Optimal Empiric Therapy Against Clinical Isolates of *Enterobacter cloacae* in an In Vitro Pharmacodynamic Model

By Harris Iacovides

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Optimal Empiric Therapy Against Clinical Isolates of *Enterobacter cloacae* in an In Vitro Pharmacodynamic Model

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Abstract

Enterobacter species are an important cause of nosocomial infections, ranking number-one in the medical intensive care unit for Gram-negative bacteremia. Isolates can exhibit high levels of resistance for agents like third-generation cephalosporins. Such resistance has been shown to confer negative mortality outcomes, as well as adds to healthcare-related economic burden by increasing hospital costs. Equally concerning, *Enterobacter* species can rapidly develop resistance during third-generation cephalosporin therapy by inducing AmpC-type β-lactamase production or selecting derepressed mutant subpopulations. Using an in vitro pharmacodynamic model (IPDM), we simulated the emergence of third-generation cephalosporin resistance in *Enterobacter cloacae* during an initial empiric ceftazidime course, and then characterized the ability of combination therapy with ciprofloxacin or gentamicin to prevent the acquisition of such resistance. Ceftazidime monotherapy consistently selected high-level resistance after 72 h of therapy whereas combination therapy prevented the emergence of resistance. Further in vivo investigation is warranted in animal and clinical trials.

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1. INTRODUCTION

Antibiotic resistance in nosocomial pathogens poses clinical dilemmas by limiting treatment options, and negatively affecting patient morbidity and mortality. *Enterobacter cloacae* is among the most frequently isolated Gram-negative bacilli in the critically ill. Clinical isolates display high levels of β-lactam resistance with rates of 15 to 40% for broad-spectrum agents such as third-generation cephalosporins. Treatment requires specific knowledge of the organism since susceptible isolates can rapidly develop resistance during the course of extended-spectrum β-lactam therapy. Such treatment failures are thought to occur due to the selection and overgrowth of stably–derepressed subpopulations expressing very high levels of chromosomal AmpC-type β-lactamase. Chow and colleagues were the first to demonstrate in a multicenter prospective trial the association between third-generation cephalosporin exposure and the isolation of antibiotic-resistant *Enterobacter* species. Of great interest however, was a subsequent finding by Schwaber and colleagues that fluoroquinolones in combination with third-generation cephalosporins were protective in preventing the isolation of a resistant *Enterobacter* isolate on first culture. Alternatively, dual therapy consisting of an aminoglycoside plus a third-generation cephalosporin demonstrated no reduction in resistance rates. These observations have lead to inconsistent recommendations for the use of antibiotic combinations for the treatment of *Enterobacter* infections in the clinical setting. Furthermore, the study of such strategies to prevent the development of resistance during therapy is limited.

The in vitro pharmacodynamic model (IPDM) is widely used in the pre-clinical study of antimicrobials and has been instrumental in characterizing pharmacodynamic indices as predictors of clinical outcome. More recently, the IPDM has been used to study novel treatment strategies for antibiotic-resistant infections. It provides the opportunity to test antibiotic therapies prior to clinical trials. As such, the emergence of resistance in clinical isolates can be re-created to determine the effects of various antibiotic regimens, alone and in combination. Even though resistance through selective pressure in *Enterobacter* species is particularly suited to study in the IPDM, no such trials have been published to date. Given the high incidence of *Enterobacter* infections and negative outcomes observed when resistance develops, IPDM research to characterize practical strategies for preventing resistance is needed.

1.1 Hypothesis

The co-administration of ceftazidime with ciprofloxacin or gentamicin will reduce selective pressures exerted by ceftazidime and prevent the emergence of antibiotic-resistant *Enterobacter cloacae*.

1.2 Research Goals

Antimicrobial combinations have been widely advocated in the prevention of resistance, however such strategies remain to be characterized and validated. Given the frequency of clinically significant *E. cloacae* infections and their propensity to acquire resistance during therapy, our goals were to:

1. Simulate the emergence of third-generation cephalosporin-resistant *E. cloacae* during an initial course of ceftazidime, using an in vitro pharmacodynamic model (IPDM) and,

2. Characterize the effects of combination therapy of ceftazidime with a fluoroquinolone (ciprofloxacin) or an aminoglycoside (gentamicin) on the acquisition of resistance.

2. BACKGROUND

2.1 Enterobacter

Enterobacter species are gram-negative, motile aerobic bacilli belonging to the Enterobacteriaceae family, known to colonize the gastrointestinal tracts of 40-80% of humans. While similar to *Klebsiella* spp., *Enterobacter* species can usually be distinguished by their motility, positive ornithine decarboxylase test and negative urease test [1]. Not all of the 14 identified species have been described as causal agents of human disease, but of those that have, *E. agglomerans, E. aerogenes, E. cloacae*, and *E. sakazakii* cause the vast majority of infections. Of these four, the major pathogenic species is *E. cloacae*, which typically arises from the normal intestinal microflora of hospitalized individuals [2]. As opportunistic pathogens, they rarely cause disease in healthy individuals. The incidence of *E. cloacae* infection has steadily increased in the past two decades, likely due to the increasing numbers of immunocompromised patients as well as those with indwelling devices. Weischer and colleagues hypothesized that *Enterobacter* species may have an enhanced affinity for implanted devices compared to other organisms [3], but were unable to demonstrate this in a follow-up study [4]. Sites of infection include the urinary tract, lung (nosocomial pneumonia), bloodstream, skin and soft tissue (burns, ulcers, surgical sites), and central nervous system (nosocomial meningitis post-neurosurgery) [5]. Risks of developing such infections include prolonged hospital stay particularly if in intensive care units, prior antimicrobial use, serious comorbidities, immunosuppression, advanced age, and the presence of foreign

devises [1, 5]. While *Enterobacter* species are twice as likely as Gram-positive organisms to cause patient mortality in sepsis [6], the contribution of virulence factors in this genus remains poorly described. Aside from endotoxin, a virulence factor common to other Gram-negative organisms, little is known about *Enterobacter*'s pathogenic potential [7]. As in other bacteria, presence of a gelatinous polysaccharide capsule outside the cell wall may play a role in serum resistance and lack of phagocytosis. Aerobactin, often produced by strains of *E. cloacae*, is thought to affect the extent of translocation of the organism from the intestinal tract, and/or boost the ability of the organism to multiply following translocation. Bacterial adherence to tissue cell cultures and expression of type-1 fimbriae may also add to the organism's virulence profile [7].

Recommended therapy for confirmed *Enterobacter* infection includes fluoroquinolones (e.g., ciprofloxacin), carbapenems (e.g., imipenem, meropenem) or fourth-generation cephalosporins (e.g., cefepime) [5]. Even when susceptible, other β-lactams including extended-spectrum penicillins (e.g., piperacillin) and third-generation cephalosporins should be avoided. Such agents are susceptible to degradation by AmpC β-lactamases induced by their presence. Furthermore, they are able to select mutant sub-populations with constitutive, high level β-lactamase production, and cross-resistance to other β-lactams. However, third-generation cephalosporins are also widely used as empiric therapy particularly in critically ill patients.[8] The optimal use of these agents, alone or in combination, is critical to minimize further increases in resistance and prolong their usefulness in the clinical setting.

2.2 Mechanisms of β-lactamase-Mediated Resistance in *Enterobacter*

In *E. cloacae*, β-lactam resistance is mediated via degradation by β-lactamase enzymes. *Enterobacter* species are intrinsically resistant to "narrow-spectrum" β-lactams due to basal, low-level enzyme production of AmpC-type β-lactamase. Wild-type *E. cloacae* secrete basal levels of β-lactamase enzyme, regulated by a chromosomal ampC gene. Thus, isolates are intrinsically resistant to ampicillin, amoxicillin, amoxicillin-clavulanic acid, first and second-generation cephalosporins, and cefoxitin. Classified as a Bush Group 1, Ambler Molecular Class C enzyme, such β-lactamases are also referred to as cephalosporinases due to their preferred substrate. Resistance occurs regardless of environmental conditions and without additional genetic alteration. A central property of AmpC β-lactamases, however, lies in their inducible nature in the presence of β-lactam antibiotics. Furthermore, antibiotic-resistant mutations occur at relatively high frequencies compared to those in other organisms. As such, resistant sub-populations are likely present in clinically significant infections and prone to selection with exposure to antibiotic pressures. Few β-lactams including carbapenems (e.g., imipenem, meropenem) and fourth-generation cephalosporins (e.g., cefepime) have enhanced permeability and structural stability which allows them to retain activity in the presence of high level AmpC expression [9].

β-lactamase Induction

AmpC β-lactamases demonstrate an inducible response to β-lactam exposure during therapy [5]. The phenomenon is observed in organisms expressing the inducible

AmpC enzyme including clinically relevant Enterobacteriaceae such as *E. cloacae, Citrobacter freundii, Hafnia alvei, Morganella morganii, Providencia rettgeri* and *P. stuartii* and *Serratia marcescens*. β-lactams demonstrate a gradient of induction potential for enzyme production [10-14]. Carbapenems and the second-generation cephalosporin subclass, cephamycins (e.g., cefoxitin, cefotetan) are among the strongest inducers. Aztreonam exhibits the weakest induction potential, with third-generation cephalosporins being classified as moderate (TABLE 1). The mechanism of induction in organisms possessing inducible AmpC β-lactamase is related to the presence of a regulatory AmpR protein. The AmpR regulator acts as a repressor to inhibit high-level ampC expression in the absence of β-lactam exposure. AmpR binds to the cytosolic precursor for peptidoglycan, UDP-muramyl pentapeptide, and is thus prevented from activating AmpC. However, with β-lactam exposure, penicillin binding proteins (PBPs) involved in peptidoglycan metabolism are inhibited and breakdown products of the murein structure accumulate. It appears that strong versus weak inducers differ in their relative affinity for PBPs such as PBP 1a and 1b, 2, 4, 7a and 7b [10, 15-17]. The muropeptides which accumulate in the bacterial cytoplasm displace AmpR from UDP-muramyl pentapeptide. In turn, free AmpR induces ampC transcription to increase the production of β-lactamase [18]. As a key feature, the process is reversible when the β-lactam (inducer) is removed and the organism reverts to its wild-type form.

Derepression

Enterobacter also has the potential to develop resistance during antimicrobial therapy, due to the selection of highly resistant, derepressed mutants [1, 5, 19, 20]. The

mutant frequencies are higher in *Enterobacter* (10^{-5} to 10^{-7}) than in other Gram-negative organisms like *Escherichia coli* (10^{-9}) and thus are more susceptible to selective antibiotic pressure [18, 21, 22]. Early reports (Olson, Richmond, Labia, Then and Yokota) speculated the increased β-lactamase production as the cause of third-generation cephalosporin resistance but lacked the methods to identify the molecular mechanism at hand [23]. Based on *E. coli* estimates, Gram-negative bacteria recycle 40-50% of cell wall peptidoglycans per generation. Through a process involving cleaving by cytosolic AmpD amidase, 90% of the anhydromuropeptide breakdown products are recycled into new peptidoglycan. Mutations in AmpD result in accumulation of cytoplasmic muropeptides which keep AmpR in an active form, i.e. unbound to UDP-muramyl pentapeptide. The net result is mutants with constitutive hyperproduction of AmpC β-lactamase and high-level resistance to most β-lactams [16-18, 24, 25]. The mutant subpopulations are thus prone to selective antibiotic pressures. In vitro studies have demonstrated differences among antibiotics in their ability to select derepressed *Enterobacter* mutants [26]. Chan and colleagues [27] tested seven β-lactams using antibiotic-containing media. Cefamandole, ceftazidime and ceftriaxone selected derepressed mutants at a faster rate than cefoperazone, cefepime, meropenem or imipenem. In another trial, single-doses of β-lactams were tested in a peritonitis mouse model using four *E. cloacae* strains [28]. After 24 hours, peritoneal samples were plated on antibiotic-containing agar. Again, third-generation cephalosporins, ceftazidime and cefotaxime, were most likely to select resistant subpopulations. Resistance was not detected with imipenem. Piperacillin selected resistant mutants in 0-25% of trials,

whereas ceftazidime and cefotaxime selected resistance in 33-83% and 83-100% of trials, respectively. Similar research findings remain to be validated by other investigators.

The clinical relevance of derepression in *E. cloacae* is considerable due to the incidence of infection, relatively high mutant frequency and effects of selective antibiotic pressures from targeted or empiric therapy. Furthermore, derepressed mutants display multi-drug resistance and further limit available treatment options. In such scenarios, cross-resistance to most agents in the β-lactam class of antibiotics is observed; fourth-generation cephalosporins and carbapenems retain activity since they lack vulnerability to β-lactamase attack [29]. (FIGURE 1)

2.3 Cephalosporin Antibiotics

The discovery of cephalosporin antibiotics traces back to the mid-1940s where Giuseppe Brotzu isolated what was eventually purified as cephalosporin C from the fungus *Cephalosporium acremonium* found in seawater sewage [5]. Unlike the analogous five-membered thiazolidine ring found in penicillin, cephalosporins have a six-membered dihydrothiazine ring fused to the β-lactam moiety. Based on the knowledge that modifications at the 6-acylamino site of the 6-aminopenicillanic acid nucleus yielded semi-synthetic penicillins with enhanced antimicrobial activity, chemical modifications of cephalosporin C were undertaken in hopes of augmenting its potency. Treatment of cephalosporin C with acid produced, via hydrolysis, 7-aminocephalosporanic acid (7-ACA), the basic building block of future agents [5, 30].

Modifications at position 1, 3 or 7 have been the basis of the various generations of cephalosporin antibiotics. Alterations at position 7 affect antimicrobial activity, while chemical groups added at position 3 yield pharmacokinetic changes. As an example, for activity against gram-negative bacilli, replacement of the position-7 hydrogen with a methoxy group yields compounds which are more resistant to β-lactamase attack; however, this is at the expense of reduced affinity for the PBPs of gram-positive cocci [5].

The precise mechanism by which β-lactam antibiotics exert their bactericidal effects in susceptible organisms remains to be fully elucidated. The cell wall is essential

for normal bacterial growth and development, and its assembly proceeds in a specific sequence requiring the catalytic action of at least 30 enzymes. Cephalosporin antibiotics, like the penicillins, act by binding to PBPs, of which four types have been identified. Of these, transpeptidases form the cross-links of peptidoglycan in the final step of cell-wall synthesis. β-lactams act as false substrate to PBPs because the amide component of penicillins and cephalosporins is structurally comparable to the D-alanyl-D-alanine natural substrate of the enzymes. This binding in turn renders the enzyme inactive and cell-wall synthesis is affected. Exactly how cell death or lysis ensues is not completely understood [5].

Cephalosporins are polar, water-soluble drugs, available in both oral and parenteral formulations, with the exception of the fourth-generation members which are only supplied as injectable preparations. Currently, there exist four generations of cephalosporins for use in clinical practice, (TABLE 2). with distinctions between each based primarily on microbiologic spectrum of activity. The first-generation agents are mainly useful in the treatment of gram-positive infections, and for this reason are widely utilized in the treatment and prevention of skin and soft tissue infections. Second-generation cephalosporins have enhanced activity against gram-negative bacteria, such as respiratory pathogens (*Haemophilus influenzae* and *Moraxella catarrhalis*) and *Neisseria* species while maintaining activity against staphylococci and nonenterococcal streptococci. The second-generation cephalosporin subclass, the cephamycins, have reduced staphylococcal activity, but enhanced activity against some gram-negative organisms. The cephamycins are unique in that they have appreciable anti-anaerobic

activity, particularly against *Bacteroides fragilis*. Clinically, the discovery of third-generation agents represents an important development in the treatment of many nosocomial infections due to their high potency, broad spectrum of activity, favorable toxicity profile, and enhanced pharmacokinetics such as good CNS penetration. As such, they are considered the drugs of choice for many serious infections in hospitalized individuals. They have been particularly valuable in the treatment of infections caused by penicillin-resistant gram-negative bacilli, such as hospital-acquired pneumonias. Stable derepression of the AmpC β-lactamase in Gram-negative organisms such as *E. cloacae* is the most common scenario conferring resistance to third-generation cephalosporins. While the precise role of induction with respect to clinical treatment failures remains to be elucidated, the emergence of resistance through the selection of derepressed mutant sub-populations is common when infections are treated with third-generation cephalosporin monotherapy [31]. As described above, the third-generation cephalosporins seem to be more efficient at selecting these mutants relative to the other cephalosporins.

The recent past has seen a significant surge in the incidence of organisms producing β-lactam deactivating enzymes. An ever-expanding list of extended-spectrum β-lactamases (ESBLs) and metallo-β-lactamases (MBLs) is eliminating the availability of such drugs for the treatment of infections once susceptible to these therapies. The occurrence of such resistance is significant since cross-resistance among most members of the class is frequent. In the case of MBLs, cross-resistance among unrelated classes of drugs, such as aminoglycosides and fluoroquinolones is common, thus greatly limiting

the available choices of agents for the treatment of such infections. Since dissemination of genes encoding ESBL and MBL resistance is thought to occur as a result of expanded-spectrum cephalosporin use in a particular region, optimal use of these agents is crucial. Also, the high frequency of AmpC β-lactamase-producing organisms conferring resistance to third-generation cephalosporins further highlights the need for the proper use of these agents. Efforts to minimize increasing rates of resistance as a means of salvaging current antimicrobials should be a priority.

2.4 Surveillance Data

Intensive care units (ICUs), an environment under constant antibiotic pressure, are a prime reservoir for *Enterobacter* infections. In ICUs, it is the most common cause of Gram-negative bacteremias, responsible for 10% of infections [32, 33] (FIGURE 2). *Enterobacter* is the second most frequent cause of ICU Gram-negative surgical site infections and pneumonias after *Pseudomonas aeruginosa*. With respect to hospital-wide surveillance, *Enterobacter* species are a common cause of all hospital pneumonias, bacteremias, surgical site infections and UTIs, ranking number two after *E. coli*. The National Nosocomial Infections Surveillance System has shown a relative stability in the incidence of *Enterobacter* infections from 1986 to 2004 [8].

Enterobacter species are intrinsically resistant to penicillin, ampicillin, amoxicillin, and most first and second-generation cephalosporins. Furthermore, they are frequently resistant to broad-spectrum β-lactam agents due to the constitutive overproduction of AmpC β-lactamase, or due to the acquisition of extended-spectrum β-lactamase (ESBL) genes on mobile genetic elements such as plasmids. Rates of resistance to third-generation cephalosporins have been fairy constant over the previous decade, but remain high in the range of approximately 25-40 % [8, 34-37]. Such rates are highest in parts of the world where antibiotic resistance is common, such as Italy, Greece, and Asia [36, 38]. Locally, *Enterobacter* resistance has remained stable since 2000, with resistance to third-generation cephalosporins (ceftazidime and cefotaxime/ceftriaxone) hovering in the 15-20% range. (FIGURE 3) While rates have

not dramatically increased in North America in the last five years, third-generation cephalosporin use remains high, representing the most frequently prescribed drug-class in the medical intensive care unit [8]. As such, efforts geared at the optimal use of these agents are necessary to minimize further increases in resistance and ultimately to prolong the utility of these drugs.

2.5 Clinical Complications of Third-Generation Cephalosporin-Resistant *Enterobacter*

Enterobacter species are among the most frequently isolated gram-negative organisms in hospitalized patients [32, 39]. The clinical implications of resistant *Enterobacter* infections include increased mortality risk and longer and costlier hospital stays [32]. Cosgrove and colleagues examined the health and economic outcomes of patients infected with third-generation cephalosporin resistant isolates. Mortality, length of stay and hospital costs were compared in 46 cases where third-generation cephalosporin resistance developed from an initially susceptible culture. Patients in whom resistance developed demonstrated a 5-fold higher mortality rate (RR=5.02, P=0.01), and had hospital stays that were 1.5-fold longer and costlier (9 days longer, P=<0.001 and $29,000 more, P=<0.001, respectively). More recently, Kang et al [40] conducted a retrospective analysis of 183 patients with *Enterobacter* bacteremia and compared 30-day mortality rates in patients infected with third-generation cephalosporin resistant *Enterobacter* species to those with susceptible isolates. Once again, resistance predicted negative outcomes. Multivariate analysis showed that third-generation cephalosporin resistance was an independent risk factor related to 30-day mortality (OR: 3.69; 95% CI, 1.01-13.52; P= 0.049). Furthermore, resistance was associated with treatment failure at 72 h more often than controls: 33/97 (34%) treatment failures in the susceptible group vs. 49/86 (57%) in the resistant group (P=0.002).

Evidence that previous third-generation cephalosporin use confers significant risks for the subsequent isolation of *E. cloacae*-resistant isolates serves as important

information necessitating that these agents are used properly. This is especially illustrated by the negative outcomes that have been demonstrated after resistance develops.

2.6 Enterobacter resistance in the clinical setting

Individuals harboring resistant *Enterobacter* isolates comprise two different patient scenarios: (i) those in whom resistance is detected on first isolation, and (ii) those in whom an initially susceptible isolate subsequently develops resistance *during* antimicrobial therapy [22, 32].

Initially Resistant Isolate

To investigate the first possible scenario, Jacobson et al. conducted a prospective case- controlled observational study of 386 patients from whom AmpC organisms (*Enterobacter* species, *Pseudomonas aeruginosa, Citrobacter* species and *Serratia marsescens*) were isolated [41]. Of those, 70/386 (18%) were resistant to extended-spectrum cephalosporins, with *Enterobacter* species being the most frequent (46/70, 66%). Compared with the respiratory tract, isolates recovered from blood had a 5.98-fold increased chance of resistance. Previous ceftazidime use was associated with a 6.17-fold increase in the likelihood of resistance (P=0.004); previous cefotaxime or ceftizoxime use demonstrated a 3.08-fold increase (P=0.008). Also, the mean number of previous antibiotics was significantly higher in resistant versus susceptible isolates (3.2 versus 1.8 drugs, P=<0.032). Interestingly, a linear relationship was demonstrated between the number of days of therapy with cefotaxime or ceftizoxime and the likelihood of the development of resistance, however even one day of ceftazidime therapy put the patient at risk of resistance.

More recently Lee and colleagues studied 414 patients with *Enterobacter* bacteremia and risk factors for the subsequent isolation of a third-generation cephalosporin resistant isolate [39]. The median number of antibiotics in the previous 30 days was again associated with resistance to extended-spectrum cephalosporins (OR= 1.8, CI= 1.2-2.6). More specifically, resistance was strongly associated to previous use of extended-spectrum cephalosporins (OR= 5.0, CI= 2.5-10.2). Interestingly, patients previously treated with an extended-spectrum cephalosporin-aminoglycoside combination had no less chance of subsequent isolation of a resistant isolate.

In another trial [22], Schwaber and investigators compared two third-generation cephalosporins to piperacillin/tazobactam to see if either posed a unique risk for subsequent isolation of a resistant isolate. In total, 2,788 patients were retrospectively studied and divided into two groups: broad-spectrum cephalosporin, ceftazidime or ceftriaxone (n = 2,341) or piperacillin-tazobactam (n= 447). Each cohort was followed from the day of receipt of either treatment until the isolation of a broad-spectrum cephalosporin-resistant isolate in a clinical culture. The majority of isolates were *E. cloacae* (80%) with the remainder *E. aerogenes*. Distribution of species and sites of infection were similar between the two cohorts, with respiratory cultures accounting for the majority of isolates (approximately 50%). Investigators found an equivalent risk of 2% for each the broad-spectrum cephalosporin and broad-spectrum penicillin groups. In other words, regardless of which drug was previously given, patients had a 2% chance of *de novo* isolation of broad-spectrum cephalosporin-resistant *Enterobacter* strains. Intensive care unit stay was found to be a strong predictor of resistance (RR= 4.53, P=

<0.001), as was having had a surgical procedure (RR= 1.97, P= 0.015). Of great interest however was the finding that fluoroquinolones were protective in preventing the emergence of resistance to broad-spectrum cephalosporins (RR=0.24, *P*=0.003) when given in combination with either of the two treatments; aminoglycosides on the other hand did not confer protection (RR= 0.98, P= 0.95).

In follow-up, Schwaber and colleagues further analyzed 282 patients retrospectively [42], with third-generation cephalosporin-resistance in the three most implicated gram-negative hospital pathogens with resistance to these agents to determine if fluoroquinolones were protective: *Enterobacter* spp (n= 203), *Pseudomonas aeruginosa* (n= 50) and *Klebsiella pneumoniae* (n= 29). Three classes of antibiotics were significantly associated with isolation of a resistant isolate: β-lactam/β-lactamase inhibitor combinations (HR= 2.52; P= <0.001), ureidopenicillins (HR= 2.55; P= 0.002) and third-generation cephalosporins (HR= 2.84; P= <0.001). Once again, fluoroquinolones were protective (HR= 0.4; P=0.005). This data has important implications given the very high frequency of use of these agents as empiric antibiotic therapy for severe nosocomial infections. The reliance on third-generation cephalosporins and fluoroquinolones in the treatment of hospitalized patients must not be ignored since their indiscriminate use will lead to further increases in resistance. As such, strategies which confer protection with respect to limiting the emergence of resistant isolates are essential and will be the determining factor that dictates how long these agents will be useful in the treatment of infectious diseases.

Resistance During Therapy

In the case of emergent resistance during the course of antibiotic therapy, Chow and colleagues [20] conducted a multicenter prospective observational trial of 129 patients with *Enterobacter* bacteremia. Of 31 patients who had been prescribed a broad-spectrum cephalosporin for an initially susceptible isolate, 6 developed resistance during therapy (19%). This association was not demonstrated for any other antibiotic class: emergence of resistance in 1/89 receiving an aminoglycoside; 0/50 receiving other β-lactams (imipenem, piperacillin, ticarcillin, aztreonam, mezlocillin, ticarcillin-clavulanate). Patients receiving combination antibiotics, mainly with aminoglycosides, demonstrated no reduction in the emergence of resistance. Namely, resistance occurred in 2/12 patients on third-generation cephalosporin monotherapy and 4/19 patients on combination therapy with an aminoglycoside. However, when patients were separated and analyzed based on those "severely ill" (based on vital sign alterations, diminished mental status, mechanical ventilation and cardiac arrest) or "less ill", a mortality benefit was demonstrated for those "severely ill" who received combination therapy.

More than a decade later, Kaye et al. [19] retrospectively examined 477 patients with culture-positive *Enterobacter* isolates, susceptible to third-generation cephalosporins to examine risk factors for the emergence of resistance. Of those initially susceptible strains, 343 were *E. cloacae*, 108 were *E. aerogenes*, and 26 were other *Enterobacter* species. Patients were followed until the isolation of a third-generation cephalosporin resistant *Enterobacter* was documented, or until hospital discharge or death. They found

that in patients receiving a third-generation cephalosporin for a documented *Enterobacter* infection, 19% (as in the Chow study) subsequently cultured a third-generation cephalosporin resistant isolate. A significantly higher incidence was reported when the site of infection was blood versus urine, tissue or wounds (4/14, 29% vs. 5/ 67, 7%, respectively; P=0.04). While no molecular typing was undertaken, species and site of isolation were identical in the vast majority of cases, suggesting both susceptible and resistant isolates were the same clone. Multivariate analysis demonstrated third-generation cephalosporin exposure to be a strong, independent predictor for the development of resistance (RR= 2.3, P=0.01). Again, an important association demonstrating the protective effects of fluoroquinolones was shown, decreasing the risk for the emergence of broad-spectrum cephalosporin resistance (OR=0.4, *P*=0.03). Furthermore, patients receiving combination therapy consisting of a broad-spectrum cephalosporin plus either an aminoglycoside or imipenem demonstrated a trend towards decreasing risk for the emergence of resistance, however this finding was not deemed statistically significant (RR= 0.5, P=0.38 and RR= 0.5, P= 0.32, respectively).

In an attempt to prospectively study a combination regimen, Fussle and colleagues studied 38 mechanically ventilated ICU patients with pulmonary infections caused by *E. cloacae* [43]. Patients were given cefotaxime 2 g and tobramycin 80 mg both intravenously every 8 hours. Tracheobronchial secretions were monitored daily with subsequent bacterial enumeration and MIC determinations made every 3 days. Of patients receiving this regimen, 18 of 38 (47%) cultured a resistant *Enterobacter* isolate

within 6 days. No comparative regimen was studied however thus leaving any beneficial effects of the combination regimen relative to monotherapy undefined.

These clinical trials, with the Chow study in particular, have formed the basis of knowledge that third-generation cephalosporins may confer a unique risk in the clinical setting for selecting *E. cloacae*-resistant mutants, showing that the rate of emergence of resistance during treatment can approach 30% in bacteremia. The small size of the Chow study however may have greatly limited its ability to accurately define the precise risk conferred by cephalosporin monotherapy. Furthermore, the size of the trial may have underestimated the benefits of combination therapy, particularly since a trend of improved survival was shown for those patients deemed "severely ill. Despite being retrospective in nature, the large Kaye trial documented a similar risk as the Chow study for the emergence of resistance, but with respect to bacteremia, demonstrated that a higher risk was observed (29% vs. 19% in the Chow trial). Furthermore, Kaye and colleagues were the first to report the potential protective benefits of fluoroquinolone combination therapy. While the risks conferred by cephalosporin monotherapy stand little debate, whether or not optimal combination therapy for such infections could prevent the emergence of resistance until directed therapy can be administered remains an elusive question that requires further investigation.

2.7 Antimicrobial Combinations

The role of antimicrobial combinations in the treatment of infectious diseases has undergone much debate. Specifically, weather or not dual therapy improves patient outcomes or lessens the likelihood for the emergence of resistance remains to be definitively proven. Unlike for human immunodeficiency virus (HIV) and tuberculosis infections, the use of more than one agent for the specific purpose of minimizing the emergence of resistance in severe Gram-negative infections remains to be verified. In the case of *Enterobacter*, Chow and colleagues showed an improved survival for severely-ill patients given combination therapy versus monotherapy, as already discussed, however the rate of the emergence of resistance was similar between monotherapy and combination therapy groups. Leibovichi et al. conducted an observational prospective trial of 2,124 patients with gram-negative bacteremia [44]. Of 1,878 patients that were available for follow-up, no difference in mortality was demonstrated between β-lactam monotherapy or combination therapy with an aminoglycoside, except for neutropenic patients (O.R.= 0.2, CI= 0.05-0.7). The emergence of resistance however was too rare an event to analyze and thus remained unanswered by the trial. A recent meta-analysis of β-lactam/aminoglycoside combinations versus β-lactam monotherapy failed to demonstrate any advantages for combination therapy with respect to limiting the emergence of resistance [45]. However, only one of the 8 studies analyzed [46] included *Enterobacter* species as a predominant organism (along with *Staphylococcus aureus, Haemophilus influenzae*, and *Acinetobacter* species) in the patient population. Furthermore,

monotherapy arms consisted of either ceftazidime or cefoperazone- a third-generation agent shown to less readily select resistance in *Enterobacter* [27].

The ability of combination therapy to affect the emergence of resistance in animal models has been demonstrated. Michea-Hamzehpour and colleagues [47] were able to show the effects of dual therapy in a mouse peritonitis model of *Enterobacter* infection. Single doses of ceftriaxone led to increasing MIC's at 24 hours (10 of 14 mice), however combination therapy with amikacin or pefloxacin significantly decreased this effect (7 of 19 mice and 0 of 20 mice, in the amikacin + ceftriaxone or amikacin + pefloxacin groups, respectively). A follow-up study by the same authors demonstrated similar results [48]. A more recent trial of experimental pneumonia in rats [49] failed to demonstrate any emergence of resistance with either mono- or combination therapy using cefepime, ceftazidime and imipenem alone or combined with amikacin. The authors correctly hypothesized that this may have been due to the low inoculum size used in the study or that the duration of experiments (60 hrs) may not have been sufficiently long enough to select a resistant subpopulation.

Thus, while animal models have demonstrated some success in showing a beneficial effect for the use of combination therapy to prevent the emergence of resistance, this has not translated to similar findings in the clinical setting. As discussed however, an analysis specific to *Enterobacter* has not been undertaken in a prospective manner. Given the strong preliminary animal data supporting such a benefit, more work is needed to determine the role of such an approach for this clinically important

organism, particularly with the limited availability of antimicrobials for severe antibiotic-resistant Gram-negative infections.

2.8 IPDM Research

Traditional methods of determining antibiotic activity have almost exclusively relied upon static measures of antimicrobial pharmacodynamics such as the minimum inhibitory concentration, MIC, of the microorganism. Here, the isolate being studied is exposed to fixed concentrations of antimicrobial agents in decreasing two-fold dilutions to determine the concentration at which visible growth is inhibited; depending how high or low this value is relative to achievable serum concentrations with standard dosing, the organism is determined to be either resistant or sensitive as defined by The Clinical and Laboratory Standards Institute (CLSI) susceptibility breakpoint guidelines. The MIC alone however fails to mimic the in vivo setting where fluctuating antibiotic concentrations exert their effects on a bacterial population. The goal of antimicrobial pharmacodynamic studies is to link the achieved unbound drug concentrations at the site of infection with bacterial eradication and/or clinical cure. Except in the rare case of constant intravenous infusion, antibiotics are administered periodically (e.g. every 12 hours), thus producing a serum peak after administration, and a trough immediately prior to the next dose.

The in vitro pharmacodynamic model (IPMD) was developed to overcome the limitations of static drug exposure in hopes of more closely simulating in vivo pharmacokinetics to allow for optimal characterization of antibiotic selection and dosing [50]. Measures of antimicrobial activity can be linked to in vivo concentration

profiles to allow for an in vitro study environment that is more closely representative of the treatment of human infection. (FIGURES 4 & 5)

Pharmacokinetics refers to the mathematical time course of drugs in the body as related to the processes of absorption, distribution, metabolism and excretion. Specific equations have been derived which help approximate and predict resultant drug levels in the human body following any given dose. Using a one-compartment model, the body is treated as a single space in which a given dose of drug is evenly distributed and eliminated. In this situation, the drug concentration in the body can be described with the collective information from three values: the dose (mg of drug injected as a bolus), the volume of distribution (Vd) (the volume or space in which the dose distributes) and the elimination rate constant (ke) (the overall removal of drug from the body compartment by all processes- metabolism and excretion).

Drug elimination that is proportional to the amount of drug in the compartment is described by the process of first-order elimination. At higher drug concentrations more drug is removed, and elimination of drug tapers as the concentration drops.
The drug concentration in the body compartment can be determined during any time point with the use of the equation:

$$\mathbf{C = C_0 \times e^{-ket}}$$

Where, C = the drug concentration at a specific time point (t),

C_0 = the peak concentration immediately following a bolus dose, and

ke = the elimination rate constant.

Another fundamental value in the description of first-order kinetics is the elimination half-life, or $t_{1/2}$. This represents the time required for the drug concentration in plasma to fall to half its original value. It can be determined from the following:

$$\mathbf{ln\ (C_0 / C_{1/2})\ =\ ke\ x\ t_{1/2}}$$

Where C_0 = initial concentration, and

$C_{1/2}$ = concentration after one half-life (or $\mathbf{ln\ 2 / ke = 0.693 / ke = t_{1/2}}$)

Finally, the clearance (Cl) of drug represents an important kinetic value that describes how quickly a drug is removed from the body compartment. It represents the volume of plasma that is cleared of drug during a specified time interval (e.g. per min). Essentially, it reflects how much drug is being removed by the kidneys (and the other non-renal organs of elimination) per unit of time (e.g. mL/min).

With $\mathbf{dose = C\ x\ Vd}$, then

$$\mathbf{ke\ x\ Vd = Cl}$$

Several models have been described. In the one-compartment, multiple-dose IPDM, antibiotic levels decline mono-exponentially from the infection flask according to the equation:

$$\mathbf{C_t = C_0\ x\ e^{-ket}}$$

Where C_t = concentration at any given time point, and

ke = elimination rate constant, calculated by Cl, or IPDM flow rate/Vd or volume of infection flask.

A computerized peristaltic pump delivers sterile nutrient broth via flexible tubing to the central chamber (infection flask) at a rate determined by the half-life of the antibiotic being studied. The infection flask consists of a central fixed-volume chamber into which a standard inoculum of logarithmic-phase bacteria is injected. Antibiotic is subsequently injected into the infection flask, with the net result being a drug-concentration vs. time profile similar to that achieved in the clinical setting- that is, immediately following antibiotic administration, an antibiotic peak concentration is achieved followed by drug elimination from the chamber into a waste vehicle at a rate equal to the antibiotic's half-life. During the course of an experiment, samples are drawn from the infection flask, serially diluted in sterile normal saline and plated onto appropriate media agar for determination of colony counts. The IPDM serves as a dynamic system by which in vivo antibiotic exposures can be studied. It enables the more precise simulation of human pharmacokinetics, something that is more difficult to model in animal studies. A continuous nutrient support further enables a researcher to carryout prolonged experiments, allowing for the analysis of multiple samplings over the course of several days (48 to 72 hours). This allows one to better define the usefulness of specific dosing regimens and aids in the selection of individual agents with maximal bacterial kill in the hopes that improved outcomes will be achieved in the clinical arena. It also allows for the ability to study high inoculum sizes, so as to better study antibiotic-resistant organisms that may develop resistance at relatively low frequencies, e.g. 1 in 10^8 bacterial cells. Lastly, it allows for the study of various combinations of agents, so as to determine those which confer optimal outcome measures versus those which may be neutral or antagonistic.

The IPDM possesses certain limitations however. Lack of host defenses, given the in vitro environment, may be regarded as a drawback of the IPDM. Absence of factors such as neutrophils, immunoglobulins, complement and cytokines may under-predict the effects or value of a given drug or dosing regimen in an otherwise healthy individual [50]. That being said, the IPDM thus most closely mimics infection in the neutropenic host, i.e. in a patient without immune support. This patient population comprises a challenge to clinicians since outcome is strongly governed by the selection of the correct drug in the correct dose; appropriate therapy is crucial since without this, infection can rapidly disseminate [5]. Technical problems are also possible with the use of the IPDM. The formation of biofilms within the surface of the glass apparatus has been described [51]. However, the occurrence as such may actually be a better in vitro representation of bacteremia since human bloodstream infections originate secondarily from the shedding of bacteria at an initial "biofilmed" source, e.g. indwelling central venous catheter [52, 53]. Other disadvantages of the IPDM relate to technical problems which may arise from contamination of the apparatus, clotting of tubing or adherence of bacteria in the media. These issues can generally be controlled by careful and meticulous technique and through experienced execution, so as to allow for consistent inoculum size, sampling methods, etc.

More recently, the IPDM has become increasingly used as a system for studying the emergence of antibiotic-resistant organisms [54-59]. Methods of preventing the selection of resistant phenotypes can be studied using various doses and combinations over extended intervals. Studies to date have mostly focused on

resistance in *Pseudomonas aeruginosa*, as well as Gram-positive organisms (*Streptococcus pneumoniae, Staphylococcus aureus*). In the case of *Enterobacter*, the IPDM serves as an ideal system for the study of resistance. Since it is well appreciated that the emergence of resistance during the treatment of *E. cloacae* infections occurs as a direct result of antibiotic pressure, the IPDM in vitro environment nicely allows for the characterization of methods for its prevention.

Only one published trial to date has investigated the emergence of resistance in *Enterobacter* using the IPDM. Palmer and colleagues [60] examined the pharmacodynamics of cefepime, ceftazidime, cefotaxime, and ceftriaxone against *S. aureus, E . aerogenes and Klebsiella pneumoniae* using an IPDM. An isogenic pair of *E. aerogenes* was used in the study, obtained from a patient who received ceftazidime for an initially sensitive isolate that developed resistance during therapy. Subsequent to ceftazidime treatment, a resistant isolate was cultured that was stably derepressed for a Bush type-1 cephalosporinase. However, in the IPDM, no regimen selected for resistance using the ceftazidime-sensitive isolate. The authors point out that this may have been due to the duration of the experiment (48 hours) which may have not been sufficiently long enough to allow a resistant subpopulation to emerge. To date, no published studies have looked at the emergence of resistance in *E. cloacae* using the IPDM. Furthermore, no studies have investigated methods for its prevention. More data is strongly needed to guide the development of a prospective clinical trial.

3. MATERIALS & METHODS

3.1 Isolates, antibiotics and media

Clinical *E. cloacae* blood isolates were identified using our institution's microbiology laboratory database (Microscan®, Dade Diagnostics, Mississauga, ON). Of those isolates, three were initially selected for the purposes of the study. In preliminary experiments to determine the relative ease of resistance selection of each isolate, it was found that Isolate 2 lacked the ability to develop resistance following ceftazidime monotherapy. Therefore, another isolate, Isolate 4 was chosen and used for the study. The three isolates (Isolates 1, 3 and 4) were transferred to skim milk in cryo-vials for storage at –70 ° C. MICs were confirmed by E-Test® (AB Biodisk, Solna, Sweden) for the antibiotics being studied (TABLES 3a and 3b). Standard 0.5 McFarland bacterial suspensions (0.5 x 10^8 cfu/ml) were prepared for both test isolates as well as *P. aeruginosa* ATCC # 27853 as quality control to check for strip accuracy. Five single colonies from a fresh overnight plate were transferred to 1 ml sterile 0.9% normal saline and visually adjusted to a 0.5 McFarland standard turbidity using a reference control tube. A sterile cotton-tipped swab was used to inoculate a Mueller-Hinton agar plate with a uniform bacterial lawn of growth. Once dried, E-Test® strips containing a ready-to-use predefined gradient of antibiotic were laid onto the agar. Precise MIC determinations were performed by reading the MIC directly from the strip after incubation at 35 °C f or 20 hours. All drugs were initially active against the isolates tested, as determined by MIC and as defined by CLSI susceptibility breakpoint guidelines. For use in the IPDM experiments, clinical formulations of ceftazidime,

ciprofloxacin and gentamicin were obtained from GlaxoSmithKline, Bayer and Sabex respectively. Mueller-Hinton broth (Difco Laboratories, Detroit, MI), cation-adjusted (CS-MHB) with 25 mg/L of calcium and 12.5 mg/L of magnesium was used for the IPDM experiments.

Stock plates were made by sub-culturing the skim-milk freezer stocks onto Trypticase Soy Agar (TSA) plates, followed by incubation at 35 ° C for 18-24 hours. The first plate obtained from the freezer stock was used to make a second plate by transferring 5-10 colonies onto a fresh TSA plate; this was done to ensure organisms were fully active following –70 ° C storage. This second plate was used for experimental purposes, and refrigerated at 4 ° C. After four weeks at 4 ° C, new stocks were obtained from the –70 °C main stock.

3.2 In vitro pharmacodynamic model (IPDM)

A one-compartment IPDM was used to simulate *E. cloacae* bacteremia in an immunocompromised host. All individual parts of the IPDM were prepared in advance, by wrapping each piece in autoclave bagging, sealing with masking tape, and sterilizing via autoclaving. The large volumes of broth in the nutrient supply flasks were allowed to cool and stored at incubation temperature for 24 hours to ensure sterility.

Using the clinical isolates, bacterial suspensions were prepared (as per MIC preparation) and adjusted to a 0.5 McFarland's standard density from fresh overnight plates; 2.5ml was injected into the 250 mL "infection" flask to yield a starting inoculum of approximately 1 x 10^6 cfu/mL. Sufficient time was allowed (0.5 hour) to ensure bacteria were in logarithmic growth phase. Clinical doses of ceftazidime 2 grams every 12 hours alone and in combination with ciprofloxacin 400 mg every 12 hours or gentamicin 6 mg/kg high-dose every 24 h were studied to simulate three days of therapy TABLES 4 & 5. In the model used, it is assumed that following an intravenous bolus dose, drug absorption is instantaneous. The resultant plasma concentration is assumed to be uniform, with the magnitude dependent on the volume in which the dose is distributed. The half-life used (3 hours) was chosen so as to achieve a concentration-time profile similar to that observed with clinical dosing in humans. FIGURES 6-8

Population pharmacokinetic-predicted peak concentrations of unbound, steady-state serum levels were studied: C_{max}= ceftazidime 150 mcg/mL, ciprofloxacin 3 mcg/mL, gentamicin 20 mcg/mL. (TABLE 6)

A computer-driven pump (Masterflex, Cole-Parmer, Chicago, IL) using flexible tubing delivered sterile CS-MHB through the infection compartment yielding the appropriate antibiotic half-life. Flow rates to deliver broth through the IPDM were determined using mean population-expected pharmacokinetic parameters of ceftazidime:

Clearance (L/h) = ke (h-1) x Volume of distribution (L)

Calibration of flow rates were performed at the onset of experiments (time 0 hour), and verified again at 24, 48 and 72 hours by measuring waste from the infection flasks. If intra-experimental flow rates differed by greater than 5% the data was excluded.

IPDM samples were collected from central compartments at 0, 24, 48 and 72 hours in preliminary experiments to determine the necessary amount of time needed for a resistant sub-population to be selected. Since no resistance was documented at 24 hours, variable resistance was documented at 48 hours, and near-uniform resistance was documented at 72 hours, three days of therapy was chosen for the study. Samples at time 0 and 72 hours were used to determine bacterial counts (CFU/mL) and check MICs. Bacterial colony counts were determined by serially diluting 100 mcL samples in 900 mcL sterile normal saline at 4 °C and plating onto solid TSA plates. Plates were incubated for 24 hours, then counted at the dilutions containing viable colonies between 10 and 100. The lower limit of detection was 1×10^2 CFU/mL. MICs were repeated for bacteria recovered at 72 hours. Samples identified as resistant at 72 hours were further tested by replating 5-10 colonies on fresh, antibiotic-free TSA plates for 5 consecutive days. MICs were then rechecked and compared to 72 hour values to determine if that

particular experiment represented a reversibly-induced or stably-derepressed population. All monotherapy experiments were conducted ten times for each isolate, and nine times for each isolate/antibiotic combination on separate occasions.

3.3 Bioassay

Predicted antibiotic trough concentrations were checked and validated by an *Escherichia coli* bioassay for the ceftazidime monotherapy experiments TABLE 7. A standard curve was constructed using the pharmaceutical ceftazidime formulation (GlaxoSmithKline, 280mg/mL) diluted at various concentrations. A 0.5 McFarland standard was prepared for *E. coli* ATCC # 25922 and swabbed onto TSA to yield a uniform bacterial lawn, as per MIC procedure. Once dried, a 10 mcL sample was dropped onto the center of the plate, allowed to dry, and incubated overnight at 37° C for 18-24 hours. The zone of inhibition was measured and recorded in millimeters (mm). For each concentration tested, the procedure was repeated ten times, with the values averaged and used to make the standard curve. With respect to testing ceftazidime levels in the IPDM, samples at the end of the dosing interval were taken (5 mL) to determine the trough concentration. (i.e. at 24 hours, prior to administration of the morning dose). This sample was centrifuged at 5000 rpm for 20 min. The supernatant (3 mL) was then further spun for 20 min. A TSA plate was swabbed with a 0.5 McFarland standard of *E. coli* ATCC # 25922 as per procedure above. A 10 mcL aliquot of the supernatant was then dropped in the center of the TSA plate, allowed to dry for 10 min and incubated overnight at 37° C for 18-24 hours. The zone of inhibition was measured and compared to the values obtained from the standard curve, so as to determine the ceftazidime concentration at that time point.

3.4 Data Analysis

Pre-treatment and post-treatment MICs were performed by E-Test®, as described above. Seventy-two hour samples were tested to check for changes in MIC. Resistance was defined as per Clinical and Laboratory Standards Institute (CLSI) susceptibility breakpoint guidelines as follows: ceftazidime MIC $\geq$ 32 mcg/mL, ciprofloxacin MIC $\geq$ 4 mcg/mL and gentamicin MIC $\geq$ 16 mcg/mL. For samples displaying resistance at 72 hours, bacteria were plated for five consecutive days on antibiotic-free TSA. MIC's were then re-checked, and those organisms retaining resistance were described as stably-derepressed mutants, whereas those reverting back to pre-treatment MIC's were described as inducible populations. In the case of the isolation of a derepressed subpopulation, MIC's were often reported as >256 mcg/mL, due to the upper limit of the E-Test® strips. The Mann-Whitney test was used to compare BK statistically between two groups: experiments in which resistance developed during the 72 hours of therapy compared to experiments in which bacteria remained susceptible during therapy.

4. RESULTS

4.1 Ceftazidime Monotherapy Experiments

Ceftazidime monotherapy selected high-level ceftazidime resistance after 72 hours of therapy for all three isolates studied. Specifically, 10 of 10 trials for Isolate 1, and 9 of 10 trial for each of Isolates 3 and 4 led to significant increases in MICs as follows: Isolate 1 from an initial MIC of 0.75 mcg/mL to 128 - >256 mcg/ml, Isolate 3 from an initial MIC of 0.5 mcg/mL to >256 mcg/mL and Isolate 4 from an initial MIC of 0.38 mcg/mL to 128 - > 256 mcg/mL. Resistance was stably derepressed (as determined by re-plating 72 hour IPDM samples for 5 consecutive days on antibiotic-free TSA) in 10 of 10 trials for Isolate 1 (100%), 8 of 9 trials for Isolate 3 (89%) and 5 of 9 trials for Isolate 4 (56%). Net bacterial kill (BK) at 72 hours was similar for monotherapy experiments. Net BK at 72 hours for the two experiments not leading to the development of resistance (one trial for each of Isolates 2 and 3) exceeded 3 $\log_{10}$ CFU kill (3.1 and 3.3 $\log_{10}$ kill, respectively). All monotherapy experiments otherwise (except for 1 trial with Isolate 4) led to bacterial re-growth at 72 hours. (TABLE 8) (Figure 9)

4.2 Combination Therapy Experiments

Despite differences in BK, combination therapy consistently prevented the emergence of resistance (TABLES 10 & 11). Ceftazidime + ciprofloxacin suppressed resistance in 100% of cases for all three isolates (9 trials each for Isolates 1, 3 and 4). MIC values did not change during combination therapy with values remaining the same as initial. BK at 72 hours was greatest for ciprofloxacin combination experiments for Isolates 1 and 3 (both averaging 2.9 $\log_{10}$ CFU kill). Interestingly, no BK was documented for Isolate 4 in the ciprofloxacin combination experiments (average -0.1 $\log_{10}$ CFU kill) however, the emergence of resistance was uniformly prevented in all 9 trials. (TABLES 10 & 11) (Figure 10)

Average BK for the gentamicin combination experiments varied widely: 2.9 $\log_{10}$ CFU kill for Isolate1, -0.3 $\log_{10}$ CFU kill for Isolate 3 and zero kill for Isolate 4. In one trial with Isolate 4, resistance was documented at 72 hours, but was lost after re-plating on TSA for five days. For the remainder of experiments (9 of 9 for Isolate 1, 9 of 9 for Isolate 3 and 8 of 9 for Isolate 4), the development of resistance at 72 hours was uniformly prevented. (TABLE 12) (Figure 11)

The antibacterial activity was significantly reduced for experiments in which resistance developed during the 72 hours of therapy compared to those in which bacteria remained susceptible. Overall, there was net bacterial growth of 1.5 +/- 0.9 $\log_{10}$ CFU/mL versus mean BK of 1.3 +/- 1.9 $\log_{10}$ CFU/mL ($p < 0.0001$).

5. DISCUSSION

5.1 Discussion of Results

This study represents the first simulation of the emergence of resistance in *E. cloacae* using the IPDM, after three days of ceftazidime monotherapy. Our results are consistent with the literature describing the risks conferred by third-generation cephalosporins on the selection of β-lactam-resistant *Enterobacter* mutants. Unique to our results is the clear advantage of combination therapy in the prevention of such resistance.

Clinically, the ability to minimize the development of resistance is paramount in ensuring successful patient outcomes. To do so, antibiotic selection and dosing strategies which optimize antibiotic concentrations at the site of infection are essential for ensuring a rapid bactericidal action for life-threatening infections. In the case of *Enterobacter* however, what may appear as optimal antibiotic therapy in fact drives the selection of resistance. As β-lactam agents, third-generation cephalosporins should be used in doses, and for infections that maintain their concentration profile above the MIC of the microorganism, and are thus referred to as time-dependent agents (i.e. outcome is based on ensuring that a minimum time period is spent above the MIC of the microorganism). Specifically, for severe infections, the drug concentration at the site of infection should exceed 80% of the dosing interval [5]. In our study, ceftazidime concentrations were well above the MIC's of the test isolates for the entire dosing interval. However, in the case of the isolation of stably resistant mutants, this allowed the eradication of the

susceptible bacterial population and subsequent overgrowth of derepressed mutants. It is not surprising that ceftazidime monotherapy consistently selected resistance in the IPDM, since the effect of the immune system is totally absent. Relative to the observations from clinical trials, our rates of resistance acquisition are comparatively higher. While the emergence of resistance with third-generation cephalosporin monotherapy has been reported to occur at rates between 19-29% in bacteremia, we consistently selected resistance at 72 hours in the IPDM. This represents two different scenarios: the isolation of an AmpC reversibly-induced population, or the isolation of a stably-derepressed population. However, when replated, the rates of stable resistance were different between the three isolates (100%, 89%, and 56%). The varying rates illustrate how the ability to select resistance in *Enterobacter* is distinct for each antimicrobial exposure. This has been shown in studies where molecular typing of *E. cloacae* strains failed to identify the dissemination of a single clone amongst bacteremic patients with ceftazidime-resistant isolates in a given institution [61]. As such, each patient's exposure to a specific regimen may or may not lead to the development of resistance. Such differences may be expected since the composition of each patient's *Enterobacter* population may vary. The IPDM serves as a model for bacteremia in the immunocompromised host and as such may under predict the utility of a particular dosing regimen in other individuals. Given the fact that three days were necessary to effectively select resistance, it seems possible then that in a patient with immune support, 48 hours of therapy may be enough to allow the infection to be cleared.

The available literature on combination therapy for the prevention of resistance in *Enterobacter* is limited. The choice of agents has mainly been with β-lactam/aminoglycoside combinations. Of note, aminoglycoside combination therapy was administered using thrice or twice daily dosing, raising concerns for sub-optimal pharmacokinetic/pharmacodynamic (PK/PD) target attainment. As a concentration-dependent antibiotic class, aminoglycosides are well appreciated to provide maximum benefit with respect to patient outcomes when administered in doses that achieve adequate peak concentration to MIC ratios that exceed ≥10 [5]. Based on *Enterobacter* susceptibility data (Gentamicin $MIC_{50/90}$ = ≤2/≤2 mcg/mL) multiple daily dosing can fail to achieve such targets since suggested peaks are in the range of 4-10 mcg/mL. Furthermore, a large proportion of patients with *Enterobacter* infections (25-50%) in the Kaye and Schwaber trials were treated for lung infections, a site argued to further limit achievable aminoglycoside concentrations [5]. Such limitations in clinical trials may have underestimated protective effects that the two-drug regimen might have otherwise conferred on an infection like bacteremia, where optimal once daily aminoglycoside dosing could have been used. Conversely, the protective effect described for fluoroquinolone therapy might be explained by the high concentrations that are achieved in the epithelial lining fluid of the lungs for that particular drug class. In that situation, both susceptible and mutant sub-populations could be eradicated since their MICs are not affected by AmpC hyperproduction.

Interestingly, lack of resistance at 72 hours for the combination regimens studied could not be explained by bacterial kill at that time point. Considering all monotherapy

and combination therapy trials, BK at 72 hours was significantly compromised for experiments in which resistance developed (i.e., 1.5 +/- 0.9 $\log_{10}$ CFU/mL of net growth versus 1.3+/- 1.9 log10 CFU/mL of bacterial kill). However, it is important to consider that most monotherapy trials with ceftazidime resulted in resistance whereas all but one combination therapy trial maintained susceptibility. Furthermore, there was variable BK demonstrated within the combination therapy trials, despite a lack of resistance at 72 hours for the 53/54 trials conducted. Bacterial re-growth at 72 hours thus represents the overgrowth of the susceptible bacterial population, and occurred for half of the regimens studied (Isolate 4 in the ciprofloxacin group, and Isolates 3 and 4 in the gentamicin group). A few explanations are possible. Firstly, the ability to completely eradicate the infection from the IPDM may be due to the inherent difficulty in treating *Enterobacter* infections. This may be illustrated by the high mortality rates for *Enterobacter* bacteremia, which range between 20 and 46% [2]. Furthermore, the development of biofilm in the IPDM may have further limited the ability of the administered antibiotics to fully sterilize the infection flask.

Our results serve as useful data to help guide optimal antibiotic selection in the empiric treatment of *Enterobacter* infections in critically ill patients. *Enterobacter* bloodstream infection comprises nearly 20% of gram-negative bacteremias in the intensive care unit. If one includes *S. marcescens,* an organism with the same Amp-C-type β-lactamase, 28% of gram-negative bacteremias are caused by one of these two organisms. This along with the fact that third-generation cephalosporins represent the most heavily used antimicrobial drug class in the ICU necessitate that these agents are

used properly so as to maintain their effectiveness. Our results provide preliminary data to support the use of combination therapy as a means of preventing the emergence of resistance, until a bacterial cause is isolated; at that point, directed therapy can be tailored for that specific infection. The ability to prevent the development of resistance may not only improve patient outcomes, but also serve as a means of prolonging the utility of the entire drug class.

Study Limitations

While the results of the study lend strong support to the use of combination regimens in the empiric therapy of *E. cloacae* infection, a few limitations in the in vitro design should be highlighted. As mentioned, the development of biofilm in the IPDM has been described as a factor which may prohibit full sterilization of the infection flask. Experience with this system has shown this to be of particular concern in studies that extend beyond 48 hours. As such, differences in measures of bacterial kill with the same isolates at the 72 hour time point may reflect this effect. Furthermore, differences between the three isolates, with respect to BK, may also reflect an inherent strain-specific aptitude for biofilm formation. Overgrowth of bacteria at the 72 hour time point for monotherapy trials showed that a mixture of both induced and derepressed organisms were present. However, overgrowth of bacteria at the 72 hour time point for combination regimens was likely explained by biofilm formation by susceptible organisms. Future methods to prevent and, or detect biofilm formation could improved the use of the IPDM in extended experiments, e.g. $\geq$72 hours. This, along with a more intensive sampling

schedule, may help describe the changing bacterial population and shed light on the bacterial composition of the biofilm.

Antibiotic carry-over when plating diluted samples may be a means whereby trace amounts of antibiotic present in an IPDM sample affect bacterial counts. This is of particular concern in trials where large amounts of bacterial kill necessitate plating of relatively undiluted samples. However, in the case of experiments with bacterial regrowth, the large number of serial dilutions needed would be unlikely to affect the recorded counts. Furthermore, in IPDM growth controls where no antibiotic is present, experience has shown the bacterial load to plateau at values of approximately 1×10^8 $\log_{10}$ CFU/mL. Techniques to totally remove antibiotic in future trials, such as through chelation and/or filtration may be helpful.

Lastly, the study was conducted using only three clinical isolates, each taken from different patients (NICU, emergency, and SICU). The isolates were within the expected MIC range based on $MIC_{50/90}$ ($\leq$0.25 mcg/mL/<32 mcg/mL) [5], thus representative of a typical *Enterobacter* strain. Furthermore, the isolates had similar MIC values, thus minimizing any potential problems which may have arisen due to varying susceptibilities. Regardless, a study using a larger number of isolates may provide more information, and certainly add to the ability to apply the results to the clinical arena.

5.2 Future Directions

The present study has shown a clear advantage for the use of ciprofloxacin or gentamicin in combination with ceftazidime to prevent the selection of derepressed *E. cloacae* mutants. A follow-up study could involve the use of traditional gentamicin levels, (e.g. peaks of 6 mcg/mL) to see if that dosing strategy offers the same protection. The main utility of the results generated from the study can be seen for the treatment of critically-ill patients, where ceftazidime might be used empirically as a monotherapy regimen. Future research may help determine if in fact ceftazidime indeed poses unique risks relative to other third-generation cephalosporins, or compared to other β-lactams (e.g. piperacillin).

The IPDM serves as a useful tool for studying antibiotic-resistant infections. Since the frequency of such infections may be relatively low, and/or, since the emergence of resistance may occur at unpredictable frequencies, the ability to recreate resistance in the IPDM provides a clinically-relevant in vitro representation of human bloodstream infection. The ability to simulate these infections allows a researcher to acquire information and subsequently plan future research that might otherwise be impossible.

6. CONCLUSION

We have demonstrated a significant protective effect conferred by combination therapy in the prevention of the development of resistance in *E. cloacae* following three days of simulated empiric therapy. Resistance at 72 hours following ceftazidime monotherapy occurred consistently for the three isolates studied. In contrast, the use of combination therapy with ciprofloxacin or gentamicin effectively prevented the selection of resistance. Importantly, this effect was not related to BK at 72 hours. Research evaluating antimicrobial strategies which help minimize the development of resistance is paramount given the increasing rates of antibiotic-resistant infections coupled with the lack of research and development efforts geared at the discovery of new agents.

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Table 1
AmpC β-lactamase Induction Potential of β-lactam Antibiotics*

Strong	↓	Carbapenems (meropenem, imipenem), cephamycins (cefoxitin)
		Amoxicillin, 1st-generation cephalosporins (e.g. cefazolin)
		Piperacillin
		2nd-generation cephalosporins (e.g. cefuroxime)
		3rd-generation cephalosporins (e.g. ceftazidime)
		Cefepime
Weak		Aztreonam

*Adapted from references Minami, Gootz, Sanders and Wiedemann

Table 2
Cephalosporin Antibiotics Commonly Used in Clinical Practice

First Generation	Second Generation	Third Generation	Fourth Generation
I.V.	**I.V.**	**I.V.**	**I.V.**
Cefazolin	Cefuroxime	Ceftazidime	Cefepime
	Cefoxitin	Cefotaxime	
	Cefotetan	Ceftriaxone	
Oral	**Oral**	**Oral**	
Cephalexin	Cefuroxime	Cefixime	
	Cefprozil		

Table 3a
Initial *Enterobacter cloacae* MICs (µcg/mL)

Isolate	Ceftazidime	Ciprofloxacin	Gentamicin
1	0.75	0.016	2
3	0.5	0.032	0.38
4	0.38	0.016	0.38

Table 3b
Susceptibility Profile of Clinical Isolates

Antibiotic	**Isolate 1**	**Isolate 3**	**Isolate 4**
Ceftazidime	S	S	S
Cefotaxime	**R**	S	S
Ceftriaxone	S	S	S
Gentamicin	S	S	S
Tobramicin	S	S	S
Piperacillin/Tazobactam	S	S	S
Trimethoprim/Sulfamethoxazole	S	S	S
Ampicillin	R	R	R
Cefuroxime	R	R	R
Cefazolin	R	R	R

Table 4
Population Human Pharmacokinetics for Antibiotics**

Antibiotic	Half-Life (hrs)	Protein binding (%)	Volume of Distribution (L/kg)
Ceftazidime	2	17	0.18-0.31
Ciprofloxacin	3-4	20-40	2.1-2.7
Gentamicin	2-3	0-10	0.2-0.3

** Adapted from references (Mandell, Lexicomp, USPDI, AHFS)

Table 5
Dosing in Adults with normal renal function**

Antibiotic	Dose	$_f$Peak^ & $_f$Trough (mcg/mL)	$_f$AUC$_{24h}$ # (mg x hr/L)
Ceftazidime	2 grams every 8 hours (IV)	100-170/6-10.6	1020
Ciprofloxacin	400mg every 12 hours (IV)	2.4-4.7/0.3-0.4	20
Gentamicin	4-7mg/kg (IV) high dose once daily regimen	18-24/<<1	70

** adapted from references (Mandell, Lexicomp, USPDI, AHFS)
^ subscript *f* refers to free, unbound drug concentration
# approximate AUC, calculated for a 70 kg person

Table 6
IPDM Antibiotic Dosing and Resultant Drug Levels

Antibiotic	IPDM Dose (mg)	Interval (hours)	$_f$Peak & $_f$Trough (mcg/mL)	$_f$AUC
Ceftazidime	37.5	12	150/9.4	1290
Ciprofloxacin	0.75	12	3/0.2	26
Gentamicin	5	24	20/0.1	85

Table 7
Bioassay Results for Ceftazidime Concentratrations During Monotherapy Experiments

Drug	Target (mcg/mL)	Measured (mcg/mL)
Ceftazidime	150/9	133-165/6-7.5
Ciprofloxacin	3/0.2	2.5-2.9/0.2-0.3
Gentamicin	20/0.1	17.9-19/0.04-0.11

Table 8
IPDM Results from Ceftazidime Monotherapy Experiments

(a) Isolate 1

Experiment	Initial MIC	MIC at 72 hours	MIC after replating	BK72^*
1	0.75	>256	>256	-1.7
2	0.75	128	128	-1.6
3	0.75	>256	128	-1.6
4	0.75	>256	>256	-1.7
5	0.75	192	192	-1.2
6	0.75	>256	>256	-1.9
7	0.75	>256	>256	-2.0
8	0.75	>256	>256	-2.0
9	0.75	>256	>256	-0.8
10	0.75	>256	>256	-1.5

^ bacterial kill at 72 hours, in CFU/mL
* negative values represent bacterial re-growth

(b) Isolate 3

Experiment	Initial MIC	MIC at 72 hours	MIC after replating	BK72
1	0.5	>256	128	-0.9
2	0.5	>256	0.5	-0.8
3	0.5	>256	>256	-1.7
4	0.5	0.5	NA	**3.1**
5	0.5	>256	>256	-1.6
6	0.5	>256	>256	-2.0
7	0.5	>256	>256	-1.8
8	0.5	>256	>256	-1.9
9	0.5	>256	>256	-2.0
10	0.5	>256	>256	-2.0

Table 8 (continued)

(c) Isolate 4

Experiment	Initial MIC	MIC at 72 hours	MIC after replating	BK72
1	0.38	>256	0.25	-0.6
2	0.38	192	>256	-0.4
3	0.38	192	0.38	-0.9
4	0.38	128	0.25	-1.3
5	0.38	>256	>256	-2.2
6	0.38	0.25	NA	**3.3**
7	0.38	>256	>256	-2.4
8	0.38	>256	0.25	2.3
9	0.38	>256	>256	-2.7
10	0.38	>256	>256	-2.6

Table 9
IPDM Results from Ceftazidime/Ciprofloxacin Combination Therapy Experiments

(a) Isolate 1

Experiment	CAZ MIC at 72 hours	CIP MIC at 72 hours	BK72
1	0.75	0.016	**4.6**
2	0.75	0.016	**4.3**
3	0.75	0.016	1.5
4	0.75	0.016	0.3
5	0.75	0.016	**4.0**
6	0.75	0.016	**4.1**
7	0.75	0.016	**4.2**
8	0.75	0.016	1.7
9	0.75	0.016	1.8

(b) Isolate 3

Experiment	CAZ MIC at 72 hours	CIP MIC at 72 hours	BK72
1	0.5	0.032	-0.5
2	0.5	0.032	**4.8**
3	0.5	0.032	2.4
4	0.5	0.032	1.9
5	0.5	0.032	**4.4**
6	0.5	0.032	**5.4**
7	0.5	0.032	**3.8**
8	0.5	0.032	**3.0**
9	0.5	0.032	2.4

Table 9 (continued)
IPDM Results from Ceftazidime/Ciprofloxacin Combination Therapy Experiments

(c) Isolate 4

Experiment	CAZ MIC at 72 hours	CIP MIC at 72 hours	BK72
1	0.38	0.016	-0.2
2	0.38	0.016	-0.9
3	0.38	0.016	-0.9
4	0.38	0.016	-0.7
5	0.38	0.016	0.0
6	0.38	0.016	0.8
7	0.38	0.016	0.2
8	0.38	0.016	0.7
9	0.38	0.016	0.6
10	0.38	0.016	-0.3
11	0.38	0.016	0.1

Table 10
IPDM Results from Ceftazidime/Gentamicin Combination Therapy Experiments

(a) Isolate 1

Experiment	CAZ MIC at 72 hours	GENT MIC at 72 hours	BK72
1	0.75	2	**4.1**
2	0.75	2	1.1
3	0.75	2	0.4
4	0.75	2	**3.7**
5	0.75	2	**4.4**
6	0.75	2	0.1
7	0.75	2	-0.2
8	0.75	2	**4.5**
9	0.75	2	0.6

(b) Isolate 3

Experiment	CAZ MIC at 72 hours	GENT MIC at 72 hours	BK72
1	0.5	0.38	-0.6
2	0.5	0.38	-0.4
3	0.5	0.38	-0.2
4	0.5	0.38	-0.3
5	0.5	0.38	0.0
6	0.5	0.38	-0.1
7	0.5	0.38	-0.1
8	0.5	0.38	-0.2
9	0.5	0.38	-1.1

Table 10 (continued)
IPDM Results from Ceftazidime/Gentamicin Combination Therapy Experiments

(c) Isolate 4

Experiment	CAZ MIC at 72 hours	GENT MIC at 72 hours	BK72
1	>256*	0.38	-0.8
2	0.38	0.38	1.9
3	0.38	0.38	-0.4
4	0.38	0.38	-0.3
5	0.38	0.38	0.1
6	0.38	0.38	0.1
7	0.38	0.38	-0.1
8	0.38	0.38	0.7
9	0.38	0.38	-1.0

*reverted back to pre-treatment MIC after replating

Table 11
Summary of Results

		Isolate 1	Isolate 3	Isolate 4
CAZ	Post-Tx Resistance	10/10 (100%)	9/10 (90%)	9/10 (90%)
	Stable Resistance	10/10 (100%)	8/9 (89%)	5/9 (56%)
CAZ + CIP	Post-Tx Resistance	0/9 (0%)	0/9 (0%)	0/11 (0%)
CAZ + GENT	Post-Tx Resistance	0/9 (0%)	0/9 (0%)	1/9 (11%)*

*CAZ MIC was increased at 72 h, but reversed back to pre-treatment value after re-plating

Figure 1
AmpC Induction in *Enterobacter cloacae*

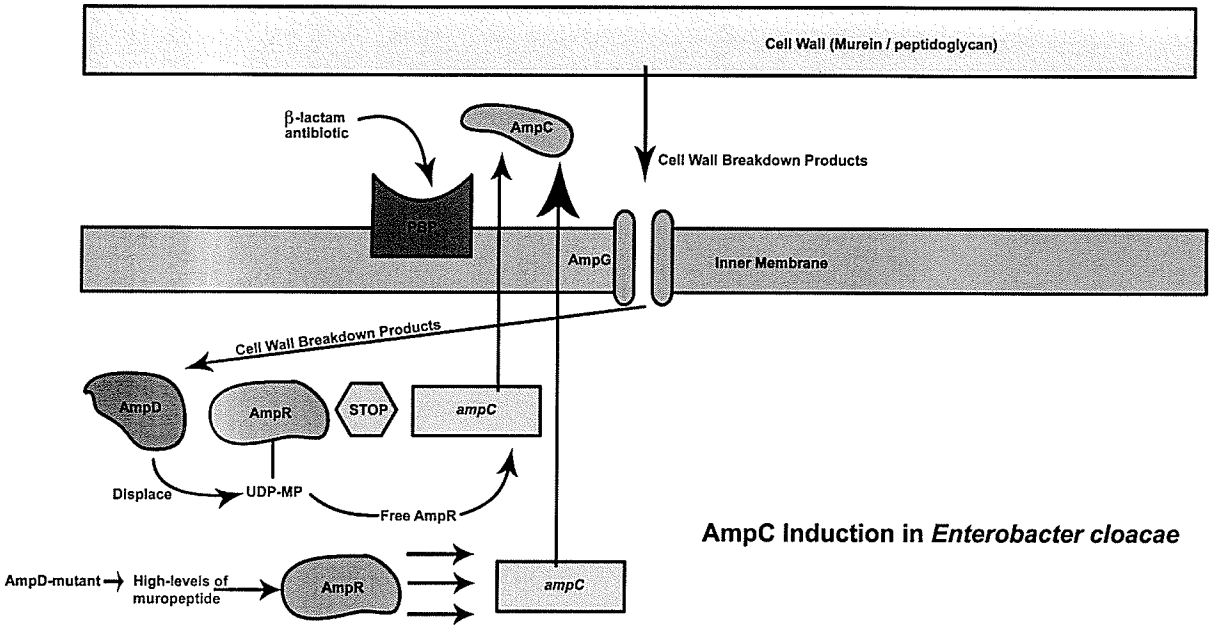

Figure 2
Percentage of Isolates Causing ICU Gram-Negative Bacteremias in 2003

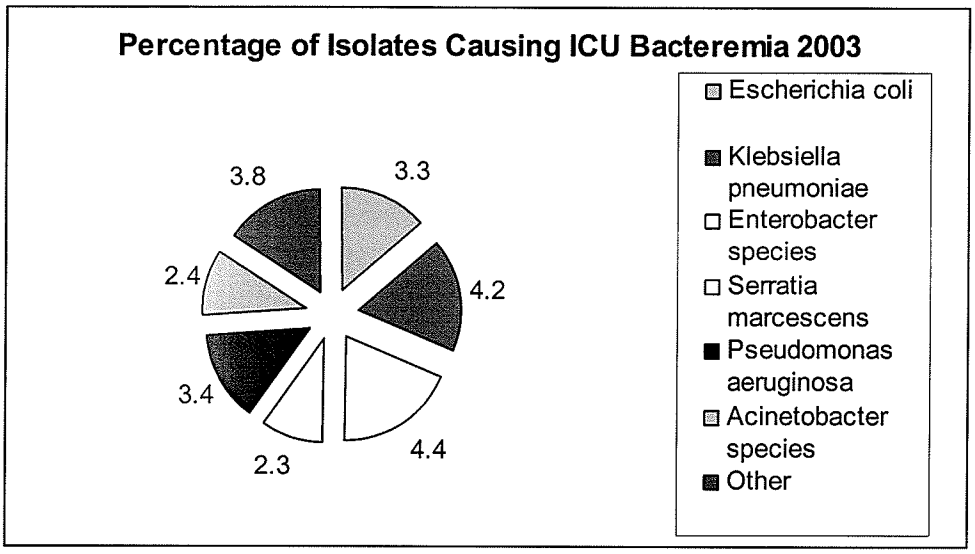

Figure 3
Comparison of Ceftazidime Resistance in *E. cloacae*- St. Boniface General Hospital Data and Mystic Surveillance Program

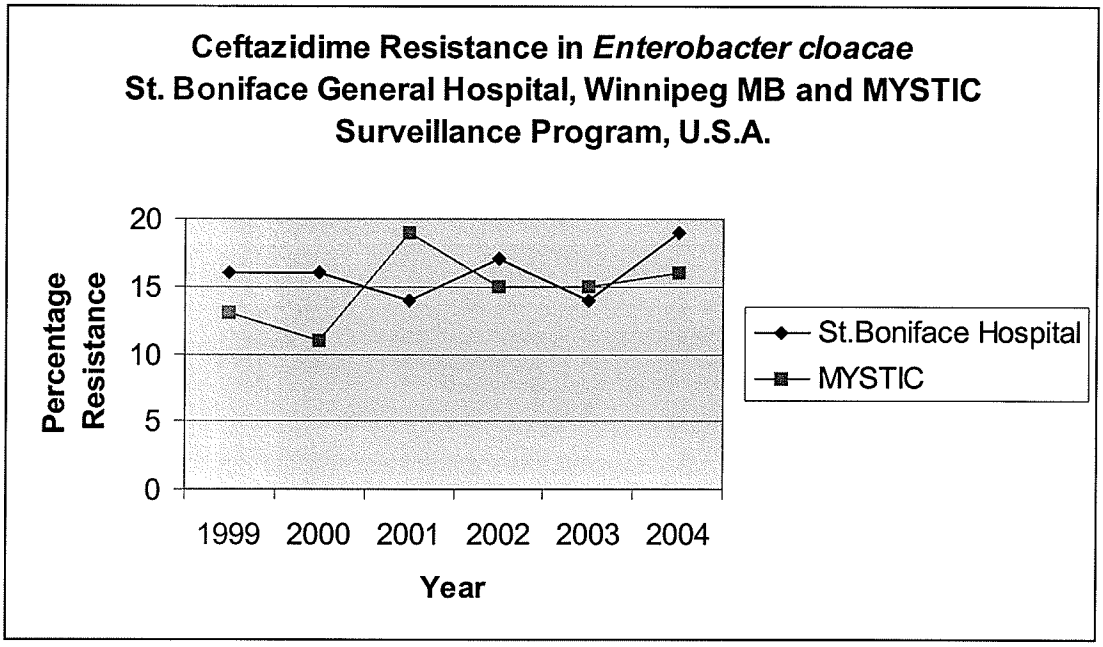

Figure 4
IPDM: Establishing the Infection

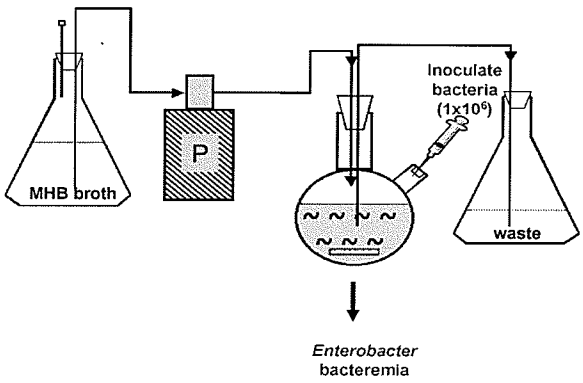

Figure 5
IPDM: Injection of Antibiotic to Simulate Human Concentration Profiles

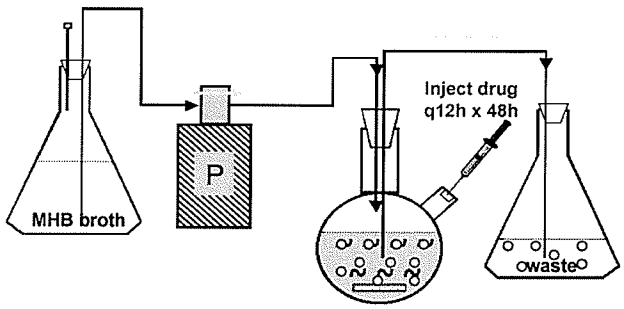

Figure 6
IPDM Ceftazidime Concentrations

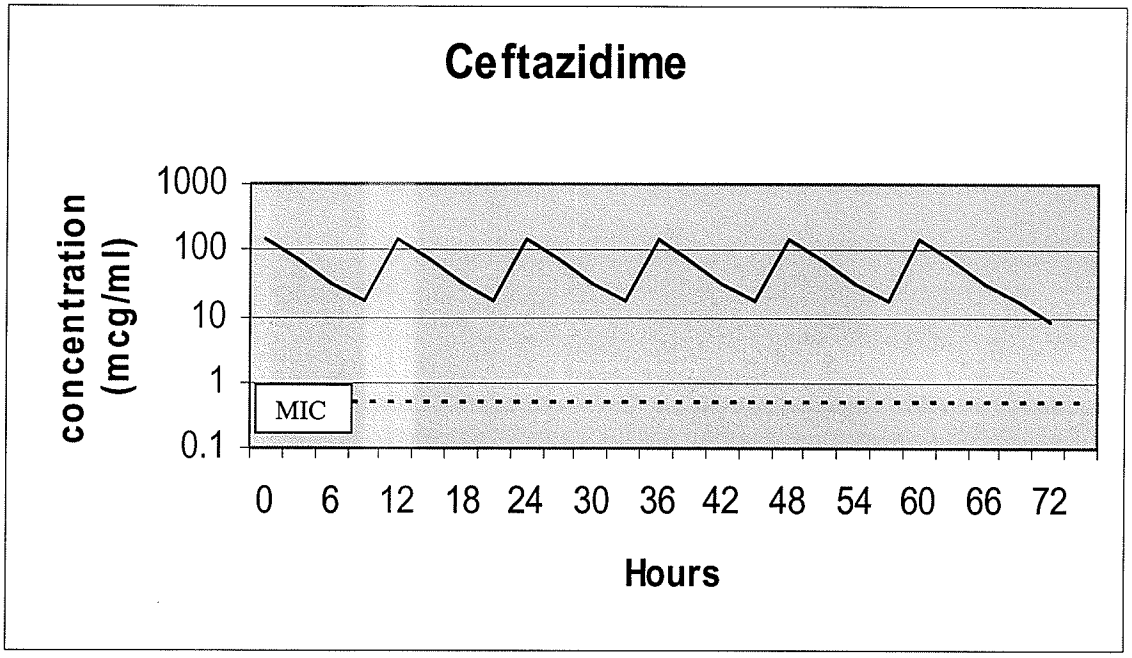

Figure 7
IPDM Ciprofloxacin Concentrations

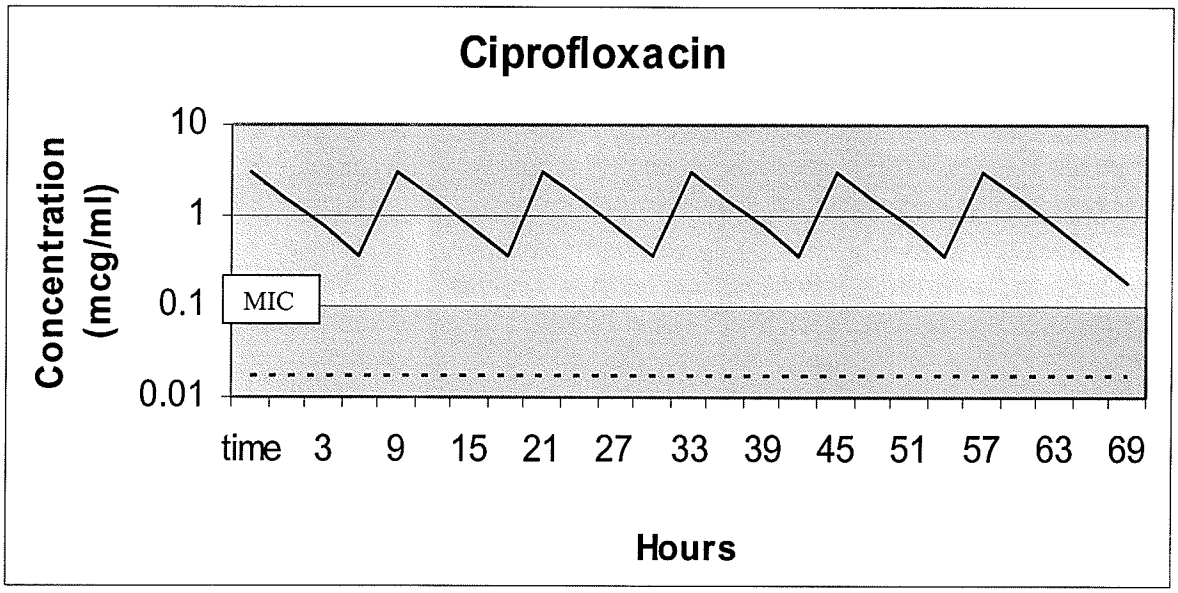

Figure 8
IPDM Gentamicin Concentrations

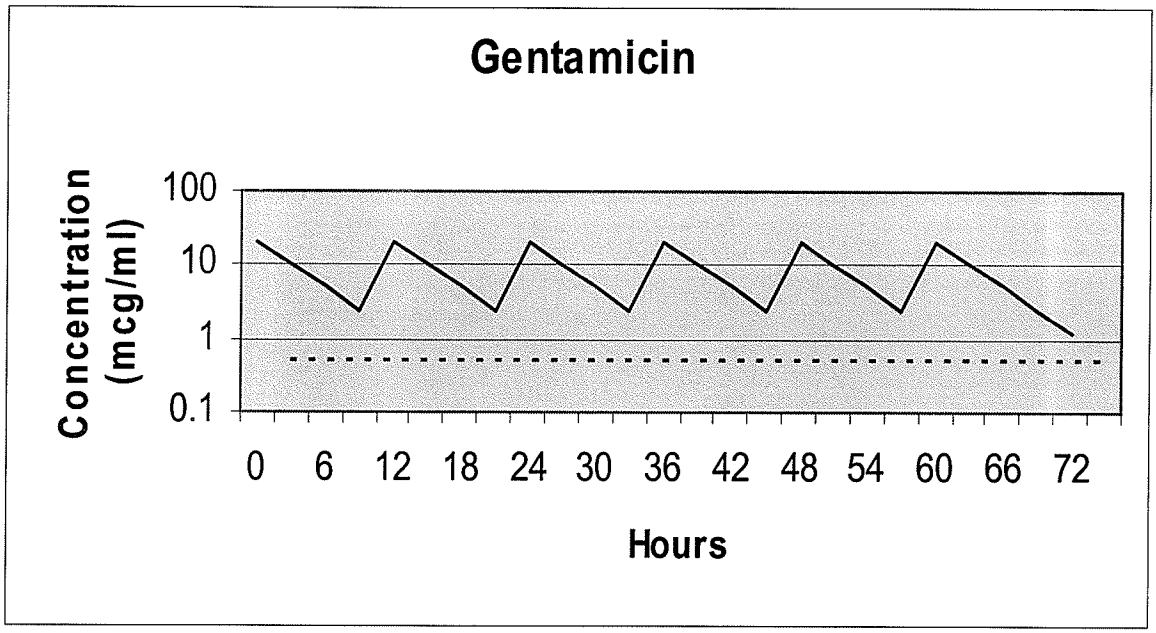

Figure 9 (a)
Ceftazidime MIC Changes for Isolate 1 in Ceftazidime Monotherapy Experiments (E)

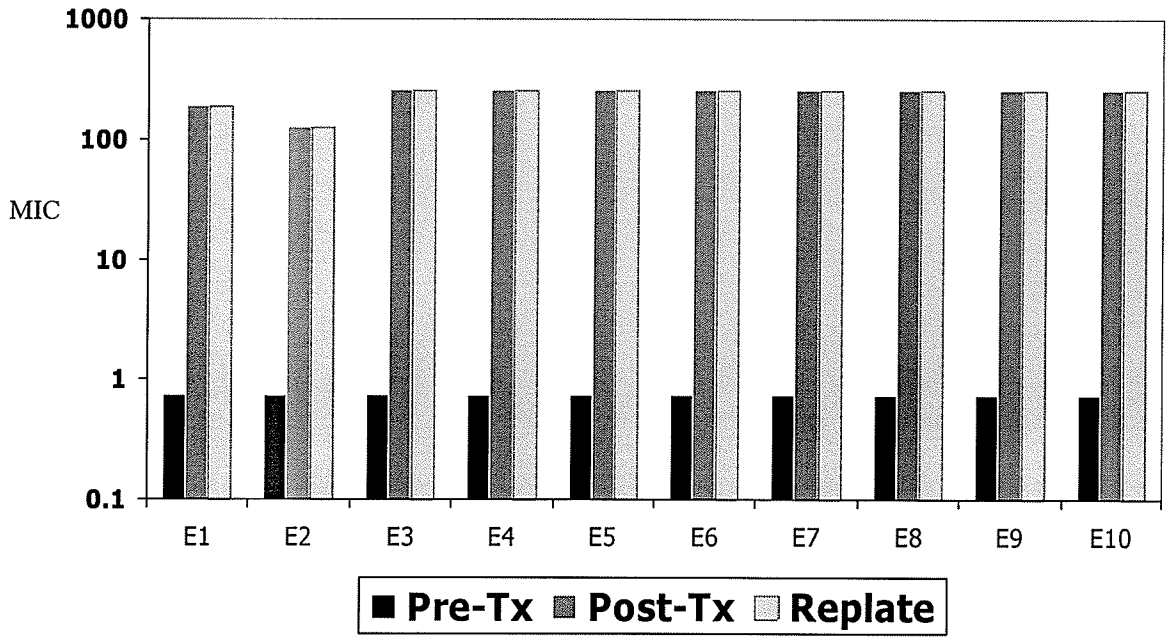

Figure 9 (b)
Ceftazidime MIC Changes for Isolate 3 in Ceftazidime Monotherapy Experiments (E)

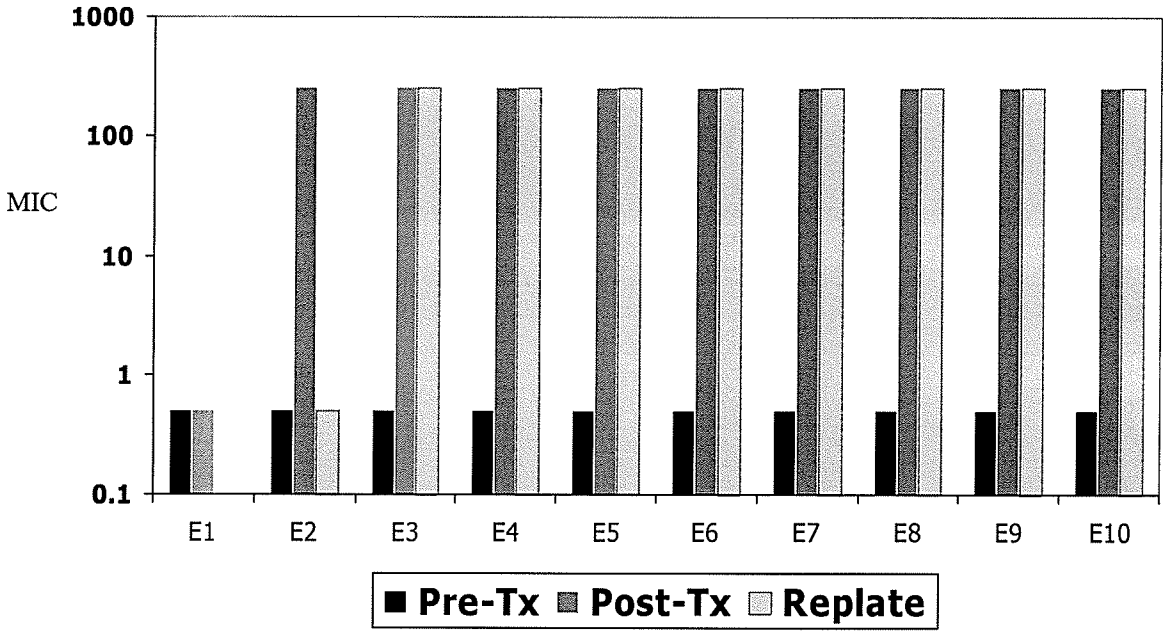

Figure 9 (c)
Ceftazidime MIC Changes for Isolate 4 in Ceftazidime Monotherapy Experiments (E)

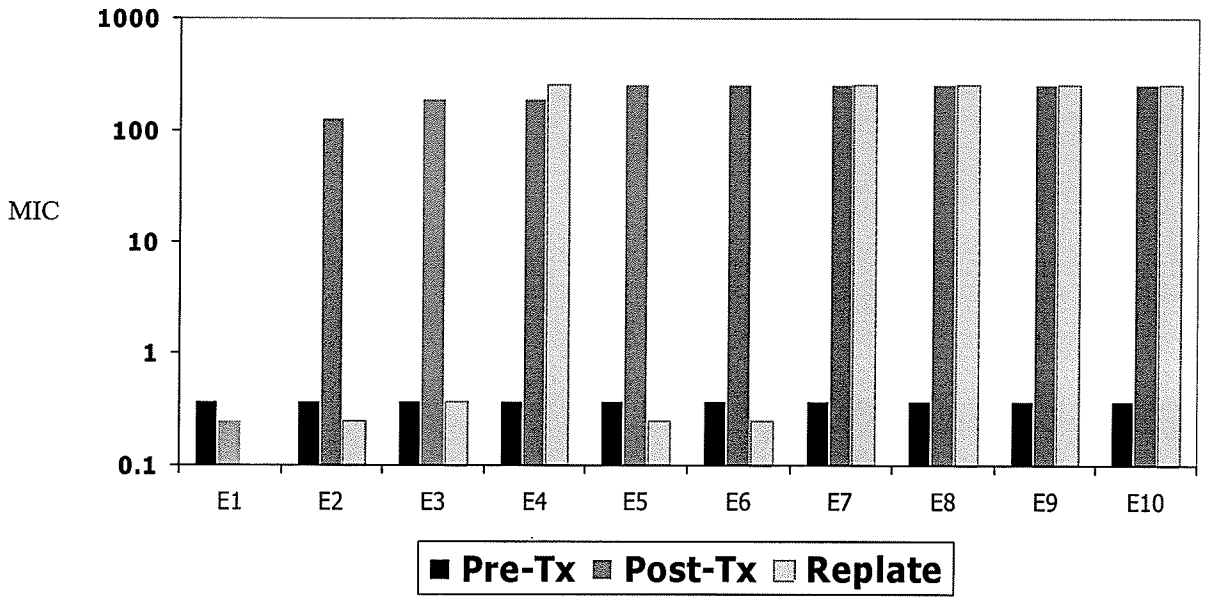

Figure 10 (a)
Ceftazidime MIC Changes for Isolate 1 in Ceftazidime + Ciprofloxacin Combination Experiments (E)

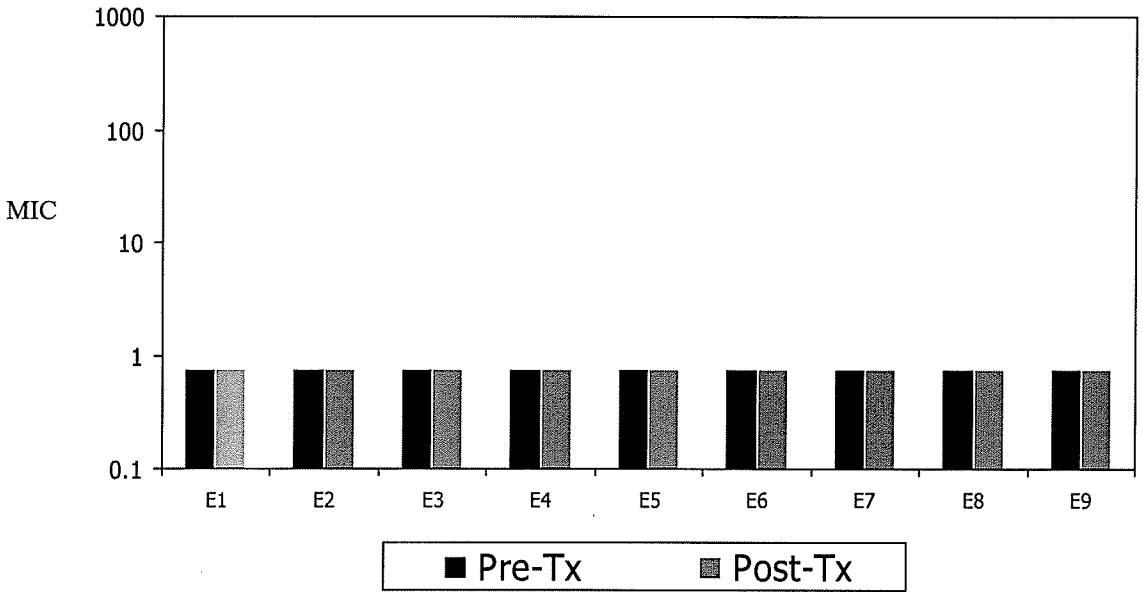

Figure 10 (b)
Ceftazidime MIC Changes for Isolate 3 in Ceftazidime + Ciprofloxacin Combination Experiments (E)

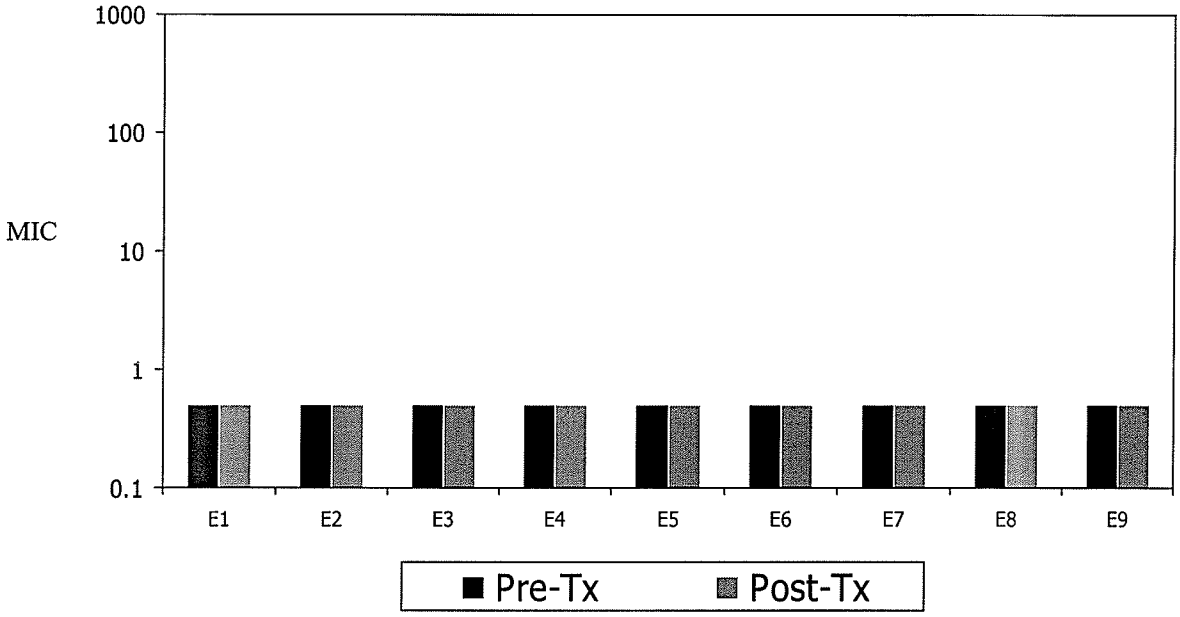

Figure 10 (c)
Ceftazidime MIC Changes for Isolate 4 in Ceftazidime + Ciprofloxacin Combination Experiments (E)

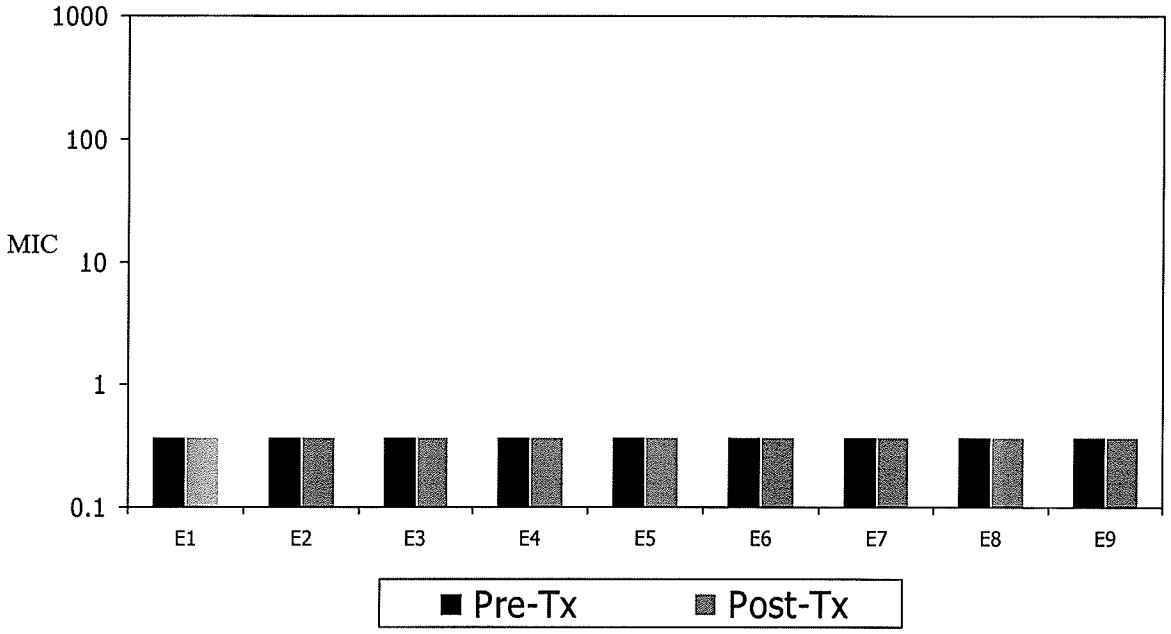

Figure 11 (a)
Ceftazidime MIC Changes for Isolate 1 in Ceftazidime + Gentamicin Combination Experiments (E)

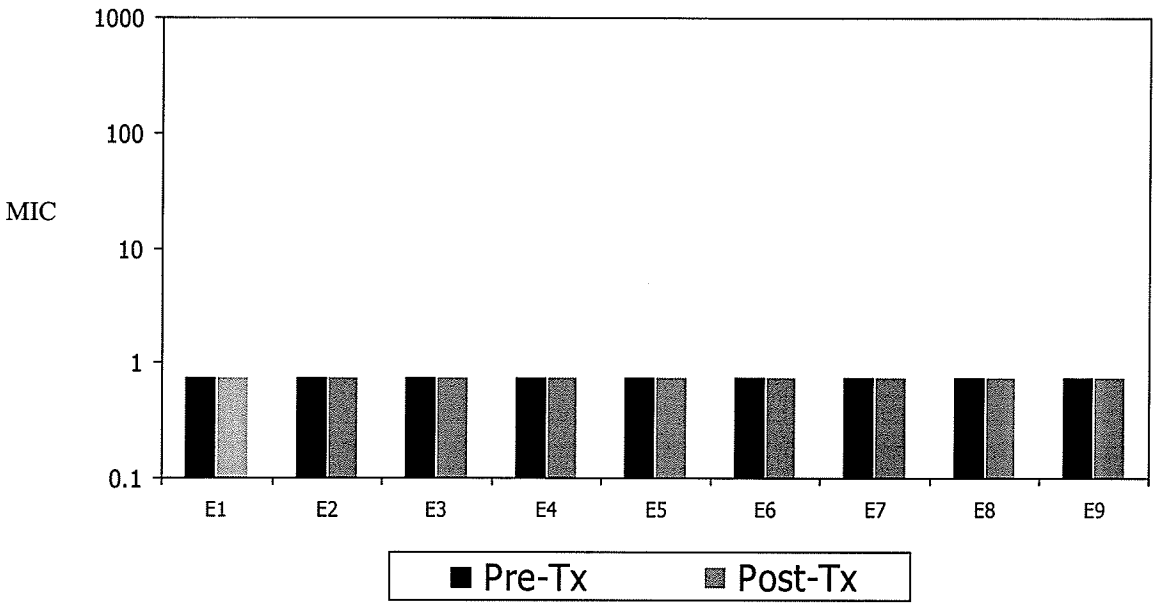

Figure 11 (b)
Ceftazidime MIC Changes for Isolate 3 in Ceftazidime + Gentamicin Combination Experiments (E)

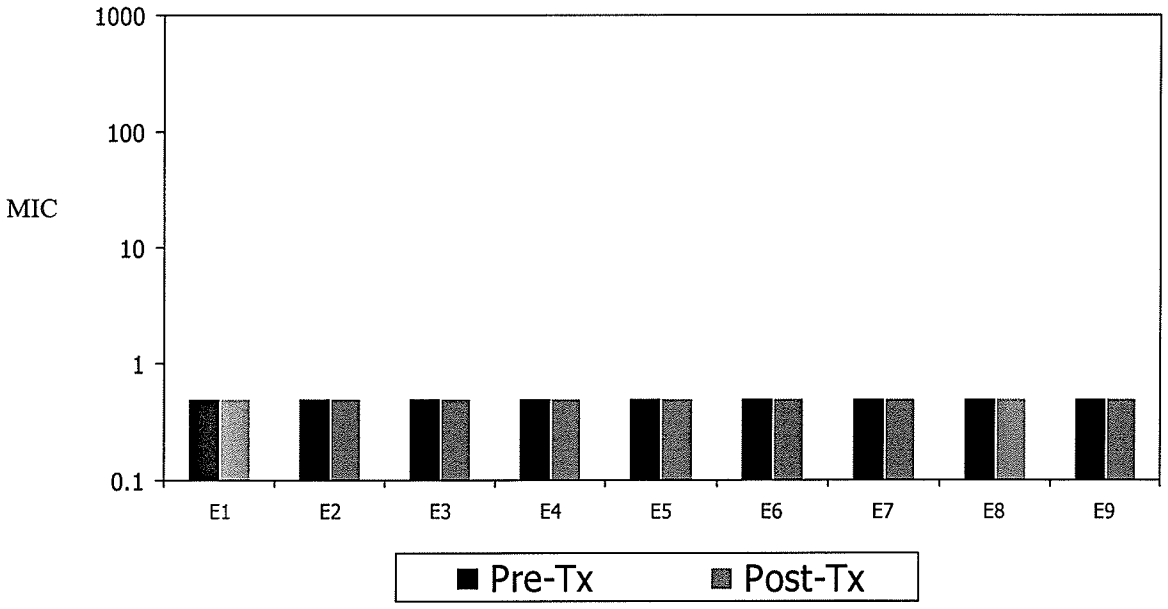

Figure 11 (c)
Ceftazidime MIC Changes for Isolate 4 in Ceftazidime + Gentamicin Combination Experiments (E)