

**Bactericidal Activity of an Ultra-High Dose of Gentamicin against  
Gram Positive and Gram Negative Bacteria**

**in an *In Vitro* Pharmacodynamic Model**

**By**

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**Abstract:**

Septic shock due to bacterial infections is one of the main causes of death in intensive care units of the developed world. To a great extent, the efforts to improve the outcomes of life-threatening infections including septic shock have focused on the deployment of antimicrobials of ever increasing potency. However, many pathogenic bacterial strains have acquired resistance to available and even recently introduced antibiotics. Alternate pharmacokinetic strategies constitute another pathway to increased antimicrobial efficacy. In this study, we have demonstrated that a single very high dose of gentamicin can eliminate sensitive and moderately resistant bacterial strains at an accelerated rate with a lower risk of regrowth in an *in vitro* pharmacodynamic model compared to standard (once daily-equivalent) dosing. This approach may be clinically viable if potential toxicity concerns can be addressed.

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<b>Abstract.....</b>	<b>ii</b>
<b>Acknowledgment.....</b>	<b>iii</b>
<b>Table of content.....</b>	<b>iv</b>
<b>List of tables.....</b>	<b>viii</b>
<b>List of figures.....</b>	<b>ix</b>
<b>List of abbreviations.....</b>	<b>x</b>
<b>1. Introduction.....</b>	<b>1</b>
<b>1.1. The clinical problem.....</b>	<b>1</b>
<b>1.2. Definitions.....</b>	<b>1</b>
<b>1.3. Sources of infection and microbiology of sepsis.....</b>	<b>3</b>
<b>1.3.1. <i>Pseudomonas aeruginosa</i>.....</b>	<b>4</b>
<b>1.3.2. <i>E. coli</i>.....</b>	<b>5</b>
<b>1.3.3. <i>S. aureus</i>.....</b>	<b>6</b>
<b>1.3.4. <i>Streptococcus pneumonia</i>.....</b>	<b>8</b>
<b>1.4. Optimizing antimicrobial therapy of septic shock.....</b>	<b>9</b>
<b>1.5. Bacterial resistance in treatment of septic shock .....</b>	<b>10</b>
<b>1.6. Aminoglycosides.....</b>	<b>12</b>
<b>1.6.1. Spectrum of activity and mechanism of action.....</b>	<b>12</b>

<b>1.6.2 Concentration-dependent killing of bacteria.....</b>	<b>13</b>
<b>1.6.3. Mechanisms of resistance against aminoglycosides.....</b>	<b>14</b>
<b>1.6.3.1. Preventing uptake.....</b>	<b>14</b>
<b>1.6.3.2. Efflux pumps.....</b>	<b>15</b>
<b>1.6.3.3. Target Alteration.....</b>	<b>15</b>
<b>1.6.3.4. Enzymatic inactivation.....</b>	<b>16</b>
<b>1.6.4 Aminoglycoside Toxicity and Side Effects.....</b>	<b>17</b>
<b>1.6.4.1.1 Otovestibular toxicity .....</b>	<b>17</b>
<b>1.6.4.1.2 Mechanism of Ototoxicity.....</b>	<b>18</b>
<b>1.6.4.1.3 Preventing / minimizing ototoxicity.....</b>	<b>19</b>
<b>1.6.4.2. Renal/ nephrotoxicity .....</b>	<b>20</b>
<b>1.6.4.2.1. Mechanism of aminoglycoside induced Nephrotoxicity .....</b>	<b>21</b>
<b>1.6.4.2.2. Risk factors leading to nephrotoxicity due to aminoglycosides .....</b>	<b>21</b>
<b>1.6.4.2.3 Preventing / minimizing nephrotoxicity.....</b>	<b>22</b>
<b>1.6.4.3. Neuromuscular paralysis.....</b>	<b>22</b>
<b>1.6.5. Other concerns about aminoglycosides .....</b>	<b>23</b>
<b>2. Aim and Hypotheses and Objectives.....</b>	<b>25</b>
<b>2.1. Aim.....</b>	<b>25</b>

<b>2.2. Study Hypotheses.....</b>	<b>25</b>
<b>2.3. Objectives.....</b>	<b>26</b>
<b>3. Material and method.....</b>	<b>26</b>
<b>    3.1. Antibiotics.....</b>	<b>26</b>
<b>    3.2. Bacterial strains.....</b>	<b>27</b>
<b>    3.3. Determination of minimum inhibitory concentration.....</b>	<b>27</b>
<b>    3.4. In vitro Pharmacodynamic Model (IPDM).....</b>	<b>28</b>
<b>        3.4.1. Bacterial inoculum.....</b>	<b>29</b>
<b>        3.4.2. Antibiotic.....</b>	<b>30</b>
<b>        3.4.3. Sample points.....</b>	<b>30</b>
<b>        3.4.4. Bacterial counts.....</b>	<b>31</b>
<b>            3.4.4.1. Serial dilution method .....</b>	<b>31</b>
<b>            3.4.4.2. Qualitative culture of broth .....</b>	<b>31</b>
<b>        3.4.5. Determination of Gentamicin concentrations at different doses.....</b>	<b>32</b>
<b>        3.4.6 Statistics.....</b>	<b>32</b>
<b>4. Results.....</b>	<b>33</b>
<b>    4.1. Minimum inhibitory concentrations.....</b>	<b>33</b>

<b>4.2. <i>In vitro</i> pharmacodynamic model (IPDM) with sensitive bacterial strains.....</b>	<b>33</b>
<b>4.3. <i>In vitro</i> pharmacodynamic model with resistant bacterial strains.....</b>	<b>35</b>
<b>5. Discussion.....</b>	<b>38</b>
<b>6. Conclusion.....</b>	<b>43</b>
<b>7. References .....</b>	<b>61</b>

## List of tables:

<b>Table 1:</b> MIC by Micro Broth dilution method.....	44
<b>Table 2:</b> Gentamicin killing of four susceptible bacterial strains in a pharmacodynamic model.....	45
<b>Table 3:</b> Gentamicin killing of four resistant bacterial strains in a pharmacodynamic model.....	46
<b>Table 4:</b> Pharmacokinetic-Pharmacodynamic parameters of supra-pharmacologic doses of gentamicin with sensitive bacterial strains.....	47
<b>Table 5:</b> Pharmacokinetic-Pharmacodynamic parameters of supra-pharmacologic doses of gentamicin with resistant bacterial strains.....	48
<b>Table 6:</b> Different sites of infection with <i>P. aeruginosa</i> , <i>E.coli</i> , <i>S. pneumonia</i> and <i>S. aureus</i> .....	49

**List of figures:**

<b>Figure 1:</b> Pharmacodynamic parameters .....	50
<b>Figure 2:</b> The time course of killing and regrowth of <i>Pseudomonas aeruginosa</i> .....	51
<b>Figure 3:</b> IVPD Model.....	52
<b>Figure 4a:</b> IPDM bacterial kill using <i>E. coli</i> ATCC 700973 .....	53
<b>Figure 4b:</b> IPDM bacterial kill using <i>P. aeruginosa</i> 44302.....	54
<b>Figure 4c:</b> IPDM bacterial kill using <i>S. aureus</i> ATCC 29213.....	55
<b>Figure 4d:</b> IPDM bacterial kill using <i>S. pneumoniae</i> ATCC 49619.....	56
<b>Figure 4a:</b> IPDM bacterial kill using <i>E. coli</i> 90900.....	57
<b>Figure 4b:</b> IPDM bacterial kill using <i>P. aeruginosa</i> 90591.....	58
<b>Figure 4c:</b> IPDM bacterial kill using <i>S. aureus</i> 91056.....	59
<b>Figure 4d:</b> IPDM bacterial kill using <i>S. pneumoniae</i> 52634.....	60

## Abbreviations

AUC.....	Area-Under-the-concentration-Curve
BK12.....	Bacterial Kill Rate over first 12 hours
BK24.....	Absolute Bacterial Kill Rate at 24 hours
Bpm.....	Beats per minute
Ca-MHB.....	Cation adjusted Mueller-Hinton Broth
CFU.....	Colony Forming Unit
CIP.....	Ciprofloxacin
Cmax.....	Maximum Concentration
CTX.....	Cefotaxime
EDP-I.....	Energy Dependent Phase-I
EDP-II.....	Energy Dependent Phase-II
GEN.....	Gentamicin
GNB.....	Gram Negative Bacilli
GPC.....	Gram Positive Cocci
HIV.....	Human Immunodeficiency Virus
ICU.....	Intensive Care Unit
IPDM.....	In vitro Pharmacodynamic Model
KR6.....	Bacterial Kill Rate over first 6 hours
LPS.....	Lipopolysaccharides
LZD.....	Linezolid
MAR.....	Multi Antibiotic Resistant
MHB.....	Mueller-Hinton Broth
MIC.....	Minimum Inhibitory Concentration
MOX.....	Moxifloxacin
MRSA.....	Methicillin-Resistant <i>S. aureus</i>

ND.....Not Determined  
OD.....Optical Density  
PaCO<sub>2</sub>.....Arterial carbon dioxide partial pressure  
SIRS.....Systemic Inflammatory Response Syndrome  
T>MIC.....Time above the Minimum Inhibitory Concentration  
 $t_{1/2}$ .....Serum half-life  
TGC.....Tigecycline  
TOB.....Tobramycin  
TSA.....Trypticase Soy Agar  
VAN.....Vancomycin  
< LD.....Less than limit of detection

## **1. INTRODUCTION**

### **1.1. The clinical problem:**

Septic shock due to bacterial infections, with a mortality rate of 30-50%, is the main cause of death in intensive care units of the developed world[1, 2] The total number of annual deaths associated with sepsis and septic shock is higher than that of first myocardial infarctions, breast cancer or human immunodeficiency virus (HIV) [3]. In addition, survivors of severe sepsis experience a lower quality of life compared with a matched population of the same age and sex [4]. Severe sepsis leads to a substantial economic burden with hospital costs that are 2 to 11 times the usual cost per patient [5]. The prevalence of sepsis in Canada is estimated to be between 27,000 and 30,000 cases per year with an overall mortality rate of 30.5% (45.2% in patients with severe sepsis and 20.9% in those with sepsis which does not progress to severe sepsis) [6]. A large multi-centre study in Canada found that the average length of stay for each patient suffering from sepsis was 6.4 days in the ICU and 29.1 days on the ward with an economic burden of \$20,528 and \$12,422 respectively (a total cost of \$32,950 for each patient) [7].

### **1.2. Definitions:**

‘Sepsis’ is defined as ‘the presence of systemic inflammatory response syndrome (SIRS) with a suspected or confirmed infection’ [8].

SIRS is defined as at least 2 of the following 4 clinical criteria in the context of an appropriate clinical insult (such as infection):

- a. core body temperature greater than 38<sup>o</sup>C or less than 36<sup>o</sup>C
- b. heart rate > 90 bpm
- c. respiratory rate greater than 20 per minute or hyperventilation with a PaCO<sub>2</sub> less than 32 mm Hg
- d. white blood cell count greater than 12,000 cells/mm or less than 4000 cells/mm, or with greater than 10% immature neutrophils in circulation

'Severe sepsis' has been defined as sepsis with evidence of organ failure.

'Septic shock', a form of severe sepsis characterized by cardiovascular failure, can be defined as a state of acute circulatory failure in which there is arterial hypotension despite adequate fluid resuscitation, resulting in tissue hypoperfusion. A minimally acceptable blood pressure cannot be maintained without the use of vasopressors. Hypotension is defined as a systolic blood pressure of less than 90 mm Hg or a drop of the systolic BP of more than 40 mm Hg from the base line. In shock, hypotension is associated with signs of poor end organ perfusion such as low urine output, altered mental state, diminished skin perfusion and increased blood lactate levels (biomarker for septic shock) [9].

### **1.3. Sources of infection and microbiology of sepsis:**

The common sources of infection in patients with severe sepsis and septic shock are the respiratory tract, blood stream (without another identifiable source), abdomen (e.g. peritonitis), urinary tract, and skin/soft tissue [10]. The respiratory system is the most common site of infection (28.8 %), followed by the gastrointestinal system (26.8%), urinary tract (24.7%), primary bloodstream infection (23.3%) and multi-site infection (23.5%) [11].

The microbiology of sepsis has changed over the past four decades. In the late 1970s through the 1980s, Gram negative bacteria were the predominant organisms causing sepsis. Subsequently, Gram positive organisms have outnumbered Gram negatives [12]. Sepsis syndromes caused by Gram positive organisms are mainly from *Staphylococcus aureus*, enterococci, and streptococci. Sepsis caused by Gram negatives is commonly caused by members of the family enterobacteriaceae, especially *Escherichia coli* and *Klebsiella pneumonia*, and by *Pseudomonas aeruginosa*, a non-enterobacteriaceae [10].

In a retrospective national epidemiologic study in the United States over a 22-year period, Gram positive bacteria accounted for 52.1% of cases of sepsis, with Gram negative bacteria accounting for 37.6%, polymicrobial infections for 4.7%, anaerobes for 1.0%, and fungi for 4.6%. Interestingly, there was a 207% increase in the number of cases of sepsis caused by fungal organisms, from 1979 to 2000 [12]. More recent epidemiologic studies, particularly those focusing on severe sepsis, showed a new trend again toward a predominance of Gram negative bacteria and a decrease in *Staphylococcus aureus*, similar to the pattern of 30-40 years ago [13]. Currently, many

different species of bacteria with various antibiotic resistance mechanisms are able to cause infections leading sepsis and related syndromes. Some of the important organisms that usually cause sepsis and septic shock are mentioned below.

### **1.3.1. *Pseudomonas aeruginosa*:**

*Pseudomonas aeruginosa* strains are mandatory aerobic Gram negative bacilli (non- enterobacteriaceae) with a size of approximately 0.5 to 0.8  $\mu\text{m} \times$  1.5 to 3.0  $\mu\text{m}$ . They use their polar flagellum for movement and are opportunistic pathogens.

Infections caused by this organism are considered to be problematic in immune-compromised patients (with high mortality rate in cases of burn, cystic fibrosis and neoplasm and ventilator-associated infections) [14]. Examples of other serious infections caused by *Pseudomonas aeruginosa* can be seen in Table 6. *Pseudomonas aeruginosa* infections are more common in neutropenic and immune-compromised individuals. Various factors are involved in their virulence, such as alginic (an antiphagocytic factor) and toxin A (diphtheria-toxin-like exotoxin). However, the most important virulence factor in bacteremia and septic shock is the endotoxin (LPS), a structural component of the cell wall, which exists in all strains of pseudomonas and, in fact, in all Gram negative organisms [15].

Treatment of infections caused by *P. aeruginosa* is often a serious clinical challenge. Because of efflux and outer-membrane impermeability of these strains, macrolides and tetracycline are not effective. They are also usually resistant to early generation penicillins and cephalosporins for similar reasons and also because of highly

active  $\beta$ -lactamases that are present in these strains. The risk of multidrug-resistant *P. aeruginosa* is a serious problem [16]. Due to multiple antibiotic resistances, antibiotic susceptibility testing is needed in order to manage *Pseudomonas aeruginosa* infections in clinical practice. In the past, combination therapy e.g. gentamicin and carbenicillin has been shown to be effective in treatment of acute infection. However, more recently, extremely potent single agents such as ceftazidime and meropenem have shown comparable results as combination therapy [17, 18].

### **1.3.2. *E. coli*:**

These are aerobic ( facultatively anaerobic) Gram negative bacilli from the Enterobacteriaceae family that are normally carried in the human bowel. The Escherichia genus is a very important member of the normal intestinal flora. *E. coli* is the most commonly isolated clinical strain in the laboratory. It is an opportunistic pathogen causing various kinds of infections such as urinary tract, surgical site and also blood stream infections (Table 6). *E. coli* usually causes cystitis, prostatitis and pyelonephritis in urinary tract and is a major problem in the clinics as well as in the community.

Some enteropathogenic strains of *E. coli* are a common cause of enteric infections, especially in less developed countries. There are 4 main serotypes of *E. coli* in these cases; classical enteropathogenic serotypes (EPEC), enterotoxigenic (ETEC),

enteroinvasive (EIEC), enterohemorrhagic (EHEC), and enteroaggregative (EAEC) strains. These are of less relevance in the developed world [19].

Optimal treatment of *E. coli* infections depends on the site of infection, its severity and the resistance of the organism. Patients with enteropathogenic strains will often require aggressive fluid hydration support. For severe invasive disease, intravenous antibiotics are required. Although monotherapy usually suffices, antibiotic combination therapy can be necessary due to the increased burden of antibiotic-resistant pathogenic *E.coli* [20]. Optimal antibiotic treatment depends on the site of infection. For meningitis, antibiotics such as 3<sup>rd</sup> generation cephalosporins, like ceftriaxone are commonly used. For pneumonia, fluoroquinolones and 3<sup>rd</sup> generation cephalosporins suffice. For serious infections with septic shock, combination therapy of a β-lactam and a fluoroquinolone or aminoglycoside yields the best survival. Other antibiotics such as doxycycline and trimethoprim-sulfamethoxazole are also used for treating infections by *E. coli* [21].

### **1.3.3. *S. aureus*:**

Staphylococci are aerobic (facultative anaerobic) Gram positive cocci with a diameter of about 1μm. *S. aureus*, the major historical pathogen, is coagulase positive. It may be a commensal in as many as 20% of humans (usually on skin or in the nares) [22]. Most of the other staphylococci have limited pathogenicity and are coagulase negative. *S. aureus* can cause infections in different organ systems, from superficial skin lesions (impetigo) and small abscesses to serious skin infections such as

furunculosis, life threatening infections such as endocarditis and nosocomial infections such as surgical wound infection and pneumonia (Table 6). Many of these can lead to sepsis and septic shock. Some strains of *S. aureus* can cause ‘Toxic shock syndrome’ which is due to the release of superantigens that broadly stimulate immune cells into the blood stream. Sometimes, enterotoxins released by the *S. aureus* can be a cause of food poisoning as well [23]. In contrast, coagulase negative staphylococci can cause endocarditis or prosthetic device infections but rarely cause septic shock [24, 25].

*S. aureus* is eliminated mainly through phagocytosis. Toxins can be neutralized by antibodies which also promote opsonization of the bacteria. However, *S. aureus* has virulence mechanisms through which it can evade the immune system. *S. aureus* produces capsule and can form biofilms. Biofilm formation is usually observed on implanted devices and indwelling catheters, thereby protecting the organism from phagocytosis by macrophages. These biofilms act as impermeable barriers and also help the organisms to escape the antibiotics. Along with biofilms, *S. aureus* produces ‘toxin A’ which also interfere with phagocytosis of the organism [26-28].

In the past, most community acquired *S.aureus* infections could be treated with penicillinase-resistant β-lactams such as oxacillin or early generation cephalosporins like cefazolin while infections by methicillin/oxacillin-resistant *S. aureus* strains (often hospital-acquired) required a glycopeptide like vancomycin [23]. More recently, methicillin-resistant clones of *S. aureus* have disseminated in the community which is problematic [29]. Multiple antibiotic resistance (MAR) is increasing rapidly among *S. aureus* strains. Serious outbreaks of methicillin-resistant *S. aureus* (MRSA) can be seen in hospitals and clinics. Most disturbingly, there have been reports of vancomycin-

intermediate resistant strains causing clinical infections in recent years. Vancomycin intermediate resistant and resistant *S. aureus* strains have been reported [30, 31].

In recent years, more than half of the *S. aureus* infections reported in the community have been due to MRSA strains [32]. Hence empiric therapy with beta-lactams and cephalosporins is not feasible. Clindamycin, trimethoprim-sulfamethoxazole (TMP-SMX), rifampin, doxycycline, or a quinolone are usually used in treating infections caused by these strains [33].

#### **1.3.4. *Streptococcus pneumoniae*:**

*S. pneumoniae* is lancet-shaped,  $\alpha$ -hemolytic (incomplete, green hemolysis on blood-agar) and can form capsules. Typically all streptococci are catalase-negative. More than 85 types of *S. pneumoniae* have been described based on the antigens expressed on their capsules. The best known virulence factor of *S. pneumoniae* is its polysaccharide capsule which protects the organism from phagocytosis.

*S. pneumoniae* can be part of the normal flora of the respiratory system but is the most frequent cause of bacterial pneumonia. It can also cause other serious infections such as meningitis and bacteremia (Table 6). The old, disabled and immune-compromised, in particular, are more vulnerable to pneumococcal infections, particularly pneumonia. The mucociliary barrier, phagocytic immune cells and anti-capsular antibodies are involved in defense against *S. pneumoniae*.

Although resistance against penicillin and other antibacterial agents is increasing, penicillin is still generally an effective remedy [34, 35]. However, cephalosporins are

recommended for serious infections requiring hospitalization [36]. Levofloxacin and moxifloxacin are also effective for pneumonia [37].

#### **1.4. Optimizing antimicrobial therapy of septic shock**

The most accepted model of septic shock suggests that this condition results from an exaggerated immune response to the presence of antigens of pathogenic microorganisms. In this model, the immune response is substantially independent of the presence of viable pathogens and progresses independently of the presence of live organisms. A recently developed alternate paradigm suggests that the central driver of septic shock is the “microbial burden” in severe infections. A corollary of this model is that rapid clearance of the pathogen from the body will be the primary determinant of survival in septic shock [38].

Optimal treatment of life threatening infections is a challenge for clinicians. Survival of critically ill patients with severe infection depends on several factors but particularly on prompt initiation of potent antimicrobial agents in order to accelerate pathogen clearance [21, 39]. Timely recognition of life-threatening infections and rapid administration of appropriate antimicrobial therapy is associated with significantly higher survival rates in patients suffering septic shock [38]. In septic shock patients, every hour delay in effective antibiotic dosing in the first six hours after onset of hypotension is associated with 7.6% per hour increase in risk of mortality [40]. Further delay in appropriate antimicrobial therapy beyond 6 hours may decrease the survival rate

to an even greater extent. Rapid antimicrobial initiation clearly optimizes early pathogen clearance [38].

### **1.5. Bacterial resistance in treatment of septic shock:**

Antimicrobial therapy of severe infections in the critically ill involves several unique factors compared to non-critically ill patients. Some of the specific issues to be considered patients with septic shock are critical illness-associated variations in drug metabolism and pharmacokinetics [41]. If not appropriately addressed, these variations can lead to treatment failure and increased mortality of critically ill and septic shock patients [42]. However, the greater concern for clinicians may be the increasing resistance of bacterial pathogens associated with septic shock. For many years, the approach for addressing resistant bacteria pathogens was to identify new antibiotics with ever greater potency. More recently, the limitations of this approach have become apparent. New agents for Gram positive infections are, for the most part, primarily bacteriostatic (i.e. slow to clear pathogens). The situation for resistant Gram negative infections is even worse; many institutions have resorted to using intravenous colistin (a polymixin), an agent that was abandoned for decades because of its very high toxic potential [43].

Antimicrobial therapy has long been recognized as a cornerstone of the treatment of critically ill patients with sepsis and/or septic shock [44, 45]. Although the initial antimicrobial choices for therapy are usually empirical, a judicious approach to selection of these drugs is very important, given the evidence of poor outcomes with inadequate or inappropriate initial treatment. In Gram negative bacteremia, appropriate initial antibiotic therapy reduces mortality rate by over 50% [46-48]. For septic shock, the

impact is even more profound. Inappropriate empiric antimicrobial therapy leads to a five-fold reduction in survival [49].

The gradual decrease in antibiotic sensitivity of major bacterial pathogens, the high frequency of relatively resistant pathogens and the altered pharmacokinetics of antibiotics in the critically ill has driven investigators to examine alternative approaches to antimicrobial dosing in patients with severe infections. Some have suggested increasing the doses of antibiotics to achieve better therapeutic levels in septic shock [38, 50]. Other potentially effective strategies include continuous infusion of  $\beta$ -lactams, or once daily ‘high-dose’ aminoglycosides [51-53]. All of these approaches fundamentally improve antimicrobial pharmacokinetics and accelerate pathogen clearance with no increase in toxicity. None-the-less, additional strategies for improving antimicrobial therapy are required. For conditions with a high mortality rate, an antimicrobial strategy that produces a measurable increase in survival with significant but manageable toxicity could be an entirely acceptable option.

Aminoglycoside antibiotics are known to exhibit concentration-dependent killing of both Gram positive and Gram negative bacteria [54, 55]. In recent years, physicians have begun to take advantage of this concentration-dependent killing by moving to once daily dosing in which the entire daily dose is administered as a single dose (e.g. 5-7 mg/kg gentamicin) [51, 56]. This appears to yield at least equivalent and perhaps higher antimicrobial efficacy with some evidence of decreased renal toxicity. However, the prospect of benefit of even higher doses, beyond 7 mg/kg per day, has not been evaluated, due to concerns about the greater potential for serious drug toxicity.

## **1.6. Aminoglycosides:**

Aminoglycosides are broad spectrum antibiotics. They exhibit bactericidal activity and have concentration dependent activity. Their use has declined in past decades due to the introduction of other antibiotics with less toxicity. Recently, because of the development of resistant strains against most available antibiotics, aminoglycosides has been resurgent. Aminoglycoside's unique characteristics give them unusual therapeutic potential in the empiric treatment of bacterial septic shock caused by Gram positive cocci (GPC) and Gram negative bacilli (GNB) [51, 57].

### **1.6.1. Mechanism of action:**

Aminoglycosides, being positively charged, bind to the negatively charged lipopolysaccharides on the cell wall. From there, they enter the periplasmic space through the outer membrane porins by passive diffusion [58, 59]. Uptake of aminoglycosides from the periplasmic space is an energy dependent process, EDP-1 (O<sub>2</sub> dependent, electron transport system). Once aminoglycoside molecules enter the cytoplasm, they bind to the 30S ribosomal subunit through another energy dependent process (EDP-II) [60-62]. Aminoglycosides bind irreversibly to 16S rRNA of the 30S ribosomal sub unit and prevent translation of proteins or induce false proof reading leading to the formation of aberrant proteins [60, 61]. Aberrant proteins may be inserted into the cell membrane leading to altered permeability of the bacterial cell [62]. This causes accumulation of aminoglycosides at higher concentration in the cell cytoplasm and further impairment in protein synthesis. Inhibition of protein synthesis alone does not confer bactericidal activity. Aminoglycosides are positively charged in

the biological fluids; once they enter the bacterial cells they competitively displace calcium and magnesium ions from the lipopolysaccharide bridges on the cell wall [63]. These ions are important for the formation of biofilms and maintenance of bacterial cell wall integrity. Once these ions are displaced, bacterial cell wall integrity is weakened and cytoplasmic content leaks into the exterior resulting in bacterial cell death.

The spectrum of activity of aminoglycosides and their potency are based on their chemical structures. They have one or several aminated sugars with glycosidic linkages to a dibasic cyclitol. As noted, aminoglycosides exert their antibacterial effects mainly by binding to prokaryotic ribosomes, causing impaired protein synthesis [64]. These agents increase their uptake into the Gram negative bacteria by disruption of Mg<sup>2+</sup> bridges between adjacent lipopolysaccharide molecules in the outer membrane [65-67].

### **1.6.2 Concentration-dependent killing of bacteria**

A specific pharmacodynamic interaction between the antibiotic and the bacteria is necessary in order to achieve successful treatment outcome. These specific relationships can be used as indicators of the antimicrobial effect of the antibiotics and/or clinical cure. Pharmacokinetic parameters such as concentration maximum ( $C_{max}$ ), serum half-life ( $t_{1/2}$ ) and 24 hour cumulative exposure to the antibiotic (area-under-the-concentration-curve [AUC]) are used by clinicians and researchers to predict the efficacy of antimicrobial agents.(Figure 1) Fluoroquinolones and aminoglycosides exhibit a concentration dependent bactericidal effect, i.e. the rate of bacterial killing increases proportionate to the maximum concentration of the antimicrobial agent (vs.

time dependent effect, which means that the bactericidal effect depends on the length of time exposure of the bacteria to the agent). Studies have shown that parameters such as  $C_{max}$  to MIC and AUC to MIC ratios are good predictors of bactericidal effects (and clinical outcomes) of the concentration dependent antibiotics such as aminoglycosides [68, 69].

As can be seen in Figure 2,  $C_{max}$  / MIC ratio is a good predictor of the antimicrobial efficacy of aminoglycosides and fluoroquinolones, T>MIC is a good predictor for beta-lactams and vancomycin and AUC/MIC is a good efficacy predictor for a wide range of antibiotics as it takes into account both the time and concentration factors [68-70].

### **1.6.3. Mechanisms of resistance against aminoglycosides:**

Bacteria have developed various mechanisms by which they evade the antimicrobial activity of aminoglycosides. The mechanisms of resistance observed are as follows;

#### **1.6.3.1. Preventing uptake:**

Aminoglycosides enter the bacterial cytoplasm by diffusion through the porins present on the outer membrane of the cell. Bacterial resistance against aminoglycosides can occur via expression of a low number of porins on the outer membrane leading to decreased uptake of compounds [71]. Another method of resistance is by mutations in the porin proteins. This can lead to altered porin structure

so that aminoglycosides cannot pass through. Once aminoglycosides are in the periplasmic space of the bacterial cell, their subsequent transport across the cytoplasmic membrane requires metabolic energy from the electron transport system in an oxygen-dependent process. Bacteria can respond to the presence of aminoglycosides in the environment and decrease the expression of these electron transport systems or shut them down [62]. Aminoglycoside uptake into the cytoplasm is inhibited leading to resistance. Some bacteria like enterococci, staphylococci, E. coli etc. are capable of surviving in oxygen poor environments (such as abscesses). In this kind of oxygen poor environment, these bacteria resist aminoglycoside antibiotics due to the anaerobic environments impact on limiting oxygen-dependent aminoglycoside transport. Obligate anaerobes are completely aminoglycoside resistant for this reason.

#### **1.6.3.2. Efflux pumps:**

Aminoglycoside resistance in bacteria can also be due to elimination of the compounds from the bacterial cytoplasm by ATP dependent efflux pumps [72]. This can affect susceptibility to the entire class of aminoglycosides. Bacterial efflux pumps are the main cause of multi drug resistance and are often observed in pathogens causing nosocomial infections (Pseudomonas, Acinetobacter etc.) [73].

#### **1.6.3.3. Target Alteration:**

Aminoglycosides bind to 16S rRNA at specific position to form a tight bond. Some pathogens (e.g. Pseudomonas and Serratia isolated in Japan), have been found to have *rmtA* and *rmtB* genes that encode rRNA methylases that modify 16S rRNA at the

aminoglycoside binding positions, conferring resistance against aminoglycosides[74]. A few years after the introduction of streptomycin, streptomycin resistant *Mycobacterium* was isolated. Resistance was due to the methylation of the aminoglycoside binding site on the 16S rRNA [75].

#### **1.6.3.4. Enzymatic inactivation:**

Both Gram positive and Gram negative bacteria resistant to aminoglycosides can express aminoglycoside modifying enzymes that will inactivate these compounds. These enzymes are either plasmid mediated or transposon mediated [76, 77]. The enzymes that modify the aminoglycoside molecules are as follows;

##### **a. Aminoglycoside acetyltransferases (AAC)**

- Catalyze acetylation of one of the four amino groups on aminoglycoside antibiotics.
- Modify the 1' & 3' positions of the 2-deoxystreptamine ring & positions 2 and 6 of the 6-aminohexose ring.

##### **b. Aminoglycoside nucleotidyl transferases (ANT)**

- Catalyze the reaction between Mg-ATP and aminoglycoside to form the O-adenylylated aminoglycoside and the magnesium chelate of inorganic pyrophosphate.
- Tobramycin and gentamycin are inactivated by this enzyme.
- affect 6' & 3' positions in streptomycin.

### **c. Aminoglycoside phosphotransferases (APH)**

- aminoglycoside kinases.
- phosphorylate aminoglycoside antibiotics which affects their ability to bind to the 16S rRNA.
- modify ‘OH’ group at 3` and 5` position.
- Primarily found in staphylococci and enterococci
- lead to broad aminoglycoside resistance

#### **1.6.4. Aminoglycoside toxicity and side effects**

Aminoglycosides are well known to be associated with both otovestibular toxicity and nephrotoxicity [78-81]. Each of the vestibular and auditory branches of eighth cranial nerve can be affected [82]. Renal toxicity effects are more common in patients with a history of renal impairment and those who receive long-term and large cumulative doses of gentamicin [83]. However, renal toxicity is reversible to a great extent [81]. In contrast, otovestibular toxicities are usually not reversible [84]. These toxicities are typically observed when aminoglycosides are used for a prolonged period though they appear independent of dosing considerations [84, 85]. Notably, the occurrence of renal toxicity is not associated with development of otovestibular toxicity [86].

The major acute toxicity with aminoglycosides is neuromuscular impairment [87, 88]. Among the neurological side effects, neuromuscular weakness and a myasthenia gravis-like syndrome are the most concerning [89].

#### **1.6.4.1. Otovestibular toxicity:**

Oto- and vestibular toxicity are among the major adverse effects associated with prolonged dosing of aminoglycoside antibiotics [84]. As noted, both are generally irreversible. Auditory toxicity is manifested as hearing loss. Vestibular toxicity may manifest variably as a combination of dizziness, ataxia, tinnitus, gait difficulties, imbalance, nausea, headache etc. [85, 90]. Gentamicin and streptomycin tend to cause more vestibular toxicity, whereas amikacin, neomycin and kanamycin tend to cause more cochlear toxicity [91].

#### **1.6.4.1. Mechanism of Ototoxicity:**

Oto- and vestibular toxicity associated with aminoglycoside therapy are distinct but overlapping clinical entities with a shared pathophysiologic etiology. Multiple risk factors for aminoglycoside-associated oto(cochlear)- and vestibular toxicity have been proposed. However in contrast to nephrotoxicity, only duration of exposure among dosing considerations (i.e. not trough levels, total cumulative dose, total daily dose, dosing frequency) has been consistently associated with risk of development of these forms of toxicity [90]. In addition to variations in the occurrence of oto- and vestibular toxicity based on the specific aminoglycoside, age has been shown to be a specific risk factor for auditory but not vestibular toxicity [92]. In addition, a gene variant has also been linked to increased risk of oto- but not vestibular toxicity [90].

The pathologic basis of injury appears similar for both forms of toxicity. In animal models, histologic evidence of aminoglycoside-induced otovestibular injury develops gradually and is manifested by loss of hair cells in the cochlear and vestibular

apparatuses [93-95]. This occurs as a consequence of aminoglycoside-generated free radicals in the inner ear [96]. The amino nitrogens and deprotonated alcoholic oxygens of the terminal amino-sugar rings of aminoglycosides participate in decompartmentalizing and chelating metal ions to produce chelated metal complexes. These chelated metal complexes are redox-active and generate reactive oxygen species (ROS) which then induce oxidative damage to biomolecules [85]. Animal experiments indicate that oxygen and nitrogen free radicals are generated in the ear during aminoglycoside dosing and induce an apoptosis cascade in the sensory hair cells [96]. One study reported that free radicals are generated through the interaction of aminoglycosides with iron and lipids and are responsible for aminoglycoside ototoxicity [97]. Recent studies in chicken tissue cultures of sensory epithelium have suggested that, with the increase in the concentration of gentamicin, there is an increase in intracellular calcium levels in these sensory hair cells suggesting influx of calcium as a mechanism of hair cell death [98-100].

#### **1.6.4.1. Preventing / minimizing ototoxicity:**

- Avoid prolonged therapy whether with multiple or once daily dosing where possible.
- Use alternative antibiotics when an option
- Monitor for initial signs of vestibular toxicity and stop administering aminoglycoside antibiotics immediately.

#### **1.6.4.2. Renal/ nephrotoxicity:**

Aminoglycosides are administered by the intravenous route and cannot be dosed via the alimentary route. They are not significantly metabolized by the liver and >90% of the drug is excreted unchanged in the urine. The concentration achieved in the cortex of kidneys is about 50 – 100 times greater than that in serum [56]. Aminoglycoside-associated nephrotoxicity is characterized by a gradual rise in serum creatinine (with concomitant decrease in creatinine clearance) and proteinuria [101-103]. Renal failure is typically non-oliguric but there is a loss of concentrating ability. The occurrence of nephrotoxicity is primarily associated with prolonged elevation of trough (rather than peak) levels [83, 104]. Increased peak concentrations appear to have limited or no impact on such toxicity due to a saturable aminoglycoside transport mechanism described below.

Aminoglycosides bind to megalin proteins present on the brush border membranes of the renal tubular cells [105]. These cells internalize the compound by pinocytosis through a saturable active transport mechanism[106]. These endosomes fuse with lysosomes present in the cell forming endolysosomes. Subsequently, aminoglycosides inhibit the phospholipases present in the lysosomes. Eventually, endolysosomes become permeable and the compound enters the cell cytoplasm at a greater concentration [107]. The higher concentration of aminoglycosides triggers apoptosis of renal tubular cells. Proximal tubular dysfunction arises causing glomerular dysfunction and vasoconstriction. There is a difference in accumulation and activation of apoptosis by different aminoglycoside molecules; for example, gentamicin and netilmicin accumulate in the kidneys at higher concentrations compared to tobramycin and amikacin.

Activation of apoptosis is high with gentamicin and tobramycin and is comparatively low with amikacin and netilmicin. Proximal tubular cells regenerate; hence, nephrotoxicity is reversible to some extent [108, 109].

#### **1.6.4.2.1. Mechanism of aminoglycoside induced nephrotoxicity:**

The concentration of aminoglycosides increases in the renal proximal convoluted tubules after each dose. Available evidence suggests that aminoglycosides trigger the production of free radicals which are in turn responsible for ischemic, toxic and immune mediated tissue injury [110]. Aminoglycoside antibiotics such as gentamicin are found to alter mitochondrial respiration. Mitochondria in the cells of the renal cortex generate reactive oxygen species like hydrogen peroxide in a dose-dependent manner with gentamicin. The formed superoxide and hydrogen peroxide in turn react with elements like iron to form more reactive oxidants leading to further injury. In a biological system, iron is present in the form of heme and is bound to proteins [111].

One study suggest that, ferritin, the stored form of iron in mitochondria, is attacked by superoxide thereby releasing iron [112]. This in turn acts as a source of metal and further reactions occur leading to formation of highly active free radicals which lead to the toxic effect. As a consequence, the severe tubular necrosis of kidney epithelial cells can be observed [111]. Because these cells can regenerate, aminoglycoside-induced renal toxicity is often reversible

#### **1.6.4.2.2. Risk factors leading to nephrotoxicity due to aminoglycosides [113]:**

- Old age
- Malnutrition

- Co-administration of furosemide, cephalosporin, amphotericin B, vancomycin, or piperacillin
- ICU patients
- Severe acute liver and renal diseases
- Decreased aminoglycoside excretion

#### **1.6.4.2.3. Preventing / minimizing nephrotoxicity:**

Nephrotoxicity can be minimized by

- Intermittent monitoring of serum aminoglycoside concentrations when administered as conventional or multiple dosing.
- Avoidance of intravascular volume depletion
- Intermittent monitoring of plasma creatinines and calculation of creatinine clearance in order to detect any significant renal injury
- Intermittent measurement of renal injury biomarkers such as urine beta-2-micro globulins [114, 115] (currently unavailable for clinical use).
- Termination of aminoglycoside dosing once there is a preliminary indication of nephrotoxicity.
- Use of free radical scavengers and iron chelators such as D-methionine to prevent free radical mediated toxicity (effective in experimental models) [96].

#### **1.6.4.3. Neuromuscular weakness:**

This is an infrequent but a potentially dangerous manifestation of acute drug toxicity. Severe neuromuscular dysfunction can cause respiratory failure and death [88].

The underlying risk factors for this effect are;

- concomitant use of neuromuscular blocking agents for anesthesia or in the intensive care unit

- concomitant neuromuscular disease (e.g. myasthenia gravis, Guillain-Barre Syndrome, critical illness polyneuropathy)
- concomitant hypocalcaemia .

Careful monitoring is essential when treating people with neuromuscular disorders such as myasthenia gravis and patients prone to hypocalcaemia. Neuromuscular paralysis can be partially reversed by administering calcium salts and potentially treated with mechanical ventilator support [89].

#### **1.6.5. Other concerns about aminoglycosides:**

There are significant issues regarding optimizing antimicrobial therapy of patients with life-threatening infections including sepsis and septic shock. In these conditions, various pathophysiological changes occur causing alteration in pharmacokinetics of antimicrobials and other drugs. At the initial stages of sepsis, there is an increase in the cardiac output causing increased clearance of aminoglycosides and other antibiotics. Capillary leakage leads to intravascular volume depletion followed by aggressive fluid resuscitation which increases extracellular water. This increase in extracellular fluid results in a marked increase in volume of distribution of aminoglycosides which are substantially restricted to that fluid compartment. Both these phenomena lead to low plasma concentration of administered aminoglycosides with standard dosing. As a consequence, higher than standard doses of aminoglycosides maybe required to achieve the target maximum concentration ( $C_{max}$ ) that yields optimal clinical response.

In addition, antimicrobial resistance in bacteria against most of the available antibiotics is of a major concern. Research and development of new and potent antibacterial agents is the primary way that society has tackled the resistance problem. However, new antibacterial agents for Gram positive bacteria as mentioned are limited in their cidal activity; most are bacteriostatic. For Gram negative bacterial pathogens, the condition is far worse as many of the hospitals have been using intravenous colistin, an antibacterial agent that was not being used for many years because of its very toxic potential [116, 117]. Alternative approaches to antimicrobial dosing (continuous infusion of  $\beta$ -lactams, once daily dosing of aminoglycosides) have been and continue to be examined [118].

#### **1.6.4.10. Ultra-high aminoglycoside dosing:**

Despite the interest and research in alternate dosing schemes, antimicrobial approaches that might substantially increase antimicrobial efficacy but have a risk of significant toxicity have not been considered. For instance, aminoglycoside antibiotics are well known and highly studied antibiotics. They have been in use for the past 60 years and have broad spectrum activity. They eliminate bacteria in a concentration dependent manner and may generate a prolonged post-antibiotic effect. For the most part, renal toxicity is associated with elevated trough concentrations during prolonged therapy and not on peak concentrations achieved in the body. Oto- and vestibular toxicity appears to occur independent of dosing considerations. Hence, the use of a single, ultra-high dose of an aminoglycoside in a patient may not have significant long-term toxic effects. Single ultra-high doses may exhibit a rapid clearance of pathogenic

bacteria and could potentially be of benefit in the survival of patients with a broad array of life-threatening infections including those with septic shock.

Given that the mortality of septic shock exceeds 50 - 70% and that over 210,000 deaths occur as a consequence of this disorder every year in the USA alone [119], an antimicrobial strategy that produces a measurable increase in survival while generating significant though manageable side effects could be an entirely acceptable option.

## **2. Aim, Hypotheses and Objectives**

### **2.1. Aim**

This study seeks to explore the potential antimicrobial efficacy of single, ultra high dose aminoglycoside therapy (gentamicin) against sensitive and resistant Gram positive and Gram negative bacterial strains in an *in-vitro* pharmacodynamic model.

### **2.2. Hypotheses**

A single, ultra-high dose of an aminoglycoside will be effective in clearing Gram positive and Gram negative bacterial strains in an *in-vitro* pharmacodynamic model.

## **2.3. Objectives**

- A.** To determine the ability of gentamicin to eliminate susceptible and moderately resistant bacterial strains using single, ultra-high doses of 10, 20 and 40 mg/kg compared to the standard 5 mg/kg once daily dose in an in vitro pharmacodynamics model
- B.** To assess the antibacterial effects of ultra-high dose gentamicin against four bacterial genera associated with septic shock at different time points up to 24 hours.

## **3. Materials and methods:**

### **3.1. Antibiotics:**

Gentamicin (Sandoz Canada Inc., Qc, Canada) tobramycin (Sandoz Canada Inc., Qc, Canada), cefotaxime sodium (Sanofi Aventis Inc., Quebec, Canada), ciprofloxacin (Bayer Inc., Toronto, Canada), Moxifloxacin (Bayer Inc., Toronto, Canada), vancomycin hydrochloride (Pharmaceutical Partners of Canada Inc., Richmond Hills, Ontario, Canada), linezolid (Pfizer Inc., Quebec, Canada) and tigecycline (Wyeth Canada inc., Montreal, Canada) were utilized for this study. All antibiotics were refrigerated at 4°C until use. Prior to each study, the desired antibiotic concentration was prepared by diluting the antibiotics in cation-adjusted Mueller Hinton Broth (Ca-MHB) (Difco, Maryland, USA). Antibiotic dilutions were prepared in 96 well plates as per the CLSI guidelines.

### **3.2. Bacterial strains:**

Four genera of bacteria were studied (*S. aureus*, *S. pneumoniae*, *P. aeruginosa* and *E. coli*). Each genus included one moderately gentamicin-resistant and one susceptible strain. Standard, sensitive bacterial strains include *S. aureus* ATCC 29213, *S. pneumoniae* ATCC 49619, *E. coli* ATCC 700973 and *Pseudomonas aeruginosa* 44302 (a clinical strain obtained from a burn infection patient with septic shock). All the resistant strains were selected clinical isolates obtained from various hospitals throughout Winnipeg, Manitoba, Canada (courtesy of Dr. George Zhanell from Health Sciences Center, Winnipeg). All bacterial strains were stored in the – 80° C freezer until used.

### **3.3. Determination of minimum inhibitory concentration (MIC):**

MICs were determined by the broth micro dilution method following the CLSI guidelines (M07-A9) [120]. MICs of Gentamicin and other standard antibiotics were determined against the eight bacterial strains used in this study. All antibiotic stock solutions were prepared following the CLSI M7-A7 method [121].

Bacterial strains were sub-cultured in Mueller Hinton Broth (Difco) overnight. The optical density of the inoculum was adjusted to 0.080 at 625 nm using spectrophotometer (spectronics-20, Genesys). The inoculum was diluted 1: 100 times and 50 µL of inoculum was dispensed into wells already containing 50 µL of Ca-MHB with the desired concentration of antibiotic to attain a bacterial concentration of  $5 \times 10^4$  CFU/well ( $5 \times 10^6$  CFU/ml). Antibiotic concentrations tested were 0.125, 0.25, 0.5, 1,

2, 4, 8, 16, 32, 64 and 128 µg. Plates were incubated at 37° C for 18 – 24 hours to determine the MICs. The lowest concentration of antibiotic at which visible growth of bacteria was inhibited is considered as MIC of that antibiotic against that specific bacterial strain. MICs of all antibiotics against the 8 bacterial strains were determined in triplicate on separate days (Table 1).

### **3.4. In vitro Pharmacodynamic Model (IPDM):**

The *in-vitro* pharmacodynamic model (substitution/ dilution model) was performed as described previously [122, 123]. Each IPDM set up consisted of a 4 L reservoir flask containing fresh, sterile Ca-MHB. Four sterile 250 mL, single side neck, round bottom organism-inoculated flasks and a 3 L waste collection flask were used in each study. The organism-inoculated flask has an inlet and outlet to facilitate the flow of media into the flask from the reservoir flask. The outlet is used to remove media from the bacteria-innocolated flask to the waste collection flask. The side neck of the bacteria-inoculated flask is plugged with a rubber septum. This side neck is used for collection of sample from the flask at different time points (Figure 3). All the IPDM experiments were set up in aseptic conditions.

All flask connections were made using sterile silicon tubing in an aseptic environment. The media in the organism-inoculated flasks was stirred with magnetic bars and maintained at a constant temperature of 37° C in a heated water bath throughout the experiment. To allow the initial filling of the inoculum-containing flasks, needle tips were inserted into the rubber septum on the side neck and metal clamps were clipped to the silicon tubing that comes out of the bacteria-containing flasks to the waste collection flask. Ca-MHB was pumped to the bacteria-inoculated flasks from the reservoir flask

using a computerized pump (Masterflex®, L/S®; Cole-Parmer Company, Chicago, Illinois, USA) set at a flow rate of 4 mL/min. After the bacteria-containing flasks were filled up to 250 mL mark (So  $V_d$  can be considered 250 ml), the flow rate ( $Cl = 0.96$  mL/min) was calibrated to dispense the media at the elimination constant of gentamicin in the critically ill patient according to this formula based on a  $t_{1/2} = 3$  hours [124]

$$Cl = \frac{\ln 2 V_d (\text{ml})}{T_{1/2} (\text{min})} = \frac{0.69 \times 250}{180} = 0.96 \text{ ml/min}$$

The needle from the rubber septum of side neck and the clamp on the silicone tubing was removed facilitating free flow of used media from the bacteria-containing flask to the waste collection flask. The reservoir flask was refilled with fresh, sterile CaMHB as required following aseptic procedures.

### **3.4.1. Bacterial inoculum:**

8 bacterial strains were tested in this model. Bacterial strains were sub-cultured on Mueller Hinton Agar plates and incubated at 37°C for 18 to 24 h. Five to ten colonies from overnight culture were suspended in 10 mL of Ca-MHB, vortexed and incubated at 37°C for 3 – 4 hours in a shaker incubator. The optical density (OD) of the inoculum was adjusted to 0.080 at 625 nm. 2.5 mL of OD adjusted inoculum was injected into each bacteria-containing flask and left for 30 minutes to allow bacteria to enter logarithmic growth phase. The initial inoculum at the start of the experiment with all the bacterial strains ranged from  $5 \times 10^5$  to  $1 \times 10^6$  CFU/mL as per previous studies [123, 125].

### **3.4.2. Antibiotic:**

Gentamicin was injected into the bacteria inoculated / infection flasks at 5 mg/kg, 10 mg/kg and 20 mg/kg doses for susceptible bacterial strains and at 5 mg/kg, 10 mg/kg, 20 mg/kg and 40 mg/kg doses for resistant strains. All the doses were a single dose over 24 hours to simulate clinical doses and concentration profiles observed in patients. Along with treated flasks, one flask was left as untreated control flask in all the studies. The peak concentration ( $C_{max}$ ) of gentamicin achieved by an intravenous dose in critically ill patients is calculated using the following formula;  $C_{max} = \text{dose (mg/kg)} / \text{volume of distribution (L/kg)}$ . The volume of distribution of gentamicin was considered as 0.3 liters/kg which is usually observed in critically ill septic patients[126]. So Peak concentrations achieved after 5 mg/kg, 10 mg/kg, 20 mg/kg and 40 mg/kg doses were 16.6  $\mu\text{g/mL}$ , 33.3  $\mu\text{g/mL}$ , 66.6  $\mu\text{g/mL}$  and 133.3  $\mu\text{g/mL}$  respectively.

### **3.4.3. Sample points:**

Just before infusing gentamicin doses, 0.5 mL of samples were withdrawn from all the bacteria-inoculated flasks to determine the initial bacterial load. Once the flasks were injected with gentamicin, the flasks were left for 10 minutes to allow the interaction of antibiotic and the bacteria. 0.5 mL of sample was collected from all the flasks after 10 minutes and this point was regarded as 0 h. The pre-calibrated infusion pump was initiated to pump fresh media into the untreated control and treated flasks at a flow rate producing the half-life of 3 h for gentamicin [124]. The amount of inflow of

media from reservoir flask into the central bacteria-inoculated flask is equal to the amount of out flow into the waste collection flask.

#### **3.4.4. Bacterial counts:**

For colony count and gentamicin concentration determinations, 0.5 mL samples were collected from the bacteria-containing flasks at 0, 15 minutes, 45 minutes, 1.5, 3, 6, 9, 12, 18 and 24 hours. Samples were serially diluted in ice cold normal saline. For enumeration of bacterial counts, two techniques are used:

**3.4.4.1. Serial dilution method:** it allows to minimization of antibiotic carry over. Aliquots of 10  $\mu$ L were plated in duplicate onto Mueller Hinton Agar plates for *E. coli*, *P. aeruginosa* and *S. aureus* and on Columbia Agar Plates (Oxoid) for *S. pneumonia* strains.

**3.4.4.2. Qualitative culture of broth:** The collected broth samples from the central antibiotic dispensed flasks and from the control flasks are diluted serially in normal saline. 100  $\mu$ L and 10  $\mu$ L of diluents were plated. Direct broth samples (100  $\mu$ L) were spread on the plates to determine the counts from broth. As serial dilution method, Mueller Hinton Agar plates are used for *E. coli*, *P. aeruginosa* and *S. aureus* and Columbia Agar Plates (Oxoid) for *S. pneumonia* strains.

The plates were incubated for 18 – 24 hours at 37<sup>0</sup> C and the mean bacterial counts were determined. Limit of detection of bacterial counts was 10 CFU/mL of media.

Viable bacterial colonies between 10 and 100 were counted using a lower limit of detection (< LD) of 10 CFU/100 µL. Log<sub>10</sub> CFU and Log<sub>10</sub> CFU reduction were calculated compared to control to determine the early bacterial kill rate over first 6 hours (KR6) for sensitive strains and for the first 12 hours (BK12) with the resistant strains and absolute bacterial kill rate at 24 hours (BK24) for all the bacterial strains.

#### **3.4.5. Determination of gentamicin concentrations:**

Gentamicin concentrations were determined using bioassay as described earlier [123, 127]. *M. luteus* ATCC 4698 was used as the test organism with a lower limit of quantification of 0.25 mg/L and were linear concentrations from 0.125 to 128 mg/L were used. The agar-well method was used to determine the zone diameters using standard concentrations of gentamicin as well as from the samples collected at different time points from treated flasks against *M. luteus*. The concentration of gentamicin was determined at various time points for each dose by comparing the zone diameters from treated groups with that of the standard zone of inhibition diameters at known concentrations. Based on these concentrations, area under the concentration curve (AUC) / MIC (mg. h /L) and peak concentration ( $C_{max}$ )/ MIC (µg/mL) were calculated for each bacteria strain using the trapezoidal rule [125, 127].

#### **3.4.6 Statistics:**

For all bacterial strains examined, bacterial log<sub>10</sub> CFU reduction and complete eradication of bacteria at the ultra-high doses gentamicin were assessed in relation to the

control 5 mg/kg (i.e. standard dose) at 3, 6, 12 and 24 h time points. Changes in log CFU/mL at incremental ultra-high doses were compared to standard 5 mg/kg dose with one-way ANOVA with Dunnett's post-test using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA. A p value < 0.05 was considered significant.

#### **4. Results:**

##### **4.1. Minimum inhibitory concentrations:**

The MICs of standard antibiotics along with gentamicin were determined and tabulated in Table 1. Susceptible bacterial strains were obtained mostly from the ATCC (American Type Culture Collection) except for the *Pseudomonas aeruginosa* 44302, which was isolated from a burn infection patient from burn unit, Health Sciences Center, Winnipeg. The MIC range of susceptible bacterial strains against gentamicin was between 0.5 and 2 µg/mL. After screening a panel of approximately 100 clinical strains that were isolated from septic shock patients by the broth micro-dilution method, strains exhibiting moderate to high MICs against gentamicin were picked up for the IPDM study. The MICs of selected resistant strains ranged from 16 to 64µg/mL.

##### **4.2. *In vitro* pharmacodynamic model (IPDM) with sensitive bacterial strains:**

Gentamicin killing of the four sensitive bacterial strains in the *in vitro* pharmacodynamic model is described in table 2. The average initial bacterial load at the start of the experiment with all the sensitive bacterial strains was  $6.22 \pm 0.22 \log_{10}$

CFU/mL. At 5 mg/kg dose of gentamicin, maximum  $\log_{10}$  CFU reductions observed against *E. coli* Bort strain (ATCC 700973), *Pseudomonas aeruginosa* 44302 and *S. pneumoniae* (ATCC 49619) are  $2.2 \pm 0.02$ ,  $2.8 \pm 0.17$  and  $4.1 \pm 0.05$  within 3 h after start of the experiment respectively. Gentamicin at 5 mg/kg dose against *S. aureus* ATCC 29213 exhibited a maximum  $\log_{10}$  CFU reduction of  $2.32 \pm 0.41$  within 1.5 h after start of the experiment. Regrowth was observed in *E. coli* starting from the 6<sup>th</sup> hour. Regrowth in all the four sensitive bacterial strains at 24 h (end point) after 5 mg/kg dose was an average increase of 1 to  $1.2 \log_{10}$  CFU/mL compared to the baseline (absolute concentration,  $7.2 \pm 0.17 \log_{10}$  CFU/mL). This indicates that the 5 mg/kg dose of gentamicin (congruent with 5-7 mg/kg standard dosing) does not completely eliminate sensitive pathogens after a single dose.

In contrast, gentamicin at high doses of 10 and 20 mg/kg eradicated all sensitive bacterial strains except *P. aeruginosa* by 45 minutes and 15 minutes after the start of the study respectively. For *P. aeruginosa*, complete eradication was observed starting at 1.5 h at 10 mg/kg dose and within 15 minutes at 20 mg/kg dose. In sensitive organisms exposed to 10 and 20 mg/kg doses, there was no regrowth observed up to the 24 hour time point. The AUC/MIC and C<sub>max</sub> /MIC values achieved with 5, 10, and 20 mg/kg doses against the sensitive bacterial strains are tabulated in table 4. In Gram negative sensitive strains; there was a statistically significant difference in  $\log_{10}$  CFU/ml between untreated controls and 5, 10 and 20 mg/kg doses starting from 12 h, 1.5 h and 45 minutes post treatment respectively with minimum p values < 0.05 (exact p values shown in figure legends) . With Gram positive strains, statistically significant difference with a p value of < 0.05 was observed between untreated controls and standard dose of 5

mg/kg starting from 6 h and with higher doses of 10 and 20 mg/kg starting from 45 minutes post treatment respectively. In both Gram positive and Gram negative strains, there was a statistically significant difference in  $\log_{10}$  CFU/ml between the standard 5 mg/kg dose compared to higher doses of 10 and 20 mg/kg starting from 1.5 h and 45 minutes respectively (Figure 4a to Figure 4d). Complete elimination of sensitive strains (below detection limit) was observed with higher doses of gentamicin within minutes after start of the experiment.

#### **4.3. *In vitro* pharmacodynamic model with resistant bacterial strains:**

Bacterial kill from gentamicin obtained against the resistant strains tested was diverse. Gentamicin killing of the four resistant bacterial strains in the *in vitro* pharmacodynamic model is described in table 3. The standard gentamicin 5 mg/kg dose, with all the four resistant bacterial strains, did not show any statistically significant difference in  $\log_{10}$  CFU/ml compared to untreated controls throughout the experiments. With *E. coli* 90900, gentamicin exhibited a maximum  $\log_{10}$  CFU reductions of  $4.15 \pm 0.24$ ,  $5.68 \pm 0.96$  and  $6.22 \pm 0.07$  at 10, 20 and 40 mg/kg doses after 3 h of start of the experiment. However, *E. coli* 90900 started to re-grow starting at 3 h with 10 and 20 mg/kg and at 6 h with 40 mg/kg dose (Figure 5a). There was a statistically significant difference between the regular 5 mg/kg dose compared to 10, 20 and 40 mg/kg high doses starting from 3 h, 1.5 h and 45 minutes with p value of  $< 0.05$  respectively.

Gentamicin at 10 and 20 mg/kg doses with *Pseudomonas aeruginosa* 90591 exhibited  $3.71 \pm 0.09$  and  $4.69 \pm 0.27$   $\log_{10}$  CFU reductions at 3 h post-dosing. Complete

eradication of *P. aeruginosa* (below detection limit) was observed with both these doses by 6 h. Regrowth was observed in 10 mg/kg and 20 mg/kg doses starting at 6 h time point. At 40 mg/kg dose, *Pseudomonas aeruginosa* 90591 exhibited a  $\geq 3 \log_{10}$  CFU reduction by 1.5 hours after administration and completely eradicated the organisms (below the detection limits) from the 3 h time point (Figure 5b) up to end of the experiment. There was a statistically significant difference between the standard 5 mg/kg dose of gentamicin and 10, 20 and 40 mg/kg high doses starting from 9 h, 6 h and 1.5 h after start of the experiment with p value of < 0.05. Gentamicin at 10, 20 and 40 mg/kg doses against *S. aureus* 91056 exhibited a maximum  $\log_{10}$  CFU reduction of  $2.15 \pm 0.68$ ,  $2.93 \pm 0.15$  and  $3.56 \pm 0.58$  compared to baseline by 3 h after start of the experiment.

With the resistant *S. aureus* strain, statistically significant difference was observed between 5mg/kg dose and 10, 20 and 40 mg/kg high doses starting from 3 h, 45 min and 15 minutes after start of experiment respectively. *S. aureus* started to re-grow starting at 3h with all the doses. By 24 hours, bacterial density in the three gentamicin dosed flasks was comparable to the untreated control (Figure 5c). Gentamicin at 10 mg/kg dose exhibited a  $\geq 3 \log_{10}$  CFU reduction against *S. pneumonia* 52634 by 6 h and the bacteria was below the detection level with 20 mg/kg dose at the same time point. However, the organism started to re-grow at both these doses. With 40 mg/kg dose, a complete eradication of *S. pneumonia* (below the detection limit) was observed starting from the 3 h time point (Figure 5d). Regrowth was not observed at this dose up to the end of the experiment. A statistically significant difference in  $\log_{10}$  CFU/ml between the regular 5 mg/kg dose of gentamicin compared to 10, 20 and 40

mg/kg high doses was observed starting from 12 h, 6 h and 45 minutes after dosing respectively.

Greater than  $3 \log_{10}$  CFU reduction is considered as bactericidal effect. Against gentamicin high resistant strains of *S. pneumonia* 52634, ultra-high doses of gentamicin at 10 and 20 mg/kg exhibited a bactericidal effect up to 12 and 24 h. Against another gentamicin high resistant strain of *P. aeruginosa*, ultra-high doses of 10 and 20 mg/kg exhibited a bactericidal effect up to 12 h after dosing. Complete elimination of both the high resistant strains was observed within 3 h after 40 mg/kg dose of gentamicin. This indicates that gentamicin high resistant strains can be eliminated at a faster rate with ultra-high doses. Against gentamicin resistant strain of *E. coli* 90900, ultra-high doses of 10 and 20 mg/kg of gentamicin exhibited a bactericidal activity up to 6 h and 40 mg/kg dose exhibited a bactericidal effect up to 9 h in the *in vitro* IPDM. Against highly resistant strain of *S. aureus* 91056, ultra-high doses of gentamicin at 20 and 40 mg/kg exhibited a bactericidal activity at 3 h post dosing.

Pharmacodynamic parameters against sensitive and resistant strains with different doses of gentamicin are tabulated in Table 4 and Table 5 respectively.

## **5. Discussion:**

Elimination of pathogens with appropriate antimicrobials is the cornerstone of treatment of life threatening infections. In recent decades the limitations of treatment

with antimicrobial agents has become evident. The effectiveness of commonly used antimicrobial agents such as  $\beta$ -lactams, aminoglycosides and fluoroquinolones has been weakened by the rapid rise in the antibiotic-resistant bacteria [125]. There has been no improvement in the survival rates of the patients with most serious infections such as septic shock over several decades [128].

Increasingly potent antibiotics have been developed and introduced to clinical practice in order to overcome this challenge. At the same time efforts have been made to improve treatment outcomes through pharmacodynamics and pharmacokinetic optimization of the existing antibiotics dosing practices; a variety of approaches exist but what all of them have in common is improving the rate of clearance of pathogens from the body thereby improving clinical outcomes[38].

In serious, life threatening infections such as meningitis, septic shock and rapidly progressive necrotizing infections, where there is a direct relationship between the risk of adverse outcomes and delay in providing the effective treatment, clearance of the pathogen by the antimicrobial agent is the key element determining the clinical outcome [38]. Research has shown that rapid initiation of appropriate antimicrobial therapy against the pathogen improves the outcomes in patients suffering with septic shock, due to the rapid clearance of the organism[40, 129]. Similarly in bacterial meningitis, clinical outcomes are improved by rapid initiation of the antimicrobial treatment [130]. Combination therapy in septic shock and bactericidal antibiotics in meningitis (and other life-threatening infections) also accelerate pathogen clearance and are associated with better survival outcomes [21, 38, 131]. Recent studies have shown that extended infusions of  $\beta$ -lactams, which improves bacterial clearance results in better clinical

outcomes in serious infections and sepsis, compared to the standard intermittent administration of these drugs [53, 132]. Some evidence also suggests that administering aminoglycosides in one daily dose may yield superior clinical results compared to the standard, 3 times daily dosing [101, 133].

Severe infections associated with septic shock are conditions with extremely high mortality. As rapid clearance of the pathogenic bacteria is the key to success in treatment, it is important to assess the merits of the unconventional antibiotic therapies. For conditions with very high mortality rates, it may be acceptable to use therapeutic regimens that improve survival rate even though they may be associated with significant but manageable toxicity.

Gentamicin is an aminoglycoside, one of the first developed classes of antibiotics. The main pharmacodynamic parameter that determines the efficacy of gentamicin is the maximum (peak) concentration ( $C_{max}$ ) to minimum inhibitory concentration ratio[134]. The importance of the  $C_{max}$ /MIC ratio was emphasized by Moore et al after conducting a clinical trial on 236 patients with Gram negative infections [55, 135]. A  $C_{max}$ /MIC ratio of 8 to 10 is associated with optimum bacterial clearance and clinical response rates in treatment of serious bacterial infections [136].

This study was designed to determine if higher than standard (ultra-high) doses of gentamicin a) show dose dependent antibacterial effects across the ultra-high dose range, b) are associated with accelerated clearance of a wide range of pathogens, and c) can clear sensitive pathogens with single dose administration. Our studies confirm all these propositions. A single ultra-high dose of gentamicin at 10, 20 and 40 mg/kg can

effectively clear sensitive and moderately resistant bacterial strains in a dose dependent fashion (with a  $\geq 3$  log<sub>10</sub> CFU reduction in blood bacterial counts) in our in-vitro pharmacodynamic model. Increasing bactericidal activity extends well beyond the range of C<sub>max</sub>/MIC ratios typically recommended for optimal clinical response. We also showed accelerated clearance of these pathogens from an IVPD model (45 minutes at the dose of 10 and 20 mg/kg gentamicin for sensitive strains other than *P. aeruginosa* and 3 hours with 40 mg/kg gentamicin for moderately resistant bacteria other than *S. aureus*). We also observed accelerated clearance of sensitive and moderately resistant bacteria (greater than 3 log<sub>10</sub> CFU) with elimination of viable sensitive organisms within minutes after ultra-high doses of gentamicin. We can reasonably anticipate an even faster clearance of the bacteria from the blood stream in vivo due to the combination of the effects of the ultra-high doses of gentamicin and an active host immune system.

Assuming that toxicity concerns are not insurmountable, these data suggest a potential alternate dosing scheme that could be utilized with aminoglycosides in severe infections associated with a high risk of death. For sensitive pathogens associated with fulminant, rapidly progressive infections with organ failure, ultra-high dose therapy could rapidly reduce the organism burden and allow faster resolution of organ injury. In addition, this approach could offer an alternate treatment approach for multi-drug resistant pathogens for which there are currently few safe options.

Ultra-high dosing of aminoglycosides has not been pursued in the past due to toxicity concerns. However, these drugs possess important properties that may allow consideration of clinical ultra-high dose regimens. Chief among these is that

antimicrobial efficacy is related to the maximum (peak) concentration achieved which we have shown also extends to the markedly elevated concentrations demonstrated in this study[136]. This suggests that we may find enhanced antimicrobial efficacy in *in vivo* with ultra-high dosing regimens. In addition, renal toxicity, the most clinically concerning toxicity in severely ill patients, appears to be dominantly associated with persistently elevated trough levels of drug [81, 136] while otovestibular toxicity appears to primarily be associated with duration of drug exposure [90]. Renal toxicity specifically has been shown to be mediated through a saturable active transport mechanism [136]. This is, in part, the basis of the broad transition to the use of once-daily aminoglycoside dosing regimens over the last couple of decades.

Since acute toxicity concerns have limited examination of ultra-high dosing options so there is remarkably little data to assess the risk and severity of acute clinical toxicity manifestations. To the limited extent such data exists, it appears that concentration-dependent neuromuscular weakness/paralysis which leads to respiratory depression may be the main dose-limiting acute toxicity [87, 137-141]. Given that mechanical ventilation is a routine in treatment protocol for critically ill patients with serious infections and septic shock, neuromuscular weakness (specifically respiratory depression) may be a manageable issue.

Our study suggests that ultra-high doses of gentamicin may potentially be beneficial for treatment of critically ill patients with severe infections caused by a broad range of pathogens including those associated with septic shock. This dosing approach can be useful in several different scenarios. Cases of infections caused by aminoglycoside-resistant community pathogens are relatively infrequent. Ultra-high

doses of aminoglycosides may be of benefit in situations of otherwise multiple drug resistant Gram positive and Gram negative bacteria, particularly in the nosocomial or health care-associated infections. Pathogens that are relatively insensitive against standard dosing could potentially be rendered sensitive when they are exposed to ultra-high dosages. Furthermore, this dosing approach may be useful for sensitive organisms causing septic shock where a single dose of drug could very rapidly clear the pathogen (albeit another antibiotic might be required for follow-up therapy to ensure pathogen eradication). Previous data suggests that accelerated pathogen clearance may improve survival rate in septic shock[38]. Ultra-high dose therapy could even have potential in bacterial biowarfare/bioterrorism threats (e.g. *Yersinia pestis*, *Bacillus anthracis*, *Burkholderia mallei*, *Francisella tularensis*, *Brucella* species) where accelerated pathogen eradication may be central to limiting rapidly progressive tissue injury and organ failure with such infections.

Further animal studies are warranted to confirm our results in an in vivo model of life-threatening infection with septic shock.

## **6. Conclusion:**

We report that a single, ultra-high dose of gentamicin at 10, 20 and 40 mg/kg can effectively clear sensitive and resistant bacterial strains with a  $\geq 3$  log<sub>10</sub> CFU reduction within early hours after administering the antibiotic in an *in vitro* pharmacodynamic model. We also observed rapid and complete elimination of sensitive bacteria within minutes after ultra-high doses of gentamicin. For resistant pathogens, we can anticipate an even faster elimination of bacteria *in vivo* due to the contribution of an intact immune response to the bactericidal effect of ultra-high doses of gentamicin. An ultra-high aminoglycoside dosing strategy may be beneficial for improving outcome for patients with life-threatening infections with septic shock. Further animal studies are warranted to confirm these results in an *in vivo* model of septic shock.

**Table 1: MIC by Micro Broth dilution method**

Organism	Strain	Minimum inhibitory concentration in µg/mL							
		GEN	TOB	CIP	MOX	CTX	VAN	LZD	TGC
<i>E. coli</i>	ATCC 700973	1	1	<0.25	<0.25	0.125	ND	ND	<0.25
<i>E. coli</i>	90900	32	128	32	32	>128	ND	ND	1
<i>p. aeruginosa</i>	44302	2	0.5	0.125	1	16	ND	ND	8
<i>p. aeruginosa</i>	90591	16	1	2	8	>128	ND	ND	16
<i>S. aureus</i>	ATCC 29213	0.5	4	0.25	<0.25	2	1	2	0.25
<i>S. aureus</i>	91056	64	>128	32	4	16	<0.25	1	0.25
<i>S. pneumoniae</i>	ATCC 49619	1	2	1	0.5	0.03	0.25	1	0.06
<i>S. pneumoniae</i>	52634	16	32	0.5	0.125	1	0.25	2	0.125

ND = not determined

**Table 2: Gentamicin killing of four susceptible bacterial strains in a pharmacodynamic model**

Organism	Dose	Time point and Log <sub>10</sub> CFU reduction					
		45 min	1.5 h	3 h	6 h	12 h	24 h
<i>E. coli</i> ATCC 700973	5 mg/kg	0.55 ± 0.12	1.04 ± 0.09	2.22 ± 0.05	2.43 ± 0.02	0.55 ± 0.16	-1.01 ± 0.10
	10 mg/kg	5.94 ± 0.32	≥ 5.94	≥ 5.94	≥ 5.94	≥ 5.94	≥ 5.94
	20 mg/kg	5.94 ± 0.32	≥ 5.94	≥ 5.94	≥ 5.94	≥ 5.94	≥ 5.94
<i>P. aeruginosa</i> Local isolate 44302	5 mg/kg	1.07 ± 0.11	1.48 ± 0.05	2.81 ± 0.17	1.12 ± 0.10	-0.13 ± 0.04	-1.21 ± 0.17
	10 mg/kg	4.79 ± 1.21	6.03 ± 0.14	6.03 ± 0.14	6.03 ± 0.14	6.03 ± 0.14	6.03 ± 0.14
	20 mg/kg	6.03 ± 0.14	6.03 ± 0.14	6.03 ± 0.14	6.03 ± 0.14	6.03 ± 0.14	6.03 ± 0.14
<i>S. aureus</i> ATCC 29213	5 mg/kg	2.10 ± 0.39	2.32 ± 0.41	1.46 ± 1.07	0.01 ± 1.53	-1.00 ± 1.32	-1.19 ± 0.42
	10 mg/kg	6.36 ± 0.30	6.36 ± 0.30	6.36 ± 0.30	6.36 ± 0.30	6.36 ± 0.30	6.36 ± 0.30
	20 mg/kg	6.36 ± 0.32	6.36 ± 0.32	6.36 ± 0.32	6.36 ± 0.32	6.36 ± 0.32	6.36 ± 0.32
<i>S. pneumoniae</i> ATCC 49619	5 mg/kg	1.96 ± 0.24	2.67 ± 0.09	4.12 ± 0.05	1.97 ± 0.40	0.12 ± 0.10	-0.69 ± 0.09
	10 mg/kg	6.35 ± 0.16	6.35 ± 0.16	6.35 ± 0.16	6.35 ± 0.16	6.35 ± 0.16	6.35 ± 0.16
	20 mg/kg	6.35 ± 0.16	6.35 ± 0.16	6.35 ± 0.16	6.35 ± 0.16	6.35 ± 0.16	6.35 ± 0.16

**Table 3: Gentamicin killing of four resistant bacterial strains in a pharmacodynamic model**

Organism	Dose	Time point and Log <sub>10</sub> CFU reduction					
		45 min	1.5 h	3 h	6 h	12 h	24 h
	5 mg/kg	0.16 ± 0.33	0.19 ± 0.58	-0.10 ± 0.63	-0.48 ± 0.72	-1.80 ± 0.28	-2.92 ± 0.70
<i>E. coli</i>							
	10 mg/kg	2.37 ± 0.11	3.39 ± 0.14	4.15 ± 0.24	3.23 ± 0.30	-0.88 ± 0.22	-2.90 ± 0.09
<b>Local isolate</b>							
<b>90900</b>	20 mg/kg	3.04 ± 0.24	4.36 ± 0.50	5.68 ± 0.96	4.02 ± 0.39	0.84 ± 0.86	-2.84 ± 0.27
	40 mg/kg	3.67 ± 0.28	5.57 ± 1.16	6.22 ± 0.07	6.18 ± 0.07	2.65 ± 0.68	-2.36 ± 0.16
	5 mg/kg	-0.23 ± 0.37	0.33 ± 0.16	1.08 ± 0.27	-0.13 ± 0.24	-1.27 ± 0.22	-2.07 ± 0.15
<i>P. aeruginosa</i>							
	10 mg/kg	-0.09 ± 0.39	0.78 ± 0.98	3.71 ± 0.09	6.09 ± 0.09	3.95 ± 0.17	-1.55 ± 0.65
<b>Local isolate</b>							
<b>90591</b>	20 mg/kg	0.49 ± 0.20	2.49 ± 0.64	4.69 ± 0.27	6.09 ± 0.09	5.32 ± 0.75	0.73 ± 1.71
	40 mg/kg	1.47 ± 0.90	3.47 ± 0.24	6.09 ± 0.09	6.09 ± 0.09	6.09 ± 0.09	6.09 ± 0.09
	5 mg/kg	-0.53 ± 0.06	-0.51 ± 0.09	-0.77 ± 0.11	-1.16 ± 0.08	-2.06 ± 0.16	-3.03 ± 0.09
<i>S. aureus</i>							
	10 mg/kg	0.94 ± 0.40	1.86 ± 1.19	2.1 ± 0.68	1.29 ± 0.29	-1.48 ± 0.79	-3.06 ± 0.24
<b>Local isolate</b>							
<b>91056</b>	20 mg/kg	1.36 ± 0.62	2.45 ± 1.34	2.93 ± 0.15	1.78 ± 0.64	-0.98 ± 0.88	-3.04 ± 0.24
	40 mg/kg	2.12 ± 0.71	2.84 ± 1.30	3.56 ± 0.58	2.80 ± 0.90	-0.34 ± 0.11	-3.00 ± 0.26
	5 mg/kg	-0.04 ± 0.01	0.01 ± 0.02	0.04 ± 0.03	-0.03 ± 0.01	-0.83 ± 0.11	-2.04 ± 0.01
<i>S. pneumoniae</i>							
	10 mg/kg	0.30 ± 0.01	1.06 ± 0.10	2.57 ± 0.75	3.12 ± 0.32	5.88 ± 0.30	0.19 ± 0.06
<b>Local isolate</b>							
<b>52634</b>	20 mg/kg	0.83 ± 0.05	1.29 ± 0.16	2.71 ± 0.69	5.88 ± 0.27	5.88 ± 0.27	5.38 ± 0.80
	40 mg/kg	1.22 ± 0.14	2.38 ± 0.24	5.88 ± 0.20	5.88 ± 0.20	5.88 ± 0.20	5.88 ± 0.20

**Table 4: Pharmacokinetic-Pharmacodynamic parameters of supra-pharmacologic doses of gentamicin with sensitive bacterial strains**

Organism	5 mg/kg		10 mg/kg		20 mg/kg	
	AUC/MIC	C <sub>max</sub> / MIC	AUC/MIC	C <sub>max</sub> / MIC	AUC/MIC	C <sub>max</sub> / MIC
<i>E. coli</i> ATCC 700973	73.5	16.5	152.8	34	302.1	66
<i>P. aeruginosa</i> 44302	36.8	8.23	76.1	17	151.0	33
<i>S. aureus</i> ATCC 29213	147	33	3.4.33	68	604.1	132
<i>S. pneumoniae</i> ATCC 49619	73.5	16.5	152.2	34	302.6	66

**Table 5: Pharmacokinetic-Pharmacodynamic parameters of supra-pharmacologic doses of gentamicin with resistant bacterial strains**

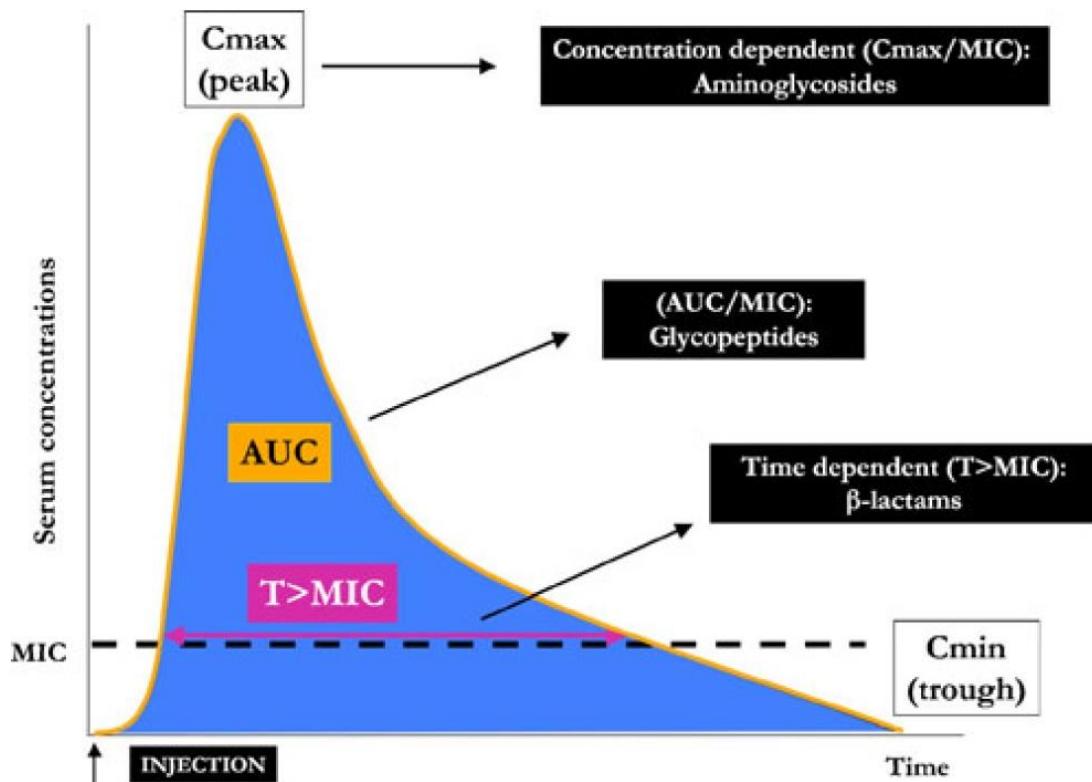
Organism	5 mg/kg		10 mg/kg		20 mg/kg		40 mg/kg	
	AUC/MIC	C <sub>max</sub> / MIC	AUC/MIC	C <sub>max</sub> / MIC	AUC/MIC	C <sub>max</sub> /MIC	AUC/MIC	C <sub>max</sub> / MIC
<i>E. coli</i> 90900	2.30	0.52	4.8	1.06	9.44	2.06	18.9	4.14
<i>P. aeruginosa</i> 90591	4.60	1.02	9.51	2.13	18.9	4.125	37.8	8.3
<i>S. aureus</i> 91056	1.15	0.26	2.38	0.53	4.72	1.03	9.44	2.07
<i>S. pneumoniae</i> 52634	4.60	1.03	9.51	2.13	18.9	4.13	37.8	8.3

**Table 6: Different sites of infection with *P. aeruginosa*, *E.coli*, *S. pneumonia* and *S. aureus***

	<i>P. aeruginosa</i>	<i>E.coli</i>	<i>S. pneumonia</i>	<i>S. aureus</i>
<b>Respiratory tract</b>	Pneumonia	Pneumonia, Sinusitis	Acute exacerbations of chronic bronchitis, Pneumonia Sinusitis	Pneumonia
<b>Bloodstream</b>	Bacteremia	Bacteremia	Bacteremia <sup>1</sup>	Bacteremia, Toxic shock Syndrome
<b>Heart</b>	Endocarditis	Endocarditis	Endocarditis and pericarditis	Endocarditis
<b>CNS</b>	Meningitis, Brain abscess	Acute meningitis	Meningitis	
<b>Ear</b>	Otitis externa and media		Otitis media	
<b>Eye</b>	Bacterial keratitis, Endophthalmitis	Endophthalmitis	Conjunctivitis	
<b>Bones and joints</b>	Osteomyelitis	Osteomyelitis, Septic arthritis	Osteomyelitis, Septic arthritis	Osteomyelitis, Septic arthritis
<b>GI tract</b>	Diarrhea, enteritis, Enterocolitis	Intra-abdominal infections, Enteric infections	Peritonitis	
<b>Urinary tract</b>	Cystitis, Pyelonephritis	Cystitis, Pyelonephritis		
<b>Skin ad Soft tissue infections</b>	Ecthyma gangrenosum	All kinds of Skin and soft-tissue infections <sup>2</sup>	Cellulitis, Myositis, Periorbital cellulitis, and Abscess	Impetigo, Scalded skin syndrome <sup>3</sup> , Folliculitis, Furuncle, Carbuncle, Thrombophlebitis, Deep tissue abscess <sup>4</sup>
<b>Endocrine glands</b>		Suppurative thyroiditis		

1. Most common manifestation of invasive pneumococcal disease
2. Especially in patients with diabetes
3. Ritter disease
4. Muscles and organs can become infected, including the parotid gland, eyes, liver, spleen, kidneys, and central nervous system; deep abscesses also may occur; fever with or without localizing pain is typical

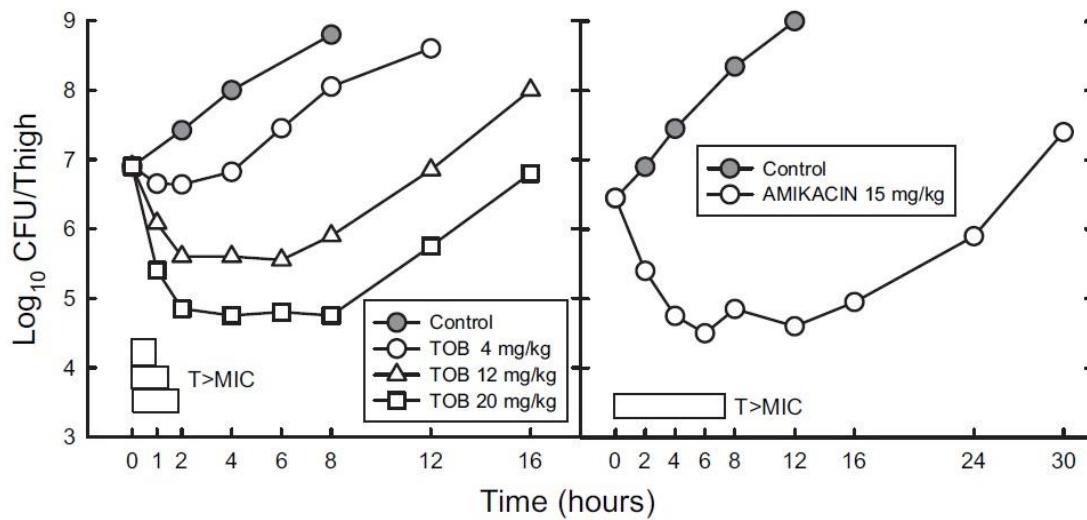
**Figure 1: Pharmacodynamic parameters:**



This is a concentration vs. time curve, showing Pharmacokinetic and pharmacodynamic parameters of three groups of antibiotics ( $\beta$ -lactams, aminoglycosides and glycopeptides). AUC Area under the curve;  $C_{\max}$  peak concentration obtained after a single dose;  $C_{\min}$  the lowest concentration before the following administration; MIC minimal inhibitory concentration

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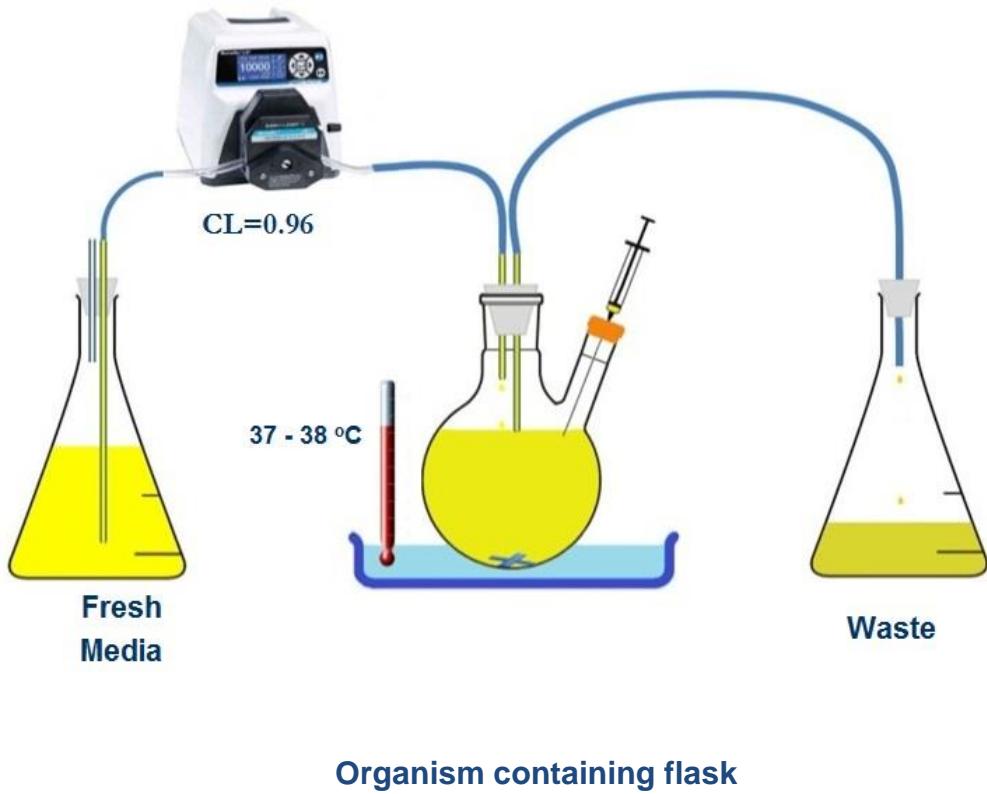
**Figure 2: Time course of killing and regrowth of *P. aeruginosa*:**



The time course of killing and regrowth of *Pseudomonas aeruginosa* in thighs of neutropenic mice following 3 doses of tobramycin (TOB) in normal mice (left panel) and amikacin in mice with renal impairment (right panel). T>MIC, time above minimum inhibitory concentration [56]

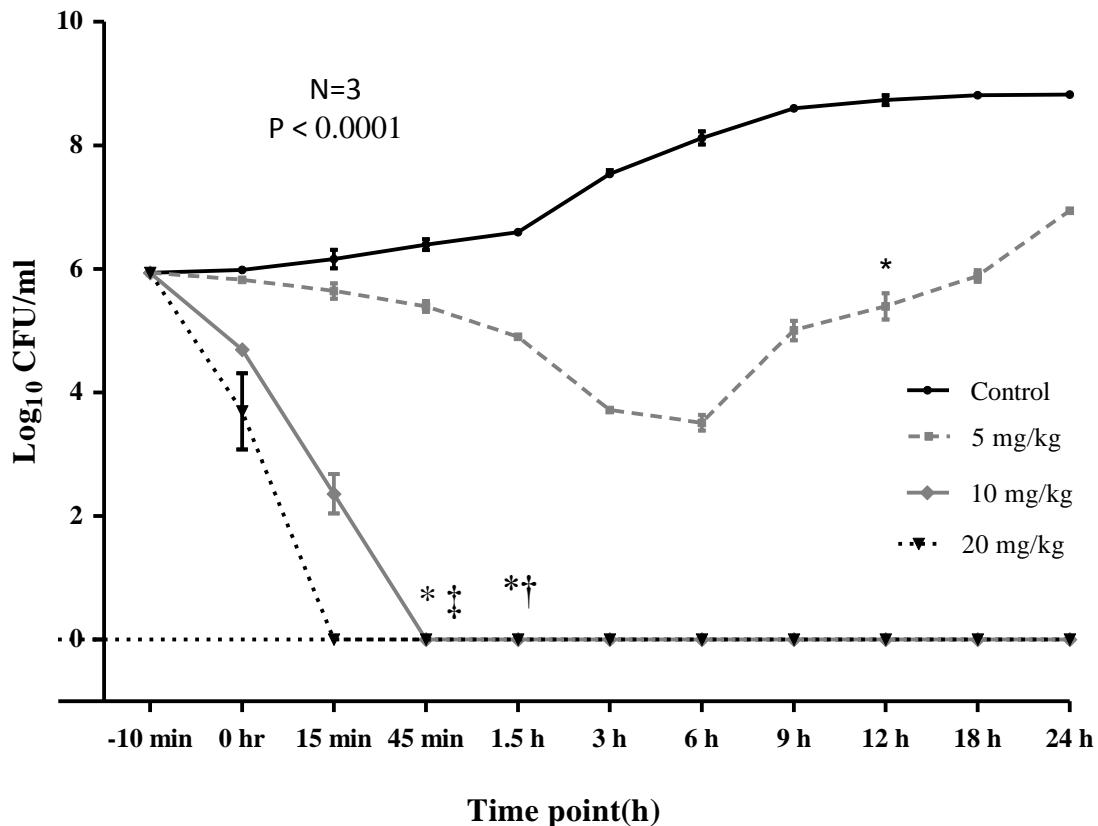
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**Figure 3: IVPD Model:**



**Figure 4: IPDM with different bacterial strains:**

**Figure 4a. IPDM bacterial kill using *E. coli* ATCC 700973  
(Gentamicin MIC - 1 µg/ml, n = 3)**



Maximum log<sub>10</sub> CFU reduction with 5 mg/kg dose was observed at 6 h after dosing.

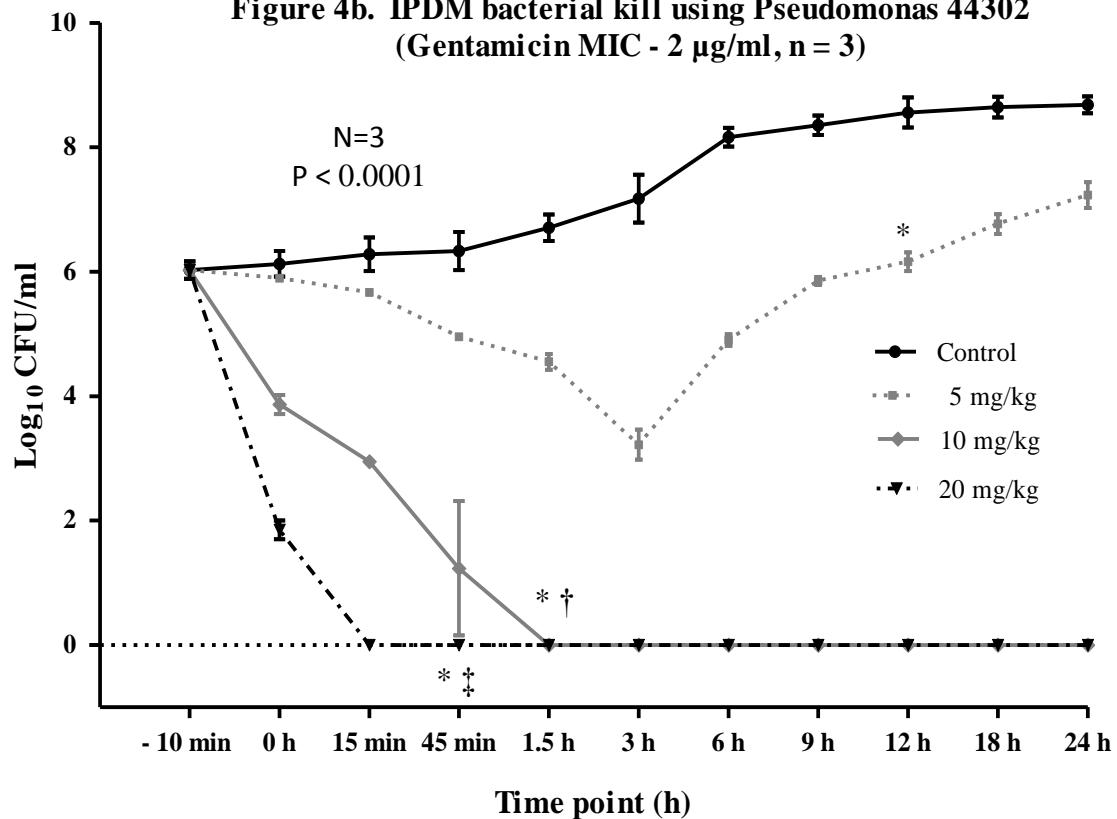
Complete elimination of *E. coli* ATCC 700973 (Bort) was observed with gentamicin at ultra-high doses of 10 and 20 mg/kg with in 45 minutes and 15 minutes after dosing respectively with no regrowth up to 24 h. Error bars represent standard deviation.

\* - p value < 0.05 between treated and control groups from specific time point to 24 h

\$ - p value < 0.05 between 10 mg/kg and 5 mg/kg dose from specific time point to 24 h

# - p value < 0.05 between 20 mg/kg and 5 mg/kg dose from specific time point to 24 h

**Figure 4b. IPDM bacterial kill using Pseudomonas 44302  
(Gentamicin MIC - 2 µg/ml, n = 3)**



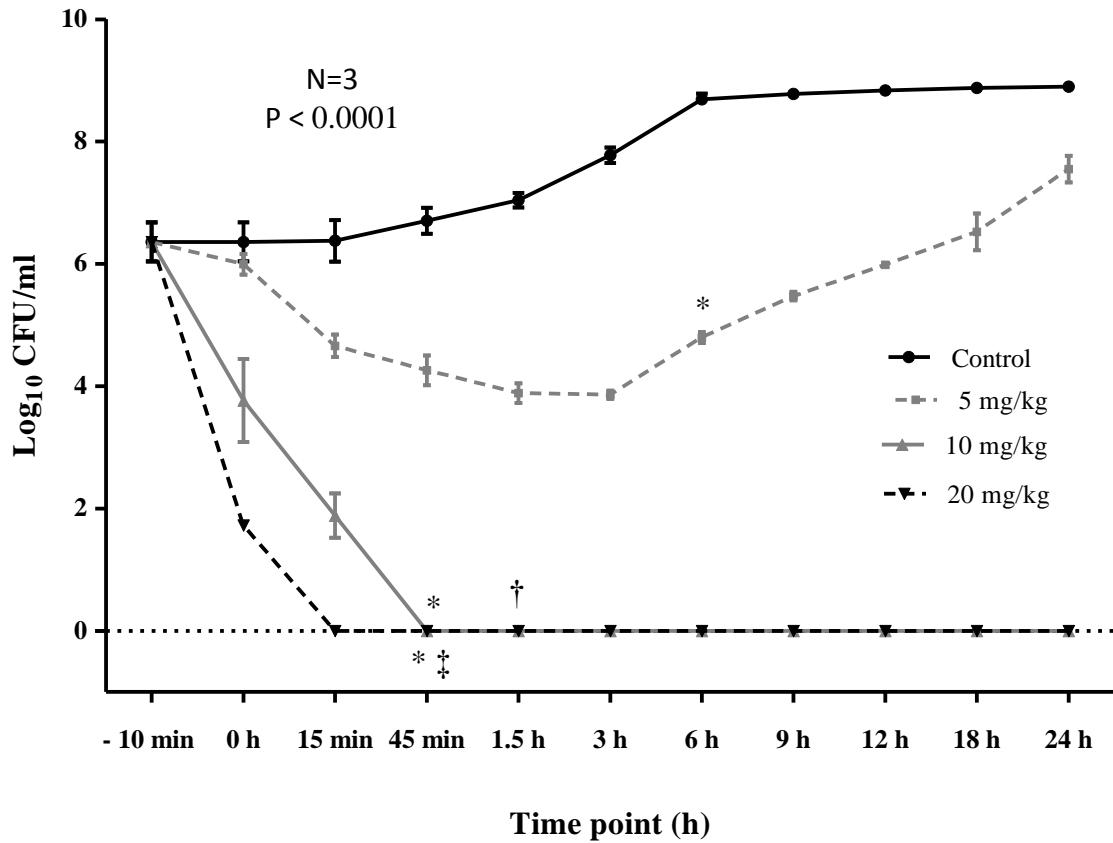
Maximum log<sub>10</sub> CFU reduction with 5 mg/kg dose was observed at 3 h after dosing. Complete elimination of *P. aeruginosa* 4430 was observed with gentamicin at ultra-high doses of 10 and 20 mg/kg within 1.5 h and 15 minutes after dosing respectively with no regrowth up to 24 h. Error bars represent standard deviation

\* - p value < 0.05 between treated and control groups from specific time point to 24 h

\\$ - p value < 0.05 between 10 mg/kg and 5 mg/kg dose from specific time point to 24 h

# - p value < 0.05 between 20 mg/kg and 5 mg/kg dose from specific time point to 24 h

**Figure 4c. IPDM bacterial kill using *S. aureus* ATCC 29213  
(Gentamicin MIC - 0.5 µg/ml, n = 3)**



Maximum log<sub>10</sub> CFU reduction with 5 mg/kg dose was observed at 3 h after dosing.

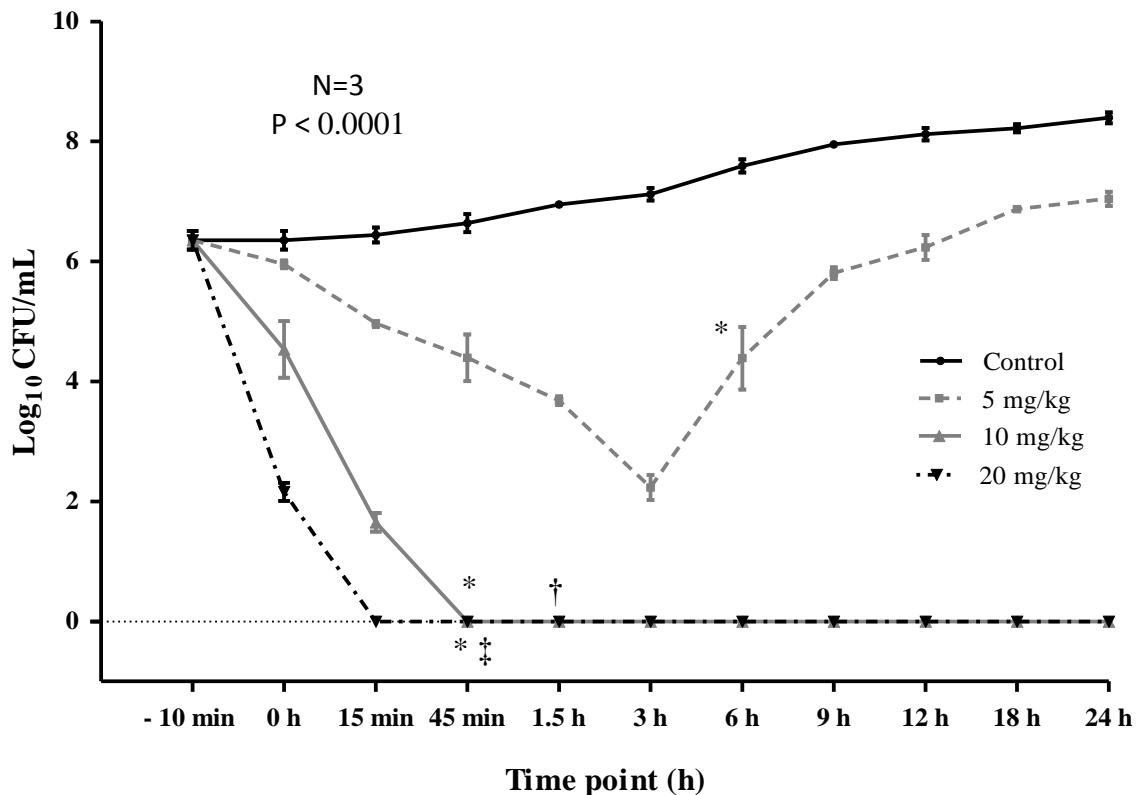
Complete elimination of *S. aureus* ATCC 29213 was observed with gentamicin at ultra-high doses of 10 and 20 mg/kg within 45 minutes and 15 minutes after dosing respectively with no regrowth up to 24 h. Error bars represent standard deviation.

\* - p value < 0.05 between treated and control groups from specific time point to 24 h

\$ - p value < 0.05 between 10 mg/kg and 5 mg/kg dose from specific time point to 24 h

# - p value < 0.05 between 20 mg/kg and 5 mg/kg dose from specific time point to 24 h

**Figure 4d. IPDM bacterial kill using *S. pneumoniae* ATCC 49619  
(Gentamicin MIC - 1 µg/ml, n = 3)**



Maximum log<sub>10</sub> CFU reduction with 5 mg/kg dose was observed at 3 h after dosing.

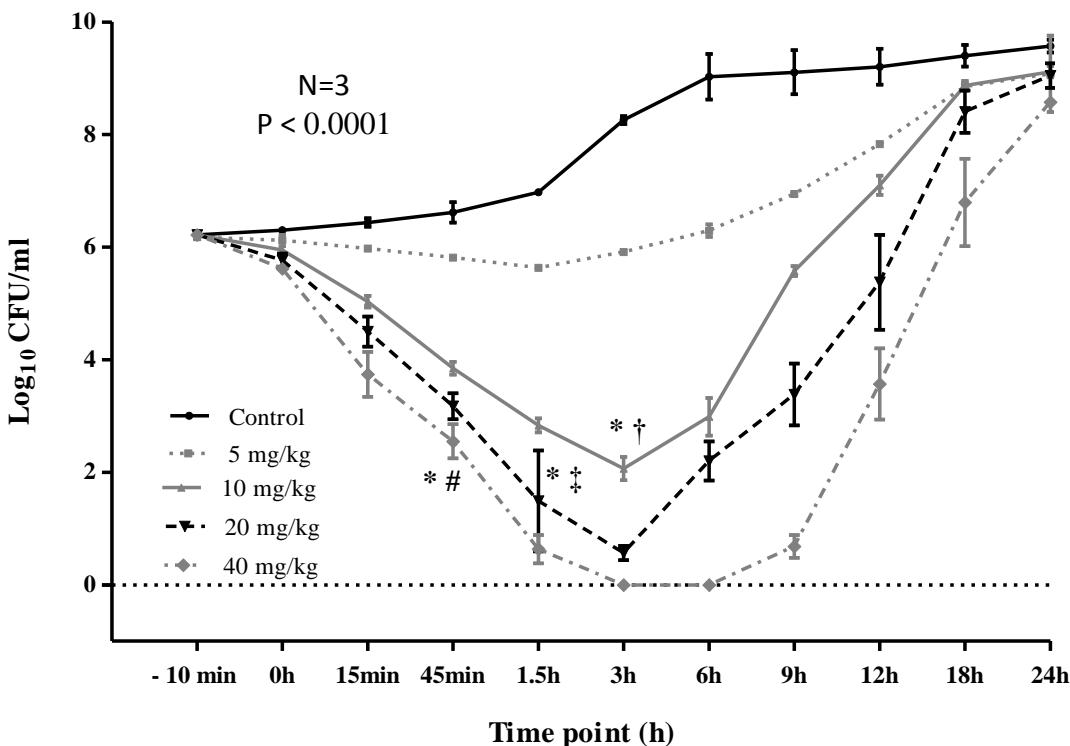
Complete elimination of *S. pneumoniae* ATCC 49619 was observed with gentamicin at ultra-high doses of 10 and 20 mg/kg within 45 minutes and 15 minutes after dosing respectively with no regrowth up to 24 h. Error bars represent standard deviation.

\* - p value < 0.05 between treated and control groups from specific time point to 24 h

\$ - p value < 0.05 between 10 mg/kg and 5 mg/kg dose from specific time point to 24 h

# - p value < 0.05 between 20 mg/kg and 5 mg/kg dose from specific time point to 24 h

**Figure 5a. IPDM bacterial kill using *E. coli* 90900  
(Gentamicin MIC - 32 µg/ml, n = 3)**



Gentamicin at 5 mg/kg dose did not exhibit any statistically significant log<sub>10</sub> CFU reduction compared to untreated control. Ultra-high doses of gentamicin at 10, 20 and 40 mg/kg exhibited statistically significant log<sub>10</sub> CFU reduction compared to untreated controls as well as with 5 mg/kg standard dose from 3 h, 1.5 h and 45 minutes to the end of experiment respectively. Regrowth was observed at all the gentamicin doses. Error bars represent standard deviations.

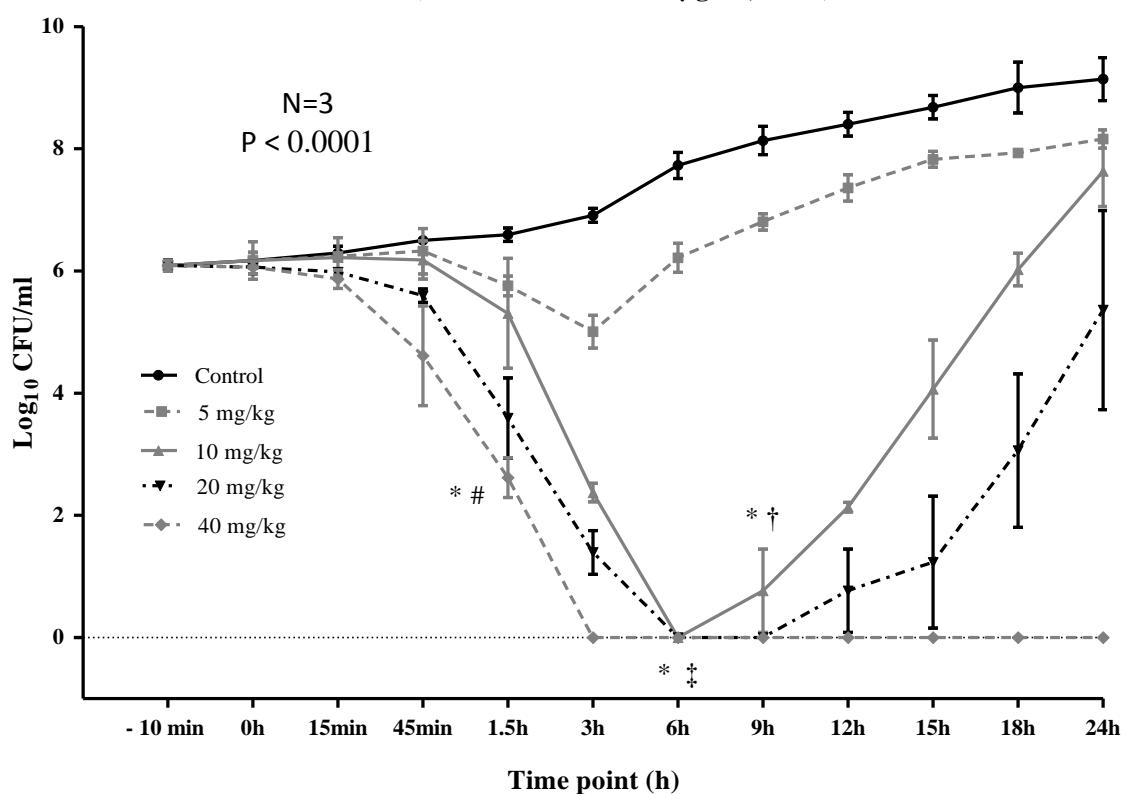
\* - p value < 0.05 between treated and control groups from specific time point to 24 h

† - p value < 0.05 between 10 mg/kg and 5 mg/kg dose from specific time point to 24 h

‡ - p value < 0.05 between 20 mg/kg and 5 mg/kg dose from specific time point to 24 h

# - p value < 0.05 between 40 mg/kg and 5 mg/kg dose from specific time point to 24 h

**Figure 5b: IPDM bacterial kill using Pseudomonas 90591  
(Gentamicin MIC- 16 µg/ml, n = 3)**



Gentamicin at 5 mg/kg dose did not exhibit any statistically significant log<sub>10</sub> CFU reduction compared to untreated controls. Ultra-high doses of gentamicin at 10, 20 and 40 mg/kg exhibited statistically significant log<sub>10</sub> CFU reduction compared to untreated controls as well as with 5 mg/kg standard dose from 9 h, 6 h and 1.5 h to the end of the experiment respectively. No regrowth was observed at 40 mg/kg dose even after 24 hrs. Error bars represent standard deviations.

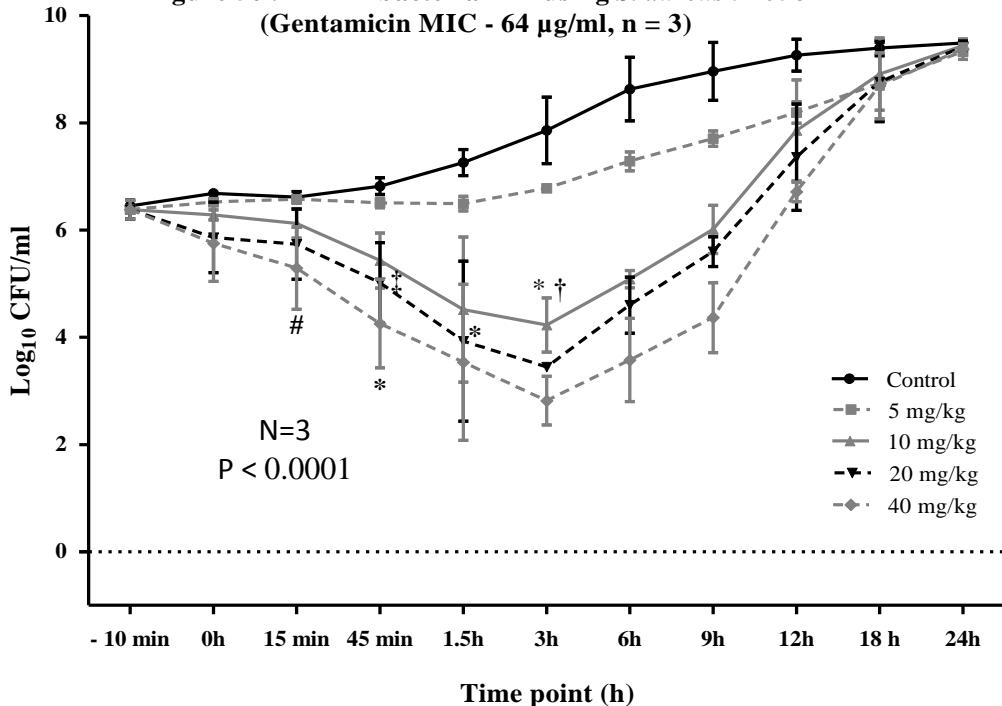
\* - p value < 0.05 between treated and control groups from specific time point to 24 h

† - p value < 0.05 between 10 mg/kg and 5 mg/kg dose from specific time point to 24 h

‡ - p value < 0.05 between 20 mg/kg and 5 mg/kg dose from specific time point to 24 h

# - p value < 0.05 between 40 mg/kg and 5 mg/kg dose from specific time point to 24 h

**Figure 5c : IPDM bacterial kill using *S. aureus* 91056  
(Gentamicin MIC - 64 µg/ml, n = 3)**



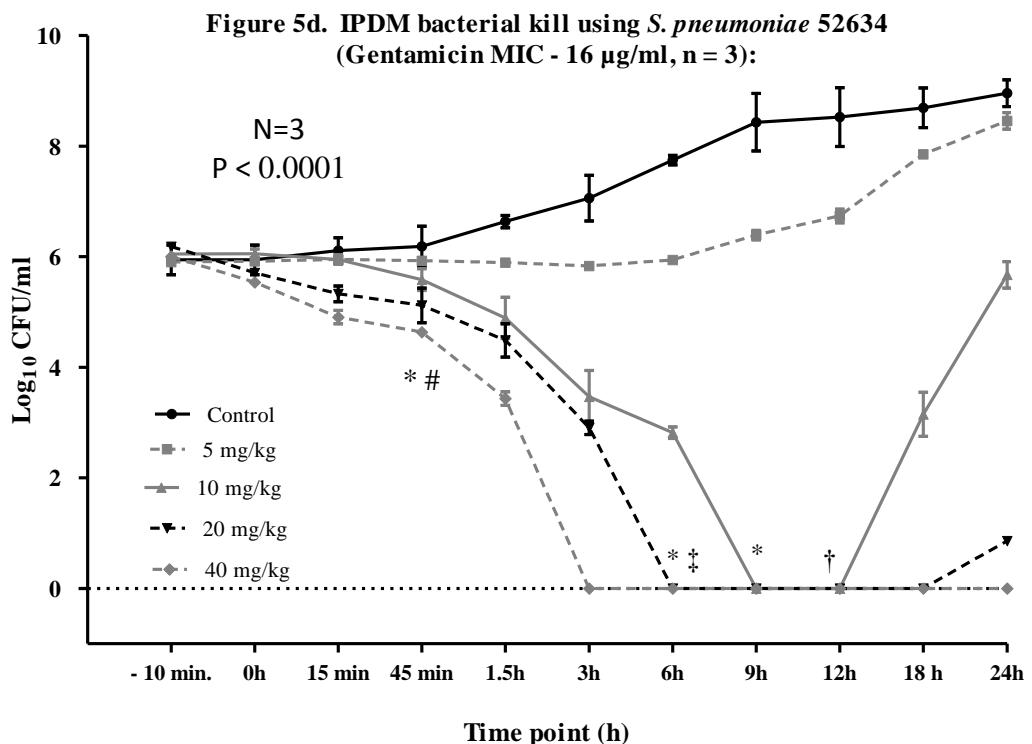
Gentamicin at 5 mg/kg dose did not exhibit any statistically significant log<sub>10</sub> CFU reduction compared to untreated controls. Ultra-high doses of gentamicin at 10, 20 and 40 mg/kg exhibited statistically significant log<sub>10</sub>CFU reduction compared to untreated controls from 3 h, 1.5 h and 45 minutes to the end of the experiment respectively. Ultra-high doses of gentamicin at 10, 20 and 40 mg/kg exhibited statistically significant log<sub>10</sub> CFU reduction compared to 5 mg/kg standard dose from 3 h, 45 minutes and 15 minutes. Error bars represent standard deviations.

\* - p value < 0.05 between treated and control groups from specific time point to 24 h

† - p value < 0.05 between 10 mg/kg and 5 mg/kg dose from specific time point to 24 h

‡ - p value < 0.05 between 20 mg/kg and 5 mg/kg dose from specific time point to 24 h

# - p value < 0.05 between 40 mg/kg and 5 mg/kg dose from specific time point to 24 h



Gentamicin at 5 mg/kg dose did not exhibit any statistically significant log<sub>10</sub> CFU reduction compared to untreated controls. Ultra-high doses of gentamicin at 10, 20 and 40 mg/kg exhibited statistically significant log<sub>10</sub> CFU reduction compared to untreated controls from 9 h, 6 h and 45 minutes to the end of the experiment respectively. Ultra-high doses of gentamicin at 10, 20 and 40 mg/kg exhibited statistically significant log<sub>10</sub>CFU reduction compared to 5 mg/kg standard dose from 12 h, 6 h and 45 minutes respectively. Regrowth was not observed with gentamicin 40 mg/kg dose up to 24 h. Error bars represent standard deviations.

\* - p value < 0.05 between treated and control groups from specific time point to 24 h

† - p value < 0.05 between 10 mg/kg and 5 mg/kg dose from specific time point to 24 h

‡ - p value < 0.05 between 20 mg/kg and 5 mg/kg dose from specific time point to 24 h

# - p value < 0.05 between 40 mg/kg and 5 mg/kg dose from specific time point to 24 h

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