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Determining the Presence of Carbapenem Antibiotic Resistance in Clinical Isolates

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DETERMINING THE PRESENCE OF CARBAPENEM ANTIBIOTIC RESISTANCE IN CLINICAL ISOLATES

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ABSTRACT

Carbapenems are β-lactam antibiotics reserved for the treatment of severe microbial infections, especially those targeting the Enterobacteriaceae. Introduced in the 1980s, carbapenems have been used successfully in hospitals, and in the 1990s resistance was discovered. Carbapenem resistance is conferred through the production of carbapenemases. In the U.S., the most common carbapenemase is *Klebsiella pneumoniae* carbapenemase (KPC). In 2012, the National Healthcare Safety Network reported a carbapenem resistance rate of 13.0% among *Klebsiella pneumoniae* infections, and indicated that the mortality rate associated with carbapenem resistant Enterobacteriaceae (CRE) infections ranged from 48.0-71.9%. According to the Food and Drug Administration (FDA), carbapenem resistance is observed when a pure culture has a minimum inhibitory concentration (MIC) ≥ 4 μg/ml as determined through antibiotic susceptibility testing (AST).In 2012, a lower MIC for carbapenem antibiotics was established for KPCs by the Clinical and Laboratory Standards Institute (CLSI) (i.e., MICs 1 - 4 μg/ml are designated resistant), but these criteria have not been endorsed by the FDA. Data are needed to determine the percentage of clinical isolates with carbapenem MIC between 1 and 4 μg/ml that are truly resistant. Determining the presence of the KPC gene is important because the use of carbapenems in patients with MIC between 1- 4 μg/ml may have poor clinical outcomes. Conversely, if lacking the KPC gene, carbapenems may still be indicated. The objectives of this study were to determine the presence of the KPC gene, the carbapenem AST profiles of clinical

isolates, and the resistance rates based on the previous and current CLSI criteria. This study involved 56 suspected CRE clinical isolates from Las Vegas, Nevada, which were analyzed by culture, AST, and polymerase chain reaction to detect the KPC gene. The prevalence of the blaKPC gene in our CRE isolates was 83.3%, and the prevalence was 94.7% among our *Klebsiella pneumoniae* isolates. Our data showed no statistically significant difference between the previous and the current CLSI criteria in defining carbapenem resistance among the Enterobacteriaceae. The results from this study helped determine the prevalence of the KPC gene and antimicrobial susceptibility profiles among CRE isolates in Las Vegas. These may be useful in improving antibiotic stewardship in Nevada.

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INTRODUCTION

The Enterobacteriaceae is a family of bacteria with many different genera and species. Members of this family are gram negative, non-spore forming, and facultative anaerobes, which include many opportunistic and pathogenic species. Most of these organisms are present in the intestinal tracts of humans and animals, while some are freely living in soil, water and sewage. Opportunistic Enterobacteriaceae cause disease when the immunity is low, and are usually in the intestinal and skin flora of humans and animals, but can produce serious infection outside their natural habitat. Clinical isolates of Enterobacteriaceae that are commonly seen in acute and long term care centers are *Escherichia coli, Klebsiella pneumoniae*, and *Proteus mirabilis* (Mahon, Manuselis, & Lehman, 2010). Other common genera include *Shigella* spp., *Salmonella* spp., *Citrobacter* spp., *Yersinia*, *Serratia* spp., etc. (Mahon et al., 2010). These organisms are notorious for causing mild to severe infections, such as cystitis, pneumonia, meningitis, bacteremia, septicemia, and wound infections, when immunity is compromised (Schwaber & Carmeli, 2008).

Klebsiella pneumoniae is a common opportunistic and nosocomial organism. It is an encapsulated non-motile gram negative bacterium found as normal flora of the human skin, mouth and the intestines. However, it is capable of causing serious infection. The distinct polysaccharide capsule of *Klebsiella pneumoniae* offers protection against phagocytosis and antimicrobial absorption, and contributes to its virulence (Mahon, Manuselis, & Lehman, 2000). The frequent colonization by *Klebsiella pneumoniae* of the respiratory tracts of hospitalized patients makes it a common cause

of lower respiratory infection, especially among immunocompromised patients, newborns and patients on respirators. *K. pneumoniae* remains the fourth and fifth most common cause of acute pneumonia and bacteremia in hospital intensive care units, respectively (Centers for Disease Control and Prevention, 2003). It can also cause serious infections outside the hospital, and has been estimated to be responsible for 6% - 8% of community acquired pneumonia (Jong, Hsiue, Chen, Chang, & Chen, 1995). Other community acquired infections caused by *K. pneumoniae* include wound infections, abscesses, and urinary tract infections.

Cephalosporins and the β-lactams are common antimicrobial agents used successfully in the treatment of infections caused by *Klebsiella pneumoniae* and other Enterobacteriaceae. In the past, 3rd and 4th generation cephalosporins were first choice in the treatment of Enterobacteriaceae infections. However, resistance of Enterobacteriaceae to these antibiotics has been well documented in recent times (Paterson et al., 2003; Saurina, Quale, Manikal, Oydna, & Landman, 2000). Carbapenems are β-lactamase inhibitor antibiotics. The parent or model compound of carbapenems is thienamycin, a compound from *Streptomyces cattleya* which was first reported in 1976 (Ratcliffe & Albers-Schonberg, 1982). Thienamycin has a hydroxyethyl side chain, a departure from the conventional structures of penicillin and cephalosporins (Papp-Wallace, Endimiani, Taracila, & Bonomo, 2011). This stereochemistry and structure are key to their extensive activity and potency. Unfortunately, thienamycin is unstable in aqueous solution, highly reactive and very

sensitive to mild base hydrolysis. This instability stimulated the search for more stable thienamycin analogs.

Carbapenems, such as Ertapenem, Imipenem, Meropenem, and Doripenem are analogs of thienamycin, and unlike thienamycin, they are more stable and less sensitive to base hydrolysis (Branch et al., 1998). They possess the widest spectrum of antibacterial activities and potency of all the β-lactam antibiotics and are effective against gram negative and gram positive organisms (Bassetti, Nicolini, Esposito, Righi, & Viscoli, 2009; Papp-Wallace et al., 2011). Carbapenems are active against the chromosomal cephalosporinases and extended-spectrum β-lactamases, both of which are found in resistant gram-negative organisms (G. A. Jacoby & Munoz-Price, 2005; Queenan & Bush, 2007). Another mechanism of action of carbapenems involves the destruction of bacterial membranes through porin proteins leading to permeability. Carbapenems form part of a new generation of antibiotics that are reserved for the treatment of severe and resistant microbial infections, especially those caused by Enterobacteriaceae. Since their discovery in 1985, carbapenems have been remarkably effective in the treatment of severe infections, and in some situations, they are regarded as the last resort for treatment of infections caused by extended spectrum β-lactamase (ESBL) organisms (Remington, 1985).

Carbapenem resistance first appeared sporadically in the mid-1990s, in places such as Spain (Corbella et al., 2000). Though the resistance was uncommon, in recent years, carbapenem resistant Enterobacteriaceae (CRE) outbreaks have been progressively increasing (Queenan & Bush, 2007; Schwaber et al., 2008). Beyond

Enterobacteriaceae, many non-fermenting gram negatives (e.g., *Pseudomonas* and *Acinetobacter* species) and some gram positives (e.g., *Staphylococcus*, *Streptococcus*, *Enterococcus,* and *Nocardia* species) are also becoming resistant to carbapenems. This pattern represents a major public health threat in our society. In the U.S., CREs were first discovered in North Carolina in 2001 (Yigit et al., 2001). Since then, outbreaks have been reported throughout the country, especially in the northeast region (Landman et al., 2007; Queenan & Bush, 2007).

Unlike Methicillin Resistant *Staphylococcus aureus* (MRSA) resistance, which is mediated by a single mechanism in a single bacterial species, the mechanisms of carbapenem resistance are complex because they involve a broad range of organisms and are mediated by different mechanisms, such as the production of β-lactamases, efflux pump and porin mutations. Carbapenemases are β-lactamases with versatile hydrolytic capacities and are capable of hydrolyzing the beta-lactam ring of carbapenems. They are also capable of hydrolyzing penicillins, monobactams, and cephalosporins. Carbapenemases are the most prominent β-lactamases to neutralize carbapenems (Nordmann & Poirel, 2002; Queenan & Bush, 2007).

Carbapenemases are enzymes that catalyze the breakdown of carbapenems. They confer resistance to carbapenems and belong to the molecular classes A, B, and D of the β-lactamases. Class A and D enzymes have a serine-based hydrolytic mechanism, while class B uses zinc in their active site. *Klebsiella pneumoniae* carbapenemase (KPC) is a class B β-lactamase and is the most common form of carbapenemase implicated in CRE outbreaks in the U.S. Sometimes sporadic outbreaks involve the New Delhi

Metallo-β-lactamase (NDM-1), a variant of the Metallo- β -lactamases (MBL). Other forms of carbapenem resistance, such as the production of AmpC-type enzyme with a combination of cephalosporinase and porin loss are increasingly becoming common (Bradford et al., 2004; Crowley, Benedi, & Domenech-Sanchez, 2002; Queenan & Bush, 2007). Other countries with KPC outbreaks include Greece, Israel, Colombia, and Puerto Rico (Leavitt, Navon-Venezia, Chmelnitsky, Schwaber, & Carmeli, 2007).

The genes coding for the KPC are located on plasmids or the mobile units of *Klebsiella* DNA. This property makes the KPC gene easily transmissible. Not surprisingly, KPC genes have been successfully isolated from other Enterobacteriaceae, such as *Escherichia coli, Serratia marcescens*, *Pseudomonas,* and *Acinetobacter baumannii* (Deshpande, Jones, Fritsche, & Sader, 2006; Patel & Bonomo, 2011; Villegas et al., 2007).

Klebsiella pneumoniae Sequence Type 258 (*ST258*), which is a single locus variant of Sequence Type 11 (*ST11*), is the most common clone or strain type of *Klebsiella* implicated in the KPC-producing *K. pneumoniae. This strain* accounts for over 70% of the CDC's *Klebsiella pneumoniae* Pulse Field Gel Electrophoresis (PFGE) database (Kitchel et al., 2009). *ST258* isolates demonstrate great diversity in molecular characteristics and epidemiology, and have been identified in KPC-producing isolates from Israel, Finland, Poland, Italy, Germany, Greece, Norway and Sweden, supporting possible international dissemination (Cuzon, Naas, Demachy, & Nordmann, 2008; Qi et al., 2011; Samuelsen et al., 2009). *K. pneumoniae* Sequence Type 14 (*ST14*) has also been identified, but it is associated with KPC-producing isolates from the Midwest and Western U.S., (Kitchel et

al., 2009). *ST11,* which is closely related to S*T258,* is the dominant KPC-producing *K. pneumoniae* in China, and it accounts for greater than 80% of China's KPC-producing *K. pneumoniae* isolates (Qi et al., 2011)

Epidemiology of CRE

CREs appeared to be uncommon before 1992; however, over the last decades CRE infections have become commonly reported (Braykov, Eber, Klein, Morgan, & Laxminarayan, 2013). CRE outbreaks have been reported in at least 43 states in the U.S. and many other countries such as Brazil, Israel, Greece, and India (Andrade et al., 2011; Centers for Disease Control and Prevention, 2012a; Gupta, Limbago, Patel, & Kallen, 2011; Pournaras et al., 2009). The Centers for Disease Control and Prevention (CDC) monitor the true incidence of infections caused by CREs; unfortunately, CRE infections are not notifiable in every state. The CDC uses information from two surveillance systems; namely, the Emerging Infection Program (EIP) and the National Healthcare Safety Network (NHSN), to monitor CRE infection incidences. The NHSN reviews data from all facilities performing surveillance for Central-Line-Associated Bloodstream Infections (CLABSI) together with data from Catheter Associated Urinary Tract Infections (CAUTIs). These infections, mainly caused by Enterobacteriaceae, such as *E. coli, K. pneumoniae, K. oxytoca, Enterobacter cloacae,* and *Enterobacter aerogenes* have showed non-susceptibility to Ertapenem, Imipenem, Doripenem or Meropenem. The EIP uses population-based CRE surveillance data from three selected sites in the U.S.

Data from the National Nosocomial Infection Surveillance (NNIS), (before it was replaced by NHSN) reported that between 1986 and 1990, the rate of non-susceptibility to carbapenem in 1,825 isolates of *Enterobacter* investigated was 2.3% (Gaynes & Culver, 1992). It also reported that the percentage of Enterobacteriaceae that were carbapenem resistant rose from 1.2% in 2001 to 4.2% in 2011 (Jacob et al., 2013). These rates vary among Enterobacteriaceae and have increased the most in *Klebsiella* species, from 1.6 % to 10.8 %. Data from The Surveillance Network (TSN), which represents an electronic repository of susceptibility test results from over 300 laboratories across the United States, demonstrated an increase from 0% to 1.2% among all CREs, and 0% to 5.3% among *Klebsiella* species (Jacob et al., 2013). Data from the Meropenem Yearly Susceptibility Test Information Collection Program (MYSTICP), between 2006 and 2007, reported that resistance among *Klebsiella pneumoniae* exceeded 8% before falling momentarily to 5.6% in 2008 (Braykov et al., 2013; Rhomberg & Jones, 2009). The NHSN, in 2004, reported a CRE rate of 10.8% among *Klebsiella pneumoniae*; that stabilized at 13% in 2012 (Hidron et al., 2008; Sievert et al., 2013). The Washoe County Health District in Nevada in 2011, reported an incidence rate of 5%, after 15 months of CRE surveillance on 111 clinical isolates tested with the Modified Hodge Test at the Nevada State Public Health Laboratory (Chen, 2011). Interestingly, the majority of the CRE isolates in Washoe County were *Enterobacter cloacae*, an organism that has shown resistance to carbapenems.

During the first half of 2012, a surveillance of CAUTIs and CLABSI in acute-care hospitals in the U.S. reported that 181 of 3,918 (4.6%) centers surveyed reported

detecting one or more infections with CREs. Long-term acute care hospitals (LTACHs) have reported a rate of 17.8% in at least one outbreak of CRE infections, while, in short-term acute care hospitals, rates were 3.9%. The highest percentage of hospitals with CREs were in the northeast U.S., where rates were up to 30%. In the 2012 NHSN report, the state of Nevada was included as one of 43 states with CRE infections in the U.S. (Jacob et al., 2013).

Detection of CRE

The most often used method for CRE detection is the measurement of Minimal Inhibitory Concentration (MIC) through automated susceptibility testing machines. MIC is a quantitative measurement of antibiotic activities, and it is defined as the minimum concentration of an antibiotic that can inhibit visible microbial growth under normal conditions (Mayer, 2010; Sievert et al., 2013). Carbapenem resistance is defined for all organisms as an MIC result of Intermediate (I) or Resistant (R) to carbapenems on any antibiotic susceptibility test (Sievert et al., 2013). According to the FDA, an organism is classified as resistant to carbapenem if the pure culture shows a microbial breakpoint with an MIC ≥ 4 μg/ml during susceptibility testing (Bulik et al., 2010). Microbial breakpoint refers to the MIC at which an organism is described as susceptible or resistant to a given antibiotic. It is important that the MIC of antibiotics be lower than their breakpoint.

Carbapenem resistance can also be determined using the disk diffusion method. A type of disk diffusion method used is the Modified Hodge Test (MHT), a form of susceptibility testing that phenotypically determines the presence of the

carbapenemase enzyme (Clinical and Laboratory Standards Institute, 2012). The MHT has been regarded as the confirmatory test for CRE; however, limitations to this method include an inability to differentiate the class of carbapenemase involved, unusable for non-fermenting organisms, long duration of testing (up to 36 hours for the results to be available), a low positive predictive valve, and variable sensitivity that ranges from 76% to 100% (Amjad et al., 2011; Haji Hashemi et al.; Mathers, Carroll, Sifri, & Hazen, 2013; Tsakris et al., 2010).

The use of boronic acid is another phenotypic test for detecting CREs and has demonstrated an excellent ability in detecting KPCs, especially among *Klebsiella pneumoniae* isolates (Doi et al., 2008). Boronic acid alone or in combination with disks containing Imipenem, Meropenem or cefepime, showed 100% sensitivity and specificity in identifying KPC producers (Tsakris et al., 2009). Unfortunately, this method is not commercially available, and like the MHT, it requires an additional day before results are available.

Real-time Polymerase Chain Reaction (PCR) is a quick, accurate and effective method of CRE detection; it can detect CRE resistance genes, such as the blaKPC, the NDM-1, MBL, and the AmpC genes (Endimiani et al., 2010). PCR and other molecular tests have the highest specificity in identification and confirmation of the underlying carbapenemases (Nordmann et al., 2012). The sensitivity of real-time PCR in detecting the blaKPC gene ranges from 92.9% to 96.4% while the specificity is as high as 99.6% (Francis, Wu, Della-Latta, Shi, & Whittier, 2012). However, the high cost and the technical expertise required, in addition to the fact that only the target gene (e.g.,

carbapenemase gene) amplifies, are significant limitations to the use of PCR and other molecular tests in CRE detection (Yang & Rothman, 2004).

A significant problem in CRE laboratory detection is the fact that some bacterial isolates carry the KPC gene, while having susceptible, but elevated MICs (Clinical and Laboratory Standards Institute, 2009). This means that some isolates producing carbapenemase may test susceptible to carbapenems. In these situations, CREs will not be identified, thus posing an infection control problem. This has caused some automated AST methods to fail in detecting low levels of carbapenemase resistance.

To tackle this problem, the Clinical and Laboratory Standards Institute (CLSI), in 2009, published a recommendation that carbapenem susceptible Enterobacteriaceae with susceptible, but elevated MIC or with a reduced disk diffusion zone, be tested for the presence of the carbapenemase enzyme using the Modified Hodge Test. Furthermore, in 2010, the CLSI officially changed the carbapenem resistance criteria to ensure that KPC-producing organisms were not missed. This, also, removed the need for secondary testing with MHT among isolates with susceptible but elevated MICs. Therefore, a lower level of MIC for antibiotic resistance was established for CREs; these criteria were further revised in 2012 (Clinical and Laboratory Standards Institute, 2012). The previous and the current CLSI criteria are shown in Table 1.

Table 1: Performance Standards for Antimicrobial Susceptibility Testing.

	Previous Breakpoints (M100-S19)[a] MIC (μg/ml)			Current Breakpoints (M100-S22)[b] MIC (μg/ml)		
Agent	**S**	**I**	**R**	**S**	**I**	**R**
Doripenem	-	-	-	≤1	2	≤4
Ertapenem	≤2	4	≥8	≤0.5	1	≥2
Imipenem	≤4	8	≥16	≤1	2	≥4
Meropenem	≤4	8	≥16	≤1	2	≥4

S = Susceptible; I= Intermediate; R = Resistant; MIC = minimum inhibitory concentration

[a] 2009 CLSI criteria

[b] 2012 CLSI criteria

Source: Twenty Second Informational Supplement (January 2012). CLSI document M100-S22. Wayne, Pennsylvania, 2012.

Diagnostic kit manufacturers and clinical laboratories must have their kits FDA-cleared before they can be used with patient isolates; these recent changes have led to confusion (Gupta et al., 2011). As of late 2010, about 80% of clinical laboratories still use FDA-cleared AST systems with FDA breakpoints (Paxton, 2010). An Indiana Sentinel Laboratories CRE Testing Capacity Survey that studied the adoption rate of the new CLSI criteria noted that only about 37% of laboratories in Indiana used the CLSI M100-S21 criteria (Jean, 2011). In Rhode Island, a statewide assessment of the impact of the new CLSI criteria conducted in 2012 found that 100% of the 11 clinical laboratories representing all the state's acute care centers did not use the new CLSI criteria (Alexander, 2013). At the same time, only 27% of these laboratories in Rhode Island were interested in conducting the necessary verification studies to implement the current CLSI criteria. The majority of laboratories and hospitals preferred to wait until

the manufacturers update their AST systems when the FDA accepts the new CLSI criteria (Alexander, 2013). These are undoubtedly key obstacles to the widespread adoption of the new CLSI breakpoints. However, the FDA has started this ratification process by releasing guidance that informs pharmaceutical companies and manufacturers of the need to review their drug package information and revise them to meet current drug testing criteria (Food and Drug Adminstration, 2009). Unfortunately, this problem may persist for some time, because the regulatory system by which the FDA updates or modifies drug labels and information can move slowly (Paxton, 2010). Until the FDA approves the new CLSI breakpoints for AST system manufacturers, inadequate surveillance and control of CRE will continue to pose a problem. In the meantime, it is recommended that institutions and clinical laboratories adopt the new CLSI criteria if they can conduct an in-house confirmatory susceptibility testing (Paxton, 2010).

Relevance to Public Health

CREs and KPCs are particularly important in public health because of the high mortality associated with their infections and the tendency to spread beyond health centers (Bratu et al., 2005; Patel, Huprikar, Factor, Jenkins, & Calfee, 2008). In terms of cost of treatment, an estimated $21 billion - $34 billion are spent annually in the treatment of CREs in the U.S. (Spellberg et al., 2011).

Some major risk factors for acquiring CRE are exposure in health care facilities, erratic use of antibiotics, a history of recent organ transplant (including stem-cell transplantation), the use of mechanical ventilation and longer hospital stay. These risks are also independently associated with higher mortality due to CREs (Patel et al., 2008).

Other risk factors associated with higher mortality among patients with CRE infections include deteriorating health status, intensive care unit admission, and the use of invasive medical instrumentation, etc., (Falagas et al., 2007; Schwaber et al., 2008). A mortality rate of 48% has been reported among patients with CRE infections, and a crude mortality rate as high as 71.9% has been reported by some researchers (Patel et al., 2008).

There is a high potential for extensive spread of the carbapenem resistance gene from one Enterobacteriaceae into another within a health institution or to the community through mobile transmissible genetic components on the KPC gene (Watanabe, Iyobe, Inoue, & Mitsuhashi, 1991; Yigit et al., 2001). CREs are mainly seen in long term acute care (LTAC) centers. This suggests that LTACs could be a potential reservoir for CREs (Perez et al., 2010). Although the focus of CRE prevention has been on acute care settings (long and short term), there have been documented cases of CREs in non-acute care settings, for example, in long term care facilities, such as nursing homes and assisted living facilities (Urban et al., 2008). Therefore, limiting prevention and control efforts to acute care centers might be counterproductive.

CREs are not a problem limited to individual facilities. They can affect entire communities and nations, which highlights the role of public health in this issue. Public health organizations have the capacity to reach across all care institutions and to improve community situational awareness with regards to CREs and coordination of prevention efforts. For example, in Israel, a centrally coordinated effort by the Israel

Carbapenem-Resistant Enterobacteriaceae Working group has been helpful in decreasing the incidence of CREs (Schwaber et al., 2011).

The implementation of the new CLSI's MIC guidelines and breakpoints can have a significant impact on carbapenem AST reports from clinical diagnostic laboratories, with associated alterations in antibiotic prescription by clinicians (MacKenzie et al., 2007). Therefore, accurate laboratory information, better knowledge of the patient's history, and current information about CREs are all necessary to avoid uncertainties in carbapenem AST reports (Endimiani et al., 2009).

In Nevada, data are needed to determine the percentage of isolates with carbapenem MIC from the current CLSI breakpoints that are truly resistant to carbapenems, by the presence of the resistant KPC gene (Endimiani et al., 2009). The objectives of this study were to determine the presence of the KPC gene and the carbapenem antimicrobial resistance profiles of clinical isolates from health care facilities in Nevada for the purpose of determining if there is a difference in the rate of resistance between the previous and the current CLSI criteria.

RESEARCH QUESTIONS

1. What is the percentage of CRE isolates that are positive for the KPC gene using the current CLSI criteria for resistance to carbapenems?
2. What is the mechanism of resistance seen in CRE isolates?
3. Is there a benefit in adopting the new CLSI carbapenem guidelines?

HYPOTHESES

H_{o1}: There is no difference between carbapenem resistance in clinical isolates with the previous breakpoints and the current breakpoints.

H_{A1}: There is a difference between carbapenem resistance in clinical isolates with the previous breakpoints and the current breakpoints.

H_{o2}: There is no difference in carbapenem susceptibility profiles among KPC gene positive isolates between the current and the previous CLSI breakpoints.

$H_{A2:}$ There is a difference in carbapenem susceptibility profiles among KPC gene positive isolates between the current and the previous CLSI breakpoints.

MATERIALS AND METHODS

Test Organisms

Pure cultures from clinical isolates suspected of being carbapenem-resistant Enterobacteriaceae (de-identified of patient data) were obtained from Quest Diagnostics in Las Vegas, Nevada, and were transported to the Emerging Diseases Laboratory (EDL) at the University of Nevada, Las Vegas, for analysis. Enterobacteriaceae strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and were used as quality control (QC) organisms. Two of these ATCC organisms are carbapenem resistant and possess the KPC gene, while four of these strains are susceptible to carbapenems and do not possess the KPC gene (Table 2).

Table 2: Test Organisms Obtained from the American Type Culture Collection (ATCC).

Test Organism	**ATCC#**	**blaKPC gene**
Klebsiella pneumoniae	13883	Negative
Klebsiella pneumoniae	4352	Negative
Klebsiella pneumoniae	700603	Negative
Klebsiella pneumoniae	BAA 1706	Negative
Klebsiella pneumoniae	BAA 1705	Positive
Enterobacter hormaechi	BAA 2082	Positive

Lyophilized ATCC strains were re-suspended in nuclease-free water (HyClone Laboratories, Logan, Utah), vortexed, and cultured on Tryptic Soy Agar (TSA) (Becton Dickinson, Sparks, MD). All clinical isolates were stored at 4°C upon arrival and re-streaked onto fresh TSA plates (Becton Dickinson) within 48 hours of receipt. QC organisms and clinical isolates were incubated at 35°C in ambient air for 24-48 hours. Isolated colonies were picked and used to prepare freezer and refrigerator stocks.

Freezer stocks were prepared by transferring two to three isolated colonies to a 2 ml cryogenic tube containing 500 μl of Tryptic Soy Broth (Becton Dickinson) and 500 μl of sterile glycerol (MP Biomedicals, Solon, Ohio). The tube was vortexed vigorously for 30 seconds or until the colonies were completely re-suspended, followed by incubation in a rotary shaker at 35°C for 30 minutes and 175 rpm. Freezer stocks were stored at -70°C. Refrigerator stocks were prepared by re-streaking an isolated colony from the 24-48 hours TSA plate onto a TSA slant (Becton Dickinson), incubated overnight at 35°C ambient air, and stored at 4°C.

Two to three isolated colonies from the 24-48 hours TSA plate were transferred to a micro-centrifuge tube containing 400 μl of nuclease free water (HyClone

Laboratories). The suspension was vortexed for 15 – 30 seconds for proper homogenization, and stored at -70°C for DNA extraction.

DNA Extraction

Bacterial DNA was extracted using the MoBio PowerSoil DNA extraction kit (MoBio, Carlsbad, CA) according to the manufacturer's instructions with one exception; 200 µl of sample (instead of 0.25g) was used for extraction. The DNA extract was eluted in 100 µl of the C_6 buffer solution provided, and stored at –70°C until ready for use.

Template DNA Concentration

After DNA extraction, the amount of DNA in each sample extract was measured with a Spectronic ^{TM} Genesys 10 BIO UV- Visible spectrophotometer (Thermo Fisher Scientific, Madison, WI) using the nanoCell accessory – 0.2mm path length. Briefly, 1.0 µl of the sample extract was used, after zeroing the spectrophotometer with the C_6 solution from the PowerSoil DNA extraction kit. The absorbance was set at 260/280 nm, with 320 nm reference wavelength and 2500 dilution factor. Samples were measured in duplicate and DNA concentrations were expressed in ng/µl. All samples with DNA concentration of 21 ng/µl and above were diluted using Tris-EDTA (TE) buffer (Teknova, Hollister, CA) prior to the PCR assay. This enabled the DNA in the PCR to maintain a concentration between 10 – 100 ng (Life Technologies).

Real-Time Polymerase Chain Reaction (PCR)

The 7900 HT Fast PCR System (Applied Biosystems, Foster City, CA) was used for detection and amplification of the blaKPC gene. Primers and a fluorescent probe specific for the blaKPC gene were identified from the literature that produced a 246-bp

amplicon (Hindiyeh et al., 2008; Tenover et al., 2006). The master mix was prepared using TaqMan 1X Universal Master Mix (Branchburg, New Jersey), sterile Nuclease Free Water (Promega, Madison, WI), 0.3 µM of forward primer - 5'-GAT ACC ACG TTC CGT CTG G-3' (Hindiyeh et al., 2008), 0.3 µM of reverse primer - 5'-GCA GGT TCC GGT TTT GTC TC-3' (Tenover et al., 2006), and 0.2 µM of probe -6-carboxyfluorescein-5'-AGC GGC AGC AGT TTG TTG ATT G-3'-6 carboxytetramethylrhodamine (Hindiyeh et al., 2008). Primers were obtained from Eurofins MWG Operon (Huntsville, AL), and the probe was obtained from Life Technologies.

All PCR assay reactions were performed in duplicate, using a total volume of 25 µl, containing 5 µl of template DNA (i.e., 10-100 ng) and 20 µl of the master mix solution. Positive and negative controls were included with each PCR assay. Following amplification, the results were analyzed on the PCR computer using the SDS ver. 3.0 software. Once amplification was completed, the level of amplification was reported by the software as the mean Cycle Threshold (C_T) value of replicate samples. C_T refers to the PCR cycle at which fluorescence (i.e., amplification product) is first detected. A sample was considered positive by real-time PCR and possessed the blaKPC gene, if the C_T crossed the threshold before the threshold cycle of 40. A C_T value of 40 or undetermined represents no target DNA present. Samples that tested undetermined for the blaKPC gene were re-analyzed using an Internal Positive Control (IPC) (Life Technologies) to determine if there were inhibitors present in the reaction.

Internal Positive Control

A commercially available TaqMan exogenous Internal Positive Control (Life Technologies) was used to detect PCR inhibition and rule out false negatives. The kit included 10X Exogenous IPC Primer and Probe (VIC™ Probe) mix, 10X Exogenous IPC Blocking Reagent, and 50X Exogenous IPC DNA. The blaKPC PCR assay was run with the internal positive control; thus, absence or a decrease in amplification of the IPC DNA in each duplex PCR indicated the presence of PCR inhibitors. Several dilutions (i.e., 10^{-1} to 10^{-3}) of selected DNA samples were tested, to determine and eliminate potential PCR inhibitors.

Antimicrobial Susceptibility Testing (AST) and Microbial Identification

All clinical isolates were subjected to carbapenem Antibiotic Susceptibility Testing (AST) using gram-negative (GN) AST and identification cards for the Vitek 2 Compact system (bioMerieux, Durham, NC) following the manufacturer's protocol. AST-GN69 and AST- XN06 Vitek 2 Gram negative cards were used for AST, and for microbial identification, Vitek 2 GN ID card ref. 21341 was used (Appendices A and B). Briefly, the clinical isolates were sub-cultured (from the refrigerator stocks or from the freezer when there was no growth) onto TSA and incubated for 18-24 hours at 35°C in ambient air. A cell suspension of each sample with optical density of 0.5 – 0.63 McFarland Standard was prepared. The suspension was loaded onto the ID and AST cards in the biological safety cabinet, and then transferred to the Vitek 2 Compact machine for analysis. The results of the susceptibility profile were analyzed on the Vitek 2 system computer using software version 5.04 (bioMerieux) according to the U.S. Food and Drug

Administration (FDA) (previous CLSI breakpoints) and the Current CLSI carbapenem susceptibility breakpoints (Table 1). Furthermore, the Advanced Expert Analysis (AES) was applied to our analysis to determine the phenotype of carbapenem resistance implicated in our isolates. AES uses the knowledge base of the Vitek 2 system ver. 5.04 to determine resistance profile, resistance phenotype, and therapeutic interpretation of the results. AES uses all information available rather than MIC values alone to determine resistance. In some cases, isolates may have susceptible MIC yet be classified as non-susceptible by AES.

Statistical Analysis

Results were analyzed to determine if there was any significant difference between carbapenem susceptibility and non-susceptibility among individual antibiotics when using the previous and the current CLSI breakpoint criteria. We also determined if there was a statistically significant difference between the carbapenem susceptibility profiles among blaKPC gene positive isolates when using the previous and the current breakpoints. For our data, a non-parametric analysis (i.e., the Fisher Exact Test) was used. All analyses were conducted using SPSS ver. 22.0 (IBM, Armonk, New York).

RESULTS

Test Organisms

Fifty-six isolates were received and sub-cultured on TSA, and incubated for 24-48 hours. Of these, one did not grow after various attempts to culture it. Two additional

isolates had the same identification number; therefore, one of these was not analyzed. In total, 54 out of 56 isolates received were included in our analysis. These isolates were recovered from urine (catheter, and clean catch; n=27), sputum (n=16), bronchiole (n=1), wound (n=5), abscess (n=1), blood (n=2), abdominal fluid (n=1), and unspecified location (n=3).

Template DNA Concentration

The amount of DNA present in 100 µl of each DNA extract was measured after DNA extraction. The mean DNA concentration of all of the isolates from duplicate measurements varied from 8.8 ng/µl to 131.3 ng/µl (Table 3). Subsequently, all DNA extracts with a concentration greater than 21 ng/µl were diluted. The final DNA concentration in 5 µl of sample used for the PCR assay ranged from 10.7 ng to 94.0 ng.

The UV- Visible spectrophotometer determines the DNA concentration by using the ratio of DNA absorption at 260 nm to absorption of RNA at 280 nm. The average 260/280 absorption ratio measured ranged from 0.033 to 8.188 (Table 3).

Real- Time Polymerase Chain Reaction (PCR) Analysis

All isolates with mean C_T values <40 were regarded as positive, and thus considered to harbor the blaKPC gene. Known KPC gene negative ATCC strains (i.e., *K. pneumoniae* ATCC 13883, *K. pneumoniae* ATCC 4352, *K. pneumoniae* ATCC BAA 1706, and *K. pneumoniae* ATCC 700603) all tested PCR negative (undetermined) for the presence of the blaKPC gene (Table 3). All known KPC gene positive strains obtained from ATCC (i.e., *Enterobacter hormaechei* ATCC BAA 2082 and *Klebsiella pneumoniae*

ATCC BAA 1705) tested positive for the KPC gene with mean C_T values of 17.1 and 18.8, respectively (Table 3).

Ten out of 54 isolates produced undetermined (i.e., negative) results with PCR and were regarded as negative for the blaKPC gene. Additional PCR analyses performed with the Internal Positive Control (IPC) on isolates that were negative showed inhibition in several blaKPC gene negative isolates. Subsequently, serial dilution (1:10 and 1:100) of the inhibited samples resulted in a positive blaKPC gene by PCR (Table 4). The prevalence rate of blaKPC gene among 54 suspected CRE isolates received from Quest Diagnostics in Las Vegas was 83.3%, and the prevalence of the blaKPC gene among *Klebsiella pneumoniae* isolates (identified with the Vitek 2 Compact instrument) was 93.6%.

Table 4: Internal Positive Control (IPC) PCR Results of blaKPC Gene Negative Isolates

Sample	**Dilution**	**blaKPC (Mean Ct Value; n=2)**	**IPC (Mean Ct; n=2)**
NTC	N/A	undetermined	31.85
Klebsiella pneumoniae ATCC BAA 1705	1:10	18.80	undetermined
	1:100	22.53	undetermined
	1:1000	26.85	32.11
CRE 004	Undiluted	undetermined	undetermined
	1:10	undetermined	32.68
	1:100	undetermined	32.66
CRE 021	1:10	undetermined	31.31
CRE 029	Undiluted	undetermined	undetermined
	1:10	undetermined	32.34
	1:100	undetermined	31.74
CRE 033	Undiluted	undetermined	34.07
	1:10	undetermined	32.20
CRE 036	1:10	undetermined	30.90
CRE 039	Undiluted	undetermined	33.53
	1:10	undetermined	31.99
CRE 040	1:10	undetermined	31.12
CRE 042	Undiluted	undetermined	undetermined
	1:10	22.02	undetermined
	1:100	25.79	35.26
CRE 050	1:10	undetermined	32.07
	1:100	undetermined	31.84
CRE 052	1:10	undetermined	31.50

NTC = No Template Control

Identification of Isolates

Microbial identification carried out on CRE isolates using the Vitek 2 ID card No. 21341 identified the following organisms with at least 94% confidence (Table 5): *Klebsiella pneumonia pneumoniae* (n=46), *Escherichia coli* (n=2), *Enterobacter aerogenes* (n=2), *Citrobacter freundii* (n=2), *Acinetobacter baumannii* (n=1), and *Proteus mirabilis* (n=1).

Table 5: Organisms Identified by Vitek 2 ID Analysis.

Organisms Identified	**N**
Acinetobacter baumannii	1
Citrobacter freundii	2
Klebsiella pneumoniae	46
Proteus mirabilis	1
Enterobacter aerogenes	2
Escherichia coli	2

Antibiotic Susceptibility Testing (AST)

The Vitek 2 instrument was used for AST of ATCC reference samples and CRE isolates, and these were analyzed using the current and the previous CLSI MIC breakpoints. Our reference samples included three known KPC negative species from ATCC (*Klebsiella pneumoniae* ATCC 13883, *Klebsiella pneumoniae* ATCC 4352, and *Klebsiella pneumoniae* ATCC 700603), and two known KPC positive species (*Enterobacter hormaechei* ATCC BAA 2082 and *Klebsiella pneumoniae* ATCC BAA 1705). Carbapenem non-susceptibility (i.e., resistance) was concluded for all samples with intermediate or resistant MIC results. Analysis of the ATCC negative reference samples using both

criteria (previous and current CLSI breakpoints), showed susceptibility to all carbapenems. All KPC positive ATCC controls were non-susceptible to all carbapenems (Table 6).

Table 6: ATCC blaKPC Positive and Negative Organisms and their AST Results.

Test Organism	CLSI Criteria[a]							
	2009	2012	2009	2012	2009	2012	2009	2012
	Ertapenem		Imipenem		Doripenem		Meropenem	
K. pneumoniae ATCC 13883	S	S	S	S	S	S	S	S
K. pneumoniae ATCC 4352	S	S	S	S	S	S	S	S
K. pneumoniae ATCC 700603	S	S	S	S	S	S	S	S
E. hormaechi *ATCC* BAA 2082	R	R	I	R	nd	R	R	R
K. pneumoniae ATCC BAA 1705	R	R	R	R	nd	R	R	R

ATCC- American Type Culture Collection; S = Susceptible; I = Intermediate; R= Resistant; nd = no criteria available for 2009.

[a] 2009 represents previous criteria and 2012 represents current criteria.

Ertapenem Susceptibility Testing

According to the Vitek 2, Ertapenem use was not indicated for clinical use in one of our 54 CRE isolates. This resulted in 53 isolates that were analyzed with the previous and current CLSI criteria for this antibiotic. Using current breakpoints (CLSI M100-S22) for susceptibility interpretations, 48 of our 53 isolates (90.6%) showed resistance (i.e., non-susceptibility) to Ertapenem, and 5 isolates (9.4%) were susceptible for this antibiotic (Table 7). However, when using the previous breakpoints (CLSI M100-S19), 47 isolates (88.7%) were resistant and 6 isolates (11.3%) were susceptible to Ertapenem.

Transitioning to the current CLSI criteria for this antibiotic would result in a change in resistance rate from 88.7% to 90.6%.

Imipenem Susceptibility Testing

Using the current Imipenem breakpoints, 49 of 54 isolates (90.7%) were classified as resistant, and five isolates (9.3%) were classified as susceptible (Table 7). In terms of the previous breakpoints, Imipenem resistance occurred in 46 of 54 isolates (85.2%), with 8 isolates (14.8%) classified as susceptible. Transitioning to the current CLSI criteria for Imipenem would result in a change in resistance rate from 85.2% to 90.7%.

Doripenem Susceptibility Testing

According to the Vitek 2, Doripenem was not indicated for clinical use in two of our 54 CRE isolates. This resulted in 52 isolates that were analyzed with the previous and current CLSI criteria for this antibiotic. Doripenem MIC interpretations with the current breakpoints classified 47 of 52 isolates (90.4%) as non-susceptible and 5 isolates (9.6%) as resistant (Table 7). Doripenem is a fairly new carbapenem antibiotic; therefore, there were no susceptibility interpretations published for it in the previous CLSI breakpoints. Thus, we cannot make a comparison between the previous and current carbapenem breakpoints.

Meropenem Susceptibility Testing

For Meropenem, resistance was reported in 49 of 54 isolates (90.7%) when using the current CLSI breakpoints, but with the previous breakpoints Meropenem resistance was reported in 47 of 54 (87.0%) isolates, with 7 isolates (13.0%) showing susceptible to

this antibiotic (Table 7). Transitioning to the current CLSI criteria for Meropenem would result in a change in resistance rates from 87.0% to 90.7%.

Table 7: Individual Carbapenem Susceptibility Among all CRE Isolates Analyzed.

Individual carbapenems	Previous Breakpoints (M100-S19)				Current Breakpoints (M100-S22)			
	Susceptible		Non-susceptible		Susceptible		Non-susceptible	
	N	%	N	%	N	%	N	%
Ertapenem[a]	6	11.3	47	88.7	5	9.4	48	90.6
Imipenem	8	14.8	46	85.2	5	9.3	49	90.7
Doripenem[b]	nd	nd	nd	nd	5	9.6	47	90.4
Meropenem	7	13.0	47	87.0	5	9.3	49	90.7

[a]Ertapenem use was not indicated for 1 isolate. [b]Doripenem use was not indicated for 2 isolates. nd = Doripenem interpretation was not defined in the Previous Breakpoints.

Ertapenem Susceptibility of blaKPC Gene Negative Isolates

Of the nine CRE isolates that were negative for the blaKPC gene, the Vitek 2 analysis indicated that eight of these should have been treated with Ertapenem (Table 8). When using the previous breakpoints, six of these isolates were susceptible to Ertapenem (i.e., two isolates were non-susceptible or resistant). When the MICs were analyzed with the current breakpoints, five were classified as susceptible to Ertapenem and three as resistant. Therefore, there was only one blaKPC gene negative isolate which reported susceptibility to Ertapenem with the previous criteria, but changed to resistant with the current criteria (Table 9).

Imipenem Susceptibility of blaKPC Gene Negative and Positive Isolates

All nine of the blaKPC gene negative isolates were indicated for treatment with Imipenem. When analyzed with the previous CLSI criteria, seven isolates were classified as susceptible to Imipenem, while two isolates were resistant (Table 8). With the current breakpoints, five of the nine isolates were susceptible to Imipenem, while four were resistant. Transitioning to the current CLSI criteria would result in two blaKPC gene negative isolates previously reported as susceptible for Imipenem that now would be reported as resistant with the current breakpoints (Table 9). On the other hand, only one blaKPC gene positive that was susceptible to Imipenem when using the previous breakpoints changed to resistant with the current breakpoints (Table 9).

Table 8: Individual Carbapenem Susceptibility Among blaKPC Gene Negative Isolates.

Individual carbapenems	**Previous Breakpoints (M100-S19)**		**Current Breakpoints (M100-S22)**	
	Susceptible	**Non-Susceptible**	**Susceptible**	**Non-Susceptible**
Ertapenem	6	2	5	3
Imipenem	7	2	5	4
Doripenem	nd	nd	4	3
Meropenem	7	2	5	4

nd = Doripenem interpretation was not defined in the previous breakpoints

Table 9: Susceptibility Changes (Susceptible to Non-Susceptible) Between the Previous CLSI Criteria and the Current Criteria Among blaKPC Genes (Negative and Positive).

Individual carbapenems[a]	**blaKPC Gene Negative**	**blaKPC Gene Positive**	**Total number of changes**
Ertapenem	1	0	1
Imipenem	2	1	3
Meropenem	2	0	2

[a] Doripenem susceptibilities were not compared.

Doripenem Susceptibility of blaKPC Gene Negative Isolates

For blaKPC gene negative isolates that were indicated for treatment with Doripenem (n= 7), analysis with the current CLSI breakpoints reported only four isolates as susceptible to Doripenem while three were resistant (Table 8). We cannot make a comparison between the previous and current breakpoints for this antibiotic because there were no susceptibility interpretations published for it in the previous CLSI breakpoints (Table 9).

Meropenem Susceptibility of blaKPC Gene Negative Isolates

When using the previous breakpoints, Meropenem susceptibility was seen in seven of nine blaKPC gene negative isolates, while two isolates were resistant to this antibiotic (Table 8). With the current breakpoints, five isolates were susceptible to Meropenem, and four were resistant. Transitioning to the new CLSI criteria would result in two blaKPC gene negative isolates previously reported as susceptible to Meropenem that now would be reported as resistant with the current breakpoints (Table 9).

Carbapenem Susceptibility of blaKPC Gene Positive Isolates

Among blaKPC gene positive isolates, individual carbapenem susceptibility profiles did not vary much between the previous criteria and the current CLSI criteria (Table 10). All blaKPC gene positive isolates (n=45) reported resistance to Ertapenem and Meropenem across the two criteria. Thus, non-susceptibility rate among blaKPC gene positive isolates continued to be the same at 100% for Ertapenem and Meropenem, when using the previous and current criteria. However, Imipenem showed

a change in resistance rate from 97% to 100% when comparing the previous to the current CLSI criteria (Table 10). No data comparisons were possible for Doripenem; however, the blaKPC gene positive isolates showed a resistance rate of 98% for this antibiotic (with the current criteria) (Table 10).

Table 10: Individual Carbapenem Non-Susceptibility in blaKPC Gene Positive Isolates (n= 45).

Individual carbapenems	**Previous Breakpoints (M100-S19)**		**Current Breakpoints (M100-S22)**	
	Susceptible	**Non-Susceptible**	**Susceptible**	**Non-Susceptible**
Ertapenem	0	45 (100%)	0	45 (100%)
Imipenem	1	44 (97.8%)	0	45 (100%)
Doripenem	nd	nd	1	44 (97.8%)
Meropenem	0	45 (100%)	0	45 (100%)

nd = Doripenem interpretation was not defined in the previous breakpoints

Mechanisms of Antibiotic Resistance

Many isolates reported different and more than one resistance phenotype when the Expert Analysis (AES) from the Vitek 2 instrument was applied to our AST analysis. The resistance phenotypes (or mechanism of resistance) reported for all isolates were different depending on the AST card used (Table 8). Analysis with the GN 69 AST card reported the following resistance phenotypes for our isolates: Extended Spectrum Beta-Lactamase (ESBL), Impermeability (carbapenems and cephamycins), carbapenemase (Metallo- or KPC), Penicillinase (acquired or wild type), and High Level AmpC (HL-CASE) (Table 11).

Analysis with the GN XN 06 AST card showed the following resistance phenotypes: ESBL, Impermeability (carbapenems and cephamycins), carbapenemase

(Metallo- or KPC), Inhibitor Resistant PASE (IRT or OXA), Penicillinase, and High Level AmpC (Table 11). Regardless of the AST card used, the three most common resistance mechanisms observed were: ESBL (87%), Impermeability (85.2%), and carbapenemase (83.3%).

Table 11: Carbapenem Resistance Phenotypes Implicated in all Isolates (n=54).

Carbapenem Resistance Phenotype[a]	**GN 69 AST Card (N; percentage)**	**GN XN 06 AST Card (N; percentage)**
Extended Spectrum Beta- lactamase	47 (87.0%)	47 (87.0%)
Impermeability (carbapenems and cephamycins)	46 (85.2%)	48 (88.8%)
Carbapenemase (Metallo- or KPC)	45 (83.3%)	49 (90.7%)
Penicillinase (acquired or wild type)	2 (3.7%)	1 (1.9%)
High Level AmpC (HL-CASE)	2 (3.7%)	1 (1.9%)
Inhibitor Resistant PASE (IRT or OXA)	0 (0%)	1(1.9%)

[a] Most isolates exhibited more than one phenotype.

The nine isolates that were negative for the blaKPC gene were identified (Table 12). According to the Vitek 2 Advanced Expert Setting Analysis (AES), resistance phenotypes for these isolates were ESBL, Impermeability, carbapenemase (Metallo- or KPC), Penicillinase (acquired or wild type), High Level-Case (amps), and Inhibitor Resistant PASE (IRT or OXA) (Table 12).

Table 12: Carbapenem Resistance Phenotype Implicated in all blaKPC Negative Isolates.

Organisms ID	**N**	**Resistance Phenotype (GN 69/XN 06 AST Card)**
Acinetobacter baumannii	1	Impermeability
Citrobacter freundii	1	carbapenemase (Metallo- or KPC)
Klebsiella pneumoniae	3	Penicillinase, Inhibitor Resistant PASE (IRT or OXA), ESBL, Impermeability
Proteus mirabilis	1	ESBL/carbapenemase (Metallo- or KPC)
Enterobacter aerogenes	2	ESBL/HL-Case (AmpC)[a], Impermeability, carbapenemase (Metallo- or KPC)
Escherichia coli	1	Penicillinase/ Inhibitor Resistant PASE (IRT or OXA)

[a]HL-Case (AmpC) – High Level AmpC

Isolates that Changed from Susceptible to Non-Susceptible Between 2009 and 2012 Breakpoints

Five isolates changed from susceptible to non-susceptible in at least one carbapenem when using the current criteria. Four of these where blaKPC gene negative (Table 13). Carbapenemase (Metallo- or KPC) were implicated as the mechanism of resistance in four of these. Other resistance mechanisms implicated included ESBL, AmpC, and Impermeability. Specifically these isolates included three non-*Klebsiella* species and two *Klebsiella pneumoniae*.

Table 13: List of Isolates that Changed in AST Between the Previous and the Current Breakpoints

Isolates	Organism ID	blaKPC status	Carbapenem implicated	AES Resistance Phenotypes
CRE 021	*Citrobacter freundii*	Negative	Imipenem, Meropenem	Carbapenemase (Metallo- or KPC)
CRE 033	*Proteus mirabilis*	Negative	Meropenem	ESBL, Carbapenemase (Metallo-or KPC)
CRE 050	*Enterobacter aerogenes*	Negative	Imipenem	AmpC, Impermeability, Carbapenemase (Metallo-or KPC)
CRE 052	*Klebsiella pneumoniae*	Negative	Ertapenem	ESBL, Impermeability
CRE 053	*Klebsiella pneumoniae*	Positive	Ertapenem	ESBL, Impermeability, Carbapenemase (Metallo- or KPC)

Susceptibility to other Antibiotics

In our study, susceptibility of CRE to gentamicin, ceftazoxime and cefepime were relatively high at 57%, 44% and 41%, respectively.

Statistical Analysis

Results were analyzed to determine if there was any significant difference between carbapenem susceptibility and non-susceptibility among individual antibiotics when using the previous and the current breakpoint criteria. Statistical analysis using Fisher's Exact Test on AST with the previous and current criteria for Ertapenem, Imipenem, and Meropenem were not significantly different (p-values > 0.05). Therefore, we failed to reject the null hypothesis (H_{01}) of equal proportion in carbapenem resistance between the previous and the current CLSI breakpoint criteria.

Statistical analysis to determine if there was a significant difference between the carbapenem susceptibility profiles among isolates with the blaKPC gene when using the previous and the current breakpoints were not significantly different (p-values > 0.05).

We failed to reject the null hypothesis (H_{o2}) that there is no difference in carbapenem susceptibility profiles among KPC gene positive isolates between the current and the previous CLSI breakpoints.

DISCUSSION

In 2012, the Clinical and Laboratory Standards Institute (CLSI) published updated editions of its antimicrobial susceptibility testing (AST) standards. The updated criteria are intended to detect emerging bacterial resistance. Periodic updates of these guidelines are necessary because bacteria acquire resistance to antibiotics over time, and using the most current knowledge ensures that infections are treated consistently and fosters good antibiotic stewardship. In recent times, automated AST systems have become the most common method of conducting susceptibility testing; these instruments measure the minimal inhibitory concentration of antibiotics using the CLSI guidelines. The FDA considers non-susceptibility to antibiotics as MIC results that are intermediate or resistant according to fixed breakpoints; in addition, the agency is responsible for approving the use of CLSI criteria with automated AST systems. The lower MIC for carbapenem antibiotics established for KPCs in 2012 by the CLSI have not been endorsed by the FDA. Therefore, data were needed to estimate the percentage of clinical isolates that are potentially resistant to carbapenems by determining the presence of the KPC gene. Detecting the presence of the KPC gene is important because, the use of carbapenems determined by using the current breakpoints for infections may

have poor clinical outcomes if the gene is present. Conversely, if the KPC gene is absent, treatment with carbapenems may still be indicated. The objectives of this study were to determine carbapenem AST profiles, resistance rates based on the previous and current CLSI criteria, and the presence of the KPC gene in CRE isolates.

In this study, carbapenem resistance among CRE isolates was mainly seen among *Klebsiella* pneumoniae (87%), followed by *E. coli*, *Enterobacter aerogenes*, and *Citrobacter freundii*, each with 3.7%. *Acinetobacter baumannii* and *Proteus mirabillis* (1.9% each) were also identified in our study as being resistant to carbapenems. Our results are similar to other published articles on CREs in which *Klebsiella* spp. are the most commonly reported Enterobacteriaceae with non-susceptibility to carbapenems. In a study by Jacob *et al.* (2013), the most commonly reported organism among 72 CRE isolates were *Klebsiella* species (n=49), with *Enterobacter* species and *E. coli* contributing 14 and 10 isolates, respectively (Jacob et al., 2013). We also observed that urine samples (27 out of 54) were the most common site of CRE isolation, followed closely by respiratory isolates (17 out of 54). Jacob *et al.* also identified CREs in urine samples more frequently (89%) than in other sites.

The prevalence rate of the blaKPC gene was 83.3% among suspected CRE isolates from different healthcare centers in Nevada, determined in our study. Isolates identified as *Klebsiella pneumoniae* comprised the majority of our CRE isolates, and 93.6% of these had the blaKPC gene. These rates and findings are similar to other studies in the U.S. that reported the blaKPC gene as the most commonly implicated gene in carbapenem resistance among the Enterobacteriaceae (Deshpande, Rhomberg, Sader, & Jones,

2006). Also, the prevalence of the blaKPC gene among *Klebsiella pneumoniae* identified in our study was similar to that reported in other studies published in the U.S., and abroad (Nordmann, Cuzon, & Naas, 2009). A study (Shanmugam, Meenakshisundaram, & Jayaraman, 2013) in India reported a blaKPC gene prevalence rate of 67.4% among CREs, and other recent studies have reported a blaKPC gene prevalence between 82% and 100% (Mosca et al., 2013; Raghunathan, Samuel, & Tibbetts, 2011; Shanmugam et al., 2013). Although CREs have been recently reported in Nevada, to our knowledge, this is the first published report of prevalence of KPC-producing Enterobacteriaceae isolated from patients in southern Nevada.

The CLSI and the Centers for Disease Control and Prevention recommend that CRE isolates with antibiotic resistant profiles or elevated, but susceptible profiles, be confirmed with the Modified Hodge Test (Clinical and Laboratory Standards Institute, 2009). While the Modified Hodge Test has deficiencies, it has remained the first step in detecting carbapenemase activity in clinical isolates in many facilities for isolates with elevated MIC, and thus, it is particularly important as part of an early infection control program. In this study, we used a PCR-based assay as an alternative to verify carbapenem resistance among CRE isolates.

The recently published CLSI standards for carbapenems have been met with mixed reception. Some researchers have argued that the change may only increase the false positive results in AST analysis, and thus lead to unnecessary and expensive treatments (Po-Yu Liu et al., 2014). In our study, we found no difference between carbapenem resistance in clinical isolates with the previous and the current breakpoints.

Also, there was no difference between susceptibility profiles among blaKPC gene positive isolates when using the two criteria. Therefore, we conclude that the current CLSI criteria may not offer additional benefit in the fight against CREs. Our results are similar to others reported in the literature that showed either no change between the two breakpoints or unnecessary increase in the estimation of carbapenem resistance (Hombach, Bloemberg, & Bottger, 2012; Metwally, Gomaa, Attallah, & Kamel, 2013).

We used the Expert Analysis (AES) available with our automated AST instrument to determine the resistance phenotype of isolates that tested negative for the blaKPC gene, yet tested non-susceptible (resistant) to carbapenems during our AST analysis. The AES reported Impermeability, carbapenemase (metallo- or KPC), ESBL, and HL-Case (AmpC), as the commonly involved mechanisms of resistance among blaKPC gene negative isolates using both the previous and current criteria. Impermeability is the inability of an antibiotic to penetrate the cell wall of the organism. Carbapenemases and Extended Spectrum β-lactamases (ESBL) work by hydrolyzing the β-lactam structure of the antibiotic (David L Paterson & Bonomo, 2005). While ESBL do not hydrolyze carbapenems and cephamycins, carbapenemases hydrolyze all groups of β-lactams. High Level Case (HL-Case) AmpC's mechanism of resistance involves hydrolysis of β-lactams, especially cephalosporin through a plasmid mediated cephalosporinase (George A Jacoby, 2009). Because our PCR assay was designed to detect only the blaKPC gene, it is possible that Metallo-beta-lactamase (MBL) may still be involved in isolates with carbapenemase (metallo- or KPC) phenotypes. The MBL is a less common carbapenem resistance mechanism, but has been increasing in prevalence in recent

reports (Centers for Disease Control and Prevention, 2010; Rolain, Parola, & Cornaglia, 2010; Tijet et al., 2011).

Other carbapenem resistance phenotypes that we observed were Impermeability and HL-Case (AmpC), and these were mainly seen in non-*Klebsiella pneumoniae* organisms. These findings are in agreement with several published reports showing that Impermeability and AmpC enzymes are carbapenem resistance phenotypes commonly implicated in other Enterobacteriaceae such as C*itrobacter* spp., *Enterobacter* spp., and *Proteus mirabilis* (Mainardi et al., 1997; Mammeri, Nordmann, Berkani, & Eb, 2008). Our study also supports that ESBL is commonly involved in resistance mechanisms of CREs along with carbapenemase (Thomson, 2010).

Because the amount of antibiotics used on patients and the development of resistance are directly proportional, increased reports of Enterobacteriaceae resistant to carbapenems and reduced MIC breakpoints (current CLSI criteria) will increase the number of Enterobacteriaceae determined to be resistant to at least one agent in any antimicrobial category. Therefore, it is anticipated that clinicians and healthcare workers will most likely prescribe increased doses of carbapenems or other antimicrobial classes which may lead to more resistance (Magiorakos et al., 2012).

Infections caused by CREs are difficult to treat, but with early detection in combination with prompt implementation of infection prevention and control practices, the high morbidity and mortality rate associated with CREs may be reduced (Cohen et al., 2011; Kochar et al., 2009). The CDC categorically recommend through their CRE toolkit, an aggressive implementation of infection prevention and control strategies

once CREs are detected (Centers for Disease Control and Prevention, 2012b). Prevention interventions, such as patient isolation, active surveillance cultures, patient cohorting, and education, have been effective in decreasing the incidence of CREs (Ciobotaro, Oved, Nadir, Bardenstein, & Zimhony, 2011; Debby Ben-David et al., 2010).

In terms of chemotherapy, the limited treatment alternatives that exist for patients with CRE infections include the use of combination therapies involving carbapenems, tigeycline, colistin, amikacin, and polymixin (Castanheira et al., 2009; Neuner et al., 2011). We determined that gentamicin, ceftizoxime and cefepime had relatively high susceptibilities among CREs in our study. Whether or not these agents demonstrate clinical success, especially in severe CRE infections, remains to be determined in vivo (Nayman-Alpat et al., 2010). In-vitro combinations of these antibiotics have shown bactericidal and synergistic effects (Bratu et al., 2005; Le, McKee, Srisupha-Olarn, & Burgess, 2011; Pankey & Ashcraft, 2011; Pournaras et al., 2011). However, higher toxicity, side effects, limited efficacy, and concerns of spread of resistance have limited their use in patients. Resistance to polymyxins and tigeycline or any known alternative have also been reported in some case-series studies, and suggest a fresh concern of pan-antibiotic resistance among CREs (Elemam, Rahimian, & Mandell, 2009).

Our study had several strengths and some limitations. The number of isolates included in the study was limited; however, our pilot study did provide data that can be utilized in further studies. Molecular detection of the blaKPC gene only detected isolates that express the KPC gene and may have underestimated the presence of other resistance mechanisms implicated in CREs, such as the MBL, the OXA, and the AmpC

enzymes in blaKPC gene negative isolates. Because *Klebsiella pneumoniae* carbapenemase is the most implicated carbapenemase in the U.S., in a resource limited setting, targeting the KPC gene will be a more efficient way to detect and confirm carbapenem resistance. We did not perform the MHT as confirmation for carbapenemase activity in our CRE isolates as recommended by the CDC; however, we were able to use a PCR-based assay as an alternative to verify the *Klebsiella pneumonia* carbapenemase gene.

CONCLUSIONS

Based on our findings, an understanding of the enzymatic mechanisms and other phenotypes of resistance mediated by CREs is important in the prevention of these organisms. Infections due to isolates that showed impermeability and concomitant production of ESBLs may be managed with carbapenems if their MICs are in the susceptible range. Although the implementation of the current CLSI breakpoints for carbapenem AST can make results more comparable worldwide, especially if it corresponds with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) system. However, evidence-based studies should validate these revised guideline changes before their general implementation. It is also important that clinicians and laboratories are aware of the implications of the revised antibiotic susceptibility testing reports in clinical practice, such as the effects on antibiotic prescription and antibiotic stewardship. In addition, to ensure proper detection of

emerging resistance, species-related zones of inhibition or MIC breakpoints should be published as a means to targeted control of CREs.

The results of this study are expected to provide health care providers and infectious disease specialists an informed interpretation of susceptibilities and antibiotic recommendations. It also serves as an important step towards developing targeted strategies to control the spread of CREs in our communities. These results will help to improve antibiotic stewardship in Nevada by determining the prevalence of the KPC gene among CRE isolates from different health institutions in Las Vegas.

In conclusion, timely intervention, such as good infection control practices, rapid detection, and prudent use of antibiotics will ensure that the spread of carbapenem resistance among organisms is kept under control. Future studies should include a real-time PCR assay that is capable of detecting multiple resistance genes to provide rapid and accurate detection of carbapenem resistance.

APPENDICES

Appendix A – Vitek 2 GN69 Card Information

Antibiotics tested
ESBL Confirmation Test
Ampicillin
Amoxicillin/ Clavulanic Acid
Ampicillin/Sulbactam
Piperacillin/Tazobactam
Cefazolin
Ceftazidime
Ceftriaxone
Cefepime
Ertapenem
Imipenem (new formula)
Gentamicin
Tobramycin
Ciprofloxacin
Levofloxacin
Nitrofurantoin
Trimethoprim/Sulfamethoxazole

Appendix B – Vitek 2 GN XN06 Card Information

Antibiotics tested
Ticarcillin/Clavulanic Acid
Piperacillin
Cefalotin
Cefuroxime
Cefuroxime Axetil
Cefotetan
Cefoxitin
Cefpodoxime
Cefotaxime
Ceftizoxime
Aztreonam
Doripenem
Meropenem
Amikacin
Nalidixic Acid
Moxiflaxacin
Norfloxacin
Tetracycline
Tigecycline

Table 3: DNA Concentrations and PCR Results for CRE Isolates.

Test Organism/Sample Identification	**Average 260/280 Ratio (n=2)**	**Average DNA Conc. (ng/µl; n=2)**	**DNA Conc. per PCR rxn.**	**PCR Results**	
				Mean C_T Value (n=2)	**SD**
Klebsiella pneumoniae ATCC 13883	1.929	33.8	16.9	undetermined	N/A
K. pneumoniae ATCC 4352	1.767	103.8	51.9	undetermined	N/A
K. pneumoniae ATCC BAA 1706	1.779	37.5	18.8	undetermined	N/A
K. pneumoniae ATCC 700603	1.819	61.3	30.6	undetermined	N/A
Enterobacter hormaechei ATCC BAA 2082	1.811	131.3	65.6	17.14	0.89
K. pneumoniae ATCC BAA 1705	1.750	38.8	19.4	18.80	0.08
CRE 001	2.434	28.8	14.4	21.19	0.08
CRE 002	2.000	25.0	12.5	17.52	0.05
CRE 003	1.500	21.3	10.6	18.41	0.01
CRE 004	2.750	8.8	43.8	undetermined	N/A
CRE 005	1.734	25.0	12.5	20.48	0.08
CRE 006	2.042	17.5	87.5	18.99	0.08
CRE 007	2.665	16.3	81.3	20.22	0.25
CRE 008	1.500	13.8	68.8	28.00	2.30
CRE 009	4.500	18.8	93.8	26.41	2.13
CRE 010	5.000	12.5	62.5	27.23	0.34
CRE 011	1.500	8.8	43.8	23.48	0.09
CRE 012	1.625	16.3	81.3	26.37	1.41
CRE 013	2.355	30.0	15.0	19.97	0.07
CRE 014	1.500	11.3	56.5	20.97	0.61
CRE 015	0.625	21.3	10.7	20.71	0.14
CRE 016	0.834	18.8	94.0	31.70	0.53
CRE 017[a]	2.095	25.0	12.5	20.69	0.17
CRE 018	1.200	17.5	87.5	23.90	1.24
CRE 019[a]	2.200	12.5	62.5	25.15	1.30
CRE 020	2.084	23.8	11.9	19.61	0.15
CRE 021	8.188	25.0	12.5	undetermined	N/A
CRE 022	5.000	17.5	87.5	25.10	0.38
CRE 023	1.684	22.5	11.3	20.79	0.20
CRE 024	3.179	27.5	13.8	20.28	0.00
CRE 025	1.000	15.0	75.0	24.02	0.21
CRE 026	1.800	11.3	56.5	23.42	0.11

Test Organism/Sample Identification	Average 260/280 Ratio (n=2)	Average DNA Conc. (ng/µl; n=2)	DNA Conc. per PCR rxn.	PCR Results	
				Mean C_T Value (n=2)	SD
CRE 027	1.072	17.5	87.5	19.79	0.85
CRE 028	0.417	3.8	19.0	24.91	0.08
CRE 029	0.834	15.0	75.0	undetermined	N/A
CRE 030	1.000	20.0	100.0	25.94	0.08
CRE 031	1.272	18.8	94.0	18.12	0.13
CRE 032	1.083	16.3	81.5	21.81	0.17
CRE 033	0.650	7.5	37.5	undetermined	N/A
CRE 034	1.104	21.3	10.7	19.04	0.16
CRE 035	1.134	21.3	10.7	19.72	0.03
CRE 036	1.205	21.3	10.7	undetermined	N/A
CRE 037	1.250	25.0	12.5	19.12	0.06
CRE 038	1.526	36.3	18.2	17.49	0.07
CRE 039	1.188	20.0	100.0	undetermined	N/A
CRE 040	2.028	47.5	23.8	undetermined	N/A
CRE 041	1.258	31.3	15.7	19.26	0.07
CRE 042	1.143	17.5	87.5	undetermined	N/A
CRE 043	1.320	35.0	17.5	18.35	0.05
CRE 044	1.370	32.5	16.3	21.13	0.30
CRE 045	0.033	2.5	12.5	23.91	0.37
CRE 046	1.252	37.5	18.8	18.23	0.03
CRE 047	1.438	23.8	11.9	18.80	0.04
CRE 048	1.243	37.5	18.8	18.70	0.00
CRE 050	1.091	33.8	16.9	undetermined	N/A
CRE 051	1.774	28.8	14.3	19.59	0.02
CRE 052	2.000	30.0	15.0	undetermined	N/A
CRE 053	1.835	42.5	21.3	18.18	0.03
CRE 054	1.956	36.5	18.3	17.92	0.06
CRE 055	1.772	40.0	20.0	18.81	0.01
CRE 056	1.571	27.5	13.8	18.74	0.21

Conc. = concentration; ATCC = American Type Culture Collection; rxn. = reaction; SD = standard deviation. [a]*CRE 017* and *CRE 019* are from the same patient.

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