ABSTRACT

BALBIN, MICHELLE MACUGAY. Surveillance and Development of Detection Platform for Antimicrobial Resistance and Pathogenic Bacteria. (Under the direction of Dr. Siddhartha Thakur).

The study aimed to identify the antimicrobial resistance (AMR) determinants and virulence factors in *Salmonella* spp. and *Escherichia coli* from different anthropogenic areas in North Carolina. Soil samples were collected from different anthropogenic areas: urban and natural. Minimum inhibitory concentration (MIC) was determined by using the broth microdilution method. Whole genome sequencing and analysis were done to identify the AMR determinants and virulence factors among the isolates. The urban environment was observed to have a higher prevalence of *Salmonella* spp. and *E. coli*. The *Salmonella* spp. isolates showed resistance to Sulfisoxazole and Streptomycin, while *E. coli* was resistant to Sulfisoxazole, Cefoxitin, and Ampicillin. The WGS analysis identified *Salmonella* serotypes Schwarzengrund and Mississippi. Aminoglycoside resistance genes and IncFIB and IncFIC(FII) plasmids were detected among *Salmonella* spp. In general, *E. coli* was predominated by isolates from phylogroup B1, B2, and D. Multidrug transporter *mdfA* gene was detected in most of the *E. coli* from the natural and in all isolates from the urban environment. *FosA7* gene was detected in an isolate from a residential yard. The pCoo and pB171 plasmids were detected in urban, while col 156 and pHN7A8 plasmids were detected in natural environments. The detection of AMR determinants and virulence factors in these bacteria is significant in understanding the occurrence and even the development of AMR. The presence of these determinants in different anthropogenic areas only suggests that there is still more in the environment and the need for continuous AMR surveillance.

There are several laboratory techniques used in the research and surveillance of bacterial pathogens such as *Salmonella* spp. and antimicrobial resistance (AMR). However, these techniques require expensive laboratory facilities and highly trained individuals, creating a gap in surveillance and research especially in developing counties. Therefore, there is a need to develop a simple, rapid, specific, sensitive, and low-cost detection platform that can be used in field testing or laboratories with lesser capabilities.

Gold nanoparticles (AuNPs) are widely used in nanosensors due to their tunability, biocompatibility, and optical properties, particularly surface plasmon resonance. We designed and performed a simple, rapid, sensitive, specific, and cost-effective detection platform using functionalized AuNPs based on colorimetric assay.

In this study, we describe an enzyme-free detection method of bacterial pathogen using DNA functionalized AuNPs. Our method directly detected the *Salmonella* spp. extracted genomic DNA without prior amplification or the use of enzymes. Moreover, each step in the described method does not require expensive instruments. This detection method can be easily performed in a water bath or heat block and the results can be observed by the naked eye, eliminating the need to perform gel electrophoresis. This method can be completed within 45 minutes.

Surveillance and Development of Detection Platform for Antimicrobial Resistance and Pathogenic Bacteria

by
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DEDICATION

I dedicate this to everyone who guided and supported me along this journey.

BIOGRAPHY

Michelle Macugay Balbin was born on February 18, 1989 in Tumauini, Isabela, a province in the northern part of Luzon island in the Philippines. She attended the Regional Science High School (Region II) where she was initially exposed to research. In 2005, she attended the College of Veterinary Science and Medicine in Central Luzon State University (CLSU-CVM), Nueva Ecija where she graduated with Bachelor of Science in Animal Husbandry (2009) and Doctor of Veterinary Medicine (2011). After passing the Veterinary Licensure Exam in 2011, she joined a research group in CLSU-CVM and studied coronavirus in swine. Being interested in infectious diseases in livestock, in 2013, she transferred to the Animal Health Unit of the Philippine Carabao Center (PCC) National Headquarters and Gene pool, a livestock research agency under the Department of Agriculture. In 2014, she became a recipient of Thailand's National Center for Genetic Engineering and Biotechnology (BIOTEC) Advanced Training program where she was stationed at King Mongkut's University of Technology in Thonburi. Her training program focused on the development of DNA nanobiosensors for the infectious disease diagnosis. In 2016 she was awarded with the Fulbright Scholarship Program to pursue her graduate studies in the US. In 2017, she joined the Comparative Biomedical Sciences program in the College of Veterinary Medicine, North Carolina State University. Her research focused on the antimicrobial resistance in different anthropogenic areas in North Carolina and development of a DNA-based nanosensor detection platform, under the mentorship of Dr. Siddhartha Thakur. She recently joined the Food and Agriculture Organization (FAO) of the United Nations AMR project in the Philippines (GCP/GLO/710/UK).

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And to Him, for all His blessings.

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CHAPTER 1: Literature Review

1.1 Introduction

The increasing problem of antimicrobial resistance (AMR) is causing an alarm worldwide, posing a risk both in humans, animal health, and the environment [1-3]. According to an eminent economist Jim O'Neil, AMR is a more certain threat than climate change [4]. With limited choices of antimicrobials, it is expected that the cost of health care will increase due to longer duration of illness, additional medical tests, and the use of more expensive drugs [5]. Currently, the estimated annual deaths due to AMR is 700,000 and by the year 2050, it is projected that this number would reach up to 10 million along with $100 trillion economic loss [6]. Recognizing this urgent problem, the World Health Organization (WHO) issued a Global Action Plan on AMR to ensure the continuity of successful treatment and prevention of infectious diseases through the responsible use of quality and effectively safe medicines. To achieve this goal, surveillance programs and research has been launched to further understand AMR.

Different research and surveillance programs are being implemented in many countries, providing more comprehensive data on diversity as well as the mechanisms of AMR [1,7]. The majority of these studies have focused on AMR in clinical and agricultural environments such as hospitals and livestock farms. This left a knowledge gap regarding AMR determinants and virulence factors [8-10] found in the natural environment as well as in areas subjected to anthropogenic impacts.

In AMR surveillance and research studies, different laboratory techniques are being used to gather relevant information. However, not all laboratories have the capability and facility to conduct research bringing a huge gap in the epidemiological surveillance data, especially in

developing countries where most of the emerging AMR determinants are reported. One of the WHO's strategic objectives on the Global Action Plan on AMR is the development of diagnostic tools (Objective 5) to help medical practitioners better identify and treat the pathogens causing infections [11]. Therefore, a need to develop for a simple, rapid, specific, sensitive and low-cost detection platform with an efficiency comparable to the existing methods that can be used in laboratories with lesser capabilities. Recent advances in research and technology have facilitated the development of platforms that focuses on eliminating the need for sample transportation and further processing. The early and accurate detection of these pathogens can reduce the financial cost by up to 20-35%, increase food production and food safety, and improve global economy [12]. More importantly, these diagnostic platforms can improve the quality of data and pace of reporting [13,14] which is important in tackling the issues related to AMR [15].

1.2 Antimicrobial Resistance in the Environment

The environment plays a significant role in the emergence and transmission of resistant bacteria. Soil is a vast source of AMR determinants as it has very diverse microbial composition, varying with the biochemical and geographical gradient [16] as a result of constant selective pressure. The majority of the antimicrobials being used today were initially isolated from the natural environment, particularly from the soil Actinomyces [6,17,18]. Normally, the antimicrobial producing organisms have determinants that would help to resist the action of the antimicrobial(s) they produce along with the other microbes found in the same environment [17,18]. Consequently, the large number of antimicrobials being discharged into the environment along with constant interaction of humans and animals not only alter the microbial community but also hasten and sustain the development of AMR and virulent pathogens [3,8,19,20] through the different selection pressures [2,8,9,21]. These selection pressures can result in changes such

as genetic mutations in the microbe's chromosome, horizontal transfer of AMR genes, and plasmids allowing the organism to survive and thrive in the environment [4] in the environment to humans and animals.

The presence of human activities in an environment increases the chance of contamination [6,8] such as spillage of antimicrobials, heavy metals, biocides and even resistant bacteria [8-10,22]. Such contamination creates a selective pressure or environmental hotspot for the development and dissemination of AMR [8,22]. In a heavy metal polluted environment, this plasmid containing resistance genes is maintained and so the AMR determinants, even in the absence of antimicrobials explaining the persistence of AMR in the environment [10]. Aside from the presence of antimicrobial producing microorganisms in the natural environment, the role of wildlife was also cited in the dissemination of resistance and pathogenic bacteria [9]. Several reports of AMR contamination in the natural environments have been associated with wildlife such as migratory birds and foxes [9,10,23].

1.3 Antimicrobial Resistance: dissemination and impact

The unwise and prudent use of antimicrobial drugs, both in animals and humans has even contributed to the rise of bacterial resistance [21,24]. Accordingly, the AMR is commonly seen in environments where microbes routinely come into contact with antimicrobials such as in hospitals and livestock farms [9,19]. It is believed that livestock animals are the most important source of AMR since they are constantly exposed to different antimicrobials as a treatment to diseases, prophylaxis and growth promotant. This has improved the food animal production and stabilized the meat supply and demand however, this approach greatly contributed to the current problem of AMR [24,25]. In the US alone, it is estimated that 80% of its antimicrobial consumption is used in food production [24]. Given the ecological nature of antimicrobials, there

has been difficulty in establishing a direct causality between the prevalence of AMR in humans, animals, and the environment [24]. However, recent studies revealed a strong correlation between AMR patterns found in the bacteria isolated from animal, human and its surrounding environment [26-30] and the antimicrobial drugs being used in animal production or farm system [26].

The emergence of the Extended-Spectrum Beta-Lactamases (ESBLs) produced by *E. coli* mainly contributed to the increase in antimicrobial resistance [21]. The ESBLs are enzymes inhibited by clavulanic acid and other inhibitors of the class A B-Lactamases, making the bacteria resistant to penicillin, cephalosporins, and monobactams. ESBL bacteria are also reported to be resistant to other antibiotics such as aminoglycosides, chloramphenicol, fluoroquinolone, tetracycline and trimethoprim-sulfamethoxazole [21]. This resistance to a wide spectrum of antibiotics created reliance on reserved antibiotics such as carbapenems [21,31]. However, there has been an increasing report of carbapenem resistance in the US, and some countries in the western part of Asia and Europe [32]. In 2010, a highly resistant Enterobacteriaceae isolated from a patient in Sweden was reported. This Enterobacteriaceae was found to carry New Delhi Metallo-B-Lactamase-1 (NDM-1) carbapenemases, making it highly resistant to many classes of antibiotics including Beta-lactams, fluoroquinolones, and aminoglycosides. Further studies on NDM-1 bacteria revealed that plasmids encoding this type of resistance were readily transmissible and prone to rearrangement, losing or gaining DNA on transfer. Interestingly, investigation of the NDM-1 bacteria emergence revealed that most of the patients in the UK had undergone elective cosmetic surgery in India or Pakistan [31]. This scenario has narrowed down the choice of antimicrobial drugs into colistin and tigecycline for the treatment of serious cases of illness [33]. In 2015, researchers from China reported the first

plasmid-colistin mediated resistance (MCR-1) in animals and humans. The emergence of MCR-1 resistant bacteria is deeply concerning and calls for an urgent re-evaluation of the use of polymyxins, especially in animal production. High prevalence was reported both in animals and humans [34] and it is believed that this type of resistance is already extensive, considering that China is one of the largest producers and exporter of swine and poultry products.

Indeed, antimicrobial resistance has rapidly spread worldwide, and humans are the most affected by this scenario. Different interrelated factors among humans, animals, and the environment play an important role in the development and persistence of antibiotic resistance. According to an eminent economist Jim O'Neil, antimicrobial resistance is a more certain threat than climate change [4]. Without effective antimicrobials, treatment of common diseases as well as the management of medical procedures (such as organ transplantation) can be prolonged or can result in death. Resistance to common antimicrobials also increases the cost of health care due to longer duration of illness, additional medical tests and the use of more expensive drugs [5,6,11]. By the year 2050, the estimated economic losses due to AMR is $100 trillion. It is estimated that 700,000 people die annually due to AMR and by 2050, it is estimated that this number would reach up to 10 million [6]. The same can happen in livestock and food production, especially in areas where intensive farming is practiced. It is estimated that there will be a 67% increase of antimicrobial use by 2030 and that one-third of this will be used in livestock production [24]. Therefore, it is only necessary to conduct continuous surveillance and monitoring programs on AMR as well as resistant bacterial pathogens to ensure the availability and effectiveness of antimicrobials in the future.

1.4 Bacterial pathogens in AMR studies

The *Salmonella* spp. and *E. coli* are the two of the most commonly reported bacterial pathogens affecting millions of humans annually [5,11,35]. These are gram-negative bacteria that are commensals and commonly found in the gastrointestinal tract of humans, livestock, birds, and even insects. While some serotypes or strains of these bacteria are host specific, others can be transmitted from animals to humans and the environment.

Salmonella is a complex group of medically important pathogens in human and veterinary medicine. This bacterium, specifically the non-typhoidal *Salmonella* (NTS) serovars are normally isolated from the intestinal tract of humans, livestock, and bird and even insects. Globally, there are an estimated 78,707,591 [5,11,35] cases annually due to NTS. In the United States, NTS is estimated to cause 1.2 million illnesses and 450 deaths annually [5,11,36]. Similarly, *E. coli* is a commensal bacterium that thrives in the intestine of the human and animal host. Some *E. coli* strains such as Shiga-toxin *E. coli* (STEC) have acquired virulence factors and properties that allowed it to survive in different hosts and environments. Globally, the estimated number of illnesses due to STEC is 1,176,854 [5,11,35] including the 265,000 reported cases in the US [36]. Disturbingly, the highest percentage of deaths due to infection from these pathogens was reported among children under 5 years of age [5,11,35].

These pathogens can easily contaminate foods as they are part of the normal flora of the food animals. The consumption of egg contaminated with NTS is reported to be the major cause of infection in humans [5,11,35]. Similarly, STEC infection is primarily due to the consumption of contaminated unpasteurized raw milk [37]. The swallowing of contaminated lake water and touching of the environment in the zoo or animal exhibits were also reported to cause infection with these pathogens.

These bacteria are known to acquire and preserve AMR determinants and virulence factors from other microorganisms allowing them to thrive in the environment. Often, the reintroduction of these resistant and pathogenic bacteria into their primary host is a health concern due to their new properties that could cause serious diseases [36]. Surveillance reports have also shown international travel and trade as major risk factors in the dissemination of exotic bacterial strains and AMR determinants [8,38]. This was highlighted when a multidrug-resistant *S.* Schwarzengrund, a predominant cause of Salmonellosis in South East Asia was reported among isolates from persons and chickens in Thailand and food imported from Thailand to Denmark and the US [38]. For these reasons, the *Salmonella* spp. and *E. coli* are often used as indicator bacteria in AMR surveillance programs and other public health-related studies.

1.5 Laboratory techniques used in AMR studies

Different laboratory techniques are being used in the surveillance of AMR and resistant bacteria. Bacterial culture is an indispensable technique used in disease investigation as well as in AMR studies. The use of selective and differential media can identify a certain bacterium [39] up to the genus level. Differentiation of species is more important in the clinical setting since this can affect the treatment regimen, medication approach, and management [25]. With regards to antimicrobial resistance, the conventional technique based on the Clinical and Laboratory Standards Institute (CLSI) is very informative, providing the antimicrobial concentration that will inhibit the growth of bacteria. Moreover, a bacterial culture is needed to obtain pure isolates for further molecular analysis. The molecular technique has always been a reliable tool in investigating diseases or antimicrobial resistance overcoming the difficulty in identification, differentiation and quantification pathogens [40]. This can provide more comprehensive information and greatly help in the monitoring and surveillance of different bacterial strains and

AMR mechanism [39]. It can also provide information on the origin and compare relatedness between pathogens or isolates. This is very important in investigating diseases that are related to travel or acquired during a visit to countries such as the case of NDM-1 bacterial infection from a patient in the UK. The Polymerase Chain Reaction (PCR) can identify and amplify DNA markers specific pathogen or plasmid encoding antimicrobial resistance. Multiplex PCR is being widely used because it can detect multiple genes at the same time. Isothermal molecular techniques such as Rolling Cycle Amplification (RCA) and Loop-Mediated Isothermal Amplification (LAMP) are rapid and equally sensitive and specific as PCR, but multiplexing is difficult using these methods [4]. Array-based detection of antibiotic resistance genes can identify multiple genes encoding AMR determinants as well as virulence factors in a bacteria genome [41]. Selection pressure can easily change the bacterial genome leading to diversity between strains or isolates. Pulse Field Gel Electrophoresis (PFGE) can identify relatedness of isolates from recent outbreaks, but not well-suited to long-term global epidemiology. Methods such as multilocus enzyme electrophoresis (MLEE) and multilocus sequence typing (MLST) are reported to be highly discriminatory. MLST can distinguish between isolates or clones by targeting fragments of the housekeeping genes, where genetic variation accumulates slowly. This can also provide information on evolutionary history, predict genotype and reveal the pattern of evolutionary descent of isolates [42,43]. With the continuous decrease in the cost of whole-genome sequencing (WGS), more bacterial genome sequences are being reported in the GenBank. Like MLEE and MLST, WGS provides information on the evolution of the isolates or origin based on its relatedness to other sequences. Moreover, WGS data has allowed researchers to understand the host's physiological responses to different organisms and prompted studies on drug discovery and design [44].

Overall, both conventional and molecular techniques are important in AMR and bacterial pathogen studies [45]. It is important to emphasize that not all laboratories are capable of these techniques, especially in developing counties [46]. These techniques require expensive facilities and highly trained individuals hence, a gap in the epidemiological surveillance and research. The availability of rapid diagnostic tests that can specifically diagnose a bacterial pathogen would not only improve the clinical management of the diseases but would also help in the control of AMR by prescription of correct antimicrobials [30,39]. Therefore, a need to develop for a simple, rapid, specific, sensitive and low-cost detection platform with an efficiency comparable to the existing methods that can be used in laboratories with lesser capabilities.

1.6 Nanosensors

Nanotechnology is a science that deals mainly with the study of matter with a dimension between 1 to 100 nm and its manipulation at an atomic and molecular scale [45]. Nanotechnology has been widely explored and studies have been conducted to evaluate its applicability in different fields especially in medicine [46,47]. One of the major interests in nanotechnology today is the nanosensors or nanobiosensors and their application in point-of-care disease diagnosis [45]. There has been continuous research on the development of simple and rapid diagnostic platforms that would allow on-site diagnosis. Moreover, nanosensors are reported to increase the specificity and sensitivity of a technique. This favors a better diagnosis of a bacterial pathogen that would not only improve the clinical management of the diseases but could also help in the control of AMR by prescription of correct antimicrobials [30].

An effective diagnostic platform requires both a detection method and a signal transducer. Detection of an analyte of AMR determinant, for example, can be done using antibody or species-specific DNA sequences or probes. A signal transducer converts the

antibody-pathogen interaction or sequence-specific DNA hybridization into a signal which can be electric (voltage, resistance, or current change) or optical (colorimetric, fluorescent, luminescent, turbidity) [49]. The electrochemical sensor is based on the detection of changes in ions in a solution or a mixture during the reaction process. The change in ions is recognized and recorded as a change in current and potential. Electrochemical biosensors are extremely sensitive and specific. The DNA-based electrochemical sensors commonly rely on the immobilization of single-strand DNA (capture probe) on a solid surface (glass slide or electrode) and the detection of a complementary strand upon hybridization. Electrochemical based detection of DNA through measurement of oxidized gold nanoparticles is widely studied and modification of this technique can further increase the sensitivity of the method. A study conducted by [50] determined the efficiency of latex particles when gold nanoparticles are deposited on its surface. The modification of the technique allowed the detection of DNA at 0.1 femtomolar (fM), which is lower compared to previous reports. The same technique was used in the detection of *Aphanomyces invadans*, a fungus that causes devastating disease in fish and reported a detection limit of 0.5 fM in synthesized linear target and 1 fM using PCR product [51]. Optical rely on the surface plasmon resonance property of nanoparticles. Nanoparticles exhibit intense and distinct colors [52] and changes in its surroundings (such as changes in ion concentrations) can be observed even thru the naked eye. This property is very suitable especially in the development of colorimetric-based detection platforms [45,53].

1.7 Gold nanoparticle and colorimetric-based sensors

Gold nanoparticle (AuNP) is the most widely used metal nanoparticle [54-56] due to its properties such as high surface area, conductivity and high compatibility to biomolecules. The AuNPs are highly tunable and has good compatibility with biomolecules. It is used widely as

drug vehicles, imaging agents in therapy, biomarkers in the pharmaceutical field, and diagnostic tools [47,57,58]. These properties have attracted researchers, especially in the medical field to further explore AuNP especially in the development of different disease detection/screening platforms [56,59-63]. In 1996, Mirkin and his research group first shown the colorimetric assay for DNA detection using oligonucleotide functionalized AuNPs. In this experiment, the researchers described a method for assembling colloidal gold nanoparticles rationally and reversibly into macroscopic aggregates. The method described attaching two batches of thiol modified oligonucleotides into the surface of 13 nm AuNPs. A solution containing oligonucleotide with complementary sequence both on its ends bind to the oligonucleotides on the surface of AuNPs. The sequence-specific recognition property of the oligonucleotide triggers the AuNPs self-assembly by creating a link between well-dispersed nanoparticles (thus the name, crosslinking method). By binding of the complementary sequence to the oligonucleotide on the AuNPs surface, the interparticle distance becomes shorter. The self-assembly of the AuNPs is immediately observed, which is a color change of the solution from red/pink to blue/gray [59]. The mechanism involved in this method was later attributed to the Brownian motion, wherein there is a random collision between nanoparticles [60]. This method has eventually lead to different experiments that used AuNPs interaction and specificity of DNA especially in the development of diagnostic platforms. Following the above-mentioned method, a group of Japanese researchers reported an aggregation phenomenon of DNA functionalized AuNPs induced by the hybridization of target DNA without cross-linking. This system reports only one kind/sequence of thiol-modified oligonucleotide functionalized on AuNPs. The hybridization of the oligonucleotide to a complementary sequence as well as the presence of a large concentration of salt affects the stability of AuNPs. The researchers reported that the aggregation of AuNP was

driven by the London-van der Waals forces between the AuNPs, wherein in this method, the repulsive interaction is greatly reduced [60].

A well-dispersed AuNP can be seen as red to pink in color with absorbance spectra of 520 nm. The decrease in the distance of AuNPs in a solution would cause a shift of plasmon resonance which is observed as a change in color from red to blue. Changes in the environment around the AuNPs can lead to aggregation which could be observed directly due to the color change of the solution from red to blue [55,60-66]. A salt solution such as Magnesium Chloride can destabilize the well-dispersed AuNPs because of the loss of electrostatic charges on the surface of the particle [67]. This causes a decrease in the distance between AuNPs as a result of particle coupling, followed by aggregation. Recent studies reported that the hybridization of oligonucleotide functionalized AuNPs on a target DNA could stabilize the AuNPs even after addition of salt solution [54,62,66,68,69,70].

During the last decade, there has been an increase in the utilization of this method as an indicator after PCR amplification eliminating the agarose gel electrophoresis step [65]. Other researchers reported the same method in the detection of pathogens amplified through loop-mediated isothermal amplification (LAMP) [68,69-71]. Molecular methods generate a large amount of product, which is negatively charged due to the phosphate backbone of the DNA. Specific hybridization of the oligonucleotide-functionalized gold nanoparticle to its complementary sequence would allow the AuNPs to stabilize. Salt induced aggregation which is seen as color change is prevented due to maintained electrostatic repulsion between the AuNP. This repulsion between AuNPs is further enhanced by a large number of hybridized negatively charged DNA on its surface [55,68-70]. In contrast, there is AuNP aggregation which is seen as a shift in color in samples or a solution that does not contain complementary DNA. The

electrostatic repulsion between the AuNP in the solution is disrupted therefore causes aggregation [62,66,68-70]. Colorimetric-based detection of DNA is an attractive method because it is rapid and cost-effective. It enables visual or direct detection of DNA without the use of sophisticated equipment.

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1.8 References

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CHAPTER 2: Antimicrobial resistance and virulence factors profile of *Salmonella* spp. and *Escherichia coli* isolated from different environments exposed to anthropogenic activities

Presented here is the manuscript titled: "Antimicrobial resistance and virulence factors profile of *Salmonella* spp. and *Escherichia coli* isolated from different environments exposed to anthropogenic activities" submitted in the Journal of Global Antimicrobial Resistance. Additional data are presented in the Appendix.

2.1 Abstract

The study aimed to identify the antimicrobial resistance (AMR) determinants and virulence factors in *Salmonella* spp. and *Escherichia coli* recovered from different anthropogenic areas in North Carolina. Soil samples were collected from different anthropogenic areas: urban and natural. Minimum inhibitory concentration (MIC) was determined by using the broth microdilution method. Whole genome sequencing and analysis were done to identify the AMR determinants and virulence factors.

A higher prevalence of *Salmonella* spp. and *E. coli* was detected in the urban environment. The *Salmonella* spp. isolates showed resistance to Sulfisoxazole and Streptomycin, while *E. coli* was resistant to Sulfisoxazole, Cefoxitin, and Ampicillin. *Salmonella* serotypes Schwarzengrund and Mississippi were identified based on WGS analysis. Aminoglycoside resistance genes and IncFIB and IncFIC(FII) plasmids were detected among *Salmonella* spp. In general, *E. coli* was predominated by isolates from phylogroup B1, B2, and D. Multidrug transporter *mdfA* gene was detected in majority of the *E. coli* from both the urban (100%) and natural (84.5%) environment. *FosA7* gene was detected in an isolate from a residential yard. The pCoo and pB171 plasmids were detected in urban, while col(156) and pHN7A8 plasmids were detected in natural environments.

The detection of AMR determinants and virulence factors in these bacteria is significant in understanding the occurrence and even the development of AMR. The presence of these determinants in different anthropogenic areas suggests the need to conduct longitudinal studies for comparing the profile of pathogens across different environments.

2.2 Introduction

The emergence, persistence, and continuous spread of antimicrobial resistance (AMR) is considered as one of the greatest threats to humans (1,2,3). Currently, the annual deaths due to AMR are estimated to be 700,000 (4) and by 2050, it is projected that this number could reach up to 10 million (4) and an economic loss amounting to $100 trillion. Recognizing this urgent problem, the World Health Organization (WHO) issued a Global Action Plan on AMR to ensure the continuity of successful treatment and prevention of infectious diseases through the responsible use of quality and effectively safe medicines (4).

The environment plays a significant role in the emergence and transmission of AMR determinants and pathogenic bacteria (2). The constant interactions of humans, animals, and the environment can give rise to selection pressures leading to changes that would help an organism to survive such as mutations, horizontal transfer of AMR genes, plasmids, and virulence factors (1,3,5). The frequent exposure of the environment to antimicrobials have created hotspots for the selection, proliferation, and spread of AMR determinants (1,5,6). As for pathogenic bacteria such as *Escherichia coli* O157:H7, the increased virulence happens when there is gene loss or silencing, insertion, and rearrangement which occurs under the same mechanisms associated with the acquisition of AMR determinants in the environment (5). Accordingly, AMR is commonly seen in environments where microbes routinely come into contact with antimicrobials such as in hospitals and livestock farms (3,5). The majority of the previously conducted studies

have focused on clinical and agricultural environments. This left a knowledge gap regarding AMR determinants and virulence factors (1,3,6,7) found in the natural environment as well as in areas subjected to anthropogenic impacts. *Salmonella* spp. and *E. coli* are the two of the most commonly reported bacterial pathogens affecting millions of humans annually (2). While some serotypes or strains of these bacteria are host specific, there are others that can be transmitted from animals to humans and to the environment. These bacteria are known to acquire and disseminate AMR determinants and virulence factors allowing them to thrive in the environment (1). Often, the reintroduction of these resistant and pathogenic bacteria into their primary host is a health concern due to their new properties that could cause serious diseases (3). The *E. coli* O157:H7 for example, is a pathogenic strain that is well adapted in the environment (5,8). For these reasons, the *Salmonella* spp. and *E. coli* are often used as indicator bacteria in AMR surveillance programs and other public health-related studies (1).

In our study, we isolated *Salmonella* spp. and *E. coli* from different anthropogenic areas in North Carolina and identified the AMR determinants as well as virulence factors through antimicrobial susceptibility testing (AST) and whole genome sequencing (WGS). The information gathered from this study is important in understanding the occurrence and persistence of resistance determinants and pathogenic bacteria in the environment.

2.3 Materials and methods

2.3.1 Sample collection and bacteria isolation

A total of 70 soil samples were collected from different anthropogenic areas in North Carolina: urban (n=29) and natural (n=41). Urban environments included garden landscape (n=3), residential yard (n=14), and indoor potted plant (n=12) while natural environment

includes Forest A (n=14), Forest B (n=8), Forest C (n=5), Forest D (n=5), and potting soil mix (n=9).

Prior to bacterial isolation, each of the soil samples was sieved and mixed. The *Salmonella* spp. was isolated using Xylose-Lysine-Tergitol (XLT-4) selective media. Subsequently, 5 colonies from each positive plate were subjected to biochemical tests using Triple Sugar Iron agar slant (TSI agar), Lysine Iron Agar (LIA) and Urea agar slant (9,10). For *E. coli*, each sample was streaked into a McConkey agar (10,11). From each plate, multiple (n=3) colonies were picked for further isolation until a pure culture was obtained. Confirmation of *Salmonella* spp. isolates were performed through amplification of the *invA* gene (9) while *the 16s rRNA* gene was used to identify *E. coli* (12).

2.3.2 Resistance determination

The AST was performed by the broth microdilution method in a 96-well gram-negative sensititre plate containing a panel of 14 antimicrobials (CMV3AGNF Gram Negative NARMS plate, Trek Diagnostic System, Cleveland, OH, USA). The Minimum Inhibitory Concentration (MIC) was interpreted based on the Clinical and Laboratory Standards Institute standards (CLSI) [9,10].

2.3.3 Analysis of antimicrobial resistance genes (ARGs)

The presence of ARGs among the isolates was analyzed through the amplification of ARGs by polymerase chain reaction (PCR). The presence of integron was also determined by the amplification of the integron gene-1 (*intI 1*) (9).

2.3.4 Whole genome sequencing

The bacterial isolates that were subjected to sequencing were selected based on their phenotypic characteristics and the presence of ARGs. DNA extraction was carried out using the

Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufacturer's instructions. The quality and concentration of the extracted DNA were determined using the NanoDrop™ 2000/2000c Spectrophotometer (Thermo Scientific, USA) and Qubit 3.0 Fluorometer (Thermo Fisher Scientific, USA). DNA libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina, USA) following the manufacturer's instructions. The resulting DNA libraries were purified using AMPure XP beads (Beckman Coulter, USA) and re-quantified using the Qubit 3.0 Fluorometer (Thermo Fisher Scientific, USA). Sequencing was performed on the MiSeq System using v2 sequencing reagent kits (Illumina, USA) (9).

2.3.5 Data assembly and analysis

Raw sequences were assembled using the CLC Genomic Workbench (QIAGEN Bioinformatics, USA). Draft genomes were annotated using Rapid Annotation using Subsystem Technology (RAST) (http://rast.theseed.org/FIG/rast.cgi). Serotype and phylotype of the isolates were determined through pubMLST (https://pubmlst.org/bigsdb), SeqSero (http://denglab.info/SeqSero), and EnteroBase (https://enterobase.warwick.ac.uk/). The presence of plasmid and virulence genes were determined using PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/), VirulenceFinder (https://cge.cbs.dtu.dk/services/VirulenceFinder/), and Virulence Finder Database (http://www.mgc.ac.cn/VFs/main.htm). Resistance genes were determined through ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) (9) and Comprehensive Antibiotic Resistance Database (CARD) (https://card.mcmaster.ca). The phylogenetic tree was constructed using GrapeTree (https://enterobase.warwick.ac.uk/) and visualized through iTOL (https://itol.embl.de/).

2.4 Results

2.4.1 Prevalence of *Salmonella* spp. and *E. coli*

The prevalence of *Salmonella* spp. in soil samples from the urban and natural environments was 10.34% and 2.44% respectively. In *E. coli*, the prevalence was observed to be 62.1% in urban and 26.8% in the natural environment. From these positive soil samples, a total of 20 *Salmonella* spp. (urban, n=15; natural, n=5) and 66 *E. coli* (urban, n=41; natural, n=25) isolates were obtained and analyzed.

2.4.2 AMR in *Salmonella* spp. and *E. coli*

The *Salmonella* spp. from the urban environment were resistant to Streptomycin (66.67%) and Sulfisoxazole (46.47%) while those from the natural environment were resistant to Sulfisoxazole (100%) (Appendix 1). Similarly, *E. coli* isolated from the urban environments showed the highest resistance to Sulfisoxazole (78.05%), followed by Ampicillin (7.32%), and Cefoxitin (4.88%). Isolates from the natural environment only showed resistance to Sulfisoxazole (80%) (Table 2.1).

2.4.3 AMR determinants and virulence factor analysis

The *Salmonella* spp. from the urban environment carries *fox* (80%), *strA* (66.67%), and *strB* (66.67%) genes while all the isolates from the natural environment encoded for *fox* (100%) gene. With *E. coli,* the ARGs *sul1 (4.89%)* and bla_{CMY-2} (4%) were detected from the urban and natural environments, respectively.

Based on the WGS analysis, we identified *Salmonella* serotypes Schwarzengrund (64.28%) and *Salmonella* Mississippi (35.71%) from the urban environment (n=14) while the isolates from the natural environment (n=5) were Mississippi (100%). All *S.* Mississippi carried

aaa(6')-Iaa gene while *S.* Schwarzengrund has *aac(6")-Iaa, aph(6)-Id,* and *aph(3')-Ib*. All *S.* Schwarzengrund isolates were detected to carry IncFIB and IncFIC(FII) plasmids (Appendix 2).

Similarly, the WGS analysis of *E. coli* isolates from the urban environment predominantly belonged to phylogroups A (26.67%), B1 (21.67%), D (20%), and B2 (13.33%). The *E. coli* from the natural environment (n=13) were determined to be from phylogroups B1 (38.46%), B2 (38.46%), D (7.69%), and E (7.69%). There were 23 virulence genes identified among the isolates, while only 9 were detected at a frequency >10%. These genes include *gad* (glutamate decarboxylase), *lpfA* (long polar fimbriae), *iss* (increased survival serum), *air* (enteroaggregative immunoglobulin repeat protein), and *eilA* (*Salmonella HilA* homolog) among others. Additionally, virulence factors such as *iroN* (Salmochelin), *espA* (secreted proteins), and *tsh* (temperature-sensitive hemagglutination) were identified. The *mdfA* gene was detected in 100% and 84.5% of *E. coli* isolates from the urban and natural environment, respectively (Figure 2.1). A new gene *FosA7* conferring resistance to Fosfomycin was detected in an isolate from a residential yard. The activity of *the FosA7* gene was assessed by amplifying and cloning the segment into *E. coli* (TOP10). Using E-test (Liofilchem, USA), the donor isolate and the transformed *E. coli* showed resistance to Fosfomycin (MIC, >256 ug/mL). Its relatedness to other Fosfomycin resistance genes was analyzed using MAFFT (https://mafft.cbrc.jp/alignment/software) (Figure 2.2). The pCoo and pB171 plasmids were detected in isolates from a residential yard and garden landscape, respectively. While col(156) and pHN7A8 plasmids were detected from the natural environments, Forest A and D.

2.5 Discussion

The development and transmission of resistant bacteria have long been recognized (2,7,13), especially in clinical and agricultural settings where selective pressure and AMR

determinants are expected to be high (13). This study identified AMR determinants and virulence factors in *Salmonella spp.* and *E. coli* from different anthropogenic areas in North Carolina using WGS.

S. Schwarzengrund was detected in our isolates from the urban environment. *S.* Schwarzengrund was identified as one of the frequently detected serotypes in non-human, non-clinical sources in the US (14). There have been reports of disease outbreak, food contamination, (14) and multidrug resistance from this serovar (15). Moreover, we detected IncFIB and IncFII plasmids, which were reported to be significantly associated with *S.* Schwarzengrund from humans, animals, and manure treated environment (15).

The *E. coli* isolates predominantly belonged to phylogroups B1, B2, and D, which are known to thrive in various ecological niches, including soil (8). Several strains from these phylogroups are commensals and pathogenic and often has large genomes that codes for AMR determinants and virulence factors, allowing them to adapt and survive in different environments (5,6). The plasmids pCoo and pB171 were detected in the urban environment while col(156) and pHN7A8 were identified in the natural environment. Both pCoo and pB171 are virulence plasmids associated with enterotoxigenic *E. coli* (ETEC) and enteropathogenic *E. coli* (EPEC), respectively and are often detected in cases of travelers and infantile diarrhea (16). The plasmids col(156) is associated with extraintestinal pathogenic *E. coli* (ExPEC), an infectious strain affecting both humans and animals, particularly birds (16). The pHN7A8, a multi-resistance plasmid from *E. coli* of animal origin in China and recently in Bolivia, was detected in our isolate from a forest. Although we did not discover ARGs, it is essential to note that this plasmid is classified as F33:A-:B-plasmid which is a crucial vector of $bla_{\text{CTX-M-55/-65}}$, $bla_{\text{NDM-1}}$, *FosA3*,

and *rmtB* resistance genes and has the capability to acquire markers involved in plasmid replication or stability (16).

E. coli has intrinsic and acquired genes that degrade and resist toxic compounds such as biocides and metals (3,4,18), allowing their survival in the environment. The *mdfA* is a multidrug efflux protein, and its overexpression results in resistance to several antimicrobials and organic cations (3). The *FosA7* gene is a new ARG that was detected from *S*. Heidelberg from broiler chickens in Canada, was discovered in our *E. coli* isolate from a residential yard. *FosA7* confers resistance to Fosfomycin, a broad-spectrum antibiotic that is used to treat uncomplicated UTI and extensively drug-resistant (XDR) gram- bacteria. Fosfomycin resistance has been reported in bacterial isolates from human and animal origin in China, Japan, France, and recently the US and Canada (19), but was not reported in isolates from the environment.

The majority of the antimicrobials being used today were initially isolated from the natural environment, particularly from the soil Actinomyces (6,7,20). The antimicrobial producing organisms have determinants that would help to resist the action of the antimicrobial(s) they produce along with the other microbes found in the same environment (3,6,20), and so it is not surprising to detect AMR determinants in the environment (3,18). However, the proximity of humans and its activities in the environment greatly influences the dynamics in the microbial community, genetic variation, resistance selection, and possible emergence of novel mechanisms of resistance (6).

Soil is a huge source of AMR determinants considering its diverse microbial composition that varies depending on the geographical and biochemical gradient (20) as a result of constant selective pressures exerted in the environment. A large amount of antimicrobials being discharged into the environment along with continual interaction of humans and animals does

not only alter the microbial community but also hasten and sustain the development of AMR and virulent pathogens (1,5,6). Urban environments are continuously being exposed to different anthropogenic activities making it more vulnerable to changes that may shape the composition of the bacterial community. The presence of human activities in an environment increases the chance of contamination (1,8) such as spillage of antimicrobials, heavy metals, biocides, and even resistant bacteria (1,3,6,18). Such contamination creates a selective pressure or environmental hotspot for the development and dissemination of AMR (1,5,18). Heavy metals and biocides, for example, was identified to play an essential role in the maintenance and spread of AMR determinants. Specific plasmids can contain genes that confer resistance to several compounds such as antimicrobials, heavy metals, and biocides. In a heavy metal polluted environment, this plasmid containing resistance genes is maintained and so the AMR determinants, even in the absence of antimicrobials explaining the persistence of AMR in the environment (6).

Salmonella spp. and *E. coli* are commensal bacteria in humans and animals, but these bacteria are also well adapted in the natural environment. Aside from the presence of antimicrobial producing microorganisms, the role of wildlife was also cited in the dissemination of resistance and pathogenic bacteria (3). Several reports of AMR contamination in the natural environments have been associated with wildlife such as migratory birds and foxes (2,3,6). It is essential to understand the occurrence and development of AMR in the environment as there is a continuous rise of resistant and pathogenic bacteria. The presence of these determinants in different anthropogenic areas only suggests the need for constant surveillance.

2.6 Accession numbers

The paired-end reads used in this study were deposited in the National Center for Biotechnology Information (NCBI) under the Bioproject accession numbers **PRJNA293224** and **PRJNA293225** for *Salmonella* spp. and *E. coli* isolates, respectively.

2.7 Abbreviations

MIC, Minimum Inhibitory Concentration; ETEC, Enterotoxigenic *E. coli*; EPEC, enteropathogenic *E. coli;* ExPEC, Extraintestinal pathogenic *E. coli;* UTI, urinary tract infection; MDR, multidrug-resistant; XDR, extensively drug-resistant; MAFFT, Multiple alignment using fast Fourier Transform; AMP, Ampicillin; AUG2, Amoxicillin/Clavulanic acid; AXO, Ceftriaxone; AZI, Azithromycin; CHL, Chloramphenicol; CIP, Ciprofloxacin; FIS, Sulfisoxazole; FOX, Cefoxitin; GEN, Gentamycin; NAL, Nalidixic acid; STR, Streptomycin; SXT, Trimethoprim/Sulfamethoxazole; XNL, Ceftiofur; TET, Tetracycline; RY, residential yard; IPP, indoor potted plant; GL, garden landscape; FA, Forest A; FB, Forest B; FC, Forest C; FD, Forest D.

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coli and *Klebsiella pneumonia* isolated from animals, foods products and humans in China. mSphere 2018;3:e00137-18.

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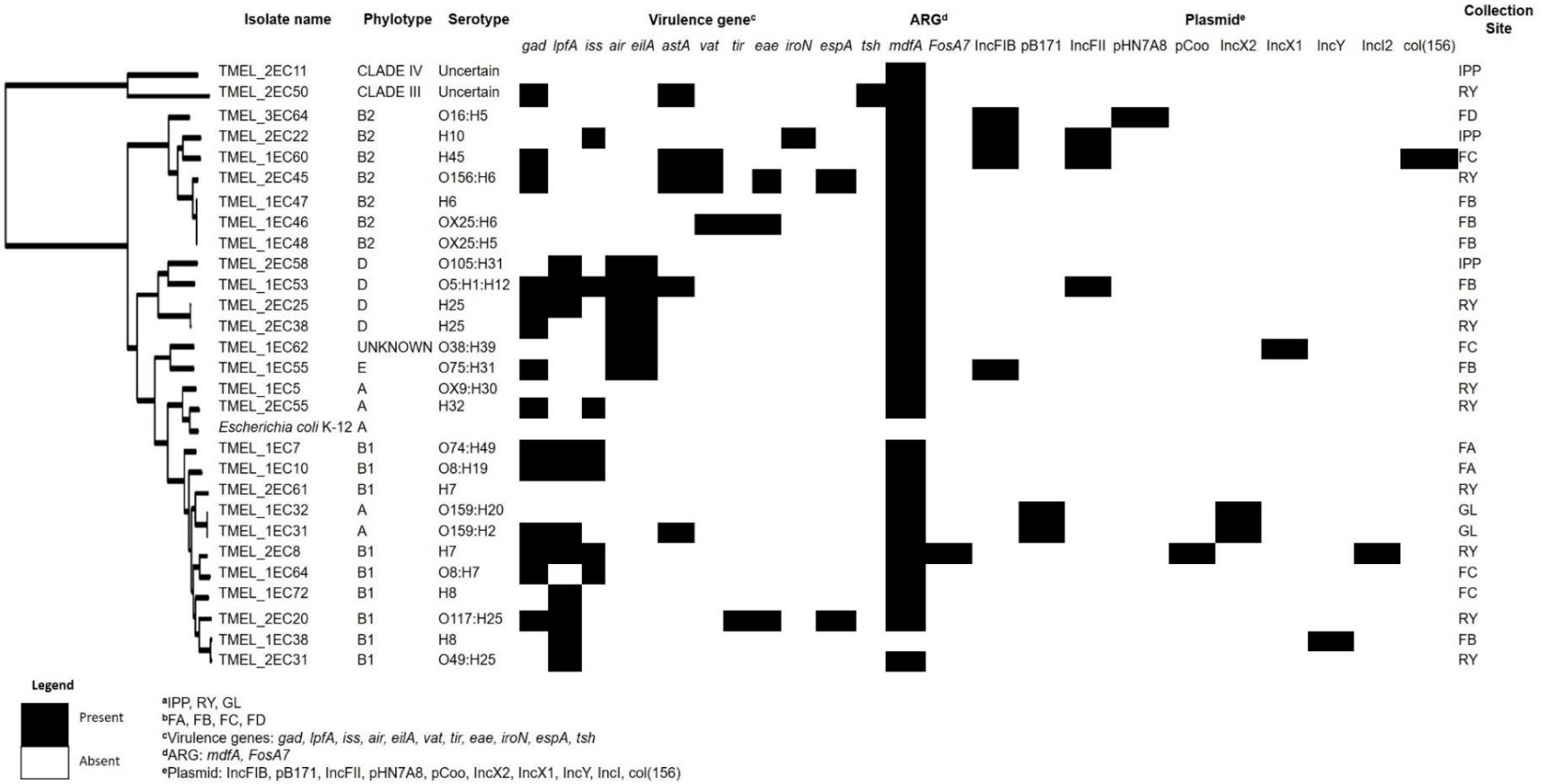
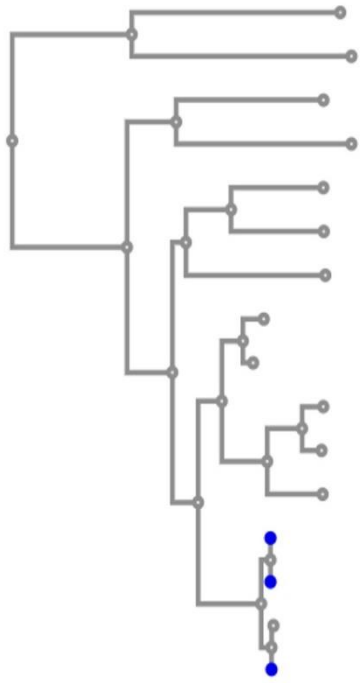


Figure 2.1 Phylogenetic tree and genotypic characteristics of E. coli isolated in the urban[a] and natural[b] environment. The Phylogenetic tree was generated based on SNP found in the isolates. The *E. coli* str. K-12 substr. MG1655 **(NC000913)** was used as a reference. The phylotypes of the isolates were determined using ClermonTyping (EnteroBase) while the virulence factors, ARGs, and plasmids were analyzed using VirulenceFinder, ResFinder, and PlasmidFinder respectively.



Fos gene	Source	
	Bacteria species	Country
FosA5	*Escherichia coli*	China
FosB1	*Staphylococcus haemolyticus*	Japan
FosB3	*Enterococcus*	China
FosX	*Listeria monocytogenes*	United Kingdom
FosC	*Achromobacter denitrificans*	South Korea
FosC2	*Escherichia coli*	Japan
FosK	*Acinetobacter soli*	Japan
FosA1	*Serratia marcescens*	Spain
FosA2	*Enterobacter cloacae*	Canada
FosA3	*Escherichia coli*	Japan
FosA4	*Escherichia coli*	Japan
FosA6	*Escherichia coli*	USA
FosA7	*Salmonella enterica*	Canada
FosA7_WGS	*Escherichia coli*	USA (*This research*)
FosA	*Escherichia coli*	Brazil
FosA7_PCR	*Escherichia coli*	USA (*This research*)

Figure 2.2 Phylogenetic relationship Fosfomycin resistance genes. Fosfomycin resistance gene has been detected in different species of bacteria from different countries. The sequences from this study, *FosA7*_WGS and *FosA7*_ PCR clustered with the *FosA7* genes from Canada and Brazil. The Fosfomycin determinants accession numbers are *FosA1*, **FJ829469**; *FosA2*, **EU487198**; *FosA3*, **AB522970**; *FosA4*, **AB908992**; *FosA5*, **KP143090**; *FosA6*, **KU25459**; *FosA7*, **LAPJ01000014**; *FosA*, **MK043330**; *FosB*, **ABS73480**; *FosB3*, **NG_050412**; *FosC*, **DQ112222**; *FosC2*, **NG_047891**; *FosK*, **NG_047898**; *FosX*, **LT795756**; and *FosA7* from this study **SAMN10396967.**

Table 2.1 Resistance and MIC distribution (squashtogram)[a] of *E. coli* isolates *form* urban (n=41) and natural (n=25) environment

AM[b]	Anthropogenic area	%R[c]	Distribution of MICs in µg/mL (%)														
			0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512
GEN	Natural	0					0.0	16.0	36.0	4.0	40.0	4.0	0.0					
	Urban	0					0.0	46.3	39.0	4.8	2.4	7.3	0.0					
STR	Natural	0								0.0	0.0	56.0	36.0	8.0	0.0			
	Urban	0								0.0	29.2	58.5	7.3	4.8	0.0			
AUG2	Natural	0							0.0	8.0	64.0	28.0	0.0	0.0				
	Urban	0							2.4	24.4	68.3	4.9	0.0	0.0				
FOX	Natural	0						0.0	0.0	12.0	44.0	40.0	4.0	0.0				
	Urban	4.9						0.0	0.0	0.0	53.6	41.5	0.0	**4.9**				
XNL	Natural	0				0.0	8.0	84.0	8.0	0.0	0.0	0.0						
	Urban	0				0.0	14.6	78.1	2.4	0.0	4.9	0.0						
AXO	Natural	0					100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0			
	Urban	0					100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0			
FIS	Natural	80.0											0.0	0.0	16.0	0.0	4.0	**80.0**
	Urban	78.1											0.0	0.00	9.8	9.8	2.4	**78.1**
SXT	Narural	0				60.0	24.0	12.0	4.0	0.0	0.0							
	Urban	0				82.9	12.2	2.4	2.4	0.0	0.0							
AZI	Natural	0				0.0	0.0	0.0	0.0	8.0	76.0	16.0	0.0					
	Urban	0				0.0	0.0	0.0	0.0	19.5	65.9	14.6	0.0					
AMP	Natural	0							0.0	24.0	64.0	12.0	0.0	0.0				
	Urban	7.3							4.9	34.2	48.8	4.9	0.0	**2.4**	**4.9**			
CHL	Natural	0								0.0	4.0	24.0	64.0	8.0				
	Urban	0								0.0	0.0	14.6	85.4	0.0				
CIP	Natural	0	44.0	56.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0							
	Urban	0	78.1	19.5	2.4	0.0	0.0	0.0	0.0	0.0	0.0							
NAL	Natural	0						0.0	0.0	0.0	44.0	56.0	0.0	0.0				
	Urban	0						0.0	0.0	68.3	26.8	4.9	0.0	0.0				

Table 2.1 (continued)

TET	Natural	0	100.0	0.0	0.0	0.0
	Urban	0	100.0	0.0	0.0	0.0

[a] Areas with white background and numbers (%) indicate the range of dilutions tested for each antimicrobial. Areas with solid-white background fall outside the range of tested concentrations. Numbers (%) in bold font indicate the percentages of isolates with resistance measured on the broth microdilution plates.

[b]Antimicrobial: AMP, Ampicillin (1-32 ug/mL); AUG2, Amoxicillin/Clavulanic acid (1/0.5-32/16 ug/mL); AXO, Ceftriaxone (0.25-64 ug/mL); AZI, Azithromycin (0.12-16 ug/mL); CHL, Chloramphenicol (2-32 ug/mL); CIP, Ciprofloxacin (0.015-4 ug/mL); FIS, Sulfisoxazole (16-256 ug/mL); FOX, Cefoxitin (0.5-32 ug/mL); GEN, Gentamycin (0.25-16 ug/mL); NAL, Nalidixic acid (0.5-32 ug/mL); STR, Streptomycin (32-64 ug/mL); SXT, Trimethoprim/Sulfamethoxazole (0.12/2.38-4/76 ug/mL); XNL, Ceftiofur (0.12-8 ug/mL); TET, Tetracycline (4-32 ug/mL)

[c] % Resistant isolates to each of the antimicrobial

CHAPTER 3: Enzyme-free detection of bacterial pathogen through use of functionalized gold nanosensors

Presented here is the manuscript titled: "Enzyme-free detection of bacterial pathogen through use of functionalized gold nanosensors" will be submitted (the present year 2019) to Biochemical and Biophysical Research Communications for peer review and publication.

3.1 Abstract

There are several laboratory techniques used in the research and surveillance of bacterial pathogens such as *Salmonella* spp. and antimicrobial resistance (AMR). However, these techniques require expensive laboratory facilities and highly trained individuals, creating a gap in surveillance and research especially in developing counties. Therefore, there is a need to develop a simple, rapid, specific, sensitive, and low-cost detection platform that can be used in field testing or laboratories with lesser capabilities.

Gold nanoparticles (AuNPs) are widely used in nanosensors due to their tunability, biocompatibility, and optical properties, particularly surface plasmon resonance. We designed and performed a simple, rapid, sensitive, specific, and cost-effective detection platform using functionalized AuNPs based on colorimetric assay.

In this study, we describe an enzyme-free detection method of bacterial pathogen using DNA functionalized AuNPs. Our method directly detected the *Salmonella* spp. extracted genomic DNA without prior amplification or the use of enzymes. Moreover, each step in the described method does not require expensive instruments. This detection method can be easily performed in a water bath or heat block and the results can be observed by the naked eye, eliminating the need to perform gel electrophoresis. This method can be completed within 45 minutes.

3.2 Introduction

Bacterial pathogens, particularly the non-typhoidal *Salmonella* (NTS) is one of the most commonly reported bacterial pathogens affecting millions of humans annually [1-3]. Globally, there are an estimated 78,707,591 cases and 59,153 deaths annually due to NTS. In the United States, NTS is estimated to cause 1,027,561 illness and 450 deaths annually [1,2,4]. Disturbingly, the highest percentage of deaths were reported among children under 5 years old [1-3]. Apart from consumption of NTS contaminated foods, the environment, water, and hygiene play an important role in infection, transmission, and dissemination of this pathogen. Moreover, *Salmonella* spp. can acquire and disseminate antimicrobial resistance (AMR) determinants and virulence factors which complicates the scenario. Globally, it is estimated that 700,000 people die annually due to AMR and by the year 2050, it is projected that this number would reach up to 10 million with an estimated economic loss of $100 trillion [5].

Research and surveillance programs to monitor bacterial pathogens and AMR were established and are being implemented in many countries. Laboratory techniques such as the standard bacterial culture and polymerase chain reaction (PCR) are widely used to provide information on the occurrence of bacterial pathogens and AMR. Bacterial culture is an indispensable technique in the bacterial identification and determination of resistance. Moreover, a bacterial culture is needed in obtaining pure isolates for further molecular analysis such as pulse-field gel electrophoresis (PFGE) which is time consuming and not suited to long-term global epidemiology [6]. The PCR has been a reliable tool in overcoming the difficulty in identification, differentiation, quantification bacterial pathogens [7], and have provided a more comprehensive information in AMR mechanisms. Currently, the cost of whole-genome sequencing (WGS) has been continuously decreasing, thus encouraging the curation of genome

sequences in the GenBank. However, not all laboratories are capable of these techniques, especially in developing counties. These techniques require expensive facilities and highly trained individuals, creating a gap in bacterial pathogens and AMR surveillance and research. The World Health Organization (WHO) has launched its Global Action Plan on AMR to ensure the continuity of successful treatment and prevention of infectious diseases. One of the WHO's strategic objective on the Global Action Plan on AMR is to encourage development and production of affordable diagnostic tools (Objective 5) [2,3,5].

One of the major interests in the biomedical sciences nowadays is the nanosensor or nano biosensor and their application in point-of-care (POC) diagnosis [8] of pathogenic organisms, genetic defects, and even harmful chemicals [9-11]. The gold nanoparticles (AuNPs) have been widely explored in the field of nanosensors due to its unique properties such as biocompatibility to different molecules [11,14,15,18-21], tunability, and surface plasmon resonance (SPR) in the visible range depending on size and shape [7,8,14,17,22-24]. The AuNPs are known to appear ruby red in color due to SPR, which is a distant-dependent effect observed based on the interaction of light and electrons surrounding the particle [7]. The changes in the AuNPs surrounding environment such as pH, salt concentration or high ionic concentration results to decrease in the interparticle distance and/or aggregation thus exhibiting colorimetric changes [8,20,24,27-29] that can be directly observed as color shift from red-pink to blue-gray due to plasmonic coupling [18,19,24,30,31]. As a result of aggregation, the position of the AuNPs SPR shifts from 520 nm to new band at 640-650 nm [7,18,19,24,26]. The AuNPs can be stabilized in solutions either electrostatically through the presence of repulsive charges on the surface of the particles, or sterically by surface coating with molecules such as polymers [8,22,25,26]. There has been a continuous research on the development of simple and rapid diagnostic platforms that

would allow on-site diagnosis. The DNA-functionalized AuNPs have been increasingly applied in the development of sensitive and selective biosensors [17,21]. In this study, the presence of *Salmonella* spp. extracted genomic DNA in the mixture and its hybridization to the DNA functionalized AuNPs prevented AuNPs aggregation even after the addition of $MgCl_2$. Therefore, the color of the mixture is retained (ruby red) in positive samples. In contrast, a color change from ruby red-pink to blue-gray indicated non-hybridization of the functionalized AuNP or non-detection of the target DNA [8] (Figure 3.1).

3.3 Materials and Methods

3.3.1 Reagents

Analytical-grade tetrachloroauric acid trihydrate salt ($HAuCl_4 \cdot 3H_2O$), magnesium chloride (anhydrous, ≥98%), hydrochloric acid (ACS reagent, 37%), and saline sodium citrate (20X concentrate) were purchased from Sigma-Aldrich (Missouri, USA). The phosphate buffered saline (Gibco solution, pH 7.4) was purchased form Fisher Scientific (Pittsburg, Pennsylvania, USA) and UltraPure DNase/RNase-Free Distilled Water (Waltham, Massachusetts, USA).

3.3.2 Thiol-modified oligonucleotide and extracted genomic DNA

The 5’ thiol-modified oligonucleotide (Thiol- GCA GCC ATG CTA TTC AGC AGT) was designed from the DNA-binding protein H-NS using NCBI BLAST, OligoCalc (biotools.nubic.northwestern.edu/Oligocalc.htm), and UNAFold (unafold.rna.albany.edu/) [32] and synthesized by Integrated DNA Technologies, Inc. (IDT, Iowa, USA). The genomic DNA was extracted using commercial kits and through boiling. DNA extraction was carried out using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germany) and MasterPure Gram Positive DNA purification Kit (Lucigen, USA) following the manufacturer’s instructions. Genomic DNA

extraction by boiling was performed by subjecting a mixture of a loopful (1 μL inoculating loop) of an overnight grown bacterial culture and UltraPure DNase/RNase-Free Distilled Water at 95 ^{o}C for 10 minutes. After boiling, the mixture was centrifuged at 15,000 x g for 1 minute. The supernatant was transferred into a new sterile microcentrifuge tube and stored at 4 ^{o}C until used.

3.3.3 Synthesis of citrate-stabilized gold nanoparticles

The AuNPs were synthesized using the standard Turkevich method [30,33]. In a clean erlenmeyer flask, 10 mL of 25 mM Gold (III) tetrachloro hydrate was prepared using UltraPure DNase/RNase-Free Distilled Water. The solution was left to boil while continuously being stirred using a teflon-coated magnetic bar. 50 mL of 38.8 mM trisodium citrate was added slowly, enough to maintain boiling of the solution. A color change in the mixture was observed until it turned to ruby-red or wine red, which indicated a successful synthesis of AuNPs. The newly synthesized AuNPs were continuously boiled and stirred for 15 minutes before it was left to cool down in room temperature and stored at 4°C until use.

3.3.4 pH-assisted functionalization of AuNPs with thiol-modified oligonucleotide

The pH-assisted functionalization of AuNPs with thiol-modified oligonucleotide was done following the previously published protocols [23,27] with minor modifications. In a 1.5 mL sterile microcentrifuge tube (MCT), 10 μL of 100 um thiol-modified oligonucleotide was mixed into a 1000 μL of citrate-stabilized AuNPs. A 100 μL of sodium citrate-HCl buffer (100 mM, pH 3.0) was added into the mixture and vortexed immediately to avoid AuNPs aggregation. The pH of the solution was determined using a pH paper and adjusted to 3.0 by the addition of HCl (37% solution) into the mixture. To remove the unreacted probes, the solution was centrifuged at 14000 rpm for 25 minutes and the supernatant was replaced with 500 μL of 0.5X SSC buffer.

This step was repeated twice and the resulting functionalized AuNPs were dispersed with 0.5x SSC buffer to a final volume of 250 mL and was stored at 4 ^{o}C until used.

Agarose gel electrophoresis assay was performed to evaluate the functionalization of AuNPs. After mixing of 10 μL of AuNPs (blank) and/or functionalized AuNPs with 3 μL of loading dye, the mixture was loaded on a 2% agarose gel buffered with 0.5X Tris-acetate-EDTA (TAE) buffer. The color change in the AuNPs as well as its migration in the agarose gel during electrophoresis was observed. To examine the stability of the functionalized AuNPs, we used the freeze-thaw cycle method [34] with minor modifications. We placed the tubes containing the functionalized AuNPs in a bucket with dry ice for ~10 minutes (or until frozen) and thawed back at room temperature for ~15 minutes or until completely thawed. The color change was then observed.

3.3.5 Optimization of enzyme-free direct detection conditions

Initially, 5 μL of extracted genomic DNA was mixed with 5 μL of the functionalized AuNPs and incubated at 65 ^{o}C for 30 minutes. After incubation, the mixture was left at room temperature for 10 minutes and added with 1 μL of 0.1M of $MgCl_2$.

To determine the optimum hybridization temperature, the mixture of 5 μL of extracted genomic DNA and 5 μL of functionalized AuNPs was hybridized at 60 ^{o}C, 63 ^{o}C, and 65 ^{o}C for 30 minutes. Similarly, the mixture was incubated for 10, 20, and 30 minutes to determine the optimum hybridization time. The optimum volume of the sample was also determined by adding 2.5, 5, and 7.5 μL of the bacterial extracted genomic DNA into the mixture. The functionalized AuNPs were also added at different volumes into the mixture from 2.5, 5, and 7.5 μL. The effect of salt was also determined by adding varied volumes of 0.1M $MgCl_2$ at 0.25, 0.5, and 1 μL into the mixture after incubation.

The minimum concentration of *Salmonella* spp. extracted genomic DNA that can be detected and distinguished by naked eye was determined by subjecting varied concentrations of extracted genomic DNA of a known *Salmonella* spp. positive isolate into the reaction mixture. The changes in the absorbance and position of the lambda peak were measured using UV-Vis spectroscopy using NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA). The correlation of each of the described protocol conditions and UV-Vis measurements was computed using the JMP Pro 14.1.0 software (SAS Institute Inc., Cary, NC, USA).

3.3.6 Detection of *Salmonella* spp.

The ability of the described method to detect extracted *Salmonella* spp. genomic DNA was evaluated. A set of 50 extracted genomic DNA, which consist of 30 *Salmonella* spp. and 20 other bacterial species extracted genomic DNA were subjected to the described method and PCR. The tests result was compared to the bacterial culture data. The ability to correctly identify the true positive (sensitivity) and the true negative (specificity) was performed using a 2x2 contingency table. To determine whether the described method, PCR, and bacterial culture marginal frequencies or test results are equal, a McNemar's test at α 0.05 was performed using the SAS 9.4 (SAS Institute Inc., Cary, NC, USA). The Cohen's Kappa statistics (κ) was also used measure the interrater agreement and the result was interpreted as follows: values ≤ 0 indicating no agreement and 0.01 – 0.20 as none to slight, 0.21 – 0.40 as fair, 0.41 – 0.60 as moderate, 0.61 – 0.80 as substantial, and 0.81-1.0 as almost perfect agreement [35].

3.4 Results and Discussion

The synthesized AuNPs using the standard Turkevich methods exhibit a ruby red color. Using UV-Vis spectroscopy, the synthesized AuNPs' lambda peak was observed at 518-519 nm (Figure 2) which indicates a 10-15 nm diameter size of the AuNPs [19,22,24,31]. We

functionalized the AuNPs by attaching a target-specific thiol-modified oligonucleotide on its surface following pH-assisted method using citrate buffer (pH 3.0) [27]. The citrate buffer (pH 3.0) minimizes the charge repulsion between the AuNPs and thiol-modified oligo thereby facilitating the functionalization of AuNPs [10,25,27,28]. Following pH-assisted functionalization of AuNPs, there was a slight shift in the lambda peak from 518-519 nm to 525 nm (Figure 3.2A). The slight shift on the lambda peak explains the change in the dielectric properties following functionalization of AuNPs [17]. The non-functionalized AuNPs and DNA-functionalized AuNPs were tested by agarose gel electrophoresis. We found that the non-functionalized AuNPs were aggregated in cationic-containing agarose gel running buffer and did not migrate from the well. The DNA-functionalized AuNPs were not aggregated, can be seen in red color, and migrated from the agarose gel well (Figure 3.2B). The results show that we successfully modified the surface of AuNPs [21,27,28]. With polymer capped nanoparticles, aggregation can be reversible due to steric stabilization [25]. This mechanism explains why the functionalized AuNPs retained its red color after it was subjected to the freeze-thaw cycle method (Figure 3.2C). This only indicates strong attachment of thiol modified oligonucleotide on the AuNPs surface [25].

Following a series of experiments, we determined the optimized conditions for this method. We observed that the AuNPs were stable at 63 ^{o}C and 65 ^{o}C hybridization temperature following addition of $MgCl_2$. Throughout the experiment, we decided to use 65 ^{o}C over 63 ^{o}C as a hybridization temperature, based on the knowledge that temperature affects the specificity of nucleic acid hybridization, and higher temperature enables near-optimal single-base discrimination [36]. The red color of the mixture was also retained after 30 minutes of hybridization time. This observation was supported by the lambda peak at 535 nm using UV-Vis

spectroscopy. The volumes of DNA sample and functionalized AuNPs in the mixture were both optimal at 5.0 μL. As for the volume of salt solution, we used 0.5 mL of 0.1 M $MgCl_2$. In summary, the described method consists of the following steps and conditions: mixing of 5.0 μL sample DNA and 5.0 μL of functionalized AuNPs, hybridization at 65 ^oC for 30 minutes, cooling at room temperature for 10 minutes, addition of 0.5 μL of 0.1 M $MgCl_2$, and color change observation.

The optimized protocol for this method was used to detect extracted *Salmonella* spp. genomic DNA at varied DNA concentration. The shift in the lambda peak position from 520 nm to 650 nm was observed as the concentration of the DNA in the solution decreases (Figure 3). In our experiment, the lowest concentration of extracted *Salmonella* spp. genomic DNA that can be detected and distinguished by the naked eye was 23.5 ng/μL. At this concentration of the extracted genomic DNA, the mixture changed color from ruby red to purple. There was a good coefficient of correlation between DNA sample concentration and absorbance ($R^2=0.806$) with a linear equation of *absorbance (650/520 nm) = 0.14+ 0.0002*(DNA concentration)*.

We evaluated the performance of the described method by subjecting a set of 50 extracted genomic DNA samples. Using the described method, we detected 36 and 14 positive and negative extracted genomic DNA for *Salmonella* spp., respectively. With PCR, we obtained 30 and 20 positive and negative DNA samples, respectively. The test results were compared with the bacterial data, in which the sensitivity and specificity obtained were 90% and 55%, respectively. Using McNemar's test, it was determined that there was no significant difference between the performance of the described method and PCR (p=0.0833). Using Cohen's Kappa statistics, our findings demonstrated substantial agreement (K=0.7148) between the 2 raters (Table 1).

Several nanosensor-based methods have also been reported for the detection of *Salmonella* spp [13,14], However several of these nanosensor-based methods still require pre-amplification, restriction enzymes, and expensive instruments. Rapid diagnostic methods are crucial for overcoming the device cost and providing simple, fast, easy-to-use diagnostic tests especially in laboratories with lesser capabilities. The field of nanosensors is believed as one of the future directions in the detection of *Salmonella* spp. [13] and other infectious and genetic related diseases. Direct detection of dsDNA would have an obvious advantage, however, this technology has been inaccessible using the currently available techniques [12]. The recent works are still a proof-of-concept experiments and partially technological [12]. Scientists are trying to increase the benefits and applicability of these nanosensors [14] but the actual application is rather limited because of the immature stage of the technology development [12,13].

In this study, we describe an enzyme-free detection method of bacterial pathogen using functionalized AuNPs. The described method directly detected the bacterial extracted genomic DNA without prior amplification or the use of enzymes. Moreover, each step in the described method, from the functionalization of AuNPs to the detection of bacterial extracted genomic DNA does not require special instruments or laboratory set-ups. This detection method can be easily performed in a water bath or heat block and the results can be observed by the naked eye, eliminating the need to perform gel electrophoresis. This method can be completed within 45 minutes.

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State University and LaBean Research Group, Material Science and Engineering, North Carolina State University, Raleigh, North Carolina, USA.

3.6 Ethical approval

Not required

3.7 Competing interest

All authors declare no conflict of interest

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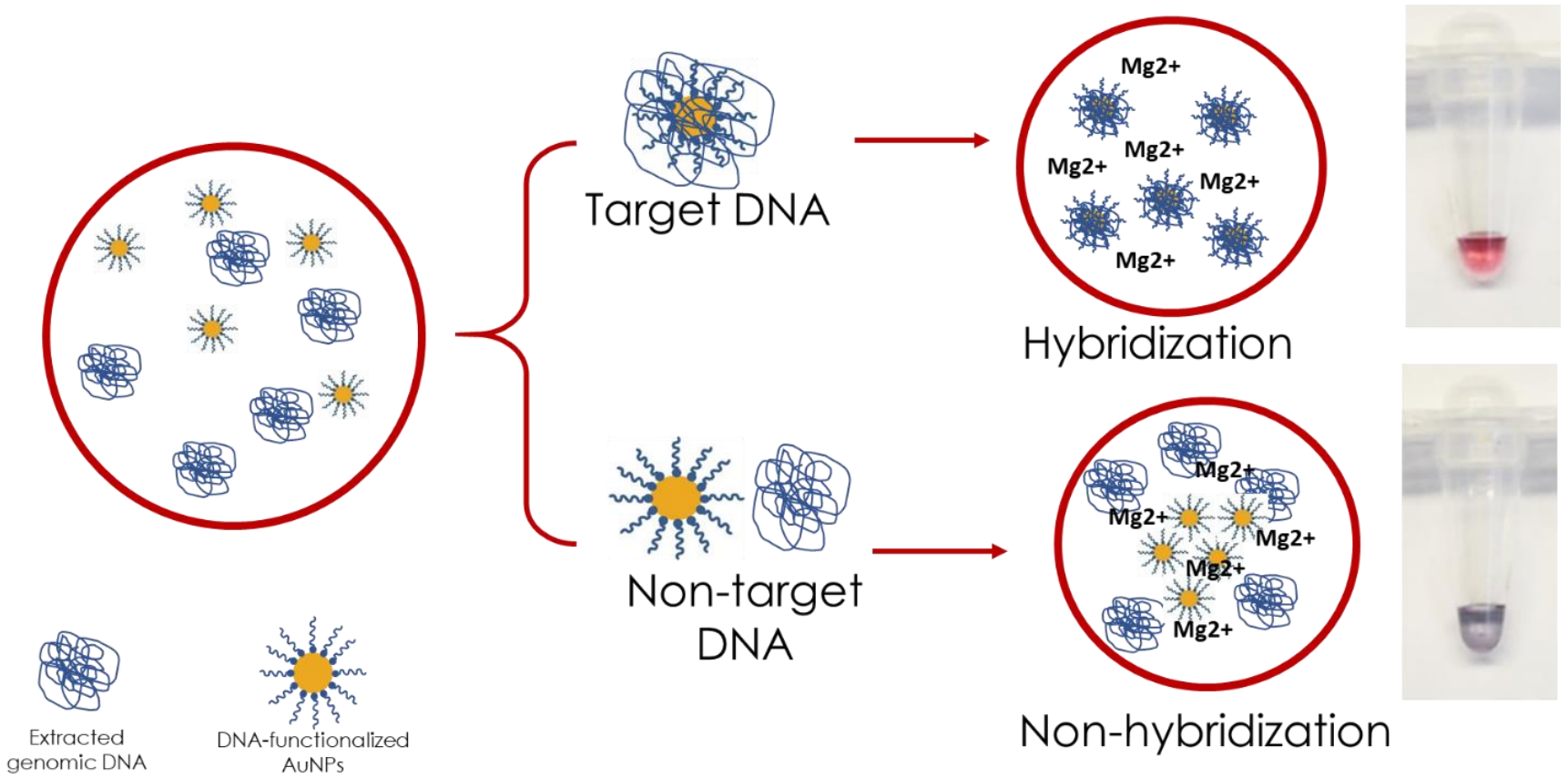

Figure 3.1 Working principle of the described method.

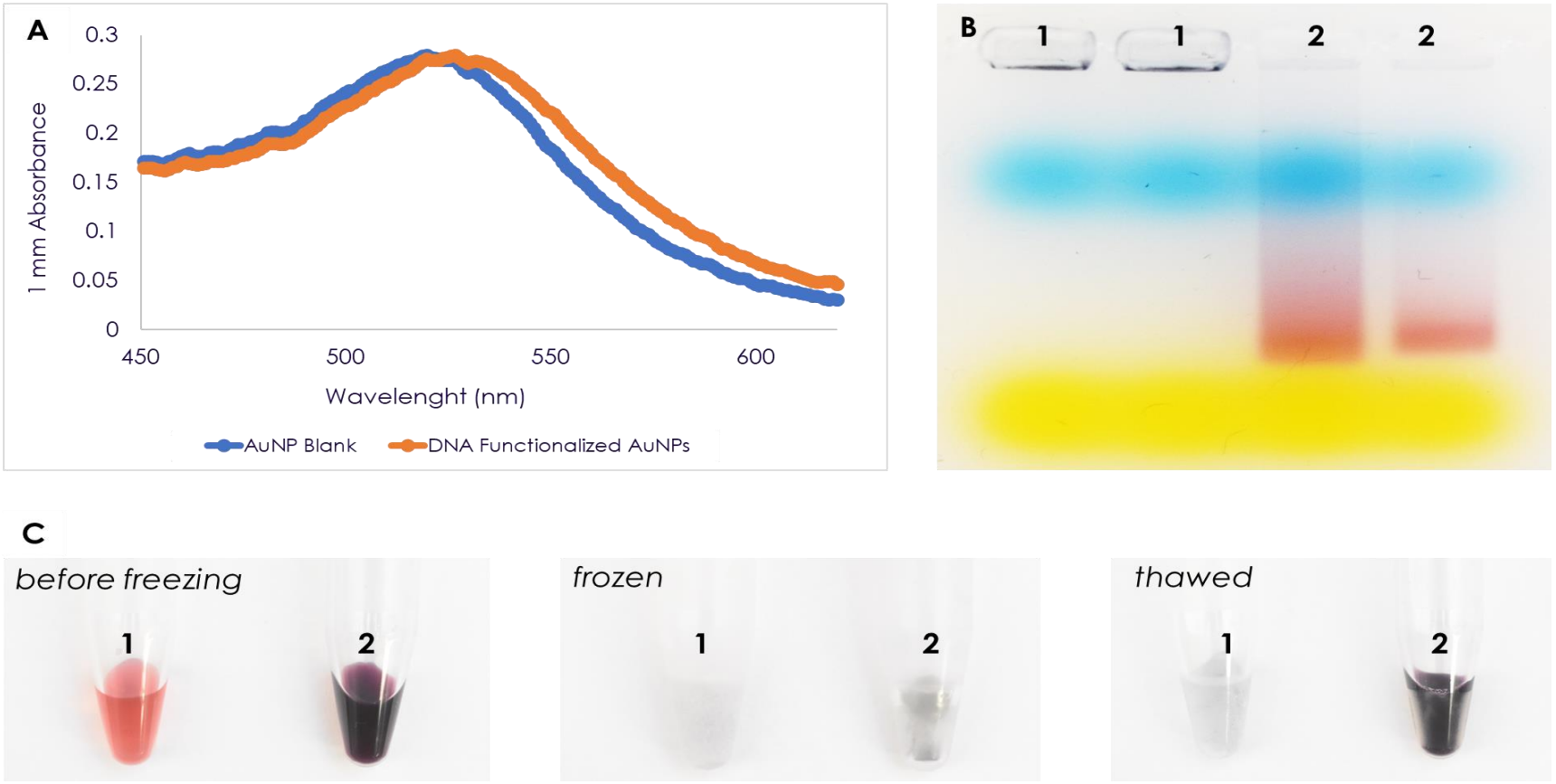

Figure 3.2 Characterization of AuNPs. There was a slight shift in the SPR peak of the functionalized AuNPs as compared to the unmodified/blank AuNPs as shown in UV-vis spectroscopy (A). With agarose gel electrophoresis (B), the unmodified/blank AuNPs became aggregated and remained on the well (1-2), whereas the functionalized AuNPs retained its red color and have migrated from the well (3-6). With freeze-thaw cycle method (C), the unmodified/blank AuNPs (tube 1) became aggregated while the DNA-functionalized AuNPs (tube 2) retained its red color.

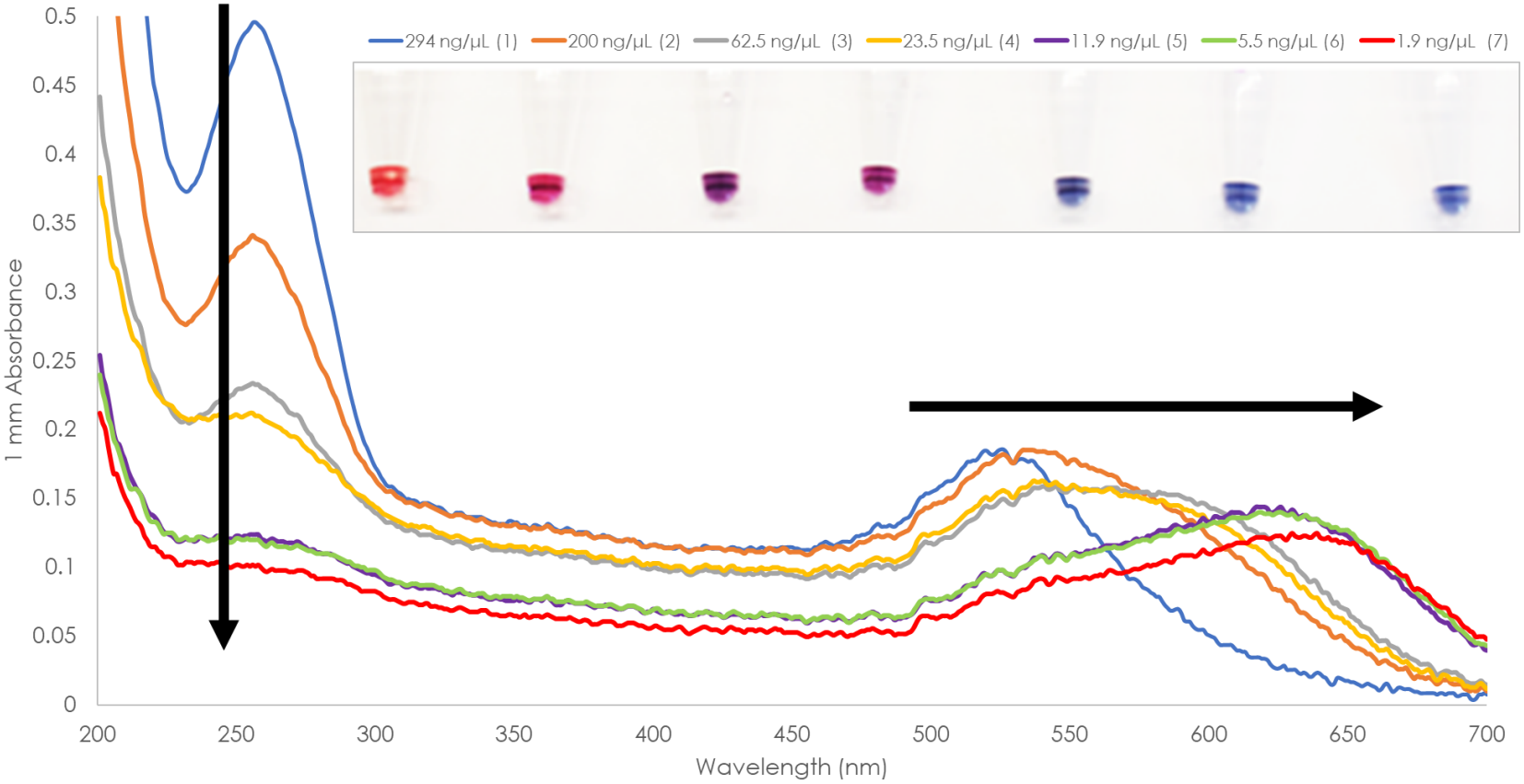

Figure 3.3 Detection of extracted Salmonella spp. genomic DNA at different concentrations. The color can be observed to change from ruby red to bluish-gray and the lambda peak moves from 520 nm to 650 nm as the extracted DNA concentration decreases.

Table 3.1 Analysis of the performance of this method in comparison to bacterial culture and PCR.

Analysis	Results		
	This method vs. Bacteria culture	This method vs. PCR	PCR vs. Bacteria culture
Sensitivity	90%	90%	96.7%
Specificity	55%	55%	95%
McNemar's Test (α=0.05)	0.0833	0.0833	1
Kappa Cohen's test	K= 0.7148		

* Significant *p-value* at less than α 0.05

APPENDICES

Appendix A. Resistance and MIC distribution (squashtogram) of *Salmonella* spp. isolates from urban (n=15) and natural (n=5) environment[a]

AM[b]	Anthropogenic area	%R[c]	Distribution of MICs in µg/mL (%)														
			0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512
GEN	Natural	0					20.00	80.00	0.00	0.00	0.00	0.00	0.00					
	Urban	0					0.00	46.67	46.67	6.67	0.00	0.00	0.00					
STR	Natural	0								0.00	20.00	20.00	60.00	0.00	0.00			
	Urban	66.67								0.00	0.00	0.00	33.33	0.00	**20.00**	**46.67**		
AUG2	Natural	0							20.00	80.00	0.00	0.00	0.00	0.00				
	Urban	0							46.67	53.33	0.00	0.00	0.00	0.00				
FOX	Natural	0						0.00	0.00	0.00	20.00	40.00	40.00	0.00				
	Urban	0						0.00	0.00	33.33	26.67	33.33	6.67	0.00				
XNL	Natural	0				0.00	0.00	0.00	80.00	20.00	0.00	0.00						
	Urban	0				0.00	0.00	6.67	86.67	6.67	0.00	0.00						
AXO	Natural	0					100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			
	Urban	0					100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			
FIS	Natural	100											0.00	0.00	0.00	0.00	0.00	**100.00**
	Urban	46.47											0.00	0.00	46.67	0.00	6.67	**46.67**
SXT	Narural	0				100.00	0.00	0.00	0.00	0.00	0.00							
	Urban	0				100.00	0.00	0.00	0.00	0.00	0.00							
AZI	Natural	0				0.00	0.00	0.00	0.00	0.00	0.00	20.00	80.00					
	Urban	0				0.00	0.00	0.00	0.00	0.00	66.67	13.33	20.00					
AMP	Natural	0							0.00	100.00	0.00	0.00	0.00	0.00				
	Urban	0							20.00	80.00	0.00	0.00	0.00	0.00				
CHL	Natural	0								0.00	0.00	0.00	100.00					

Appendix A. (continued)

	Urban	0								0.00	0.00	33.33	66.67		
CIP	Natural	0	0.00	20.00	80.00	0.00	0.00	0.00	0.00	0.00	0.00				
	Urban	0	73.33	26.67	0.00	0.00	0.00	0.00	0.00	0.00	0.00				
NAL	Natural	0						0.00	0.00	0.00	100.00	0.00	0.00	0.00	
	Urban	0						0.00	0.00	0.00	100.00	0.00	0.00	0.00	
TET	Natural	0									100.00	0.00	0.00	0.00	
	Urban	0									100.00	0.00	0.00	0.00	

[a] Areas with white background and numbers (%) indicate the range of dilutions tested for each antimicrobial. Areas with solid-white background fall outside the range of tested concentrations. Numbers (%0 in bold font indicate the percentages of isolates with resistance measured on the broth microdilution plates.

[b]Antimicrobial: AMP, Ampicillin (1-32 ug/mL); AUG2, Amoxicillin/Clavulanic acid (1/0.5-32/16 ug/mL); AXO, Ceftriaxone (0.25-64 ug/mL); AZI, Azithromycin (0.12-16 ug/mL); CHL, Chloramphenicol (2-32 ug/mL); CIP, Ciprofloxacin (0.015-4 ug/mL); FIS, Sulfisoxazole (16-256 ug/mL); FOX, Cefoxitin (0.5-32 ug/mL); GEN, Gentamycin (0.25-16 ug/mL); NAL, Nalidixic acid (0.5-32 ug/mL); STR, Streptomycin (32-64 ug/mL); SXT, Trimethoprim/Sulfamethoxazole (0.12/2.38-4/76 ug/mL); XNL, Ceftiofur (0.12-8 ug/mL); TET, Tetracycline (4-32 ug/mL)

[c] % Resistant isolates to each of the antimicrobial

Appendix B. Phylogenetic tree and genotypic characteristics of *Salmonella* spp. isolated in the urban and natural environment. The Phylogenetic tree was generated based on SNP found in the isolates. The *Salmonella* Schwarzengrund str. CVM19633 (**NC_011094**) was used as a reference. The serotypes of the isolates were determined using SISTR(EnteroBase) and SeqSero while the ARGs and plasmids were analyzed using ResFinder and PlasmidFinder, respectively