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Methods for Detection of and Therapy for Carbapenem-Resistant Enterobacteriaceae

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A thesis submitted to the faculty of
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Master of Science

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ABSTRACT

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As antibiotic resistant bacterial strains are becoming more prevalent in healthcare settings, it is necessary to find alternative methods of detecting and treating these infections. One of the antibiotic resistant strains of interest is the carbapenem-resistant *Enterobacteriaceae* (CRE). CREs have the ability to evade some of the most potent antibiotics currently in use and employ carbapenemases to negate the effect of antibiotics. The three most common carbapenemase genes, found in carbapenem-resistant *Enterobacteriaceae* along with a gene found only in *Escherichia coli* were chosen to create a qPCR assay for rapid detection of resistant infections. The carbapenemase genes are KPC, VIM and NDM and the *E. coli* gene is *uidA*, a β-glucuronidase gene. Consensus sequences were obtained from each of the genes to account for the many variants of each gene. We were able to triplex the assay and test it against a library for twenty isolates varying by which gene they contain. Additional research has been conducted on the library of carbapenem-resistant *Enterobacteriaceae* using bacteriophages or phage. The Phage Hunters class isolated and identified twenty phage that infect *K. pneumoniae*. Out of the twenty phage, seven phage were able to effectively infect carbapenem-resistant *K. pneumoniae.*

Keywords: carbapenem-resistant, carbapenem-resistant *Enterobacteriaceae*, qPCR, multiplex qPCR assay, bacteriophage, phage therapy

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Introduction

Rising occurrence of antibiotic resistance

The World Health Organization (WHO) has recently published a list of the top twelve antibiotic resistant pathogens that pose the greatest threat to human health and kill millions each year. Among these "superbugs" are the carbapenem-resistant *Enterobacteriaceae* (CRE) [1]. The management of bacterial infections can no longer be done through use of safe, cheap and plentiful antibiotics [2]. Figure 1 is a timeline of the antibiotics introduced and how quickly antibiotic resistance has been identified (Figure 1). The need for new treatments for these superbugs has never been higher, and due to the lack of the development of new antibiotics, these antibiotic resistant strains will become increasingly prevalent [2-5]. The rate of antibiotic discovery has declined dramatically since the 1940s-1960s [6]. Indeed, multi-drug resistance is becoming more commonplace amongst bacterial pathogens. This is particularly alarming in the case of Gram-negative pathogens, as fewer treatment options exist for these infections [3]. CREs are of the highest concern because of their resistance to carbapenems, which are last resort antibiotics used to treat multi-drug resistant infections. In these types of infections, there are few, if any, treatment methods.

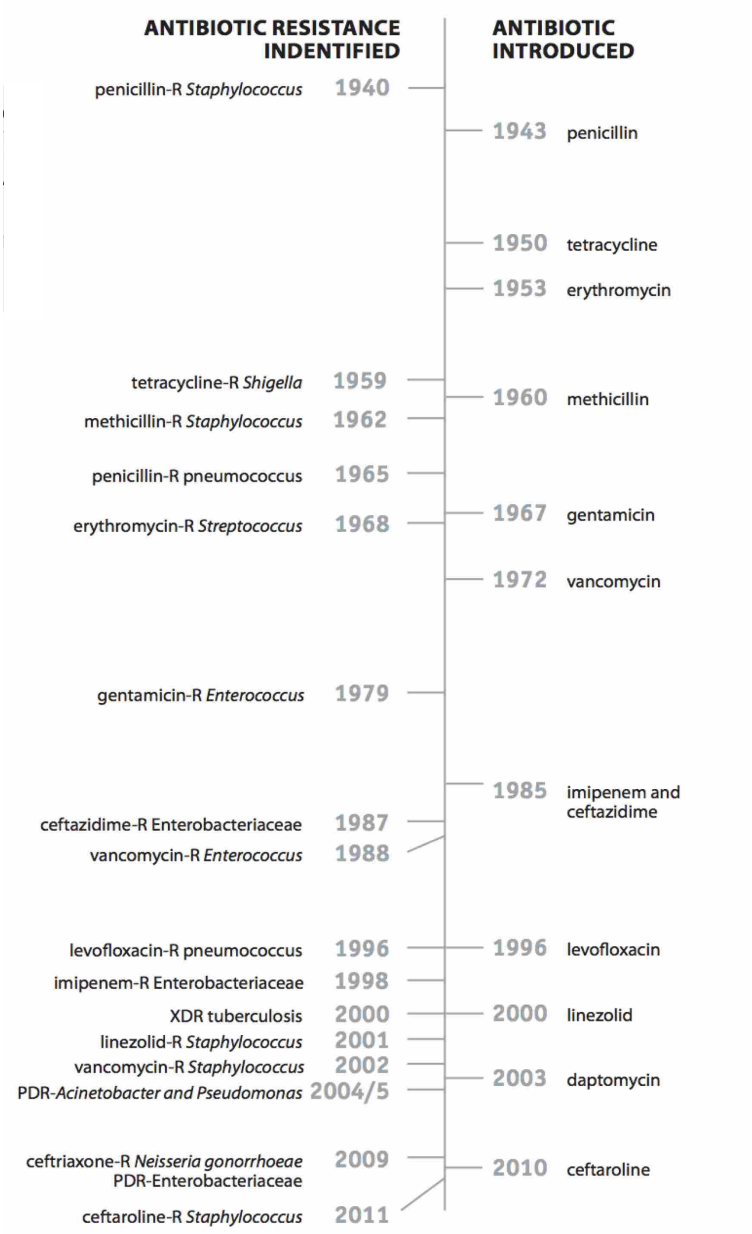

Figure 1: Timeline depicting when antibiotics were introduced and when antibiotic resistance was observed From: *Antibiotic Resistance Threats in the United States, 2013* from the Centers for Disease Control and Prevention

Enterobacteriaceae Family

The *Enterobacteriaceae* family encompasses many bacteria that are commonly isolated from clinical cultures, including *Escherichia coli* and *Klebsiella pneumoniae.* Members of the *Enterobacteriaceae* family are Gram-negative bacilli that the natural

inhabitants of the gastrointestinal tract [7]. *Enterobacteriaceae* are facultative anaerobes and are non-spore forming. This family is extremely relevant because they are a common cause of community-associated and healthcare-associated infections [8]. Currently, infections caused by carbapenemase-producing *Enterobacteriaceae* are generally healthcare-associated, but as these infections are becoming more common, community-associated infections are starting to emerge [8]. This is a substantial threat because carbapenems have traditionally been used in the treatment of infections caused by extended-spectrum β-lactamase producing *Enterobacteriaceae* and are still considered to be a last line of defense against *Enterobacteriaceae* [8].

Carbapenem-Resistant Enterobacteriaceae Infection Rate

Patients with CRE infections face a serious life-threatening disease. In 2013, the Centers for Disease Control and Prevention (CDC) estimated that there were 140,000 healthcare-associated *Enterobacteriaceae* infections that occurred in the US, and approximately 9,300 (~7%) of these infections were caused by CREs [6]. Approximately half of all bloodstream CRE infections result in death [5, 6]. One problem with diagnosing these infections stems from the fact that many members of the *Enterobacteriaceae* family are commonly found in hospitals and initially appear as common nosocomial infections which are treated with broad-spectrum antibiotics. These broad-spectrum antibiotics effectively destroy any commensal microbes that may help prevent further infection, allowing the CREs to quickly proliferate and flourish [6]. Additionally, in the era of worldwide travel, CRE infections are being reported throughout the world (Figure 2) [9].

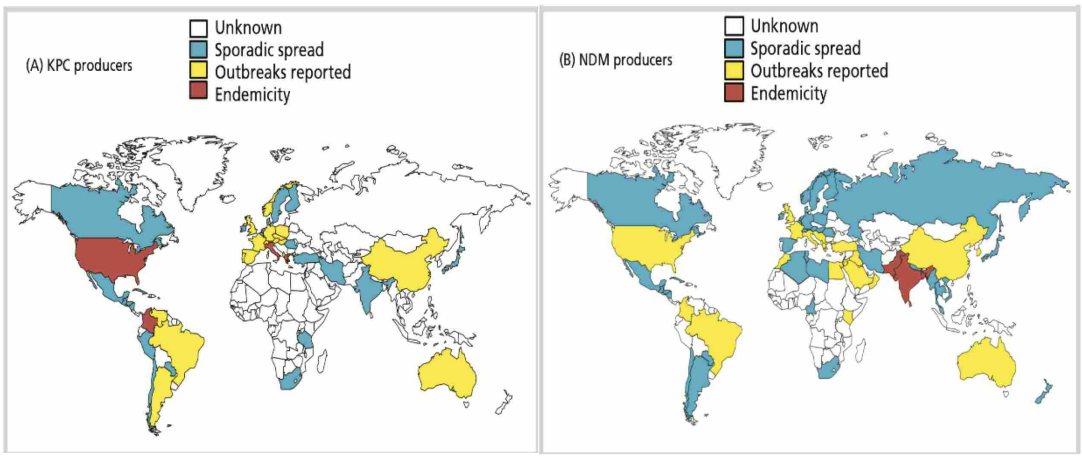

Figure 2: Worldwide distribution of carbapenemases. A) *K. pneumoniae* carbapenemase producers in *Enterobacteriaceae.* B) New Delhi metallo-β-lactamase producers in *Enterobacteriaceae.*
From: Bonomo, R.A., et al., *Carbapenemase-Producing Organisms: A Global Scourge.* Clinical Infectious Diseases, 2018. 66(8): p. 1290-1297.

Carbapenems, Polymyxins, Tigecycline

With the current slow rate of antibiotic discovery, the number of deaths from antibiotic-resistant infections are steadily on the rise [1-4, 10]. Carbapenems are no exception and resistant isolates are becoming increasingly common. Carbapenems belong to the β-lactam family of antibiotics which is the largest and most important class of clinically used antibiotics. β-lactams are effective at blocking the enzymes that crosslink the peptidoglycan of the bacterial cell wall. Carbapenems fall under the β-lactam family due to the similar chemical structure and mechanism of action. Each member of the β-lactam family contains a β-lactam ring characterized by a cyclic amide with a nitrogen atom attached to the β-carbon (Figure 3). Carbapenems act as a mechanism-based inhibitor of the peptidase domain of penicillin binding proteins (PBPs) and as well as other peptidase reactions. The key factor contributing to the efficacy of carbapenems is their ability to bind multiple different PBPs [11]. The ability to bind to many different PBPs allows for the weakening of the peptidoglycan, causing the cell to burst due to osmotic

pressure. Carbapenems are typically used as last resort drugs in treating multi-drug resistant infections [12, 13]. There are few treatment options after carbapenems are discovered to be ineffective, with polymyxins and tigecycline being two of the few options left to patients [14].

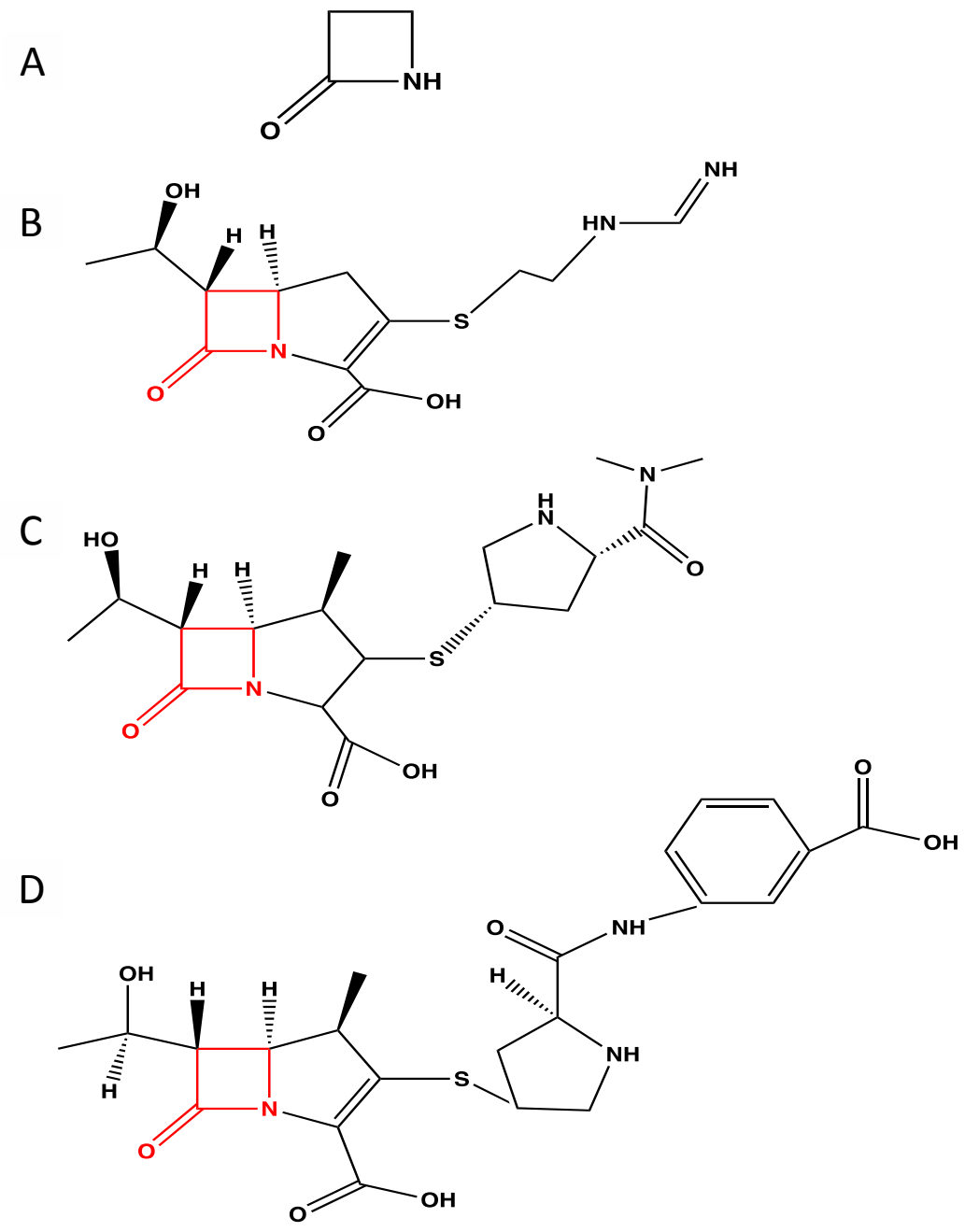

Figure 3: Chemical structures of the carbapenem family. The β-lactam is shown in red. A) β-lactam ring, B) structure of imipenem, C) structure of meropenem, D) structure of ertapenem.

The antibiotic class of polymyxins consists of five chemically different compounds: A, B, C, D, and E (colistin), with polymyxins B and E being used in clinical practice [15]. Colistin is used orally for bowel decontamination and topically as a powder for skin infections and has shown effectiveness against most Gram-negative bacteria [15]. However, colistin has been linked to being the cause of both nephrotoxicity and neurotoxicity while being used as treatment against multidrug resistant bacteria, especially in patients that already present with renal insufficiency [15]. This nephrotoxicity can be reversible after the discontinuation of the drug [16, 17]. The neurotoxicity caused by the colistin is usually reported in 4%-6% of patients and can manifest as a spectrum from numbness in the limbs to loss of control body movements [17]. In *in vitro* studies, it has been suggested that when a CRE is exposed to a polymyxin as a monotherapy, it may lead to emergence of resistance and should be administered in conjunction with other agents [18].

Tigecycline is related to tetracycline and is used as a bacteriostatic drug [17-19]. Tigecycline is unique for its ability to be used as monotherapy for coverage of several drug-resistant pathogens when first-line therapy fails [19]. Other potential advantages of tigecycline include its value as an alternate treatment option in patients who have allergies to penicillin, and no adverse effects on kidneys have been observed [19]. However, in 2010, the FDA issued a warning about tigecycline regarding increased mortality risk based upon a meta-analysis of 13 phase III and IV trials [17, 19]. However, there is increased resistance when tigecycline is used as treatment for CRE infections [17]. Though both of these drugs have been used as a monotherapy, a paper by Tumbarello it al. suggests that a

triple-drug regimen that included tigecycline, a polymyxin and a carbapenem was significantly linked to a reduced risk of death [20].

It would seem counterintuitive to use a carbapenem as a choice of therapy against a CRE, but recent studies suggest that use of a carbapenem is useful, especially when administered as prolonged or continuous infusions or in combination with other agents [18, 20]. Polymyxins have been limited by their toxicity and lack of availability in some parts of the world, but smaller studies show that combined treatment of a polymyxin with β-lactamase inhibitor combinations can be effective [18].

Bacteriophage as a Method of Therapy

With the emergence of profoundly antibiotic resistant pathogens, combined with low drug discovery rates, it is apparent that development of novel treatments is necessary [21]. One of the oldest methods for treating bacterial infections has been bacteriophage or phage [21-23]. In the early 1900s, phage were recognized as a way to treat bacterial infections (commonly termed as phage therapy) by Frederick Twort and Felix d'Herelle [22-24]. D'Herelle specifically used phage suspensions to treat infection such as dysentery, which at the time had no other consistently effective treatment. His success lead to a period of widespread enthusiasm for phage therapy in humans [23, 25]. With the advent of antibiotics in western medicine, the use of phage as the treatment of choice for bacterial infections had diminished until very recently [26, 27].

Phage are bacterial viruses that play a profound role in the evolution of their host [21]. Whole genome sequencing of bacteria has revealed that phage elements contribute significantly to sequence diversity and can potentially influence pathogenicity [21]. Because of the effect that phage have upon their host, the phage has the ability to infect

and, in many cases, kill bacterial cells (Figure 4) [23]. Another benefit of phage therapy is that bacteriophages cannot infect mammalian cells but instead specifically target bacteria. This specificity is highly refined, and each phage will only attack one species or a single strain of bacterium [25]. Because of the specificity of phage, another added benefit of phage is that they are ubiquitous in the environment, and are ten times more numerous than bacteria, making them the most abundant "life" form on earth [21, 23]. This allows for the ease of isolating and specificity of phage.

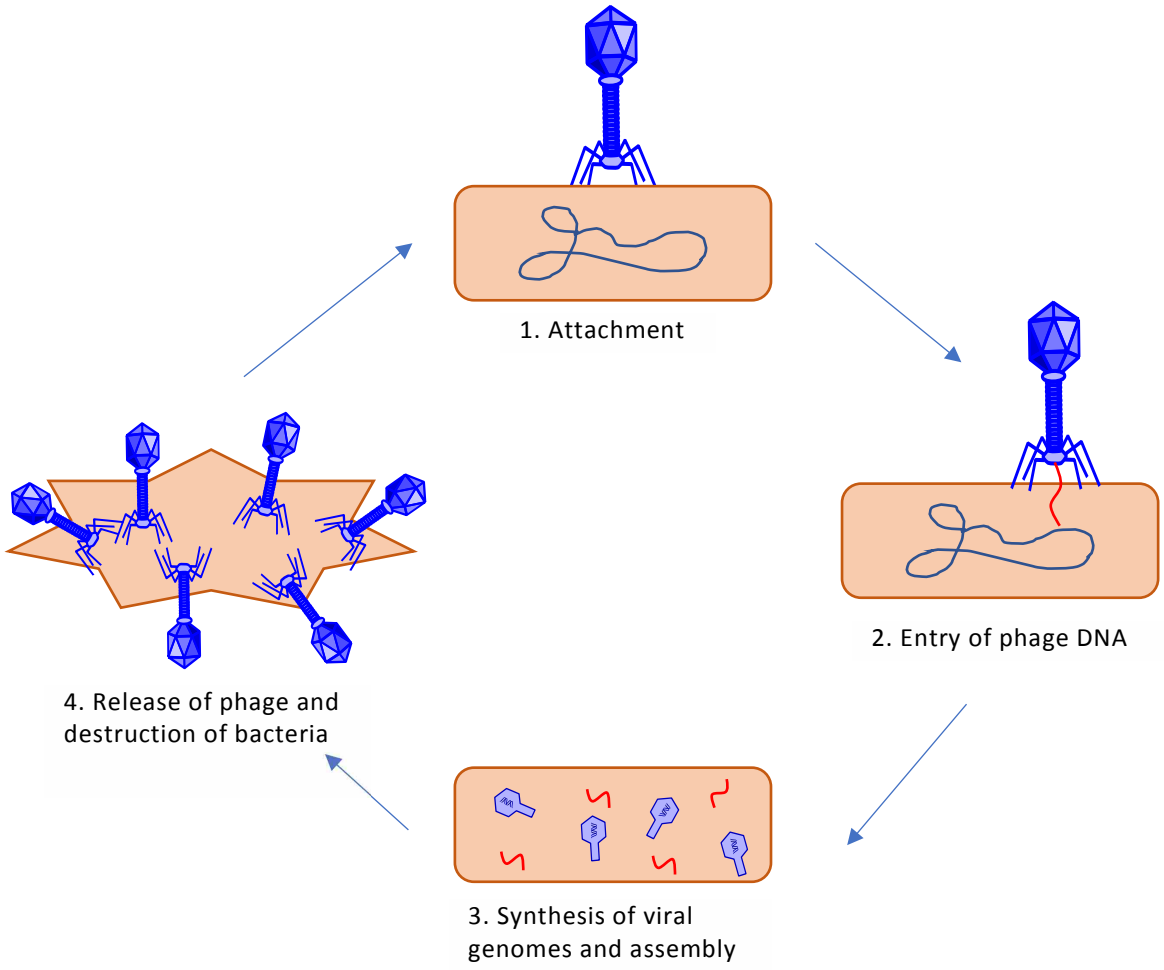

Figure 4: Lifecycle of a lytic bacteriophage. The phage will attach to a bacterium, following the attachment, the phage will insert its DNA into the bacterium's DNA. The bacterium will continually make the phage genome and assemble the phage proteins. After the bacterium has assembled the phage, the phage will lyse the cell.

Non-carbapenemase Mechanisms of Antibiotic Resistance

While carbapenemase-based resistance alone is concerning, it can become more so with the idea that carbapenemase-based resistance may work in conjunction with other mechanisms of antibiotic resistance. Antibiotic resistance can be mediated through several mechanisms with the following three being most common [26]. The first involves mechanisms that minimize the intracellular concentrations of the antibiotic due to poor penetration through the bacterial membrane (altered porins) or active drug efflux pumps. The second group involves mechanisms that modify the antibiotic target, either by genetic mutation or post-translational modification. The third group employs mechanisms that inactivate the antibiotic by hydrolysis or modifications, such as carbapenemases hydrolyzing β-lactam rings [26].

One mechanism alone is enough to cause resistance, but increased resistance could result from combinations of these. For example, if membrane permeability of antibiotic decreased, the bacterium may be resistant. But if there was an enzyme present in the cytoplasm that inactivated what little antibiotic entered the cell, the bacterium would be even more resistant. Current literature suggests that there may be mobile genetic elements that can be acquired by a bacterium that results in altered porins [27, 28]. Another study also suggests that an efflux pump system increases carbapenem resistance in CRE [29]. By identifying carbapenemase genes and any other mechanisms or resistance in CREs, it will be possible to develop more effective treatment options.

Carbapenem Resistance Mediated by Carbapenemases

Due to the speed and convenience of worldwide travel, CREs are easily being spread from country to country, and their resistance genes are increasingly being

transferred to other bacterial species [30]. The main mechanism of resistance in CREs is an enzyme designated as a carbapenemase. Carbapenemases are β-lactamases with versatile hydrolytic capacities [31]. Although known as carbapenemases, many of these enzymes recognize almost all of hydrolysable β-lactams [31]. Simply put, these enzymes recognize the β-lactam ring, cleave it, and render the antibiotic useless.

There are several classes of carbapenemases which are distinguished by the hydrolytic mechanism at the active site [31]. Class A, C and D enzymes have a serine-based hydrolytic mechanism (Figure 5A), while class B enzymes are metallo-β-lactamases (Figure 5B), reliant on a zinc ion in the active site [31, 32]. The most common class A carbapenemase is *Klebsiella pneumoniae* carbapenemase (KPC). These have the ability to hydrolyze a broad variety of β-lactams [31]. Class B, specifically New Delhi metallo-β-lactamase (NDM), is also characterized by the ability to hydrolyze carbapenems and by their resistance to the commercially available β-lactamase inhibitors while maintaining susceptibility to metal ion chelators [31]. Currently, the most common carbapenemase in the United States is KPC [30]. The metallo-β-lactamase NDM is also starting to become more common in the United States [8]. The most common carriers of these carbapenemases are *K. pneumoniae* and *E. coli.* There are other conventional methods of detecting some of the carbapenemases, but the methods vary in the fidelity of these methods. Because of diverse mechanisms and the variability of the other methods, it is obvious that there needs to a diagnostic method to quickly identify these carbapenemases.

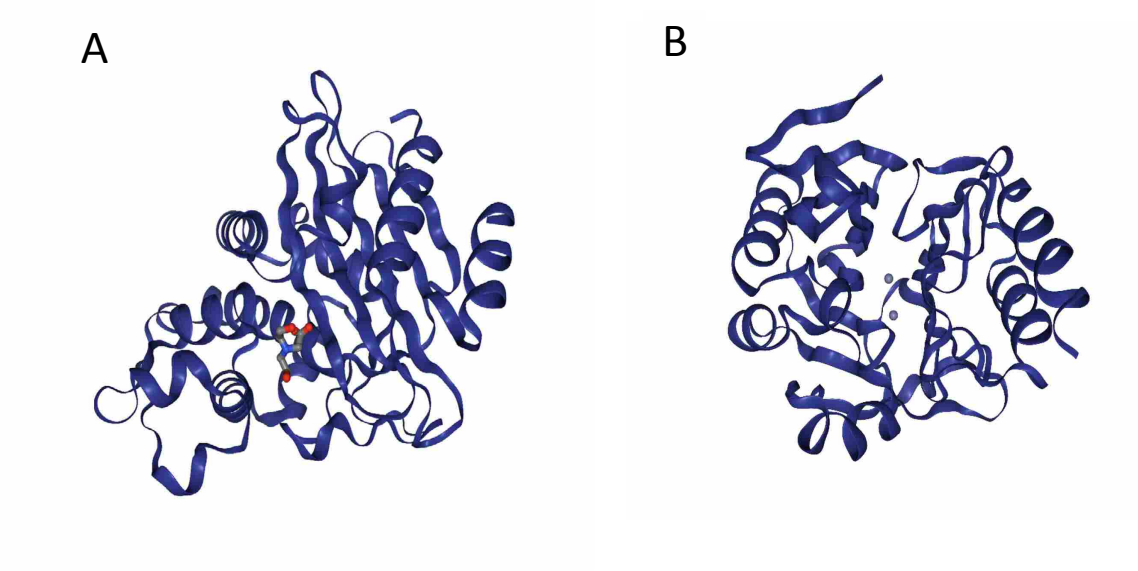

Figure 5: Protein structures of KPC and NDM. A) KPC protein structure, B) NDM protein structure.

Conventional Detection of Carbapenemases

The detection of carbapenemases in a clinical lab setting is challenging. There are several phenotypic detection methods for carbapenemases. The first is the Modified Hodge Test (MHT), which employs an agar plate, lawn-inoculated with a carbapenem-susceptible strain. A carbapenem-containing disk is placed in the middle of the plate and test organisms are streaked in a straight line perpendicular to the edge of the disk. If there is an indentation in the inhibition area where a bacterium is streaked, it indicates that the bacterium is producing a carbapenemase [33, 34]. Another evidence of a carbapenemase-producer is an elevated carbapenem minimum inhibitory concentration (MIC) test. An MIC is performed by growing the organism in different concentrations of the carbapenem and noting the lowest concentration that inhibits growth. Having an elevated carbapenem MIC is indicative of carbapenem-resistance, but full clinical resistance is not always seen [31]. If a MIC test reveals little about the isolate, a disk approximation test may be performed,

where the zone of inhibition around a β-lactam disk is altered by the action of the inhibitor on the metallo-β-lactamase in the test organism [31]. To do either an MHT or MIC test, the bacterium needs to be cultured from the patient, and then cultured again for each of these tests, requiring up to 48 hours total. When a patient is in critical condition, taking days to get a diagnosis increases the risk of death. Thus, rapid identification of these carbapenemases is critical in moving forward.

A relatively rapid technique for identifying the presence of specific genes is qPCR, which requires few bacterial cells and gives a definitive result in mere hours. There have been several assays developed which detect multiple genes in a single tube qPCR format [35, 36]. This is critical, as the turn-around time for the identification of specific carbapenemase genes will be much faster than more conventional tests for carbapenemases. The Satterfield et al. paper describes the development of a quadraplexed assay for detecting different botulinum toxin types in a single tube format using TaqMan probes with different fluorophores [36]. It stands to reason that the method could be adjusted for carbapenemase genes.

Chapter 1

A triplex real-time PCR assay for rapid detection of most common carbapenemase genes, KPC and NDM, and one of the most common carbapenemase carrier species *E. coli*

Olivia B. Tateoka, Daniel B. Nelson, Richard A. Robison

Abstract

Carbapenem-resistant *Enterobacteriaceae* (CREs) are a worldwide health concern and remain difficult to diagnose. Currently, there are several conventional methods to diagnose CRE infections, however, there is not a method for diagnosing carbapenemases and the organisms that carry the carbapenemases. The most common carbapenemase genes are *K. pneumoniae* carbapenemase (KPC) and New Delhi metallo-β-lactamase (NDM). One of the most common carriers of these carbapenemases and also a common cause of nosocomial infections is *E. coli.* Currently, there are few methods of diagnosing a CRE using phenotypic and molecular-based methods. This report describes the development of a single tube qPCR assay that uniquely identifies KPC, NDM and *E. coli.*

Introduction

Within the last decade, there has been a dramatic increase in the number of cases of carbapenem-resistant *Enterobacteriaceae* (CRE) infections. Patients with CRE infections are currently facing a serious life-threatening illness because of the inability to be effectively treated for their infection. In 2013, the Centers for Disease Control and Prevention (CDC) estimated that there were 140,000 healthcare associated *Enterobacteriaceae* infections that occurred in the US, and approximately 9,300 (~7%) of these infections were caused by CREs [6]. Because of the amount of resistance being seen,

the CDC has listed CREs as one of the most prominent groups of drug resistant microbes threatening human health in the United States [6].

The *Enterobacteriaceae* family consists of Gram-negative bacilli and are a part of the natural inhabitants of the gastrointestinal tract [7]. *Enterobacteriaceae,* such as *Escherichia coli,* are frequently the agents of serious nosocomial infections. They account for 21% of all nosocomial infections (e.g., sepsis, ~30%; pneumonia, 15 to 20%; urinary tract infections, ~90%; and intra-abdominal infections, ~90%) [37-40]. *E. coli* is one of the most common carriers of multi-drug resistance with its ability to produce extended spectrum β-lactamase [41]. It is not surprising, then, *E. coli* has the ability to pick up additional antibiotic resistance in the form of a carbapenemase.

β-lactam resistance among the *Enterobacteriaceae* is largely driven by the expression of enzymes that cleave the β-lactam ring. These β-lactamases are divided into four classes (A, B, C and D) and are classified by the Ambler system. Class A includes the active-site serine β-lactamases, class B contains the metallo-β-lactamases, class C contains the AmpC β-lactamases and class D contains the oxacillinases [9, 27, 31, 42, 43]. The genes for carbapenemases belonging to the Ambler class A, B, and C groups of β-lactamases are typically found on acquired plasmids but may also be on other transmissible genetic elements inserted into the chromosome. In the US, the CRE epidemic is driven by the rapid expansion of *Enterobacteriaceae* that express the class A *K. pneumoniae* carbapenemase (KPC) [42, 44, 45]. Another of the carbapenemases that is of clinical relevance is the New Delhi metallo-β-lactamase (NDM) [37].

CREs are hard to diagnose because they initially appear as a common nosocomial infection. Most patients are treated with a broad-spectrum antibiotic which effectively destroys any commensal microbes that may help prevent further infection. CREs become opportunistic pathogens, quickly proliferating and flourishing, causing further infections [6]. Thus, it is essential that there be quick and efficient method of detection of these CREs.

There are several detection methods of CREs that are currently available, and they are differentiated into two different groups: phenotypic and molecular based methods [9, 42]. Several of the molecular methods that have been developed include the FilmArray® Blood Culture Identification Panel (BioFire) which targets only KPC, and the Verigene® Gram-negative blood culture test (Nanosphere) which is a microarray that targets all the carbapenemases but does not accurately predict efficacy of carbapenem therapy [42]. Another method is Unyvero® P55 (Curetis AG) is a multiplex PCR device that detects 20 respiratory pathogens and has 17 drug resistance markers; however, the assay requires more study to assess its performance [42]. Finally, there is GeneXpert Carba-R (Cepheid), an assay to detect carbapenemase genes directly from rectal swabs for the rapid identification of colonized patients. The major limitation identified with this method is low positive predictive values [42].

Rapid phenotypic methods for the detection of CRE are performed on bacteria grown in pure culture. These include Carba NP, BYG Carba, and MADLI-TOF. Carba NP is among the most widely used rapid, phenotypic carbapenemase detection tests performed by clinical and research laboratories, and it is based on pH shift detected by phenol red indicator that occurs concomitant with imipenem hydrolysis [42]. The BYG Carba uses the

same principle as the Carba NP test but uses an electrochemical method to detect imipenem hydrolysis [9, 42]. MALDI-TOF can identify carbapenem degradation products following incubation of a bacterial protein extract with a carbapenem substrate [9].

Due to the variability amongst the different methods of detecting carbapenemases, and the lack of the reliability of many of these methods, it becomes obvious that a new method is required that uniquely identifies the carbapenemases and the carriers of the carbapenemases. The purpose of this study was to develop a real-time PCR assay that could quickly, accurately, and precisely detect the carbapenemases KPC and NDM and one of the top common carriers of carbapenemases, *E. coli*.

Materials and Methods

Bacterial Isolates and Culture Conditions

The bacterial isolates used in this study were acquired from the Centers for Disease Control and Prevention (CDC, Atlanta, GA) and Intermountain Healthcare (IHC, Provo, UT). Isolates were inoculated on Luria-Bertani (LB, Fisher BioReagents) agar containing 16 μg/mL of imipenem and grown at 37°C overnight prior to DNA extraction.

DNA Extraction

Following the overnight culture, total genomic DNA was extracted using the QIAmp DNA Mini kit (Qiagen) and was isolated according to the manufacturer's instructions. The DNA concentrations were measured with TBS-380 Fluorometer (Promega) using the Quant-iT PicoGreen dsDNA assay kit P11496 (Invitrogen).

Primers and TaqMan probe design

DNA sequence unique to *E.* coli was obtained from NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank). Additionally, sequences of the two carbapenemases of interest, KPC and NDM, were also obtained from GenBank. Fifty sequences of KPC and NDM were aligned and a consensus sequence was obtained using Geneious (Biomatters). The consensus sequence of both KPC and NDM were used to design primers and probes. Primers and probes used in this study are listed in Table 1. The primers and probes were designed using the *PrimerQuest* algorithms from Integrated DNA Technologies (IDT) (http://www.idtdna.com/ primerquest/Home/Index). All oligo sequences were selected for proper GC content, optimal annealing temperature, and lack of hairpin structures. A thorough NCBI BLASTn search and analysis of sequence alignments using Geneious were performed to ensure both primer and probe specificity and lack of homology with sequences from other organisms. Probes were fluorescently labelled as follows: KPC with FAM, NDM with Cy5, and *uidA* (*E. coli*) with TexasRed.

Table 1: Primer and probe sequences for KPC, NDM, *uidA*.

Target gene	Oligo	Sequence (5'→3')	Amplicon Size (bp)
K. pneumoniae carbapenemase (KPC)	Forward	CTC GAA CAG GAC TTT GGC GGC TC	173
	Reverse	GCC ACC ATG CCT GGT GTC AG	
	Probe	FAM – CGG CTC AGG CGC AAC TGT AAG TTA - BHQ	
New Delhi metallo-β-lactamase (NDM)	Forward	GGT TTG ATC GTC AGG GAT GGC G	505
	Reverse	GAT GTC GGT GCC GTC GAT CC	
	Probe	Cy5 – TGCTGGTGGTCGATACCGCCTGGAC - IAbRQSp	
E. coli, *uidA*	Forward	GGA CGC GTC GGA TCG AGT TT	123
	Reverse	GCG AAG AGG CAG TCA ACG GG	
	Probe	Cy3-CC AGA CCG ATA ACT TTA TGA CCA AGC G - IAbRQSp	

BHQ, Black Hole Quencher; IAbRQSp, Iowa Black RQ

Optimization of qPCR

Parameter variables such as the number of PCR cycles, cycle temperatures and length of annealing and replicating steps were all optimized. Primers were first evaluated using SYBR Green to optimize cycle temperatures and times. For every reaction, a master mix of 25 µL was prepared using the following: forward and reverse primers at 500 nM, 3 µL of target DNA, 13 µL of SYBR Green Select Master Mix (Thermofisher) and PCR H_2O to 25 µL. The mixtures were loaded into 25 µL Cepheid PCR tubes, and PCR was performed using a SmartCycler II (Cepheid). During the cycling phase, the annealing/extension temperatures were varied from 55°C to 65°C in single degree increments to maximize the reaction. After the optimized procedure was identified, the singleplex assays were set up as follows: 13 µL of TaqMan Multiplex Master Mix

(Thermofisher), 500 nM of each primer, 250 nM of probe with an initial denaturation at 95°C for 120 s followed by 40 cycles of 95°C for 15 s, then 61°C for 30 s. A sample was determined positive if it crossed a fluorescence threshold of 15 before cycle 40. Off-target DNA and no template were used as negative controls.

Multiplexing the three singleplex real-time assays

Once the single reaction conditions were optimized, the three assays were multiplexed (triplexed) into a single tube format. The sample volume was 25 µL as recommended by the manufacturer. 13 µL of TaqMan Multiplex Master Mix, 500 nM of KPC, NDM and *uidA* primers, 250 nM for each probe and 3 µL of target DNA and PCR-grade H_2O were added for a total solution volume of 25 µL. Thermal cycling conditions were the same as the singleplex assays. The optimized real-time protocol was evaluated using isolated DNA from 10 clinical isolates containing the KPC carbapenemase, 4 clinical isolates containing the NDM carbapenemase and 6 *E. coli* isolates.

Results

Specificity testing

The initial specificity of each primer was evaluated in separate qPCR tubes using SYBR Green to detect amplification. Specific primers yielded threshold amplification in the presence of DNA for their respective gene of interest while maintaining a steady non-amplification state when any other DNA was added. Having established that the primers were highly specific to their respective DNA targets, the SYBR Green was replaced with specific dual-labelled hydrolysis probes for KPC, NDM and *uidA*. All isolates were tested a minimum of three times, and signal thresholds were exceeded only when specific primer and probe sets were used on target DNAs containing the corresponding gene, indicating

target specificity. Out of the 20 isolates in this study, all 20 tested positive for sequences corresponding to their respective genes.

Sensitivity testing

For each isolate, tenfold serial dilutions were made of the purified genomic DNAs. For singleplex assays, the threshold sensitivities for each gene were as follows: KPC, 3.44pg; NDM, 5.51pg; *uidA*, 6.34pg (Figure 6). For the triplex assay, the threshold sensitivities for each gene were as follows: KPC, 34.4pg; NDM, 55.1pg; *uidA*, 66.3pg (Figure 7). This corresponds of about 1-2 genome copies for the singleplex assay and 50-100 genome copies for the triplex assay.

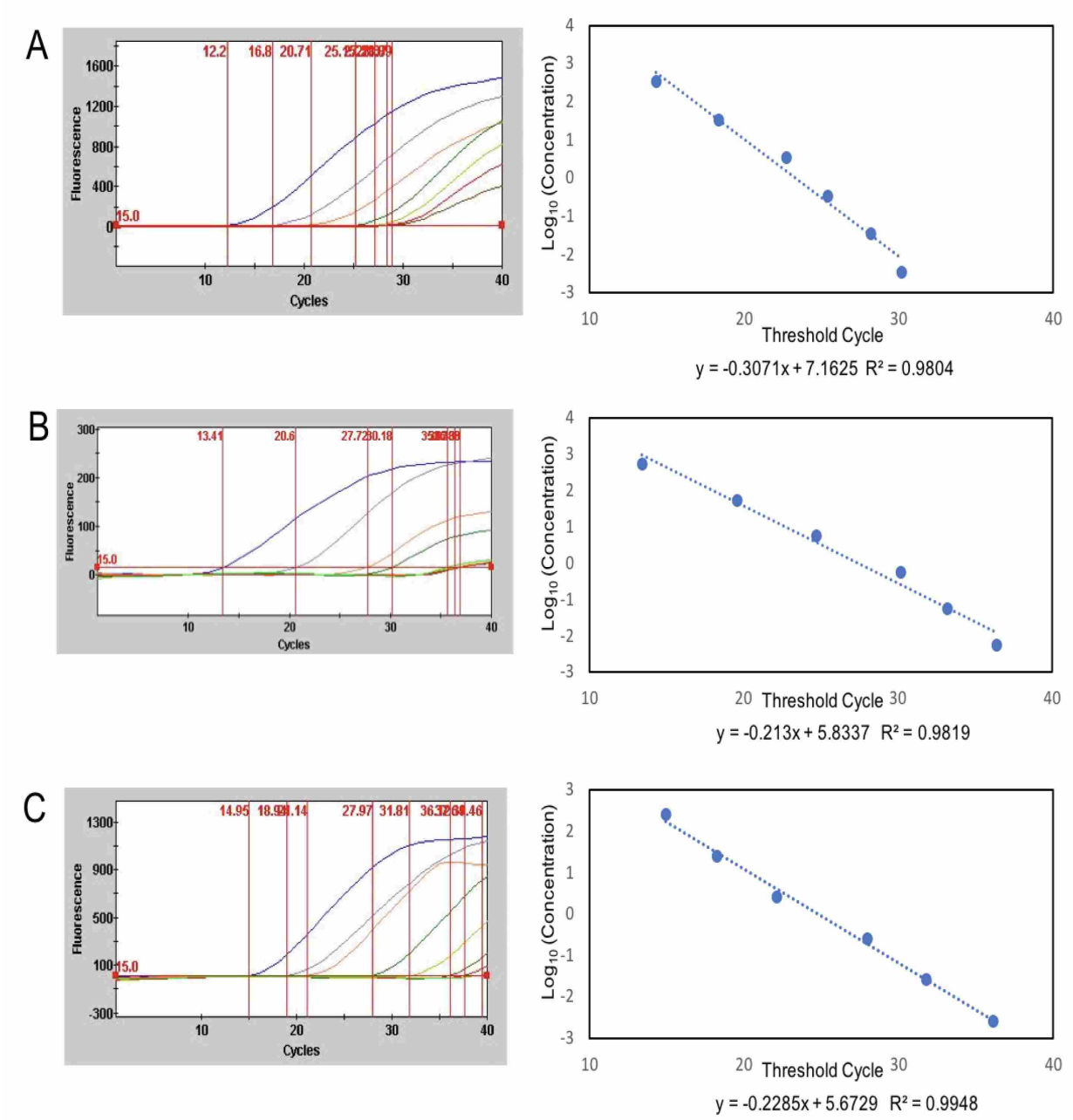

Figure 6: Sensitivity testing of singleplex assays. Detection limits of the singleplex assays and standard curves derived from serial dilutions of purified genomic DNA. A) KPC singleplex assay, B) NDM singleplex assay, C) *uidA* singleplex assay

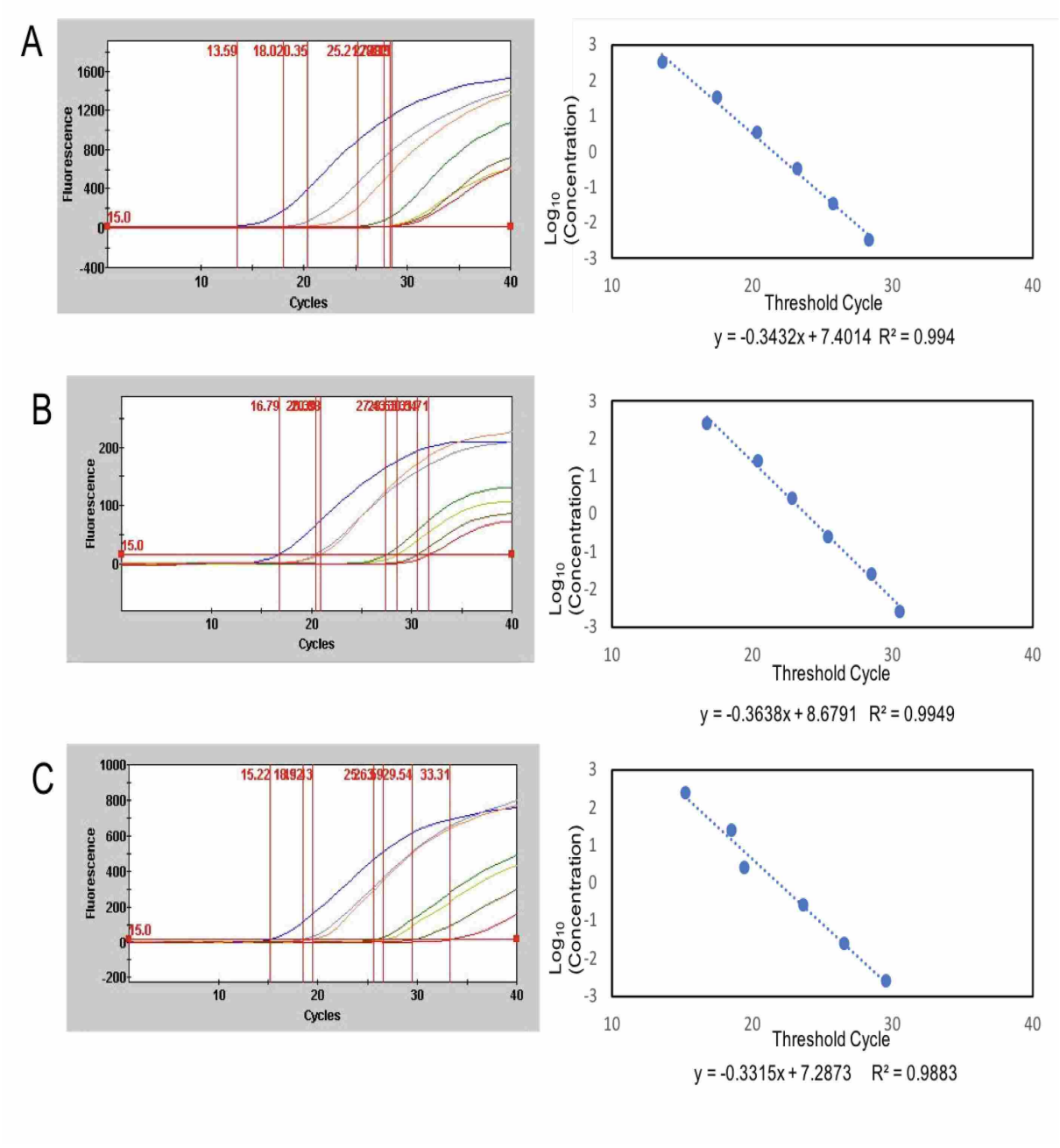

Figure 7: Sensitivity testing of multiplex assays. Detection limits of the multiplex assays and standards curves derived from serial dilution of purified genomic DNA. A) KPC multiplex assay, B) NDM multiplex assay, C) *uidA* multiplex assay.

Discussion

Carbapenem-resistant *Enterobacteriaceae* infections are a serious healthcare concern and the need for better diagnostic tools is paramount. Having an accurate PCR assay with minimal diagnostic times could decrease mortality rates. Although PCR based procedures have revolutionized microbial detection, there are limitations. It is possible to have false positives and false negatives occur [46, 47]. Some of those limitations can be minimized with good technique, and good assay design. It is possible, especially as these CREs are continually evolving, for mutations to occur in the gene of interest, which can compromise the assay.

Some studies showing false positive results have reported sensitivities beyond culture for clinical and environmental samples, that correlate with serological, radiological, and /or additional PCR-bases assays [46]. Although false positive results are considered inaccurate, they may represent the presence of unculturable *Enterobacteriaceae* species in clinical and environmental samples and may be more accurate than culture due to the inability of some bacteria to grow via existing culture methods. Additionally, real-time PCR is able to resolve some of the limitations previously described, because of the versatility and additional specificity of the internal probe. The probe technology allows for simultaneous detection of multiple targets, which can overcome the problem of potential gene mutations at a single locus. Overall, real-time PCR assay are generally considered to have a large dynamic range, low-assay variations, and high reliability [46, 48].

This assay has several advantages. First, it is able to detect the two most common carbapenemases found in the United States. Second, the assay is species-specific for *E. coli,* which can be helpful when screening for CRE infections. The triplex assay is both

sensitive and specific using purified DNA from clinical isolates. This assay could prove to be a rapid, sensitive and economical tool in detection of carbapenemases and *E. coli*. The assay has been able to further provide detection of carbapenemases in a variety of samples and could provide researchers and clinicians with a rapid and reliable means of determining carbapenemases. Further work can be done on this assay by adding in another common carrier of carbapenemases, *K. pneumoniae.* This additional probe will not only make this assay a quadraplex, but also test for the most common carbapenemases and the most common carbapenemase carriers.

Chapter 2

Evaluation of bacteriophage against clinical isolates of carbapenem-resistant *Enterobacteriaceae*

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Abstract

Bacteriophage (phage) therapy for bacterial infections was a treatment that was discovered nearly a century ago but was quickly abandoned with the advent of antibiotics. There has been renewed interest in phage therapy due to increasing occurrence of antibiotic resistance of virulent bacterial pathogens such as carbapenem-resistant *Enterobacteriaceae* (CRE). Currently, fifty percent of patients infected with CREs succumb to the infection. To explore the possibility that phage therapy could be used as treatment for these multi-drug resistant infections, twenty phage were tested against clinical isolates of carbapenem-resistant *K. pneumoniae.* Several of the phage were able to infect these clinical isolates suggesting that phage therapy may be a viable option for treating CREs.

Introduction

Klebsiella pneumoniae, a member of the *Enterobacteriaceae* family, is one of the most common Gram-negative bacteria that is responsible for hospital acquired infections, including pneumonia, bacteremia, and urinary tract infections [49, 50]. As opportunistic pathogens, *K. pneumoniae* primarily attack immunocompromised individuals who are hospitalized and suffer from severe underlying diseases such as diabetes [50]. In the United States, *Klebsiella spp.* accounts for 3-7% of all nosocomial bacterial infections, placing them among the most important infectious pathogens in hospitals [50].

A well-recognized difficulty in treating most *Enterobacteriaceae* infections is resistance to broad-spectrum antimicrobials [43]. Carbapenems have been the essential antimicrobial in treating these types of infections and until recently have been effective in treatment because resistance to carbapenems has been relatively uncommon [43]. The emergence of carbapenemases that have direct carbapenem-hydrolyzing activity has contributed to an increased prevalence of carbapenem-resistant *Enterobacteriaceae* (CRE), a high mortality rate associated with infections caused by CREs, and the potential for widespread transmission of carbapenem-resistance through mobile genetic elements [43, 51-53]. These issues combined with the limited therapeutic options available to treat patients with CRE infections, have led to the necessity of alternative treatments, such as phage therapy.

Bacteriophage (phage) were first used successfully to treat bacterial infections a decade before penicillin was discovered [21]. The ease of production and the broad spectrum of action of antibiotics became more advantageous than phage [22], thus leading to the cessation of therapeutic phage production ceased in most of the Western world [54]. However, phage have continued to be used therapeutically in Eastern Europe and in the former Soviet Union [54].

Phage therapy has been successful because phage are viruses that specifically infect and kill bacterial cells. One of the defining differences that make phage an excellent antimicrobial is their novel mechanism of action which is distinct from antibiotics. Phage have the ability to live in one of two lifecycles, lytic or lysogenic. The majority of phage use the lytic lifestyle, where the virus enters the host cell, taking control of the host in order to create the viral proteins and then lyses the host cell and the progeny is released

[54, 55]. In the lysogenic lifecycle, the phage will insert their genetic content into the host's chromosomes and remain inactive as the phage's genome is replicated alongside the host's chromosomes for an extensive period of time, until the lytic cycle is induced [55, 56]. The phage lifecycles allow for the destruction of the bacteria with very little damage to any of the surrounding bacteria.

An additional benefit of phage therapy includes the relatively small antibacterial range, resulting in phage selecting only the antibiotic resistant strains of bacteria and leaving normal microflora intact [56-58]. Phage therapy is already starting to be used in agriculture and food industries [54]. A number of *in vitro* studies have shown that phage have the potential to lyse targeted bacterial pathogens [58, 59]. In this study we evaluated lytic phage against clinical isolates of carbapenem-resistant *Enterobacteriaceae.*

Materials and Methods

Bacterial strains and culture conditions

K. pneumoniae ATCC 13883 was used as a control organism and was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Clinical isolates of carbapenem-resistant *K. pneumoniae* obtained from the CDC (Centers for Disease Control and Prevention, Atlanta, GA, USA) were designated as *K. pneumoniae* 1002002, *K. pneumoniae* 1300761, *K. pneumoniae* 20080030*, K. pneumoniae* 1002235. Additional carbapenem-resistant clinical isolates were obtained from hospital patients at Intermountain Healthcare in Utah County, UT, USA and were designated as IHC#1 *K. pneumoniae,* IHC#2 *K. pneumoniae,* IHC#3 *K. pneumoniae*. All strains were cultured in Luria-Bertani (LB) broth (Fisher BioReagents, Fair Lawn, NJ, USA) at 37°C and grown overnight.

Following the overnight culturing, strains were aliquoted at 1:10 dilution into LB broth and allowed to recover for 1 hour, ensuring that the bacteria were in exponential phase.

Antibiotic susceptibility testing (AST)

Testing was done using the microdilution method in 96 well plates to find the minimum inhibitory concentration following the Clinical and Laboratory Standards Institute (CLSI) guidelines [60]. CLSI susceptibility breakpoints (M100-S27) were used to determine susceptibility/resistance rates. All of the strains were tested against ampicillin, gentamicin, cefazolin, imipenem, chloramphenicol, and tetracycline. The antibiotics were prepared in two-fold dilutions (e.g. 2,4, 6, 8, and 16 μg/ml). The strains were incubated overnight in cation-adjusted Mueller-Hinton broth (MHB, Sigma-aldrich, St. Louis, MO, USA) in a shaking incubator at 37°C. Following overnight culturing, the strains were subcultured to reach an OD_{600} of 0.01. MHB was mixed with antibiotic and then the subcultured bacteria was added to the well. The plates were incubated for 18 hours at 37°C and the level of turbidity indicated the susceptibility of the strain to the antibiotic.

Bacteriophage propagation and titer assay

All bacteriophage were isolated by the Phage Hunters program at Brigham Young University (BYU, Provo, UT, USA). *K. pneumoniae* 13883 was grown overnight at 37°C in LB in a shaking incubator. 1 mL of overnight culture was added to 10 mL of LB. 100 μL of phage lysate (provided by Phage Hunters) was added to the 1:10 dilution and grown for 24 hours with shaking at 37°C. The bacteriophage and host were centrifuged at 6000 rpm for 20 minutes, and the supernatant was filtered through a 0.2-μm filter (Millipore) to eliminate bacterial lysates. To verify the presence of phage and titer, the supernatant was

serially diluted (1:10) to 10^{-8} dilution, and 50 μL of diluted supernatant was incubated with 400 μL host strain for 30 minutes at room temperature. After incubation, 4.5 mL of 1% molten LB agar was added to the phage and host strain and was overlaid on a LB agar plate. The plates were incubated for 18-24 hours.

Bacterial challenge assay

All the strains were incubated overnight in 10 mL of LB broth at 37°C with shaking. After the overnight incubation, the cultures were diluted 1:10 in LB broth and then allowed to recover for 1 hour, until OD_{600} reached 0.04-0.05. The strains were aliquoted at 400 μL each and 50 μL of phage were added and incubated for 30 minutes at room temperature. After incubation, 4.5 mL of molten LB agar was added to the mix and overlaid on a LB agar plate. The plates were incubated for 18-24 hours at 30°C. The presence of plaques indicated the infectivity of phage. This challenge assay was performed in triplicate.

Results

Antibiotic susceptibility testing

The results of the MIC are found in Table 2. Six of the seven isolates exhibited resistance to imipenem. These isolates also exhibited resistance to many other classes of antibiotics. All of the isolates indicated resistance to ampicillin (AMP) with 128 μg/mL not being enough to inhibit bacterial growth. Similarly, all isolates were resistant to cefazolin (CEF) up to 32 μg/mL. IHC #2 was the only isolate to be susceptible to gentamicin (GEN), where the other isolates were resistant up to 64 μg/mL. Five out of seven isolates were resistant to imipenem (IMI). IHC #2 indicated an intermediate amount of susceptibility to imipenem, where isolate 2008030 showed susceptibility to imipenem. Five of the isolates

were resistant to chloramphenicol (CAM), with varying amounts of resistance to a certain μg/mL. Isolates 1002002 and 1300761 had intermediate susceptibility to chloramphenicol. Six of the seven isolates were resistant to tetracycline (TET), with isolate 1300761 showing susceptibility. The MIC results indicate that the majority of the isolates are not only carbapenem-resistant but have multi-drug resistance as well.

Table 2: Antibiotic susceptibility testing results

	AMP [≥ 32]	CEF [≥8]	GEN [≥16]	IMI [≥4]	CAM [≥32]	TET [≥16]
IHC #1	R (>128)	R (>32)	R (>64)	R (>16)	R	R (>64)
IHC #2	R (>128)	R (>32)	S (2)	I	R (>128)	R
IHC #3	R (>128)	R (>32)	R (>64)	R (>16)	R	R
2008030	R (>128)	R (>32)	R (>64)	S (0.25)	R (>64)	R (>64)
1002002	R (>128)	R (>32)	R (>64)	R (>64)	I	R (>64)
1002235	R (>128)	R (>32)	R (>64)	R (>64)	R	R
1300761	R (>128)	R (>32)	R (>64)	R (>64)	I	S

R, resistant; S, susceptible; I, intermediate; AMP, ampicillin, CEF, cefazolin; GEN, gentamicin; IMI, imipenem; CAM, chloramphenicol; TET, tetracycline

Bacterial challenge assay and phage titer results

The list of bacteriophages tested is found in Table 3. The titer of each phage was established and is listed in Table 4. The ability of bacteriophages to infect against various clinical isolate host strains was evaluated and presented Table 5. All twenty of the phage were tested against each of the clinical isolates and only a unique few were able to infect multiple isolates. Out of the twenty bacteriophage found against *K. pneumoniae,* 2 Small had the highest versatility and was effective against all of the host strains. The next phage that was able to infect the majority of the clinical isolates was Alina. Figure 8 is representative of the plaques formed as well as a representative image of electron

microscopy done on phage. The plaques that were formed, were all lytic phage, and produced clear plaques.

Table 3: List of bacteriophage

2 Small	K. Potts	Factom	IM Groot	Inominatum
Alina	Call	Nihilmali	Big	Keltset
Penguinator	SeyesCirculi	Chronis	EMP27	Silibus
King DDD	K Nelson	Wonnacott	Alpha	Omega

Table 4: Phage titer list

Phage Name	Phage Titer
Penguinator	$5.6x10^6$
Factom	$1.6x10^6$
K. Nelson	$4.6x10^6$
Call	$1.2x10^6$
2 Small	$4.0x10^6$
Alina	$5.2x10^6$
K. Potts	$7.2x10^6$
Silibus	$6.2x10^6$
Keltset	$3.4x10^6$
SeyesCirculi	$1.8x10^6$
Big	$1.7x10^8$
Omega	$2.2x10^6$
Alpha	$1.04x10^7$
Nihilmali	$1.44x10^7$
Wannacott	$1.4x10^6$
Chronis	$7.4x10^6$
IM Groot	$1.8x10^6$
King DDD	$8.6x10^6$
Inominatum	$3.8x10^6$
EMP27	$5.8x10^6$

Table 5: List of clinical isolates and the phage that were able to infect them

2008030	1002002	1300761	1002235	IHC#1	IHC#2	IHC#3
2 Small	2 Small	2 Small	2 Small	2 Small	2 Small	2 Small
Alina	Alina	Call	Alina	Alina	Alina	Alpha
K. Nelson	Call	Factom	Big	Alpha	Alpha	Big
K. Potts	EMP27	IM Groot	Factom	Big	Big	Factom
Nihilmali	Factom	K. Potts	Inominatum	Factom	Factom	K. Nelson
Silibus	Inominatum	Keltset	K. Potts	K. Nelson	K. Nelson	K. Potts
	K. Nelson		Nihilmali	K. Potts	K. Potts	Nihilmali
	Penguinator		SeyesCirculi	Silibus	Nihilmali	Silibus
	Silibus		Silibus		Silibus	

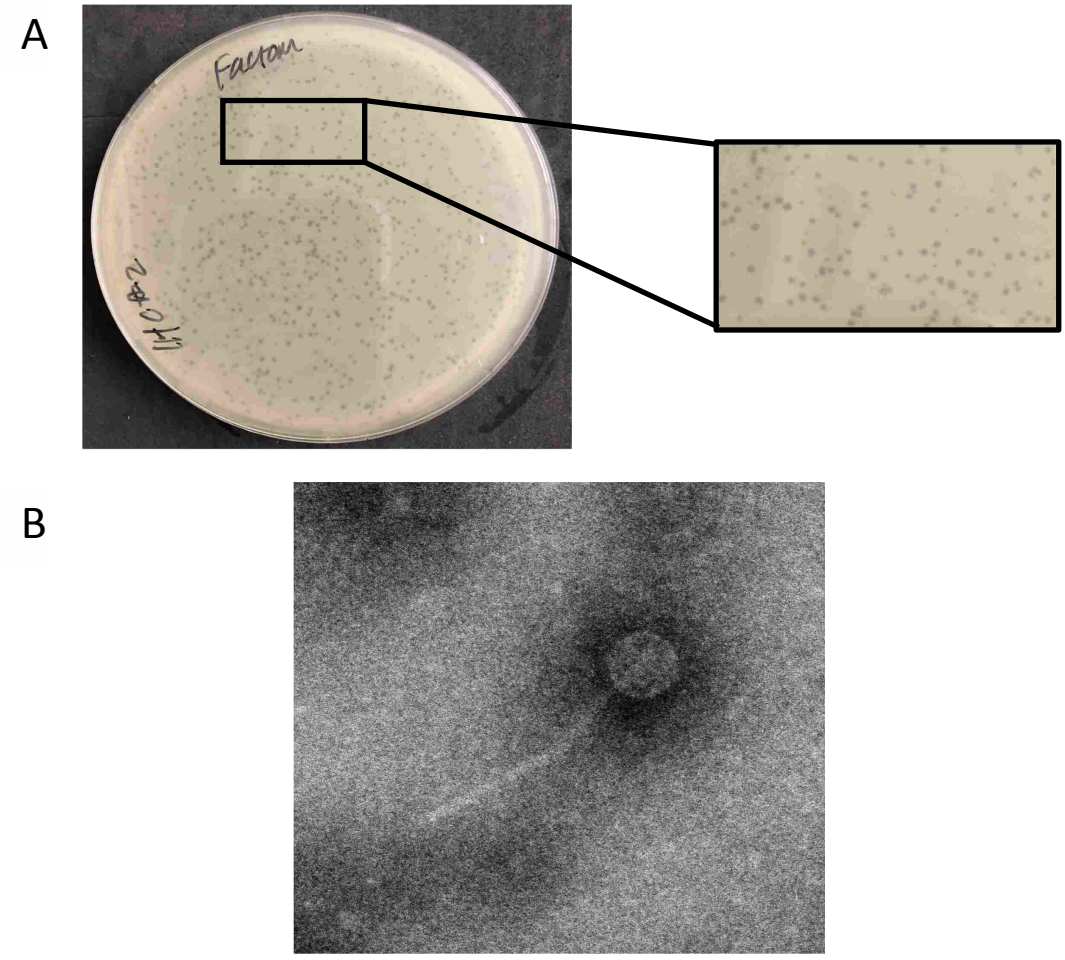

Figure 8: Representative images of phage. A) representative image of plaques formed during the bacterial challenge assay, B) representative of electron microscopy image of phage

Discussion

Hospital-acquired infections that are caused by *K. pneumoniae* are a human health problem that are prevalent worldwide [49, 61]. Since antibiotic treatments have associated restrictions and shortcomings, phage therapy is now being considered as a potential treatment and prevention for bacterial infections [49, 55]. There are several potential beneficial effects of phage therapy, including creating a combination of phage that have activity against different bacterial pathogens, ability to infect multi-drug resistant pathogens, narrow antibacterial spectrum allowing preservation of the existing microbiome, the potential for low level of side effects, and wide distribution upon systemic administration [55]. Another crucial aspect of phage therapy is the ability of the phage to be applied directly to local microflora without causing harm [23].

One of the criticisms that phage therapy faces is the ability to meet the "gold-standard" of efficacy. The lack of efficacy is likely caused by insufficient funds particularly in terms of clinical trials [23]. At present, there are few phage products that are currently in use, both in terms of commercial use (e.g., Pyophage, and Instiphage sold in the former Soviet Union) and in the form of biocontrol (e.g., OmniLytics (UT, USA) and Micreos Food Safety (The Netherlands)).

There are other things to consider as phage therapy is being considered as treatment for bacterial infections. The phage needs to be thoroughly investigated, including observing the method in which the phage are attaching to the bacteria, discerning the phage titer needed to effectively lyse bacteria, or that the phage carries and type of gene that would be beneficial to the bacteria. It may also be advantageous to examine the bacteria for

any difference in a species. For example, *K. pneumoniae* has many different capsules types that may affect the efficacy of phage attaching to the surface of a bacterium.

This study demonstrates that there are several phages that show some efficacy against clinical isolates of carbapenem-resistant *K. pneumoniae*. The phage were able to infect within a minimal amount of time and in some cases infected multiple isolates. The bacteriophage 2 Small showed versatility against several different strains of *K. pneumoniae*. 2 Small, in addition to a few other phage listed in this study, could be used treat *K. pneumoniae* infections, and the phage have the ability to be made into a "cocktail," in which many different phage work together to treat a myriad of infections. This study indicates some of the necessary evidence needed for phage therapy to transition from *in vitro* studies and into clinical studies.

Summary

We were able to assess the possibility of using bacteriophage as a therapeutic against carbapenem-resistant *Enterobacteriaceae* (CREs). Our study has validated that phage have the possibility of being used as sole treatment or in combination with antibiotics as treatment against many multi-drug resistant organisms. We were also able to create an assay that would effectively diagnose carbapenemase genes as well as diagnose if the carbapenemase is being carried on *E. coli.* This assay can effectively be used in clinical settings. The results found in each of these projects has the ability to help diagnose and treat CRE infections and have the possibility of significantly helping patients. One of the greatest hopes for the phage therapy project is that further work will be done with these phage to create a cocktail that could be effective in treating varying bacterial infections and increase better patient outcomes. Future direction with the qPCR assay would be to add another level of detection and make the assay a quadraplex assay, with the most common carbapenemases and the most common carriers of the carbapenemases.

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