	75.56	4.44	0.00	20.00
4-Fold Dilution										
Antibiotic	**Dye**	**Total Samples**	**Correct**	**Minor**	**Major**	**Very Major**	**% Correct**	**% Minor**	**% Major**	**% Very Major**
Ciprofloxacin	Presto	60	56	2	0	2	93.33	3.33	0.00	3.33
Meropenem	Presto	60	37	14	7	2	61.67	23.33	11.67	3.33
Gentamicin	Presto	60	42	13	1	4	70.00	21.67	1.67	6.67
Ciprofloxacin	XTT	57	53	0	0	4	92.98	0.00	0.00	7.02
Meropenem	XTT	57	49	5	0	3	85.96	8.77	0.00	5.26
Gentamicin	XTT	57	40	8	0	9	70.18	14.04	0.00	15.79
Cefazolin	Presto	53	44	3	2	4	83.02	5.66	3.77	7.55
Tobramycin	Presto	57	29	23	5	0	50.88	40.35	8.77	0.00
Ampicillin	Presto	58	41	12	2	3	70.69	20.69	3.45	5.17
Cefazolin	XTT	53	37	3	0	13	69.81	5.66	0.00	24.53
Tobramycin	XTT	57	40	13	0	4	70.18	22.81	0.00	7.02
Ampicillin	XTT	55	34	8	0	13	61.82	14.55	0.00	23.64
6-Fold Dilution										
Antibiotic	**Dye**	**Total Samples**	**Correct**	**Minor**	**Major**	**Very Major**	**% Correct**	**% Minor**	**% Major**	**% Very Major**
Ciprofloxacin	Presto	60	55	3	0	2	91.67	5.00	0.00	3.33
Meropenem	Presto	60	41	8	8	3	68.33	13.33	13.33	5.00
Gentamicin	Presto	60	40	19	1	0	66.67	31.67	1.67	0.00
Ciprofloxacin	XTT	58	53	2	0	3	91.38	3.45	0.00	5.17
Meropenem	XTT	58	43	9	0	6	74.14	15.52	0.00	10.34
Gentamicin	XTT	58	42	13	0	3	72.41	22.41	0.00	5.17
Cefazolin	Presto	56	44	8	0	4	78.57	14.29	0.00	7.14
Tobramycin	Presto	57	33	23	1	0	57.89	40.35	1.75	0.00
Ampicillin	Presto	60	49	8	2	1	81.67	13.33	3.33	1.67
Cefazolin	XTT	48	30	8	0	10	62.50	16.67	0.00	20.83
Tobramycin	XTT	51	33	3	0	15	64.71	5.88	0.00	29.41
Ampicillin	XTT	50	28	11	0	11	56.00	22.00	0.00	22.00

Table A.4 Data in table form for 1-week and 1-month storage at room temperature and in fridge of CRE BacPAC

1 WEEK FRIDGE										
Antibiotic	**Dye**	**Total Samples**	**Correct**	**Minor**	**Major**	**Very Major**	**% Correct**	**% Minor**	**% Major**	**% Very Major**
Ciprofloxacin	Presto	56	53	1	0	2	94.64	1.79	0.00	3.57
Meropenem	Presto	55	41	8	3	3	74.55	14.55	5.45	5.45
Gentamicin	Presto	57	41	15	1	0	71.93	26.32	1.75	0.00
Ciprofloxacin	XTT	53	51	0	0	2	96.23	0.00	0.00	3.77
Meropenem	XTT	53	45	3	0	5	84.91	5.66	0.00	9.43
Gentamicin	XTT	52	38	9	0	5	73.08	17.31	0.00	9.62
Cefazolin	Presto	55	44	5	2	4	80.00	9.09	3.64	7.27
Tobramycin	Presto	59	35	23	1	0	59.32	38.98	1.69	0.00
Ampicillin	Presto	59	49	10	0	0	83.05	16.95	0.00	0.00
Cefazolin	XTT	51	42	1	0	8	82.35	1.96	0.00	15.69
Tobramycin	XTT	53	31	11	7	4	58.49	20.75	13.21	7.55
Ampicillin	XTT	52	37	5	1	9	71.15	9.62	1.92	17.31
1 WEEK ROOM TEMP										
Antibiotic	**Dye**	**Total Samples**	**Correct**	**Minor**	**Major**	**Very Major**	**% Correct**	**% Minor**	**% Major**	**% Very Major**
Ciprofloxacin	Presto	57	55	0	0	2	96.49	0.00	0.00	3.51
Meropenem	Presto	55	16	12	27	0	29.09	21.82	49.09	0.00
Gentamicin	Presto	57	35	20	2	0	61.40	35.09	3.51	0.00
Ciprofloxacin	XTT	54	53	0	0	1	98.15	0.00	0.00	1.85
Meropenem	XTT	54	43	8	0	3	79.63	14.81	0.00	5.56
Gentamicin	XTT	52	40	10	0	2	76.92	19.23	0.00	3.85
Cefazolin	Presto	54	43	6	1	4	79.63	11.11	1.85	7.41
Tobramycin	Presto	60	26	28	6	0	43.33	46.67	10.00	0.00
Ampicillin	Presto	59	50	7	2	0	84.75	11.86	3.39	0.00
Cefazolin	XTT	52	41	3	0	8	78.85	5.77	0.00	15.38
Tobramycin	XTT	53	31	8	9	5	58.49	15.09	16.98	9.43
Ampicillin	XTT	52	33	11	0	8	63.46	21.15	0.00	15.38
1 MONTH FRIDGE										
Antibiotic	**Dye**	**Total Samples**	**Correct**	**Minor**	**Major**	**Very Major**	**% Correct**	**% Minor**	**% Major**	**% Very Major**
Ciprofloxacin	Presto	58	49	4	2	3	84.48	6.90	3.45	5.17
Meropenem	Presto	52	20	18	14	0	38.46	34.62	26.92	0.00
Gentamicin	Presto	58	42	12	4	0	72.41	20.69	6.90	0.00
Ciprofloxacin	XTT	51	45	1	2	3	88.24	1.96	3.92	5.88
Meropenem	XTT	51	45	2	0	4	88.24	3.92	0.00	7.84
Gentamicin	XTT	51	34	9	0	8	66.67	17.65	0.00	15.69
Cefazolin	Presto	54	45	4	2	3	83.33	7.41	3.70	5.56
Tobramycin	Presto	57	39	17	1	0	68.42	29.82	1.75	0.00
Ampicillin	Presto	58	50	4	3	1	86.21	6.90	5.17	1.72
Cefazolin	XTT	51	37	2	0	12	72.55	3.92	0.00	23.53
Tobramycin	XTT	55	46	6	1	2	83.64	10.91	1.82	3.64
Ampicillin	XTT	55	36	9	0	10	65.45	16.36	0.00	18.18
1 MONTH ROOM TEMP										
Antibiotic	**Dye**	**Total Samples**	**Correct**	**Minor**	**Major**	**Very Major**	**% Correct**	**% Minor**	**% Major**	**% Very Major**
Ciprofloxacin	Presto	56	49	0	5	2	87.50	0.00	8.93	3.57
Meropenem	Presto	55	13	4	38	0	23.64	7.27	69.09	0.00
Gentamicin	Presto	55	22	10	23	0	40.00	18.18	41.82	0.00
Ciprofloxacin	XTT	57	55	0	0	2	96.49	0.00	0.00	3.51
Meropenem	XTT	57	30	18	9	0	52.63	31.58	15.79	0.00
Gentamicin	XTT	57	41	13	0	3	71.93	22.81	0.00	5.26
Cefazolin	Presto	55	51	1	2	1	92.73	1.82	3.64	1.82
Tobramycin	Presto	60	35	16	9	0	58.33	26.67	15.00	0.00
Ampicillin	Presto	60	56	0	4	0	93.33	0.00	6.67	0.00
Cefazolin	XTT	52	40	2	0	10	76.92	3.85	0.00	19.23
Tobramycin	XTT	57	45	3	9	0	78.95	5.26	15.79	0.00
Ampicillin	XTT	57	44	6	2	5	77.19	10.53	3.51	8.77

Table A.5 Data in table form for urine spiked samples of CRE BacPAC

10^8										
Antibiotic	**Dye**	**Total Samples**	**Correct**	**Minor**	**Major**	**Very Major**	**% Correct**	**% Minor**	**% Major**	**% Very Major**
Ciprofloxacin	Presto	16	12	0	4	0	75.00	0.00	25.00	0.00
Meropenem	Presto	16	8	5	3	0	50.00	31.25	18.75	0.00
Gentamicin	Presto	16	9	4	3	0	56.25	25.00	18.75	0.00
Ciprofloxacin	XTT	16	12	0	4	0	75.00	0.00	25.00	0.00
Meropenem	XTT	16	6	4	6	0	37.50	25.00	37.50	0.00
Gentamicin	XTT	16	4	0	12	0	25.00	0.00	75.00	0.00
Cefazolin	Presto	14	14	0	0	0	100.00	0.00	0.00	0.00
Tobramycin	Presto	16	5	3	8	0	31.25	18.75	50.00	0.00
Ampicillin	Presto	16	14	0	2	0	87.50	0.00	12.50	0.00
Cefazolin	XTT	14	14	0	0	0	100.00	0.00	0.00	0.00
Tobramycin	XTT	16	8	0	8	0	50.00	0.00	50.00	0.00
Ampicillin	XTT	16	14	0	2	0	87.50	0.00	12.50	0.00
10^7										
Antibiotic	**Dye**	**Total Samples**	**Correct**	**Minor**	**Major**	**Very Major**	**% Correct**	**% Minor**	**% Major**	**% Very Major**
Ciprofloxacin	Presto	14	14	0	0	0	100.00	0.00	0.00	0.00
Meropenem	Presto	16	15	0	0	1	93.75	0.00	0.00	6.25
Gentamicin	Presto	16	9	7	0	0	56.25	43.75	0.00	0.00
Ciprofloxacin	XTT	15	14	1	0	0	93.33	6.67	0.00	0.00
Meropenem	XTT	15	7	6	0	2	46.67	40.00	0.00	13.33
Gentamicin	XTT	15	12	2	0	1	80.00	13.33	0.00	6.67
Cefazolin	Presto	13	13	0	0	0	100.00	0.00	0.00	0.00
Tobramycin	Presto	15	9	4	2	0	60.00	26.67	13.33	0.00
Ampicillin	Presto	15	13	0	2	0	86.67	0.00	13.33	0.00
Cefazolin	XTT	12	12	0	0	0	100.00	0.00	0.00	0.00
Tobramycin	XTT	14	7	4	0	3	50.00	28.57	0.00	21.43
Ampicillin	XTT	14	12	2	0	0	85.71	14.29	0.00	0.00
10^6										
Antibiotic	**Dye**	**Total Samples**	**Correct**	**Minor**	**Major**	**Very Major**	**% Correct**	**% Minor**	**% Major**	**% Very Major**
Ciprofloxacin	Presto	15	11	4	0	0	73.33	26.67	0.00	0.00
Meropenem	Presto	15	8	4	0	3	53.33	26.67	0.00	20.00
Gentamicin	Presto	14	11	3	0	0	78.57	21.43	0.00	0.00
Ciprofloxacin	XTT	13	11	1	1	0	84.62	7.69	7.69	0.00
Meropenem	XTT	13	5	5	0	3	38.46	38.46	0.00	23.08
Gentamicin	XTT	13	9	1	0	3	69.23	7.69	0.00	23.08
Cefazolin	Presto	14	14	0	0	0	100.00	0.00	0.00	0.00
Tobramycin	Presto	15	11	2	2	0	73.33	13.33	13.33	0.00
Ampicillin	Presto	15	15	0	0	0	100.00	0.00	0.00	0.00
Cefazolin	XTT	8	6	1	0	1	75.00	12.50	0.00	12.50
Tobramycin	XTT	8	6	0	0	2	75.00	0.00	0.00	25.00
Ampicillin	XTT	6	4	2	0	0	66.67	33.33	0.00	0.00
10^5										
Antibiotic	**Dye**	**Total Samples**	**Correct**	**Minor**	**Major**	**Very Major**	**% Correct**	**% Minor**	**% Major**	**% Very Major**
Ciprofloxacin	Presto	13	8	5	0	0	61.54	38.46	0.00	0.00
Meropenem	Presto	14	6	2	0	6	42.86	14.29	0.00	42.86
Gentamicin	Presto	14	7	7	0	0	50.00	50.00	0.00	0.00
Ciprofloxacin	XTT	12	10	0	0	2	83.33	0.00	0.00	16.67
Meropenem	XTT	12	6	3	0	3	50.00	25.00	0.00	25.00
Gentamicin	XTT	11	10	1	0		90.91	9.09	0.00	0.00
Cefazolin	Presto	14	14	0	0	0	100.00	0.00	0.00	0.00
Tobramycin	Presto	14	8	5	0	1	57.14	35.71	0.00	7.14
Ampicillin	Presto	14	14	0	0	0	100.00	0.00	0.00	0.00
Cefazolin	XTT	6	4	0	0	2	66.67	0.00	0.00	33.33
Tobramycin	XTT	6	4	0	0	2	66.67	0.00	0.00	33.33
Ampicillin	XTT	5	2	0	0	3	40.00	0.00	0.00	60.00

APPENDIX B : CHAPTER 3 SUPPLEMENTS

Figure B.1 PrestoBlue strain variation. Top: N. gonorrhoeae strains incubated for 24 hours with 1/10 PrestoBlue added at 23 hours. Bottom: N. gonorrhoeae strains incubated for 24 hours with 1/10 PrestoBlue added at 0 hours.

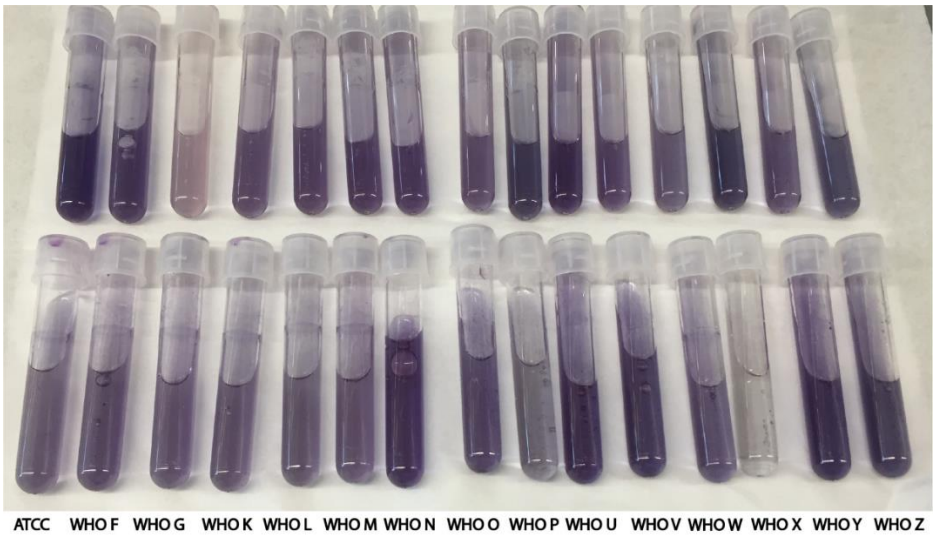

Figure B.2 MTT strain variation. Top: *N. gonorrhoeae* strains incubated for 24 hours with 0.1mg/mL MTT added at 23 hours. Bottom: *N. gonorrhoeae* strains incubated for 24 hours with 0.1mg/mL MTT added at 0 hours.

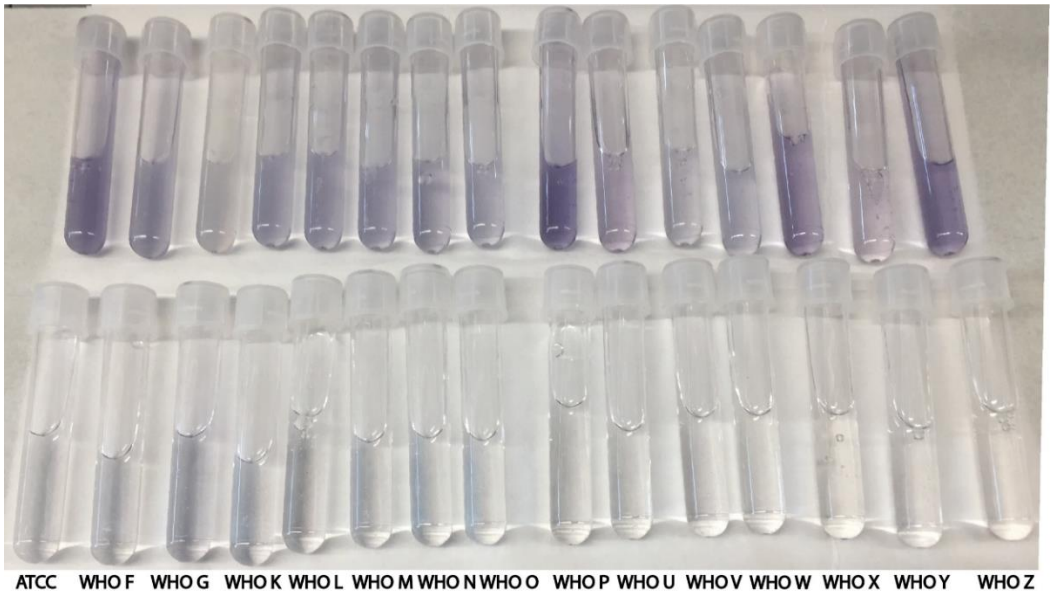

Figure B.3 NBT strain variation. Top: N. gonorrhoeae strains incubated for 24 hours with 0.1mg/mL NBT added at 23 hours. Bottom: N. gonorrhoeae strains incubated for 24 hours with 0.1mg/mL NBT added at 0 hours.

Figure B.4 XTT strain variation. Top: N. gonorrhoeae strains incubated for 24 hours with 0.1mg/mL XTT added at 23 hours. Bottom: N. gonorrhoeae strains incubated for 24 hours with 0.1mg/mL XTT added at 0 hours.

APPENDIX C CHAPTER 4 SUPPLEMENTS

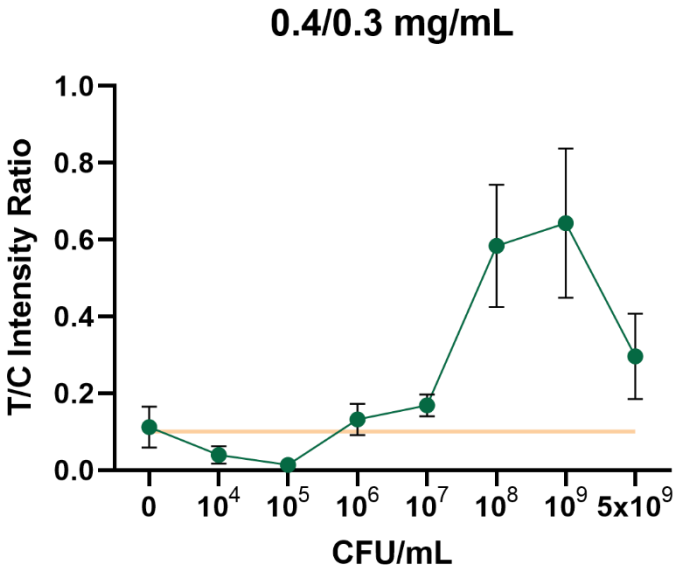

Figure C.1 LFA with test line of 0.4mg/mL and 0.3mg/mL 19962 and 7233 antibodies respectively with NG ATCC spiked in running buffer. Ratio of test line intensity to control line intensity plot versus N. gonorrhoeae ATCC 49226 bacteria concentration in CFU/mL (green). n = 3. Errors bars represent SEM. Yellow line represents positive cutoff intensity of 0.1.

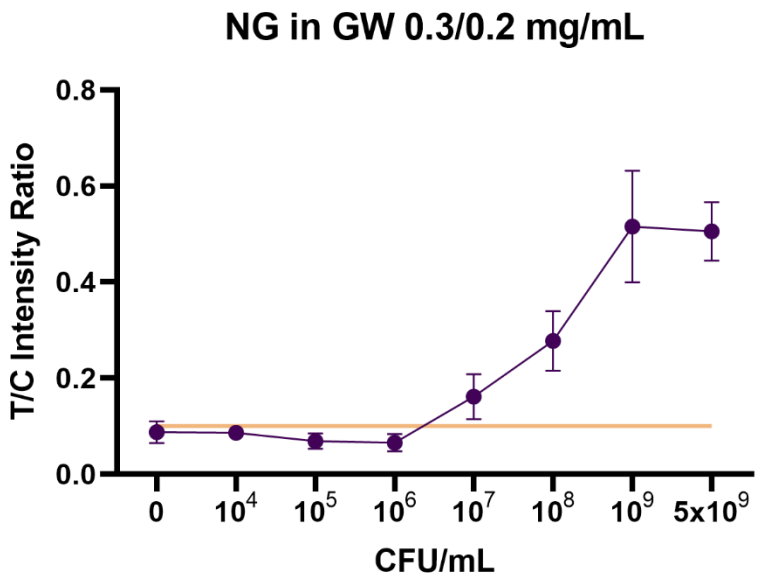

Figure C.2 LFA with test line of 0.3mg/mL and 0.2mg/mL 19962 and 7233 antibodies respectively and NG ATCC spiked in Graver Wade media. Ratio of test line intensity to control line intensity plot versus N. gonorrhoeae ATCC 49226 bacteria concentration in CFU/mL (purple). n = 3. Errors bars represent SEM. Yellow line represent positive cutoff intensity of 0.1.

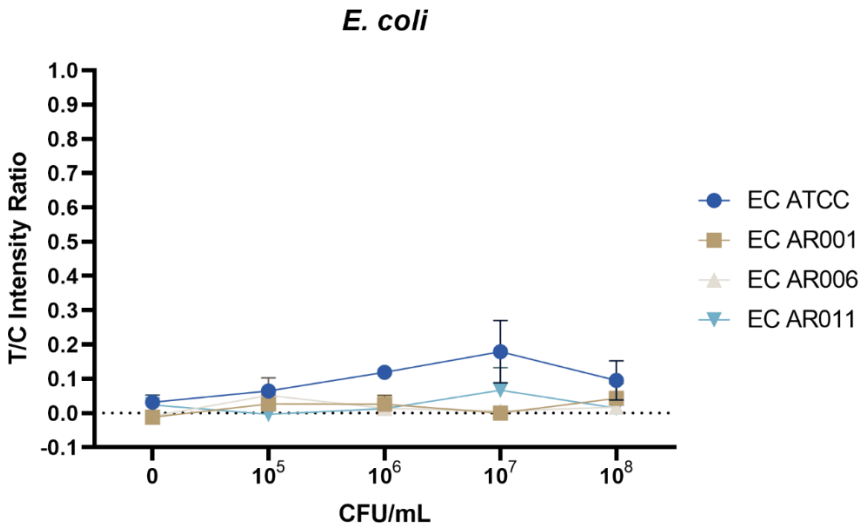

Figure C.3 Specificity of NG LFA when challenged with E. coli clinical control strain ATCC 49226, and AR001, AR006, and AR011 from the CDC & FDA AR Resistant Isolate Bank

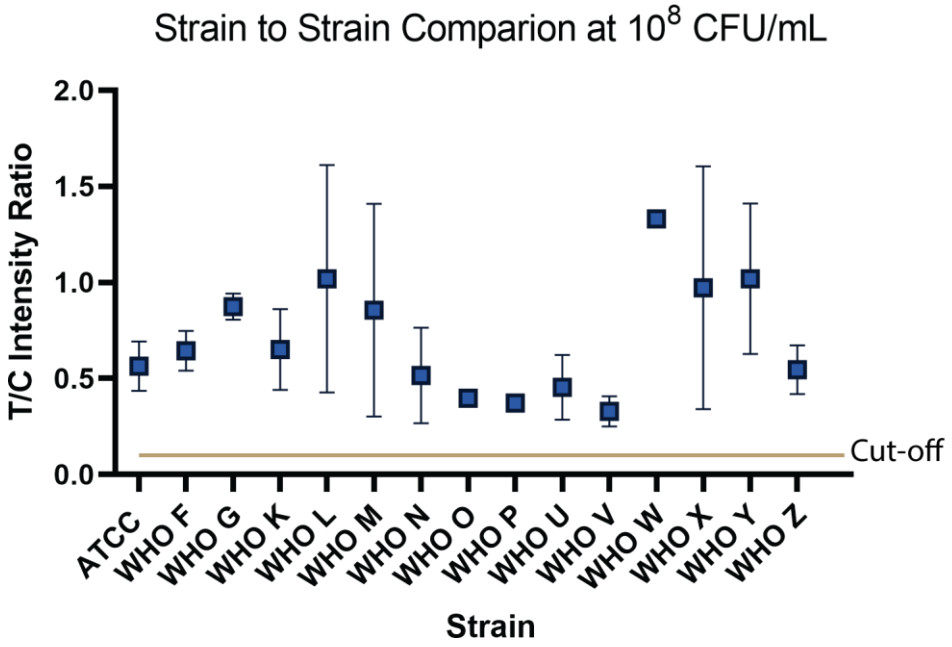

Figure C.4 *N. gonorrhoeae* WHO Panel test/C=control intensity ratio vs strain *name*

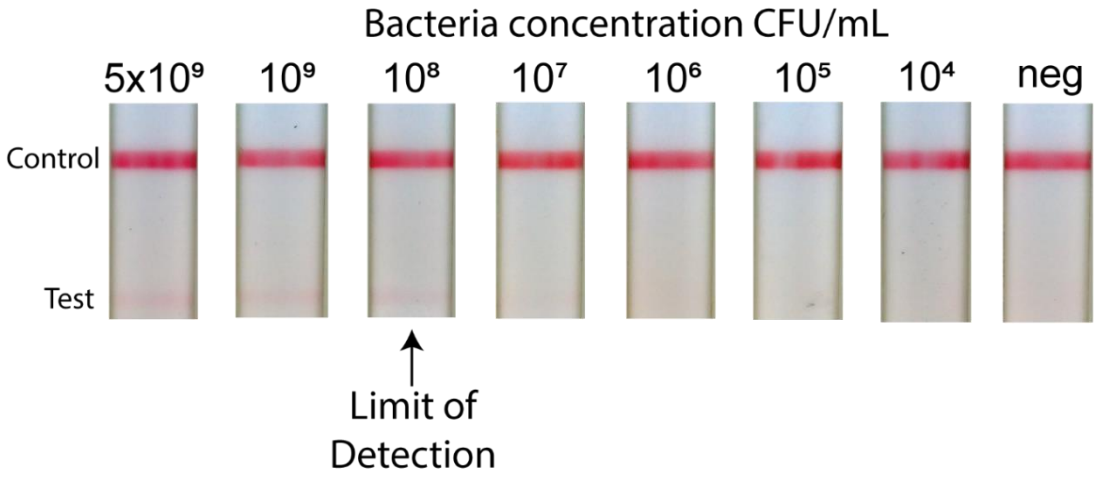

Figure C.5 Images of commercially available gonorrhea rapid test kit lateral flow strips after sampling

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