DIVERSE STRESS TREATMENTS AND ACINETOBACTER BAUMANNII PERSISTER FORMATION

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ABSTRACT

This study was aimed to investigate diverse stress treatments on Imipenem susceptible A. baumannii for persister cells formation. Isolates were assessed for persister cells presence by colony forming unit counting (CFU/ml) after exposure to Imipenem (10 µg/ml) in presence and absence of acid stress treatment (pH 6) for 0-7 hr. In vitro activity of curcumin to synergize Imipenem to kill persister cells was investigated. The frequency of hicAB and relEB of type II toxin-antitoxin (TA) loci was evaluated by polymerase chain reaction. The transcription level of hicA and relE toxin genes was assessed by quantitative reverse transcriptase polymerase chain reaction. The level of persistence varied between isolates and across the stress conditions. Imipenem treated isolates gave persister fraction of 0.07% to 0.4% whereas Imipenem + pH6 treated isolates gave 0.3% to 0.7% of the initial untreated population. No significant reduction in persistence level was observed after combination with curcumin (P value > 0.05). The hicAB and relEB loci were detected in 100% and 61.5% of Imipenem susceptible isolates, respectively. Imipenem triggered hicA toxin gene transcription while acid stress triggered hicA and relE toxin genes transcription in persister cells.

Key words: Imipenem, TA system, hicAB, relEB, curcumin.

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INTRODUCTION
The bacterial infectious diseases are treated by antibiotics. The excessive and inappropriate use of antibiotics has led to a world crisis represented by treatment failure. Due to the rapid evolution of resistance, most of the effective antibiotics turned inactive. Scientists observed that resistance is not the only cause of treatment failure, but persistence also has a roll in this failure (24). Persistence is a mechanism that bacteria use to survive stress exposure. In in-vitro or in-vivo stress conditions, a subpopulation of bacteria enters dormancy state, these cells called persisters (26). Unlike resistant cells, persisters are cells without any genetic modification that can resume growth only after stress subsides and give rise to the same susceptible populations (23). At the dormancy state, all the biological processes are turned inactive and the antibiotics intake is blocked. Due to their modes of action, antibiotics cannot attack inactive biological process, therefore; they fail to completely cure the persistent bacterial infections (7). Different bacterial infectious diseases, like tuberculosis, urinary tract infection, and air way infections are attributed to persistence (13). Under stress conditions (antibiotics or environmental stresses), regulatory proteins, stress-induced proteins, will be activated which by their turn will regulate the required mechanisms that enable bacterial cells to response to any sudden changes in their environment to ensure their survival (25). Aims are now being directed to discover new strategies to target the mechanisms of persister cells formation in order to eradicate persistence in pathogenic bacteria and to delay the appearance of resistance. Acinetobacter baumannii, aerobic, non-motile, gram negative bacteria are the major nosocomial pathogens (17). This opportunistic bacterium causes meningitis, urinary tract infection, pneumonia, and wound infections (8). It has been reported that these bacteria can form persister cells and can evolve multi drug resistant cells by undergoing genetic mutations and receiving resistance genes through recombination and horizontal gene transfer (9,11,12,28). The molecular mechanism that underling persistence in Acinetobacter baumannii is not well understood. It has been reported that toxin and anti-toxin systems (TA systems) are involved in presister cells formation in Mucobacterium tuberculosis Salmonella and E. coli (1,4,5,6 15,16). Deletion of some of TA loci in E. coli decreased persistence to antibiotics (10). The chromosomes and plasmids of Acinetobacter baumannii harbor several gene pairs of Toxin-Antitoxin (TA) systems (20). TA system consists of stable toxic proteins and instable antitoxins (either protein or RNA) that can bind to toxins or promoter and inhibit the activation or transcription of toxins (14). Under stress conditions, like antibiotics, nutrient shortage, oxidative stress, acidic condition, high temperature and plasmid loss, antitoxins are degraded while toxins are activated (19,22). Activated toxins act as stress respond elements and regulate cells growth and death by inhibiting the cellular processes, like DNA replication (targeting DNA gyrase) (35), translation (targeting tRNA, mRNA, and ribosome) (36) and energy (ATP) production (depolarizing the inner cell membrane) (26). It has been reported that TA is the main strategy Acinetobacter baumannii and E. coli use to form biofilm and persister cells through enhancing the transition from mobile state to sessile state and generating slow growing cells in a dormancy state (18). In this study, antibiotic and acidic stress treatments and the ability of clinical isolates of Acinetobacter baumannii to form persister cells were investigated. The anti persister potential of curcumin in combination with Imipenem was investigated against persister forming A. baumannii. Additionally, the prevalence of type II TA systems, hicBA and relBE (the toxins of both modules inhibit cellular translation by targeting tRNA and mRNA, respectively) loci among tested isolates chromosome and plasmid were assessed. A possible Relationship between persistence and hicA and relE genes transcription was investigated. Both Antibiotic and acid stresses induced the formation of persister cells accompanied by an increase in hicA and relE genes transcription. Combination of Imipenem and Imipenem-acid stress with curcumin showed no significant effect on persistence, indicating No synergetic anti-persistence effect of curcumin and Imipenem on persister cells.
MATERIALS AND METHODS

Bacterial Isolates: 20 isolates of Acinetobacter baumannii were isolated from wound, burned surface and urine. The isolates were identified by biochemical test and ViteK system.

Disk diffusion assay for Antibiotic susceptibility test: The inoculums of bacteria were prepared by diluting the bacterial growth to a turbidity similar to the turbidity of 0.5 McFarland. The entire surface of Muller-Hinton agar was inoculated evenly with bacteria. An IPM, Imipenem (10 µg) disk was applied and the plates were incubated at 37 °C for 10 hr.

Bacterial persistence disk test: After incubation with antibiotic disk for 10 hours, Imipenem antibiotic disk of susceptible strains were replaced with glucose containing disks to allow the regrowth and detection of surviving cells in the inhibition zone. The substituted disks were prepared by adding 100 µl of 1% glucose onto blank disks and applied directly after preparation by using sterile forceps. The Plates were incubated at 37 °C for overnight.

Imipenem Time kill assay: 1ml of Exponential growing bacteria (5 × 10^6 CFU/ml) on Luria Bertani broth (LB) media was prepared. The culture was treated with Imipenem Sample was washed and diluted 1:10 fold in LB broth and 10 µl was spotted on LB agar at 37 °C for overnight to determine the number of bacterial cells survived overtime of treatment represented by Colony forming units. 5ml of Exponential growing bacteria (5 × 10^6 CFU/ml) on Luria Bertani broth (LB) media was prepared. The culture was treated with Imipenem 10µg/ml and 100 µM curcumin. Sample was taken after 6hrs of treatment. The culture was treated with Imipenem 10µg/ml and 100 µM curcumin. Sample was taken after 6hrs of treatment. Sample was washed and diluted 1:10 fold in LB broth and 10 µl was spotted on LB agar at 37 °C for overnight to determine the number of bacterial cells survived overtime of treatment represented by Colony forming units. The CFU/ml was plotted over time of antibiotic treatments. A duplicate was used in this experiment.

Persistence and acidic condition: 1ml of Exponential growing bacteria (5 × 10^6 CFU/ml) on Luria Bertani broth (LB) media pH 6 was prepared and incubated at 37°C for 60 min. after 1 hr. of incubation at acidic condition, cells were harvested then re-suspended in LB supplemented with Imipenem 10µg/ml. samples were taken at 0,1,2,3,4,5,6,7 hrs. Each sample was washed and diluted 1:10 fold in LB broth and 10 µl was spotted on LB agar at 37 °C for overnight to determine the number of bacterial cells survived overtime of treatment represented by Colony forming units. The CFU/ml was plotted over time of antibiotic treatments. A duplicate was used in this experiment.

Effect of curcumin on Imipenem, and acidic condition+ Imipenem induced persisters: 5ml of Exponential growing bacteria (5 × 10^6 CFU/ml) on Luria Bertani broth (LB) media was prepared. The culture was treated with Imipenem 10µg/ml and 100 µM curcumin. Sample was taken after 6hrs of treatment. The culture was treated with Imipenem 10µg/ml and 100 µM curcumin. Sample was taken after 6hrs of treatment. Sample was washed and diluted 1:10 fold in LB broth and 10 µl was spotted on LB agar at 37 °C for overnight to determine the number of bacterial cells survived overtime of treatment represented by Colony forming units. 5ml of Exponential growing bacteria (5 × 10^6 CFU/ml) on Luria Bertani broth (LB) media pH 6 was prepared and incubated at 37°C for 60 min. after 1 hr. of incubation at acidic condition, cells were harvested then re-suspended in LB supplemented with Imipenem 10µg/ml and 100 µM curcumin for 6 hr. Sample was washed and diluted 1:10 fold in LB broth and 10 µl was spotted on LB agar at 37 °C for overnight to determine the number of bacterial cells survived overtime of treatment represented by Colony forming units. 10µg/ml. samples were taken at 0,1,2,3,4,5,6,7 hrs. Each sample was washed and diluted 1:10 fold in LB broth and 10 µl was spotted on LB agar at 37 °C for overnight to determine the number of bacterial cells survived overtime of treatment represented by Colony forming units.

PCR Detection of hicBA and relBE loci: 25 µl of PCR reaction contains 12.5 of 1x master mix buffer, 1 µl of 10 pmol/µl forward and reverse primers,1 µl of 100 ng/µl DNA and 4.5 µl water. The PCR reaction was carried out represented by Colony forming units. The CFU/ml was plotted over time of antibiotic treatments. A duplicate was used in this experiment.
with the following parameters: initial denaturation at 94 °C for 5 min, second denaturation at 94 °C for 1 min, 1 min of annealing at 55 °C and 56 °C for hicBA and relBE, respectively, extension at 72 °C for 50 sec. 35 cycles of amplification was applied.

PCR products were analyzed by 2% agarose in the presence of 100 bp DNA ladder. After performing gel electrophoresis, the gel was exposed to UV by using UV Transilluminator.

**Quantitative PCR:** 1 ml of bacterial growth at exponential phase treated with Imipenem (10µg/ml) and (10µg/ml) Imipenem +acid (pH 6) were centrifuged for 5 min. The precipitated cells were washed with phosphate buffer saline (pH 7) and centrifuged for 5 min. Total RNA was isolated by TRIzol RNA isolation reagent (sigma). RNA was transcribed to cDNA by cDNA synthesis kit (Bio-Rad). The transcription of hicA and relE genes was assessed by SYBR green master mix (sigma). 1 µl of reverse and forward primers, 12.5 µl SYBR green master mix, 3 µl of cDNA, and 2.5 µl of water to obtain a final volume of reaction equals 20 µl. The PCR reaction was carried out with the following parameters: initial denaturation at 94 °C for 4 min, second denaturation at 94 °C for 15 second, 50 second of annealing at 50 °C and 55 °C for hicA and relE, respectively, extension at 72 °C for 50 sec. 30 cycles of amplification was applied. Primers used in this experiment are listed in table 2. As a housekeeping gene, the 16S rRNA was used.

**RESULTS AND DISCUSSION**

**Exposure to Imipenem induces Acinetobacter baumannii persister cell formation:** *Acinetobacter baumannii* has the ability to evade various stress conditions, like antibiotics, acidic stress, oxidative stress, and nutrient depletion stress. In addition to resistance, efflux pumps and biofilm formation, part of this ability is attributed to possessing mechanisms mediating persister cells formation (21). The Imipenem susceptibility test revealed that 35% of isolates were resistant, and 65% were Sensitive (Figure 1A). According to the TD test, only 45% of the Imipenem sensitive bacteria formed persister cells in the inhibition zone after the Imipenem disk (10 µg/mL) substitution with a nutrient disk (Figure 1B).

![Figure 1. Persister cells detection by TDtest.](image-url)

(A) Inhibition zone after exposure to Imipenem 10 µg/ml (B) re-growing colonies in inhibition zone after replacing Imipenem disk with nutrient broth disk. (C) different response to Imipenem as detected by disk diffusion assay and IDtest. (35 %) Resistant, (20%) Sensitive, and (45%) Persistent

The β-lactam antibiotic (Imipenem) targets the cell wall synthesis process of the growing cells. Since persisters are cells in a dormany state, Imipenem is unable to target these cells, leaving an integral persister cells that have the ability to revive after Imipenem dissipates. Since persister cells are genetically sensitive to antibiotics, same inhibition zone diameter was obtained when the progeny that grew inside the inhibition zone was re-tested against Imipenem. This result emphasized that these are persisters not resistant cells. The time-kill assay revealed a biphasic killing curve (Figure 2 A). During the first hour of treatment with Imipenem (10 µg/mL), the susceptible cells were killed, while a small population, persister cells, survived. After 2, 3, 4, 5, and 6 hr of incubation with Imipenem, the number of persister cells represented by cell forming unit was measured. As the period of Imipenem treatment increased the number of cell forming unit was slowly decreased. Minimum persisters number was observed after 5h of Imipenem treatment and the number stabilized for up to 7hr (the end time of this experiment). Isolates showed different persister fractions ranging from 0.07% to 0.4% in Imipenem (Figure 2B). Among the tested Imipenem susceptible isolates, two isolate was of a low persister fraction (< 0.1) while seven isolates were of a high persister fraction (> 0.1) (figure 2 B).
Although Imipenem succeeded in killing 99% of the susceptible cells, it was insufficient to kill the slow growing persister cells. And It is unclear whether Imipenem triggered the formation of persister cells, or these cells were pre-existing in the population as a heterogeneous subpopulation and succeeded to evade Imipenem exposure.

Acid stress positively affects the formation of persister cells in *Acinetobacter baumannii* treated with Imipenem: Several studies have reported that environmental conditions, like oxidative stress, can increase the proportion of persister cells (33) while others, like U.V and lytic phage, can kill persisters (27,30). It was reported that in-vivo phagosome acidification induced the formation of antibiotic persister cells in *Salmonella* (31). In this study, we tried to compare the efficacy of Imipenem to kill persisters in presence and absence of environmental stress, like acidic stress. The tested *A. baumannii* isolates were exposed to acidified medium (pH 6) prior to Imipenem (10 μg/mL). Time kill assay was performed. Within the first hour, a rapid killing was observed. A stabilized minimum number of cells was observed after 5hr of incubation. The efficacy of Imipenem was antagonized by environmental acidic stress with a 2.7-fold increase in the proportion of cells survive Imipenem killing compared to that of Imipenem only treated isolates (Figure 3). This indicates that acidic stress increased persistence.

**Figure 2.** Killing curves of cultures treated with Imipenem and Imipenem in combination with acidic condition.

The plots show the killing data of 9 isolates with different persister fractions. (A) Killing dynamics during 7 hr of treatemnt with Imipenem. (C) Killing dynamics during 7 hr of treatment with Imipenem in combination with acidic condition. 1ml of samples were collected at each indicated time, washed, dilluted and spotted on agar media to count the survival colonies. (B) and (D) persister fractions of Imipenem and Imipenem in combination with acidic condition, respectively. There are differences in persister fractions between the two treatments. Isolates showed high fractions (0.3-0.7) in Imipenem incombination with acidic condition and a low fractions (0.07-0.4) with Imipenem only. reported that in-vivo phagosome acidification induced the formation of antibiotic persister cells in *Salmonella* (31). In this study, we tried to compare the efficacy of Imipenem to kill persisters in presence and absence of environmental stress, like acidic stress. The tested *A. baumannii* isolates were exposed to acidified medium (pH 6) prior to Imipenem (10 μg/mL). Time kill assay was performed. Within the first hour, a rapid killing was observed. A stabilized minimum number of cells was observed after 5hr of incubation. The efficacy of Imipenem was antagonized by environmental acidic stress with a 2.7-fold increase in the proportion of cells survive Imipenem killing compared to that of Imipenem only treated isolates (Figure 3). This indicates that acidic stress increased persistence.

**Figure 3.** The average of nine Imipenem and Imipenem in combination with acid stress treatments.
Acid stress resulted in 2.7-fold increase in proportion of cell survival in A. baumannii. Isolates showed different persister fractions ranging from 0.3% to 0.7% (Figure 2 C and D). The induction of A. baumannii persistence to Imipenem in acidic stress might be stemmed from two separate mechanisms: The activation and overexpression of toxins of TA system, which in turns arrest cell growth. Or by the activation of other mechanisms of persistence, like efflux pumps (2,34) and SOS response to DNA damage since low pH can damage DNA indirectly through the production of reactive oxygen species.

**Curcumin failed to act in synergism with Imipenem:** It was reported that combinations of curcumin, a pro-oxidant, and colistin decreased the persistence of Acinetobacter baumannii to colistin. The reduction was attributed to the down regulation of repair genes in response to Reactive Oxygen Species (ROS) generated from this combination, indicating that ROS decreases persistence of bacteria (3). While the ROS produced from Salicylate was reported to increase persistence in E.coli through decreasing membrane potential (32). Here we investigated the efficacy of curcumin in potentiating Imipenem with and without acidic stress to decrease the persistence level. Only the isolate that formed the highest percentage of persistence against Imipenem and Imipenem-acidic stress in combination was treated with curcumin (100µM) in combination with Imipenem (10 µg/ml) with and without acidic stress for 6 hr. Despite the pro-oxidant activity and efflux pumps inhibitor feature of curcumin, no synergistic activity of curcumin with Imipenem (10 µg/mL) against persistence (P value > 0.05) was shown (Figure 4).

![Figure 4. Effect of curcumin on Imipenem and Imipenem-acid in combination persisters.](image)

Acinetobacter baumannii isolate treated for 6 hours with (A) (10 µg/ml) Imipenem, (B) (100 µM) curcumin in combination with (10 µg/ml) Imipenem, (C) (10 µg/ml) Imipenem+ acid stress and (D) (100 µM) curcumin in combination with (10 µg/ml) Imipenem+ acid stress. The neutral effect of curcumin on Imipenem and Imipenem-acid stress persisters indicates that ROS is not involved in A. baumannii persistence to the tested stress.

**hicA and relE toxin genes transcription and their relation with Acinetobacter baumannii persister formation:** Since stress-related variations in persister fractions were observed, persistence genetic determinants, like type II Toxin-antitoxin loci, among isolates were tested to indicate if there is any possible correlation between toxins transcription and persistence of A.baumannii to Imipenem and acid stress. The prevalence of hicAB (chromosomal loci) and relEB (plasmid loci) genes (Table1) were 100% and 61% in Imipenem susceptible isolates of A.baumannii, respectively (Figure 5).
Figure 5. PCR result for hicAB and relBE
Toxin anti-toxin genes of Acinetobacter baumannii. 266 bp for hicAB and 433 bp for relBE compare to 100 bp marker. 1-13 samples DNA
The isolate of the highest percentage of persistence and was hicAB+ and relBE was used to investigate the transcription of hicA and relE (Table 2) toxin at identified point of time (6 hour) of treatments. Persister cells were isolated from 6 hour time point and the total RNA was isolated from these cells. The expression profile of toxin-antitoxin loci in *M. tuberculosis* showed that genes within the same toxin-antitoxin family were differentially expressed under different set of stress conditions (29), our qPCR obtained data revealed that The transcription of hicA and relE toxins of hicAB and relEB type II TA system was differentially expressed under acidic and drug stress (figure 6).

Figure 6. Transcription level of *A. baumannii* hicA and relE genes in persister cells formed after Antibiotic stress and antibiotic combined with acid stress.

Data are mean from 2 independent replicates. Total RNA was isolated. The transcription level of hicA and relE was measured by qPCR, using 16S RNA as a house keeping genes. Delta Delta Ct method was used to calculate relative change in gene transcription. hicA was expressed in both antibiotic stress and acidic stress with no significant difference (p=0.69), while relE was only expressed after exposing to acidic condition with significant difference (p=0.004) and 11.875 fold increase. This data explain the increased persister fraction in Imipenem-acid stress. This data reflect the need of *A. baumannii* persister cells to alter gene expression in response to Imipenem and acidic stress. Our data correspond with a study that was conducted to investigate the persistence of *A. baumannii* to ciprofloxacin which revealed an upregulation in hicAB gene (34), reflecting the importance of TA system in persistence of bacteria to stress factors. The active toxin will inhibit cell cycle and division either by abolishing proton motive force or by interfering the translation process, causing cells to enter the dormancy state (13). The mechanism of action of traditional antibiotics rely on cellular activity. As persisters are an inactive cells in a dormant state, traditional antibiotics fail to kill them. These facts have pushed the Scientest to search on compounds that there mechanisme of action does not rely on cellular activity and metabolic state of the targeted bacteria and have no toxic effect on human cells.

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