5-13-2016

Evaluation of an Agar Dilution Method for Identification of Extended-Spectrum Beta-Lactamase (ESBL)-Producing Klebsiella pneumoniae in the Environment

Kimberly Erukunuakpor

Follow this and additional works at: https://scholarworks.gsu.edu/iph_theses

Recommended Citation

ABSTRACT

Evaluation of an Agar Dilution Method for Identification of Extended-Spectrum Beta-Lactamase (ESBL)-Producing *Klebsiella pneumoniae* in the Environment

By

Kimberly Okiemute Erukunuakpor

April 2016

Antibiotic resistance is a serious global public health problem. ESBLs are enzymes that destroy expanded-spectrum beta-lactam antibiotics rendering these drugs ineffective. Infection with ESBL-producing *K.pneumoniae* are hard to treat and result in longer hospital stay and higher mortality rates. The Clinical Laboratory Standard Institute (CLSI) have standard methods for detection of ESBL producing strains of bacteria in infected patients to guide antibiotic therapy, reduce the risk of mortality and risk of transmission. The presence of *K.pneumoniae* and *E.coli* which produce ESBLs have been confirmed in natural environments such as soil and water but no standard methods exist to identify directly and quantify these bacteria to understand the risk of human exposure in these settings. The purpose of this research is to assess the ability of an agar dilution method, using a differential agar Bio-Rad Rapid *E.coli* 2 agar utilized in environmental water quality studies, to identify correctly ESBL-producing *K.pneumoniae*. The minimum inhibitory concentration (MIC) of ceftriaxone antibiotic for wild-type ESBL producing *K.pneumoniae* isolates were compared on Mueller-Hinton broth (MHB) and Bio-Rad Rapid *E.coli* 2 agar. Using the MIC values, the isolates were classified as susceptible, intermediate or resistant. The MIC of wild-type strains of *K.pneumoniae* were above 4μg/mL for both methods on all susceptibility tests performed. The results of this research suggest that Bio-Rad Agar dilution method performed well, correctly identifying these strains as resistant to ceftriaxone, an indication of ESBL production. The Bio-Rad agar dilution method can be considered as a viable standard method for direct identification of ESBL-producing *K.pneumoniae* in natural environments.

Evaluation of an Agar Dilution Method for Identification of Extended-Spectrum Beta-Lactamase
(ESBL)-Producing *Klebsiella pneumoniae* in the Environment

By

Kimberly Okiemute Erukunuakpor

B.Sc., Microbiology

A Thesis Submitted to the Graduate Faculty
of Georgia State University in Partial Fulfillment
of the Requirements for the Degree

Master of Public Health

Atlanta, GA 30303

APPROVAL PAGE

Evaluation of an Agar Dilution Method for Identification of Extended-Spectrum Beta-Lactamase (ESBL)-Producing *Klebsiella pneumoniae* in the Environment

By

Kimberly Okiemute Erukunuakpor

Approved:

Dr. Lisa Casanova

_____

Committee Chair

Dr. Christine Stauber

_____

Committee Member

04/25/2016

_____

Date

ACKNOWLEDGEMENTS

I would like to begin by thanking my thesis chair Dr. Lisa Casanova for her guidance and support throughout the completion of this thesis. I would like to acknowledge my committee member Dr. Christine Stauber for her patience. At this time, I would also like to express my gratitude to Dr. Sheryl Strasser and Dr. Ike Okosun for their support throughout the program. In addition, I want to thank the faculty and staff of the School of Public Health at Georgia State University for sharing their knowledge.

I want to thank my amazing family and friends for their kindness, patience and unconditional support throughout the years and especially in completing this program. Finally, I would like to thank God.

Authors' Statement

In presenting this thesis as a partial fulfillment of the requirements for an advanced degree from Georgia State University, I agree that the Library of the University shall make it available for inspection and circulation in accordance with its regulations governing materials of this type. I agree that permission to quote from, to copy from, or to publish this thesis may be granted by the author or, in her absence, by the professor under whose direction it was written, or in his absence, by the Associate Dean, College of Health and Human Sciences. Such quoting, copying, or publishing must be solely for scholarly purposes and will not involve any potential financial gain. It is understood that any copying from or publication of this dissertation which involves potential financial gain will not be allowed without written permission of the author.

<div align="right">

___Kimberly Erukunuakpor___
Signature of the Author

</div>

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER I

INTRODUCTION

1.1 Background

Antibiotics once hailed as 'miracle drugs' are fast becoming ineffective due to resistance. Antibiotic resistance is the ability of an infectious bacterial species to evade the destructive effects of an antibiotic. Following the discovery of the first antibiotic -Penicillin- bacteria evolved to resist destruction (Casey, 2012). Bacteria resist the impact of antibiotics through three mechanisms: (i) destroying or altering the antibiotic structure, (ii) stopping the entry of the antibiotic to the target site, (iii) modifying the target site of the antibiotic (Neu, 1992).

Antibiotic resistance is a serious global public health problem as it threatens the effective treatment of bacterial infections (WHO, 2014). The 2014 Antimicrobial Resistance Global Surveillance Report by the World Health Organization revealed a high prevalence of antibiotic-resistant infections, with some WHO regions reporting up to 95% bacterial resistance to the most recently developed antibiotics (WHO, 2014). In the United States, data from the National Hospital Discharge Survey revealed a surge in hospitalizations for antibiotic-resistant infections, from 37005 in 1997 to 169985 in 2006, an increase of 359% (Mainous, Diaz, Matheson, Gregorie, & Hueston, 2011). Furthermore, the Centers for Disease Control and Prevention estimates that over 2million people are infected with resistant bacteria yearly in the US, 26,000 of which lead to death (CDC, 2013). Most of these infections are common health-care associated and community-acquired infections (urinary tract infections, wound infections, bloodstream infections and pneumonia) caused by *Staphylococcus aureus* and species of the Enterobacteriaceae family which include *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*) (Carey, 2012; CDC, 2013).

Members of the Enterobacteriaceae family produce β-lactamases as resistance mechanisms. β-lactamases are bacterial enzymes that hydrolyze β-lactam antibiotics. β-lactam antibiotics are antibiotics that have a β-lactam ring in their structure such as penicillin derivatives, cephalosporins and related compounds. *E. coli*, *K. pneumoniae* and a few other members of the Enterobacteriaceae family have the ability to synthesize extended-spectrum β-lactamases (ESBLs). ESBLs are a group of β-lactamases that confer resistance to third-generation (extended-spectrum) cephalosporins (e.g., cefotaxime, ceftriaxone, ceftazidime) and monobactams (e.g., aztreonam) which were developed to fight against β-lactamase-mediated bacterial resistance to antibiotics (Paterson & Bonomo, 2005; Pitout & Laupland, 2008).

Epidemiologic studies in healthcare settings have revealed that ESBL-producing bacteria, particularly *K. pneumoniae*, constitute a grave threat. A study focused on patients with bloodstream infections found that after 72 hours of antibiotic therapy, patients infected with ESBL-producing *K. pneumoniae* had a higher treatment failure rate than patients infected with non-ESBL *K. pneumoniae*. The study also reported significantly higher mortality rate among ESBL infected patients than non-ESBL infected patients, after 21 days of therapy (Tumbarello et al., 2006). A case-control study found that among patients -with urinary tract infections, respiratory tract infections, and bloodstream infections- cases infected with ESBL-producing *K. pneumoniae* had a longer mean hospital stay than controls infected with non-ESBL producing *K. pneumoniae* (Brooklyn Antibiotic Resistance Task Force, 2002). With the threat to infection control, clinical microbiology laboratories play a significant role in the appropriate management of patients to curb the spread of ESBL-producing *K. pneumoniae* through early detection. These laboratories primarily follow standard guidelines for antimicrobial susceptibility tests published by the US Clinical and Laboratory Standards Institute (CLSI) for detection of ESBL producing organisms

(CLSI, 2014). These methods assess the ability of the infecting organism to hydrolyze different cephalosporin antibiotics on microbiological media specifically structured for detecting ESBL production in clinical specimens (Pitout & Laupland, 2008).

Numerous environmental studies, using varying methods, have explored the occurrence of ESBL-producing bacteria in places outside of healthcare settings. Several of these studies have confirmed the presence of ESBL-producing Enterobacteriaceae in environments where human-pathogen interaction may occur. A study of food samples of animal origin found that 26% contained ESBL-producing *E.coli* (Jouini et al., 2007). Zurfluh et al. (2013) detected ESBL-producing Enterobacteriaceae in up to 36% of 58 rivers and lakes sampled. ESBL-producing *K. pneumoniae* and *E.coli* have also been found in wastewater, soil and vegetables (Ben Said et al., 2015; Prado et al., 2008). These studies emphasize the importance of understanding the dissemination of ESBL-producing bacteria in natural environments as they may serve as reservoirs for these bacteria.

1.2 Purpose of study

A guideline for methods using media designed for clinical specimens exists for determination of ESBL-production in infectious agents in healthcare settings (CLSI, 2014). ESBL detection aids in guiding patient therapy, active surveillance, and development of effective public health interventions to prevent the spread of health-care associated infections by ESBL-producing bacteria. These bacteria are also known to exist in environments outside of health-care settings and may contribute to the burden of infections with ESBL-producing bacteria. However, no standard methods are available for detecting and quantifying ESBL-producing bacteria in the environment. Most importantly, microbiological media for rapid identification of ESBL-producing bacteria in the environment are needed, because media used in clinical laboratories is often not selective

enough to use with environmental samples, which may contain many other competing organisms that interfere with detection of ESBL producing bacteria. Therefore, the objective of this research is to evaluate an agar dilution method for measuring the minimum inhibitory concentration of antibiotics against ESBL-producing *K. pneumoniae* using a microbiological culture media, Bio-Rad Rapid *E. coli* 2 agar that is designed to isolate *K. pneumoniae* and *E. coli* from the environment.

CHAPTER II

LITERATURE REVIEW

2.1 Background

Antibiotic resistant bacteria constitute a threat to infection treatment and control. Current research reveals that many pathogenic bacterial species —*Klebsiella pneumoniae, Escherichia coli*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and species of Enterobacter, Salmonella, and Shigella — are now resistant to most antibiotics (WHO, 2014 Livermore, 2003). The infections caused by these bacteria are common health-care associated and community-acquired infections such as urinary tract infections, wound infections, bloodstream infections and pneumonia. However, resistance leads to more severe outcomes from these infections (Carey 2012, CDC, 2013).

Extended-spectrum β-lactamases (ESBLs) are enzymes produced by bacteria that confer resistance to expanded-spectrum antibiotics such as ceftriaxone, cefotaxime and aztreonam (Bradford, 2001). Most bacteria that produce ESBLs are members of the Enterobacteriaceae family which include *Klebsiella pneumoniae* and *Escherichia coli* (Thomson & Moland, 2000). Infection with ESBL-producing *Klebsiella pneumoniae* is associated with severe outcomes –higher risk of morbidity and mortality– compared to infection with non-ESBL producing *K. pneumoniae*, therefore, epidemiologic studies exploring the occurrence of infections with ESBL-producing bacteria have focused on this organism (Paterson & Bonomo, 2005).Furthermore, clinical microbiology laboratories play a significant role in patient management to curb the spread of this organism in clinical settings through early detection using standard guidelines published by the Clinical and Laboratory Standard Institute (CLSI, 2014).

ESBL-producing bacteria are known to exist in environments such as soil and water where human exposure may occur (D'Andrea, Arena, Pallecchi, & Rossolini, 2013). However, the contribution of these environments to the burden of infections with ESBL producing bacteria is unknown because no standard method exists to monitor and quantify these bacteria in areas outside of clinical settings.

2.2 Extended-spectrum β-lactamases (ESBLs)

Resistant bacteria synthesize β-lactamases enzymes which inactivate the effects of β-lactam antibiotics. β-lactam antibiotics are a class of antibiotics that have a β-lactam ring in their chemical structure; these include penicillin, cephalosporins and related compounds (Holten & Onusko, 2000). New antibiotics, termed 'oxyimino-cephalosporins' were developed, to address bacterial resistance to β-lactam antibiotics (Leggiadro, 1997). These new antibiotics were specially designed with a wider spectrum of activity to resist the effect of β-lactamases. However, the introduction of the new antibiotics in clinical practice saw the rapid emergence of strains of *Klebsiella pneumoniae*, *Escherichia coli*, and other gram-negative pathogens expressing new β-lactamases, termed Extended-spectrum β-lactamases (ESBLs), that were able to degrade and confer resistance to these drugs (Bradford, 2001).

"ESBLs are commonly defined as β-lactamases capable of conferring bacterial resistance to penicillins, first-, second-, and third-generation cephalosporins, and aztreonam by hydrolysis of these antibiotics, but are inhibited by β- lactamase inhibitors such as clavulanic acid" (Paterson & Bonomo, 2005). Most ESBLs are classified into three groups, TEM, SHV, and CTX-M, based on the β-lactamase enzyme they mutated from (Bush, Jacoby, & Medeiros, 1995). TEM-derived and SHV-derived ESBLs are found mainly in clinically isolated strains of *Klebsiella pneumoniae* and *E.coli* while the CTX-M-derived ESBLs are not limited to clinical isolates (Pitout, Nordmann,

Laupland, & Poirel, 2005). Epidemiologic surveillance suggests that the burden of community-associated antibiotic-resistant infections caused by ESBL-producing *E.coli* can be attributed to the expression of CTX-M-derived ESBLs. A study by Pitout et al. (2004) that examined patients with community-onset infections caused by ESBL-producing *E.coli* found that 70% of the *E.coli* strains expressed CTX-M-derived ESBLs (Pitout et al., 2004). Another study that examined ESBL-producing *E.coli* isolates from patients with community-acquired urinary tract infections revealed that 62% of the isolates were positive for were CTX-M derived ESBLs (Smet et al., 2010). Furthermore, a prospective observational study of five hospitals in the United States found that among patients with community-associated bacteremia, wound infections, and urinary tract infections caused by ESBL-producing *E.coli*, 91.3% of strains isolated were positive for CTX-M derived ESBLs (Doi et al., 2013).

2.3 ESBL-Producing *Klebsiella pneumoniae*

A review by Thomson & Moland (2000) found that members of the Enterobacteriaceae family most likely to produce ESBLs are *K. pneumoniae*, *E. coli*, *Klebsiella oxytoca*, and to a lesser extent Citrobacter, Enterobacter, Proteus, Salmonella and Serratia. Other non-enteric pathogens of public health importance also found to produce ESBLs are *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (Thomson & Moland, 2000). Worldwide, *K. pneumoniae* and *E.coli* remain the primary ESBL-producing organisms of public health importance (Pitout & Laupland, 2008). A report by the Infectious Disease Society of America revealed that ESBL-producing *Klebsiella sp* and *E.coli* were one of six major antibiotic-resistant pathogens to which new antibiotics are urgently needed (Talbot et al., 2006).

Klebsiella pneumoniae, in particular, has been the focus of epidemiological investigations into the burden of infections caused by ESBL-producing organisms in healthcare settings. ESBL-

producing *K. pneumoniae* is considered a grave threat in hospital settings because infection with this pathogen has been associated with more severe outcomes compared to non- ESBL producing *K. pneumoniae*. A case-control study by Lautenbach et al. (2001) found that median length of hospital stay was 1.76 times greater, and mortality was two times higher for patients infected with ESBL-producing *K. pneumoniae* compared to patients infected with non-ESBL producing *K. pneumoniae* (Lautenbach et al., 2001). A study focused on patients with bloodstream infections found that after 72 hours of antibiotic therapy, patients infected with ESBL-producing *K. pneumoniae* had a treatment failure rate that was two times higher than the failure rate for patients infected with non-ESBL *K. pneumoniae*. The study also reported significantly higher mortality rate among ESBL infected patients (52%) than non-ESBL infected patients (29%), after 21 days of therapy (Tumbarello et al. 2006). A case-control study found that among patients -with urinary tract infections, respiratory tract infections, and bloodstream infections- cases infected with ESBL-producing *K. pneumoniae* had a longer mean hospital stay (29 days) than controls infected with non-ESBL producing *K. pneumoniae* (11days) (Brooklyn Antibiotic Resistance Task Force, 2002). The adverse outcomes of infection with ESBL-producing *K. pneumoniae* establishes this pathogen as a serious public health threat. Therefore, it is important to detect the presence of this organism in clinical settings and areas where human-pathogen interaction may occur.

2.4 Detection and Identification of ESBL-Producing *Klebsiella pneumoniae*

Clinical microbiology laboratories play a significant role in the appropriate management of patients infected with ESBL-Producing *K. pneumoniae* through early detection and identification of this organism. The US Clinical and Laboratory Standards Institute (CLSI) have standard guidelines for detection of ESBL-production in members of the Enterobacteriaceae

family of bacteria specifically *K. pneumoniae*, *K. oxytoca*, *E.coli*, and *Proteus mirabilis* (CLSI, 2014).

The guideline outlines susceptibility testing methods – Broth dilution, Agar dilution and Disk diffusion methods – that assess the ability of these organisms to grow in the presence of different antibiotics. There are also methods outlined specifically for detecting ESBL production in clinical specimens. The standard microbiological media recommended for use on clinical specimens is the cation-adjusted Mueller-Hinton Broth (CAMHB) and the Mueller-Hinton Agar (MHA) (CLSI, 2014; Pitout & Laupland, 2008). The broth and agar dilution methods involve preparing a two-fold dilution of antibiotics in liquid (CAMHB for broth dilution) or solid (MHA for agar dilution) media, a standardized bacterial suspension of $1\text{-}5\times10^5$ colony forming units (CFU)/mL is inoculated into the liquid medium or spotted on the solid medium. Visible growth, evidenced by turbidity on the liquid medium and colony growth on the solid medium, is observed after a defined period (Jorgensen & Ferraro, 2009; Wiegand, Hilpert, & Hancock, 2008; CLSI, 2014). The disk diffusion method involves the application of a paper antibiotic disk on solid media (MHA) inoculated with a standardized bacterial suspension of $1\text{-}2\times10^8$CFU/mL, after which zones of growth inhibition around the antibiotics are measured (Jorgensen & Ferraro, 2009; CLSI, 2014).

The broth dilution and agar dilution methods provide quantitative results; that is the minimum inhibitory concentration (MIC). MIC is the lowest concentration of an antimicrobial agent that inhibits visible growth of a bacterium. The MIC is interpreted qualitatively using an MIC interpretive criteria/breakpoint that categorizes the infecting organism as susceptible, intermediate, or resistant (Jorgensen & Ferraro, 2009; Wiegand et al., 2008; CLSI, 2014). The disk diffusion method provides qualitative results based on a zone diameter interpretative criteria that categorize the infecting organism as susceptible, intermediate, or resistant (Jorgensen & Ferraro,

2009; CLSI, 2014). Results from these methods interpreted based on the MIC and zone diameter interpretative criteria or breakpoints determine antibiotic resistance. Using the CLSI guideline accurately, these methods have been shown to have a sensitivity and specificity of up to 94% and 98%, respectively, in identifying ESBL-producing *K. pneumoniae* isolates (Wiegand, Geiss, Mack, Stürenburg, & Seifert, 2007).

2.5 ESBL-producing Bacteria in the Environment

Berkner, et al. (2014) stated that "since the beginning of the antibiotic era in the first half of the 20th century, antibiotics and antibiotic resistance genes have been introduced to or have spread to almost every ecosystem on earth." The spread of antibiotic-resistant bacteria is evidenced by the detection and isolation of resistant bacteria from natural environments such as air, soil and naturally occurring water bodies and other nutrient-enriched environments such as wastewater, agricultural farms as well as agricultural products (Wellington et al., 2013).

Environmental studies, exploring the occurrence of antibiotic resistant bacteria in various environments have confirmed the presence of ESBL-producing bacteria in these environments (D'Andrea et al., 2013; Zurfluh et al., 2013). A study looking at 58 naturally occurring rivers and lakes found that 36% of these waters contained ESBL-producing *K. pneumoniae*, *E.coli* and Enterobacter (Zurfluh et al. 2013). Another study found that 26% of food samples originating from farm animals contained ESBL-producing *E.coli* (Jouini et al. 2007). Another study looking at vegetables, soil and irrigation water samples collected from 18 different farm environments found that up to 30% of all samples contained ESBL-producing *K. pneumoniae*, *E.coli*, Enterobacter and Citrobacter (Ben Said et al. 2015). These studies confirm the colonization of nutrient-rich environments by ESBL-producing bacteria. These studies have utilized various susceptibility testing methods such as the CLSI broth dilution, agar dilution and disk diffusion tests. Other

susceptibility methods utilized include antimicrobial gradient methods such as the Etest and also automated instrument systems such as the Vitek 2 system.

Wastewater, particularly hospital effluent, are described as "hotspots" for ESBL-producing bacteria (Hocquet, Muller, & Bertrand, 2016). Results from studies looking at levels of ESBL-producing bacteria in wastewater from hospitals and communities show that concentrations of ESBL-producing *E.coli* can be as high as 6×10^{11}cfu/mL (Bréchet et al., 2014; Kwak et al., 2015).

Despite the knowledge of the severe outcomes of infection with ESBL-producing *K. pneumoniae* and evidence of the dissemination of this organism and other ESBL-producing bacteria in environments where human exposure may occur, no standard methods exist for monitoring these bacteria outside of clinical settings. Studies exploring the presence of ESBL producers in the environment have utilized various methodologies making it difficult to compare outcomes from these studies and to determine the relevance of the presence of these bacteria in these areas to human health. Therefore, it is important to research a reliable susceptibility testing method which may be utilized as a standard for detecting and quantifying ESBL-producing bacteria in the environment.

Chapter III

METHODOLOGY

3.1 Bacterial Isolates

Wild-type (WT) *Klebsiella pneumoniae* were obtained from the clinical laboratory of the University of North Carolina Medical Center; these were isolated from three patients presenting at the medical center and identified as extended-spectrum ß-lactamase (ESBL) producing strains of *K. pneumoniae* that were resistant to the antibiotic ceftriaxone. These strains, labeled *K. pneumoniae* F9093593, *K. pneumoniae* G0165470 and *K. pneumoniae* G2082851, were streaked onto sheep's blood agar plates and transported to the School of Public Health Laboratory, Georgia State University.

Commercially available reference strains of bacteria, from the American Type Culture Collection (ATCC), were also obtained for use. The organisms used in this research were *Escherichia coli* ATCC 25922 (susceptible to ceftriaxone) and *K. pneumoniae* ATCC 700603 (resistant to ceftriaxone) which are routinely utilized for quality control in antimicrobial susceptibility testing.

3.1.1 Propagation of Bacterial Stocks

Pure cultures of each isolate were prepared in quantity for use for the entire project. Briefly, an isolation streak plate of each original culture on sheep's blood agar was made on Trypticase Soy Agar (TSA). One isolated colony was collected using a sterile wooden stick and inoculated into 100mL of Trypticase Soy Broth (TSB) then incubated with shaking at 37°C for 24 hours. After 24 hours, 20mL glycerol was added to the 100ml of inoculated TSB, then dispensed into 1ml tubes and stored in a refrigerator at -81°C.

12

3.2 Antimicrobial Agent

Ceftriaxone antibiotic is the antimicrobial agent utilized for this research. Ceftriaxone is a third-generation ß-lactam antibiotic, and is one of the antibiotics that ESBL producing bacteria are resistant to. Stock solutions of ceftriaxone antibiotic used were prepared using the Clinical and Laboratory Standards Institute (CLSI) procedure for preparation of dilutions of antimicrobial agents for use in susceptibility tests (CLSI, 2014).

3.3 Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial agent that inhibits visible growth of a bacterium. MICs for ceftriaxone obtained in this research are interpreted according to CLSI breakpoints for Enterobacteriaceae on ceftriaxone ($\leq$1µg/mL=Susceptible; 2µg/mL=Intermediate; $\geq$4µg/mL =Resistant) (CLSI, 2014).

3.3.1 Broth Macrodilution

The CLSI Broth Macrodilution Method was used to determine the MIC of ceftriaxone antibiotic for each bacterial isolate. It uses cation-adjusted Mueller-Hinton Broth (CAMHB), the standard medium for antimicrobial susceptibility testing. Serial two-fold dilutions of ceftriaxone, starting at 0.125µg/mL up to 256µg/ml, were tested. These dilutions are the standard used in clinical laboratories to determine MIC of an antibiotic.

For this procedure, TSA streak plates of bacterial strains were prepared and incubated at 35°C for 24 hours. After incubation, isolated colonies were inoculated into a 10ml tube of 0.9% Sodium Chloride (NaCl) till the turbidity matched a 0.5% McFarland standard, to get a cell density of approximately 1-2$\times10^8$ CFU/mL in the NaCl suspension. 150µL of the bacterial suspension was then diluted in 15ml CAMHB, to get an approximate bacterial concentration of 5$\times10^5$ CFU/mL.

The CAMHB suspension was vortexed to get a homogeneous suspension of cells, and 1ml aliquots were then inoculated into CAMHB tubes with ceftriaxone antibiotic, at each two-fold dilution. These tubes were incubated at 35°C for 20 hours after which they were checked for visible growth. Each tube was marked growth or no growth, and the MIC recorded.

3.3.2 Bio-Rad Agar Dilution

The agar dilution method is an antibiotic susceptibility testing method that involves preparing two-fold dilutions of antibiotics in a growth medium, after which a standardized bacterial suspension is spotted on the medium. MIC of the antibiotic is determined by observation of visible growth, evidenced by colony appearance on the medium, after a defined period. The CLSI Agar Dilution method for MIC determination uses Mueller-Hinton Agar as the growth medium. For this research, Bio-Rad Rapid *E. coli* 2 agar was substituted for Mueller-Hinton Agar. The Bio-Rad Rapid *E. coli* 2 agar is a differential agar medium used for the direct identification and enumeration of *E.coli* and coliform bacteria, such as *K. pneumoniae,* in environmental water quality testing. The dilutions of ceftriaxone tested were two-fold serial dilutions starting at 0.125μg/ml up to 512μg/ml.

Briefly, TSA streak plates of bacterial strains were prepared and incubated at 35°C for 24 hours. After incubation, isolated colonies were inoculated into a 10mL tube of 0.9% Sodium Chloride (NaCl) till the turbidity matched a 0.5% McFarland standard. Five spots of 1μL of the bacterial suspension were pipetted onto Bio-Rad Rapid *E. coli* 2 agar plates prepared with ceftriaxone antibiotic at each dilution. Plates were incubated at 35°C for 20 hours after which they were checked for visible growth. Each plate was marked growth or no growth, and the MIC recorded.

3.4 Spread Plate for Cell Count

The spread plate procedure was used to determine further that the correct approximate bacterial cell density per inoculum had been utilized for each experiment. Five dilutions of 100μL of the NaCl suspension was prepared in tubes containing 900μL of TSB and spread plated on TSA. TSA plates were incubated at 35°C and colonies formed on plates were counted after 24hours.

Chapter IV

RESULTS

Duplicate susceptibility tests -using the CLSI cation-adjusted Muller-Hinton Broth

Macrodilution method- for ceftriaxone antibiotic was determined for the quality control strains

utilized in this experiment. The test results are interpreted according to the CLSI breakpoints for

Enterobacteriaceae ($\leq$1µg/mL=Susceptible; 2µg/mL=Intermediate; $\geq$4µg/mL =Resistant). If the

broth macrodilution test is working properly *E. coli* ATCC 25922 should be susceptible to

ceftriaxone and *K. pneumoniae* ATCC 700603 should be resistant.

Table 4.1: Ceftriaxone MIC for Negative control (*E. coli* ATCC 25922) and Positive control (*K. pneumoniae* ATCC 700603) bacterial isolates using CLSI CAMHB macrodilution.

Bacterial Isolate	MIC (µg/mL)	
	Test 1	Test 2
E.coli ATCC 25922	0.125 (S)	0.125 (S)
K. pneumoniae ATCC 700603	8 (R)	4 (R)

*S=Susceptible; R=Resistant

Table 4.1 shows the MIC determined for these two isolates *E. coli* ATCC 25922 and *K.*

pneumoniae ATCC 700603. The MIC of ceftriaxone for *E. coli* ATCC 25922 was at the lowest

concentration tested (0.125µg/mL), showing susceptibility to ceftriaxone and for *K. pneumoniae*

ATCC 700603 was at approximately 8µg/mL, showing resistance to ceftriaxone.

Table 4.2: Ceftriaxone MIC for Wild-Type and Control isolates

TEST 1		
Bacterial Isolate	**MIC (µg/mL)**	
	CLSI Broth Macrodilution	**Bio-Rad Agar Dilution**
E.coli ATCC 25922	0.125 (S)	0.125 (S)
K. pneumoniae ATCC 700603	ND	8 (R)
K. pneumoniae F9092593	64 (R)	32(R)
K. pneumoniae G0165470	>256 (R)	>512 (R)
K. pneumoniae G2082851	256 (R)	16 (R)

TEST 2		
Bacterial Isolate	**MIC (µg/mL)**	
	CLSI Broth Macrodilution	**Bio-Rad Agar Dilution**
E.coli ATCC 25922	0.125 (S)	0.125 (S)
K. pneumoniae ATCC 700603	8 (R)	4 (R)
K. pneumoniae F9092593	16 (R)	16 (R)
K. pneumoniae G0165470	>256 (R)	>512 (R)
K. pneumoniae G2082851	256 (R)	32 (R)

TEST 3		
Bacterial Isolate	**MIC (µg/mL)**	
	CLSI Broth Macrodilution	**Bio-Rad Agar Dilution**
E.coli ATCC 25922	0.125 (S)	0.125 (S)
K. pneumoniae ATCC 700603	4 (R)	4 (R)
K. pneumoniae F9092593	256 (R)	64 (R)
K. pneumoniae G0165470	>256 (R)	>512 (R)
K. pneumoniae G2082851	256 (R)	64 (R)

*ND=Not determined

Table 4.2 presents results from triplicate tests performed to determine MIC of ceftriaxone for all bacterial isolates using the CAMHB macrodilution and Bio-Rad Agar dilution methods. For both methods, on all three tests, the MIC of ceftriaxone for the non-ESBL producing control organism (*E. coli* ATCC 25922) was below 1µg/mL. Based on the CLSI breakpoint for Enterobacteriaceae, the Bio-Rad Agar with ceftriaxone added detected the susceptibility of *E. coli*

ATCC 25922. Almost identical results are observed for the ESBL-producing control organism (*K. pneumoniae* ATCC 700603), for both methods. MIC for ceftriaxone for this organism was ≥4μg/mL on the Bio-Rad Agar with ceftriaxone added. Based on the CLSI breakpoint for Enterobacteriaceae, the Bio-Rad Agar with ceftriaxone added detected the resistance of *K. pneumoniae* ATCC 700603. The MIC results observed for the three Wild-Type Strains -*K. pneumoniae* F9092593, *K. pneumoniae* G0165470, and *K. pneumoniae* G2082851- confirmed their resistance to ceftriaxone. The MIC for ceftriaxone was above 4μg/mL for the three Wild-Type Strains on all three susceptibility tests, for both methods. The Bio-Rad Agar with ceftriaxone added performed well in identifying these strains as ESBL producers.

Chapter V

DISCUSSION

5.1 Discussion

Antibiotic resistance is a major public health issue (WHO, 2014). Resistance to antibiotics leads to therapy failure which results in severe outcomes from common treatable infections. Extended-spectrum β-lactamase (ESBL) enzymes, produced majorly by *K. pneumoniae* and *E.coli*, facilitate resistance to ceftriaxone, cefotaxime, and aztreonam considered to be among the most advanced antibiotics (Pitout & Laupland, 2008). Infection with *K. pneumoniae* strains and other bacteria synthesizing ESBLs lead to higher mortality (Lautenbach et al., 2001; Tumbarello et al., 2006). The presence of ESBL producing bacteria in natural environments, such as soil and water, have been confirmed (Ben Said et al., 2015; Jouini et al., 2007; Zurfluh et al., 2013). Currently, no standard methods exist to monitor these bacteria in natural environments. This research was undertaken to evaluate an agar dilution method, using a growth medium –Bio-Rad Rapid *E. coli* 2 agar– that is designed to isolate *K. pneumoniae* and *E. coli* from the environment.

The results of this research revealed that compared to the CLSI broth macrodilution method, the Bio-Rad agar dilution method performed well in identifying the non-ESBL producing strain of *E.coli* (susceptible to ceftriaxone) and the ESBL producing strains of *K. pneumoniae* (resistant to ceftriaxone). Based on the CLSI interpretative criteria, *E.coli* ATCC 25922 is identified on the Bio-Rad agar with ceftriaxone added as susceptible to ceftriaxone because growth inhibition was observed at concentrations below 1μg/mL. Growth inhibition for *K. pneumoniae* ATCC 700603, *K. pneumoniae* F9092593, *K. pneumoniae* G0165470, and *K. pneumoniae* G2022851 were observed at concentrations at or above 4μg/mL identifying these strains as

19

resistant to ceftriaxone. The minimum inhibitory concentration (MIC) of two isolates *K. pneumoniae* F9092593 and *K. pneumoniae* G2022851 did differ between repeat tests, with the MIC of *K. pneumoniae* G2022851 being up to 3 dilutions higher (256 on Broth macrodilution; 16 on Bio-Rad agar dilution) on test one (Table 4.2). However, the variations in MIC is typical when multiple susceptibility tests are performed.

The Bio-Rad Rapid *E. coli* 2 agar is a useful tool in environmental water quality studies for direct enumeration and identification of *E.coli* and coliforms, which includes *K. pneumoniae*, in environmental samples. The substitution of the standard Mueller-Hinton media with Bio-Rad Rapid *E. coli* 2 agar media on the agar dilution susceptibility test demonstrated a high precision level, identifying the ESBL producing and non-ESBL producing bacteria on all susceptibility tests performed. The results of this research also showed that the Bio-Rad agar had a high accuracy in identifying the three ESBL producing *K. pneumoniae* strains when compared to the Broth macrodilution results.

The use of a standard method is critical to monitor and compare outcomes from environmental studies demonstrating the dissemination of ESBL producing bacteria in the environment. Monitoring these bacteria is important to determine the relevance of their presence in natural environments where human exposure may occur. This research demonstrates the accuracy of an agar dilution method using a differential media designed for use on environmental samples. This method, similar to the CLSI standard susceptibility method for clinical samples, may be utilized as a standard for monitoring ESBL producers in the environment.

5.2 Limitations

For this research, Bio-Rad Rapid *E. coli* 2 agar was substituted for the standard Muller Hinton agar. MHA is recommended as the standard growth medium because it presents little or no interference to the activity of antibiotics being tested. From the results of this research, Bio-Rad Rapid *E. coli* 2 agar exhibited no interference to ceftriaxone antibiotic; this suggests that it may work well with other β-lactam antibiotics. However, further research is necessary to confirm that the Bio-Rad Rapid *E. coli* 2 agar indeed presents no interference to the activity of other antibiotics and is suitable as an antibiotic susceptibility testing growth medium. Additionally, the sample size for this research was restricted to three *K. pneumoniae* strains. Further research using a larger sample size as well as other strains of ESBL producing bacteria such as *E.coli*, Enterobacter, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* is critical to validate the results from this research. Furthermore, due to the small sample size this research was unable to determine the sensitivity and specificity of the Bio-Rad agar dilution method.

5.3 Future Research

The results from this research are promising. However, further evaluation of the Bio-Rad agar dilution method is necessary. Further research using β-lactam antibiotics, other than ceftriaxone, is necessary to confirm that the Bio-Rad Rapid *E. coli* 2 agar presents no interference to the activity of antibiotics and is suitable as an antibiotic susceptibility testing growth medium. Also, a larger sample size using various strains of ESBL producing bacteria such as *E.coli*, Enterobacter, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* is critical to validate the results from this research and to determine the sensitivity and specificity of the method.

REFERENCES

Ben Said, L., Jouini, A., Klibi, N., Dziri, R., Alonso, C., Boudabous, A., … Torres, C. (2015). Detection of extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae in vegetables, soil and water of the farm environment in Tunisia. *International Journal of Food Microbiology*, *203*, 86–92. http://doi.org/10.1016/j.ijfoodmicro.2015.02.023.

Berkner, S., Konradi, S., & Schönfeld, J. (2014). Antibiotic resistance and the environment--there and back again: Science & Society series on Science and Drugs. *EMBO Reports*, *15*(7), 740–744. http://doi.org/10.15252/embr.201438978.

Bradford, P. (2001). Extended-Spectrum β-Lactamases in the 21st Century: Characterization, Epidemiology, and Detection of This Important Resistance Threat. *Clinical Microbiology Reviews*, *14*(4), 933–951. http://doi.org/10.1128/CMR.14.4.933-951.2001.

Brooklyn Antibiotic Resistance Task Force. (2002). The Cost of Antibiotic Resistance: Effect of Resistance among *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* on Length of Hospital Stay. *Infection Control and Hospital Epidemiology*, *23*(2), 106–108.

Bréchet, C., Plantin, J., Sauget, M., Thouverez, M., Talon, D., Cholley, P., … Bertrand, X. (2014). Wastewater Treatment Plants Release Large Amounts of Extended-Spectrum β-Lactamase–Producing Escherichia coli Into the Environment. *Clinical Infectious Diseases*, *58*(12), 1658–1665.

Bush, K., Jacoby, G., & Medeiros, A. (1995). A functional classification scheme for beta-lactamases and its correlation with molecular structure. *Antimicrobial Agents and Chemotherapy*, *39*(6), 1211–1233. http://doi.org/10.1128/AAC.39.6.1211.

Casey, G. (2012). Antibiotics and the Rise of Superbugs. *Kai Tiaki Nursing New Zealand*, *18*(10), 20-24 5p.

Clinical and Laboratory Standards Institute (CLSI). (2014). Performance standards for antimicrobial susceptibility testing; sixteenth informational supplement. CLSI document M100–S24. CLSI, Wayne, Pa.

D'Andrea, M., Arena, F., Pallecchi, L., & Rossolini, G. (2013). CTX-M-type β-lactamases: A successful story of antibiotic resistance. *International Journal of Medical Microbiology*, *303*(6–7), 305–317. http://doi.org/10.1016/j.ijmm.2013.02.008.

Doi, Y., Park, Y., Rivera, J., Adams-Haduch, J., Hingwe, A., Sordillo, E. M., … Paterson, D. (2013). Community-associated extended-spectrum β-lactamase-producing Escherichia coli infection in the United States. *Clinical Infectious Diseases: An Official Publication Of The Infectious Diseases Society Of America*, *56*(5), 641–648. http://doi.org/10.1093/cid/cis942.

George H. Talbot, Bradley, J., John E. Edwards, J., Gilbert, D., Michael Scheld, & Bartlett, J. (2006). Bad Bugs Need Drugs: An Update on the Development Pipeline from the Antimicrobial Availability Task Force of the Infectious Diseases Society of America. *Clinical Infectious Diseases*, (5), 657.

Hocquet, D., Muller, A., & Bertrand, X. (2016). What happens in hospitals does not stay in hospitals: antibiotic-resistant bacteria in hospital wastewater systems. *Journal of Hospital Infection*. http://doi.org/10.1016/j.jhin.2016.01.010.

Holten, K., & Onusko, E. (2000). Appropriate prescribing of oral beta-lactam antibiotics. *American Family Physician*, *62*(3), 611–620.

Jorgensen, J., & Ferraro, M. (2009). Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practices. *Clinical Infectious Diseases*, (11), 1749.

Jouini, A., Vinue, L., Ben Slama, K., Saenz, Y., Klibi, N., Hammami, S., … Torres, C. (2007). Characterization of CTX-M and SHV extended-spectrum beta-lactamases and associated resistance genes in Escherichia coli strains of food samples in Tunisia. *JOURNAL OF ANTIMICROBIAL CHEMOTHERAPY*, *60*(5), 1137–1141.

Kwak, Y, Colque, P., Byfors, S., Giske, C., Möllby, R., & Kühn, I. (2015). Surveillance of antimicrobial resistance among Escherichia coli in wastewater in Stockholm during 1 year: does it reflect the resistance trends in the society? *International Journal of Antimicrobial Agents*, *45*(1), 25–32. http://doi.org/10.1016/j.ijantimicag.2014.09.016.

Lautenbach, E., Patel, J., Bilker, W., Edelstein, P., & Fishman, N. (2001). Extended-spectrum beta-lactamase-producing Escherichia coli and Klebsiella pneumoniae: risk factors for infection and impact of resistance on outcomes. *Clinical Infectious Diseases: An Official Publication Of The Infectious Diseases Society Of America*, *32*(8), 1162–1171.

Leggiadro, R. (1997). Extended-spectrum beta-lactamases and other enzymes providing resistance to oxyimino-beta-lactams. *INFECTIOUS DISEASE CLINICS OF NORTH AMERICA*, *11*(4), 875–&.

Mainous, A., Diaz, V., Matheson, E., Gregorie, S., & Hueston, W. (2011). Trends in Hospitalizations with Antibiotic-Resistant Infections: US, 1997-2006. *PUBLIC HEALTH REPORTS*, *126*(3), 354–360.

Neu, H. C. (1992). The Crisis in Antibiotic Resistance. *Science*, *257*(5073), 1064–1073.

Paterson, D., & Bonomo, R. (2005). Extended-Spectrum β-Lactamases: a Clinical Update. *Clinical Microbiology Reviews*, *18*(4), 657–686. http://doi.org/10.1128/CMR.18.4.657-686.2005.

Pitout, J., Hanson, N., Church, D., & Laupland, K. (2004). Population-Based Laboratory Surveillance for Escherichia coli–Producing Extended-Spectrum β-Lactamases: Importance of Community Isolates with blaCTX-M Genes. *Clinical Infectious Diseases*, *38*(12), 1736–1741. http://doi.org/10.1086/421094.

Pitout, J., & Laupland, K. (2008). Extended-spectrum [beta]-lactamase-producing
      Enterobacteriaceae: an emerging public-health concern. *The Lancet Infectious Diseases*,
      8(3), 159–66.

Pitout, J., Nordmann, P., Laupland, K., & Poirel, L. (2005). Emergence of Enterobacteriaceae
      producing extended-spectrum beta-lactamases (ESBLs) in the community. *JOURNAL OF*
      ANTIMICROBIAL CHEMOTHERAPY, *56*(1), 52–59.

Prado, T., Pereira, W., Silva, D., Seki, L., Carvalho, A., & Asensi, M. (2008). Detection of
      extended-spectrum β-lactamase-producing Klebsiella pneumoniae in effluents and sludge
      of a hospital sewage treatment plant. *Letters in Applied Microbiology*, *46*(1), 136–141.
      http://doi.org/10.1111/j.1472-765X.2007.02275.x.

Smet, A., Martel, A., Persoons, D., Dewulf, J., Heyndrickx, M., Claeys, G., … Butaye, P. (2010).
      Characterization of Extended-Spectrum [beta]-Lactamases Produced by Escherichia coli
      Isolated from Hospitalized and Nonhospitalized Patients: Emergence of CTX-M-15-
      Producing Strains Causing Urinary Tract Infections. *Microbial Drug Resistance*, *16*(2),
      129–34. http://doi.org/http://dx.doi.org.ezproxy.gsu.edu/10.1089/mdr.2009.0132.

Thomson, K., & Moland, E. (2001). Cefepime, Piperacillin-Tazobactam, and the Inoculum
      Effect in Tests with Extended-Spectrum β-Lactamase-Producing Enterobacteriaceae.
      Antimicrobial Agents and Chemotherapy, *45*(12), 3548–3554.
      http://doi.org/10.1128/AAC.45.12.3548-3554.2001.

Thomson, K., & Moland, E. (2000). Version 2000: the new β-lactamases of Gram-negative
      bacteria at the dawn of the new millennium. *Microbes and Infection*, *2*(10), 1225–1235.
      http://doi.org/10.1016/S1286-4579(00)01276-4.

Tumbarello, M., Spanu, T., Sanguinetti, M., Citton, R., Montuori, E., Leone, F., … Cauda, R.
      (2006). Bloodstream Infections Caused by Extended-Spectrum-β-Lactamase-Producing
      Klebsiella pneumoniae: Risk Factors, Molecular Epidemiology, and Clinical Outcome.
      Antimicrobial Agents and Chemotherapy, *50*(2), 498–504.
      http://doi.org/10.1128/AAC.50.2.498-504.2006.

US Centers for Disease Control and Prevention (CDC). (2013). Antibiotic resistance threats in
      the United States. Retrieved from http://www.cdc.gov/drugresistance/threat-report-2013/.

Wellington, E., Boxall, A., Cross, P., Feil, E., Gaze, W., Hawkey, P..… Williams, A. (2013). The
      role of the natural environment in the emergence of antibiotic resistance in Gram-
      negative bacteria. *The Lancet Infectious Diseases*, *13*(2), 155–65.
      http://doi.org/http://dx.doi.org.ezproxy.gsu.edu/10.1016/S1473-3099(12)70317-1.

World Health Organization (WHO). (2014) Antimicrobial Resistant Global Surveillance Report.
      Retrieved from
      http://apps.who.int/iris/bitstream/10665/112642/1/9789241564748_eng.pdf?ua=1.

Wiegand, I., Geiss, H., Mack, D., Stürenburg, E., & Seifert, H. (2007). Detection of Extended-Spectrum Beta-Lactamases among Enterobacteriaceae by Use of Semiautomated Microbiology Systems and Manual Detection Procedures. *Journal of Clinical Microbiology*, *45*(4), 1167–1174. http://doi.org/10.1128/JCM.01988-06.

Wiegand, I., Hilpert, K., & Hancock, R. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols*, *3*(2), 163–75. http://doi.org/http://dx.doi.org.ezproxy.gsu.edu/10.1038/nprot.2007.521.

Zurfluh, K., Hächler, H., Nüesch-Inderbinen, M., & Stephan, R. (2013). Characteristics of Extended-Spectrum β-Lactamase- and Carbapenemase-Producing Enterobacteriaceae Isolates from Rivers and Lakes in Switzerland. *Applied and Environmental Microbiology*, *79*(9), 3021–3026. http://doi.org/10.1128/AEM.00054-13.