Artículo Original

Detection of transitory resistance in *Streptococcus suis* and *Pasteurella multocida* strains from swine origin in Cuba

Detección de resistencia transitoria en cepas de *Streptococcus suis* y *Pasteurella multocida* de origen porcino en Cuba

Ivette Espinosa-Castaño✉, Michel Báez-Areas, Rosa Elena Hernández

Centro Nacional de Sanidad Agropecuaria (CENSA), Apartado 10, San José de las Lajas, Mayabeque, Cuba.

ABSTRACT: The acquired resistance requires a genetic change; either mutations or the acquisition by horizontal gene transfer. However, there are situations in which resistance is not driven by a genetic change and bacteria become transiently resistant to antibiotics. The transient and reversible resistance can be achieved by different mechanisms, such as the formation of biofilms or persistent cells, which are related to the physiological state of the bacteria when it is exposed to a stressful condition. Persistent cells represent a fraction in a bacterial population that begins a dormancy phase under adverse conditions. Unlike bacteria that resist antibiotics by genetic mechanisms, persistent cells are unable to grow in the presence of an antibiotic. *Pasteurella multocida* and *Streptococcus suis* are important pathogens in the respiratory disorders of swine production. These bacteria produce frequents infections that could be considered recurrent. This work was aimed at detecting events of transitory resistance *in vitro* in both species. Four strains, susceptible to ß-lactamic and quinolone, corresponding to each species, were selected after previously confirming their susceptibility according to the classical testing method. *P. multocida* strains were analyzed for surviving cells after exposure to 200 and 400 μgml^{-1} of Enrofloxacin and Ampicillin, respectively, while *S. suis* cells was treated with 100$\mu gml^{-1}$of Penicillin and 200μgml^{-1}Enrofloxacin during 24 and 48 hours. *S. suis* and *P. multocida* strains formed persistent cells under the action of both antibiotics until a detectable concentration of $1x10^4$ $ufcml^{-1}$. The level of persistence varies among the strains. This is the first time that the formation of persistent cells by *P. multocida* has been described and corroborates this behavior previously described by other authors in *S. suis* strains. The potentiation assay showed that it is possible to eradicate persistent cells *in vitro* through the combinations of aminoglycoside (Gentamicin) with glycerol and Gentamicin with arginine The manifestation of these transient resistance phenotypes not associated to genetic changes can explain the therapeutic failures and recurrence in respiratory infections, which usually occur subclinically, reducing lung capacity and decreasing gain in weight.

Key words: transient resistance, persistent cell, antibiotic tolerance, *Pasteurella multocida*, *Streptococcus suis.*

RESUMEN: La resistencia adquirida requiere de cambios genéticos por mutaciones o la adquisición de genes por transferencia horizontal. Sin embargo, existen situaciones en las cuales la resistencia no es producto de cambios genéticos y las bacterias se manifiestan resistentes transitoriamente. La resistencia reversible y transitoria se logra por diferentes mecanismos, como formación de biopelículas o las células persistentes, las cuales se relacionan con el estado

✉ Autor para correspondencia: *Ivett Espinosa-Castaño*. E-mail: espinosa@censa.edu.cu
Recibido: 10/2/2017
Aceptado: 2/6/2017

fisiológico de la bacteria cuando se expone a una condición estresante. Las células persistentes representan una fracción en una población bacteriana que, bajo condiciones adversas, comienza una fase de dormancia. A diferencia de las bacterias que resisten a los antibióticos por mecanismos genéticos, las células persistentes son incapaces de crecer en presencia de un antibiótico. La resistencia que se caracteriza por la supervivencia de una fracción de la población bacteriana, en presencia de un antibiótico, pero sin crecimiento, se define como resistencia transitoria y juega un papel importante en la recurrencia de infecciones. *Pasteurella multocida* y *Streptococcus suis* son patógenos importantes en los trastornos respiratorios de la producción porcina; estas bacterias producen frecuentes infecciones que pueden ser consideradas recurrentes. El presente trabajo tuvo como objetivo detectar eventos de resistencia transitoria *in vitro* en ambas especies. Se seleccionaron cuatro cepas correspondientes a cada especie después de confirmar previamente su susceptibilidad en el ensayo de difusión en agar con disco a dos fármacos utilizados para controlar ambas infecciones (ß-lactámicos y fluoroquinolona). Las cepas de *P. multocida* se analizaron en busca de células supervivientes después de la exposición a 200 y 400 μgml^{-1} de Enrofloxacina y Ampicilina, respectivamente, mientras *S. suis* se trató con 100 μgml^{-1} de Penicilina y 200 μgml^{-1} de Enrofloxacina durante 24 y 48 horas. Las cepas de *S. suis* y *P. multocida* formaron células persistentes bajo la acción de ambos antibióticos hasta una concentración detectable equivalente a $1x10^4$ $ufcml^{-1}$. El nivel de persistencia varió entre estas cepas. Esta es la primera vez que se describe la formación de células persistentes por *P. multocida* y corrobora este comportamiento previamente descrito por otros autores en cepas de *S. suis*. El ensayo de potenciación mostró que es posible la erradicación de células persistentes *in vitro* a través de las combinaciones de aminoglucósido (Gentamicina) con glicerol y Gentamicina con arginina. La manifestación de estos fenotipos de resistencia transitoria no asociados a cambios genéticos puede explicar fracasos terapéuticos y recurrencia en las infecciones respiratorias, que habitualmente ocurren subclínicamente, reducen la capacidad pulmonar y disminuyen la ganancia en el peso.

Palabras clave: resistencia transitoria, célula persistente, tolerancia antibiótico, *Pasteurella multocida, Streptococcus suis*.

INTRODUCTION

Actually the ability of microorganisms to resist antibiotics is one of the most important challenges in human and veterinary health. The consequences of the antimicrobial resistance are the impossibility of treating infections correctly, prolonged illnesses, deaths, production losses and negative consequences for food security (1,2).

The acquired resistance to antibiotics is the result of insertions, deletions and mutations in the existing genes or the acquisition of external resistance encoding elements like plasmid and tramposones. The major research approach has been directed to the acquired resistance (2). But unfortunately, the bacteria lacking of resistance genes and susceptible to antibiotics in the laboratory test such as Agar disc diffusion could reveal an unexpected behavior, which was described as "transient resistance" or "tolerance antibiotic" without the acquisition of a genetic change. The formation of biofilms or persistent cells are expressions of this transient resistance (2,3).

The biofilms are microbial communities that can be formed within soft tissues or surfaces where the bacteria are protected from the immune system or antibiotics by a layer of exopolymers (2,3,4). Persistent cells represent a small subpopulation of cells that spontaneously enter a dormant, non dividing state, therefore becoming highly tolerant to kill with lethal doses of bactericidal antibiotics, reaching this state without undergoing the genetic change (5).Therefore, the antibiotics depend on the physiological activity of the bacterial cells, interfering with the active cellular processes,

such as macromolecular synthesis, lack of effectiveness on persistent cells, which are dormant or their metabolic activity is reduced. Such cells could revert to a growing state after antibiotic treatment is ceased. Both expressions (biofilms and persistent cells) play an important role in the recalcitrance of infections (5,6).

Some bacteria that colonize the respiratory tract produce recurrent infections, probably due to expressions of transient resistance such as biofilms or persistent cells (2,3,5,6). Respiratory infections are one of the most important health problems in pig herds, due to the multifactorial nature. *S. suis* and *P. multocida* species are important pathogens, involved in causing great economic losses to the swine industry (7,8). *S. suis* is also regarded as an important zoonotic agent with a significant increase of infectious in humans (7,9). The tolerance to antibiotics by the formation of persistent cells could be a possible explanation for therapeutic failures and recurrent infections by these pathogens (9,10).

Researches about persistent cells have been focused on knowing the mechanisms that support this behavior, as well as the strategies for their eradication. In this sense, combinations of drugs have been used. The aminoglycoside (AG) activity can be potentiated by stimulating proton motive force generation in persistent cells through carbonate metabolites. This effect is known as potentiation (10,11).

The formation of persistent cells was reported for *S. suis*, (12), but not yet for *P. multocida*. While the effect of carbon metabolites combined with AG *in vitro* for the eradication of *S. suis* and *P. multocida* persistent cells has not been described yet. This work was aimed at detecting the transient resistance events specifically the production of persistent cells in *S. suis* and *P. multocida* strains from pigs in Cuba, and to evaluate the effect of carbonate metabolites in combination with AG for the eradication of persistent cells *in vitro*.

MATERIALS AND METHODS

Strains and culture conditions

The bacterial strains used in this study are part of the collection of the Animal Bacteriology Laboratory of the National Center for Animal and Plant Health (CENSA), isolated from pig with pneumonia. All *S. suis* and *P. multocida* strains were identified and serotyped as previously described (13,14,15). The bacterial stock was stored in glycerol (20%) at -20°C, and all strains were cultured on Columbia (BIOCEN) agar plates containing 5% sterile ovine blood at 37ºC for 24 hours.

Eight strains were selected from the collection, four corresponding to each species. These strains were classified as susceptible according classical testing methods for two drugs (ß-lactamic and fluorquinolone), usually used to control both infections (Table 1) according to previous results (14,16). The cultures of each *P. multocida* and *S. suis* strain were obtained in Brain Hearth broth (BHB) and Todd Hewit Broth (THB), respectively.

TABLE 1. Strains used in this study./Cepas utilizadas en el studio.

Identification	Species	Capsular serogroup
PM21	*Pasteurella multocida*	A
PM5	*Pasteurella multocida*	A
PM29	*Pasteurella multocida*	A
PM8K	*Pasteurella multocida*	A
Ss NT	*Streptococcus suis*	Non typeable
SsS2	*Streptococcus suis*	Serotype 2
SsS3	*Streptococcus suis*	Serotype 3
SsS9	*Streptococcus suis*	Serotype 9

Reagents

The antibiotics used were Ampicillin, Enrofloxacin for *P. multocida*, Penicillin-Enrofloxacin for *S. suis* and Gentamicin for both bacteria. Stock solutions were prepared from each, following CLSI recommendations (17), sterilized with sterile 0.22 µm pore diameter filters. All aliquots were stored at -20°C and protected from light. The carbon sources used (Arginine, Fructose, Glycerol, Glucose, Manytol, Rafinose, Starch, and Trealose) were purchased from Sigma-Aldrich.

Minimal Inhibition Concentration (MIC)

MIC was determined using the microdilution method (18). Briefly, an overnight culture was diluted in an inoculum of approximately 5.10^5 $CFUml^{-1}$ in Muller Hinton Broth (*P. multocida*) and Todd Hewith Broth (*S. suis*), and incubated with a 2-fold antibiotic concentration range (500-1 μgml^{-1}) from 16 to 20 h. The optical density was measured at 595 nm using a microtiter plate reader (SUMA, PR-621, Cuba), and the lowest concentration of the antibiotic that did not exceed an OD of 0.01was taken to be the MIC of that antibiotic.

Time and concentration-dependent killing experiments

Stationary phase cultures of all *S. suis* and *P. multocida* strains were obtained. The concentration of viable cells was determined by counting the colony forming units (CFUs) on Columbia agar plates supplemented with 5% ovine blood (CBA). In all cases, 0.5 ml of the culture was transferred to microcentrifuge tube, antimicrobials were added at 100-fold MIC and cultures were incubated until 48 h. For CFU determinations, 100 µl samples were taken during the antimicrobial challenge after 24 and 48 hours during long-term experiments. Cells were harvested by centrifugation and washed in 1% NaCl solution. The number of persistent cells was determined by plaque counting. Colonies were counted after incubation for 48h at 37°C. All experiments were performed with two independent biological replicates (5).

Heritability of persistence

The stationary phase cultures (5ml) were exposed to 100-fold MIC of Penicillin (*S. suis, strain* SSNT) and 100-fold MIC Ampicillin (*P. multocida* strain PM5), for 5 h. Subsequently, cells were washed in 1% NaCl solution. Surviving cells were re-suspended into 5 ml fresh broth without antibiotics until the stationary phase was reached again. They were subjected to antimicrobial treatment as described and the procedure was carried out three consecutive cycles (19).

Aminoglycoside potentiation assay

The persistent population was isolated after 18 h treatment (ß-lactamic) by centrifugation at 5000 *g* for 5 min. Cells were washed with minimal medium (M9), centrifuged, and finally re-suspended in M9 medium without any carbon source. The persistent population was then treated with gentamicin 250μgml^{-1} and 600mM following the carbon compounds: Arginine, Fructose, Glycerol, Glucose, Manytol, Rafinose, Starch, and Trealose. Controls with gentamicin without carbon source were included. Bacterial counts were carried out as previously described (20).The experiment was performed with three independent biological replicates.

Statistical analysis

Bacterial counts (CUF/ml) from each biological replicate were log10 transformed prior to statistical analysis using Microsoft Excel. Variance analysis and a comparison test of multiple Tukey ranges with a significance level of 0.05 were used to determine whether the number of surviving persistent cells was significantly different upon the different conditions with respect to the untreated control. All analyses were performed using the statistical package INfoStat 2016 (21).

RESULTS AND DISCUSSION

Although, different authors have suggested that all bacteria species have the potential capacity to form persistent cells (6,22), specific studies on the behavior of the species and their strains should be carried out, because the molecular bases explaining cell persistence events in bacteria are not fully known.

In this study, the eight strains tested were sensitive to ß-lactamic and quinolone antibiotics. The ranges of MIC values for Penicillin were 1 μgml^{-1}- 0.5 μgml^{-1} and Enrofloxacin ($2\mu gml^{-1}$-$1\mu gml^{-1}$) for *S. suis* strains, while for *P. multocida* strains, the antibiotics exhibited these value ranges: Ampicillin ($2\mu gml^{-1}$-$1\mu gml^{-1}$) and Enrofloxacin ($4\mu gml^{-1}$-2 μgml^{-1}). For the subsequent killing curve experiments, MIC values were defined as follows for *S. suis*: Penicillin (1 μgm^{-1}) and Enrofloxacin (2 μgml^{-1}). In this definition, the data found in this work, as well as the MIC values reported in other studies (23,24) for *S. suis*, were taken into account. The MIC values established for *P. multocida* were Ampicillin ($2\mu gml^{-1}$) and Enrofloxacin ($4\mu gml^{-1}$), which coincided with the criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (25).

For isolating drug-tolerant *S. suis* and *P. multocida* persisters, bactericidal antibiotics which act on the common bacterial target structures, such as the cell envelope (Ampicillin and Penicillin) or topoisomerase (Enrofloxacin) (26), were applied. CFU count of the liquid culture did not increase to a sizeable extent after the antibiotic challenge, and the strains of both pathogens exhibited a biphasic killing kinetics during the experiment. The cells, approximately ($10^9 CFUml^{-1}$) of *S. suis*, was killed until 10^7 $CFUml^{-1}$, reaching a plateau or a slow decrease of surviving cells to 10^4 CFU ml^{-1} (Figure 1A-B), similar for *P. multocida* strains (Figure 2A-B). Biphasic killing curves are the experimental hallmark of persistence and are obtained when a lethal dose of a bactericidal antibiotic is added to a bacterial population and the number of surviving cells is followed over time (27,28). All *S. suis* and *P. multocida* strains revealed a range of tolerance cells for both antibiotics, although there was a slight differences. This is consistent with the results of some authors who have found that the persistence degree among strains was different (29,30,31).

Two different types of persistent cells have been defined, type I persistent cells from stationary growth phase cultures, whereas type II persistent cells have their origin in exponential growth phase cultures (12,32,33). In this work, the bactericidal activity evaluated, using the concentration-killing curves and the antibiotic, were supplied to both *P. multocida* and S. *suis* cultures in the stationary phase. The logarithmic phase culture was not evaluated according to different previous studies on *S. suis* and other species where the number of the persistent cells observed was higher during the stationary growth when compared to the exponential grown bacteria (12,28).

Previous studies have established persistent cells are tolerant to multiple antibiotics (5). However in this study, the strains PM21 and PM29 failed to produce a detectable level of such cells after 48 hours of challenge with Enrofloxacin. But when the strains were treated with ampicillin, PM29 was tolerant, while PM21 did not survive (Figure 2 A-B). This result is consistent with the studies of Hofsteenge *et al*., 2013 (30), who did not find a correlation between persistent levels for different antibiotics among environmental *E. coli* strains. Barth *et al*., 2013 (31) neither found a correlation among the different antibiotics and the persistent cells from *Acinetobacter baumannii* clinical isolates.

Most of the transient resistance researches have been focused on the following microbial groups (*Escherichia coli*, *Mycobacterium tuberculosis*, *Pseudomona aeroginosa*, *Staphyloccus aureus*), based on the use of strains that were genetically manipulated

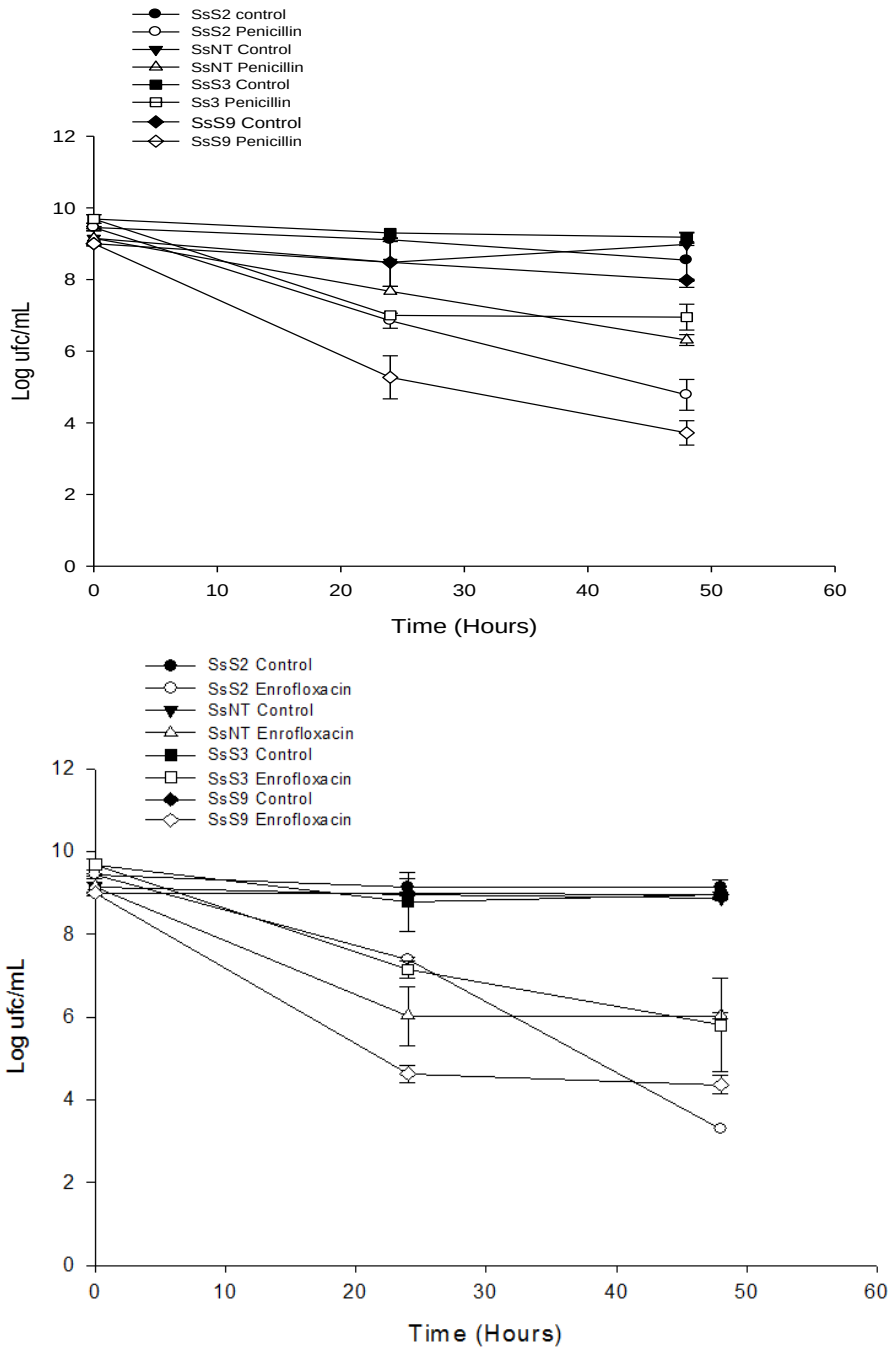

FIGURE 1. Time-dependent killing of *Streptococcus suis.* Cultures in stationary phase of each of four *S. suis* strains (Ss serotype 2, Ss non-typeable, Ss serotype 3 and Ss serotype 9) were treated with A) Penicillin 100xMCI and B) Enrofloxacin 100xMCI. An identical culture of each strain without any antibiotic challenge served as a control. Experiments were performed with two biological replicates, respectively. Error bars indicate the standard deviations./ Curvas de letalidad de *Streptococcus suis.* Cultivos en fase estacionaria de cada una de las cuatro cepas de *S. suis* (Ss serotipo 2, Ss no tipificable, Ss serotipo 3 y Ss serotipo 9) se trataron con A) Penicilina 100xMCI y B) Enrofloxacina 100xMCI. Un cultivo de cada cepa al cual no se le adicionó antibiótico se utilizó como control. Los experimentos se realizaron con dos réplicas biológicas, las barras de error indican la desviación estándar.

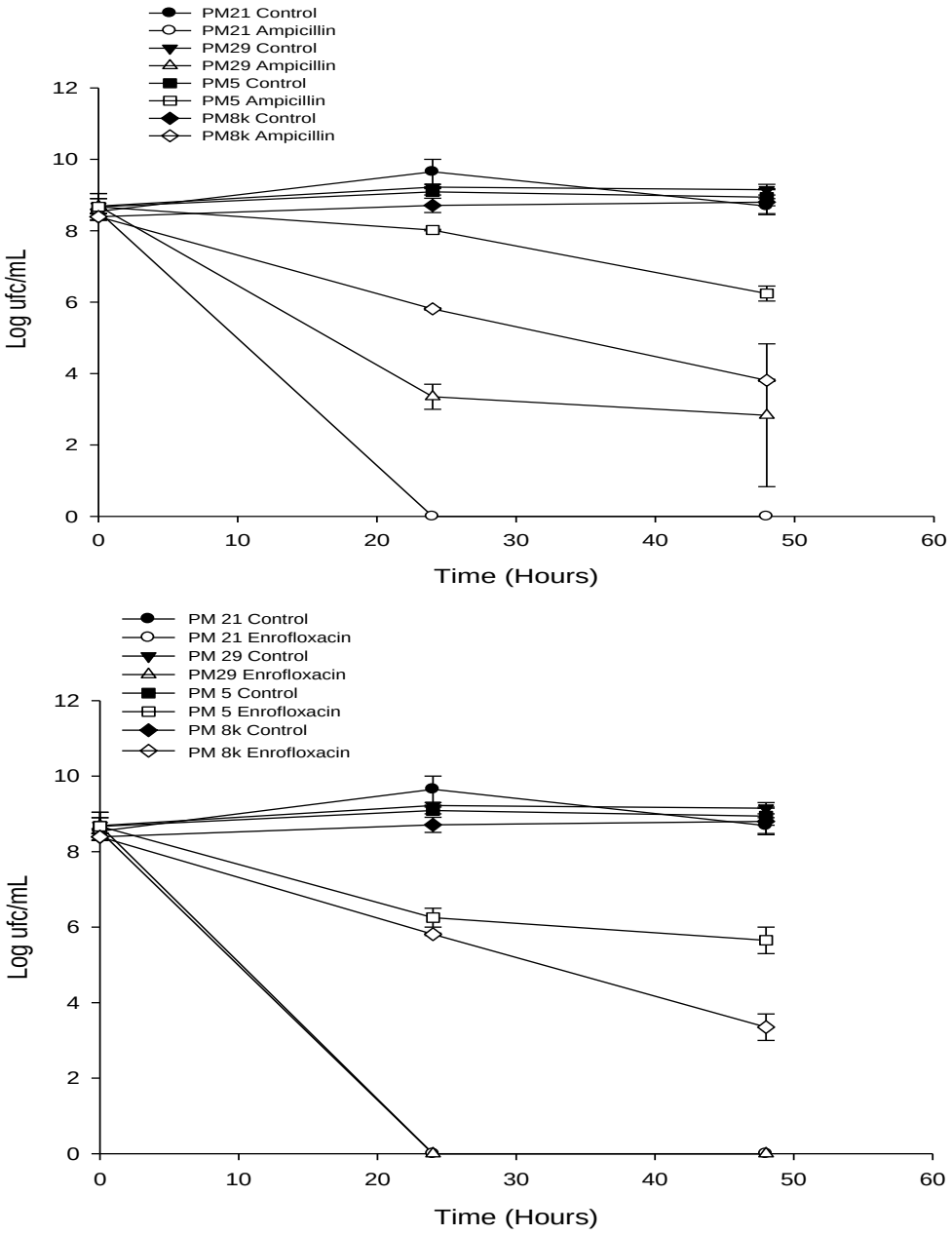

FIGURE 2. Time-dependent killing of *Pasteurella multocida.* Cultures in stationary phase of each of four *P. multocida* strains (PM5, PM21, PM29 and PM8K) were treated with A) Ampicillin 100xMCI and B) Enrofloxacin 100xMCI. An identical culture of each strain without any antibiotic challenge served as a control. Experiments were performed with two biological replicates, respectively. Error bars indicate the standard deviations./ Curvas de letalidad de *Pasteurella multocida.* Cultivos en fase estacionaria de cada una de las cuatro cepas de *P. multocida* (PM5, PM21, PM29 and PM8K) se trataron con A) Ampicillin 100xMCI y B) Enrofloxacina 100xMCI. Como control se utilizó un cultivo de cada cepa al que no se le adicionó antibiótico. Los experimentos se realizaron con dos réplicas biológicas, las barras de error indican la desviación estándar.

previously to favor an increase or decrease in the production of persistent cells (35,36). Some of these previous studies based on the use of mutant strains have demonstrated the relative and redundant nature of persistent cells (32,34,35). However the studies using collections of non-genetically manipulated strains are scarce. A study carried out in a collection of *E. coli* strains from environmental origin showed that there were differences in the amount of persistent cells derived from each strain, once they were confronted with antibiotics from different families (30). In these work, *S. suis* and *P. multocida* strains showed variations in the frequency of persistent cells.

The heterogeneous behavior showing the strains as regard the persistent cell formation may have different explanations. First, the intrinsic properties to each strain (34). Second, and according to Luidalepp *et al* (35), the bacterial cultures genetically homogeneous can generate subpopulations with different physiological properties. When stationary-phase bacteria are diluted in fresh medium, some cells start growing immediately and some later. Therefore, inoculum age had effects on the frequency of persistent cell formation. In this study, even still when the cultures were previously adjusted to a similar concentration, the strains differed in the persistent range (36,37).

In order to examine the possible heritability of phenotypic resistance, SsS3 and PM5 strains, which previously showed high persistence levels under the effect of both antibiotics (Penicillin and Ampicillin), were selected for the heritability assay. The stationary phase cultures of these strains were treated with ß-lactamics. The survival cells were inoculated in fresh medium, and a newly fraction of initial culture survived to the treatment. The same occurred for a third challenge with the antibiotic, showing a biphasic curve (Figure 3),

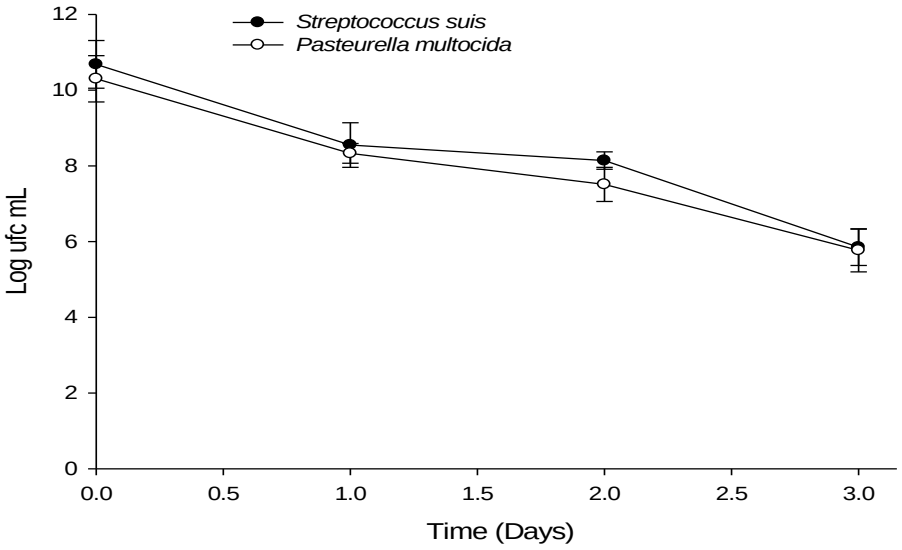

FIGURE 3. Time-dependent killing of *S. suis* (black circle) and *P.multocida* (white circle). Experiments were performed with two biological replicates and error bars indicated the standard deviations. Two persistent isolates (SsNT and PM5), which survived 24 h of treatment with 100 µgml^{-1} of Penicillin and Ampicillin, respectively, were exposed to a three 24-h treatment with 100 µgml^{-1}./Curvas de muerte dependientes del tiempo de *S.suis* (círculos negros) y *P.multocida* (círculos blancos). Los experimentos se realizaron con dos réplicas biológicas y las barras indican la desviación estándar. Dos aislados persistentes (SsNT y PM5) que sobrevivieron a un tratamiento por 24 horas con 100 µgml^{-1} de Penicilina y Ampicilina, respectivamente, se expusieron a tres tratamientos en similares condiciones.

indicating that the resistance phenotype was transient, not being the consequence of a genetic change. Each culture obtained from the persistent cells was as sensible to ß- lactamic as the parental culture. If the cells had been able to grow once the antibiotic was retired due a genetic resistance mechanism, then such behavior would have been different, the population would not have decreased and a biphasic curve would not have been obtained, because the bacterial population would have increased in each cycle.

The mechanism of persistent cell formation is not well understood and the metabolic state of these cells is debated. The main model that explains the genetic basis of the formation of persistent cells consists of a toxin-antitoxin (TA) system, inducing a dormancy state (38,39,40), and allowing cells to survive to the effects of antibiotics. TA systems (41) generally consist of a stable toxin (protein) that disrupts an essential cellular process (e.g., translation via mRNA degradation) and a labile antitoxin (either RNA or a protein) that prevents toxicity (38,39). Other model is the alarmoneguanosinetetraphosphate (ppGpp) that also directly reduces DNA replication and protein synthesis (40,41).

When a culture is treated with a bactericidal antibiotic in high doses, it is possible to find dead cells, viable but non-culturable cells (VBNCs) and persistent cells. There are current different techniques for the isolation and discrimination among these types of cells (42,43,44). In this work, normally growing strains of both *S. suis* and *P. multocida* were lysed with ß-lactamic (Penicillin and Ampicillin), respectively. The non-lysed live cells were sedimented by centrifugation and subjected to the aminoglycosides (AG) potentiation assay with the following carbon sources (Arginine, Fructose, Glycerol, Glucose, Mannitol, Rafinose, Starch, and Trealose). Figure 4 shows the effect of the combination of carbonate metabolites with AG (Gentamicine) on the persistent cells previously obtained for each of the strains of both pathogens. The persistent cells of both microorganisms treated with gentamicine and metabolite of carbon decreased with respect to the cultures only treated with gentamicine. Glycerol, arginine and starch were the carbonate metabolites that most strongly potentiated the AG activity in both Penicillin and Ampicillin persistent cells obtained from the stationary-phase cultures of *S. suis* and *P. multocida* (Figure 4). The addition of carbonate metabolites which served to generate a proton motive, forced to conduce to the incorporation of AG, therefore making the cells more susceptible to gentamicine (45).

Numerous research have corroborated that persistent cells are metabolically inactive or predominantly dormant (46). Therefore the strategies to kill these sleeping cells require compounds that enter the cell without an active transport. Some examples include DNA-cross-linking compounds as mitomicyn C or acyldepsipeptide, a protease that degrades many cellular proteins (44). Another alternative to eradicate these dormant cells consists in waking them in order to use traditional compounds as antibiotics, specifically AG (Gentamicin, kanamycin and streptomycin) in combination with carbon metabolites (41,44,45).

The results indicated that Glycerol was the metabolite that, in combination with AG gentamicine, completely eradicated the persistent cells produced by both *S. suis* and *P. multocida* strains after the treatment with ß-lactamic (Figure 4). Mehmet *et al* (46) found that glycerol was the metabolite that, in conjunction with AG, decreased different metabolic types of *E. coli* persistent cells. The combination Arginine plus G also eradicated persistent cells. Arginine is a crucial amino acid that modulate the cellular immune response during infection in the host. The importance of arginine metabolism has been reported in many pathogens like *Salmonella typhimurium*, *Helicobacter pylori*, *Mycobacterium tuberculosis*, and *Streptococcus suis*, as a source of energy and as a trigger for the polyamine synthesis required

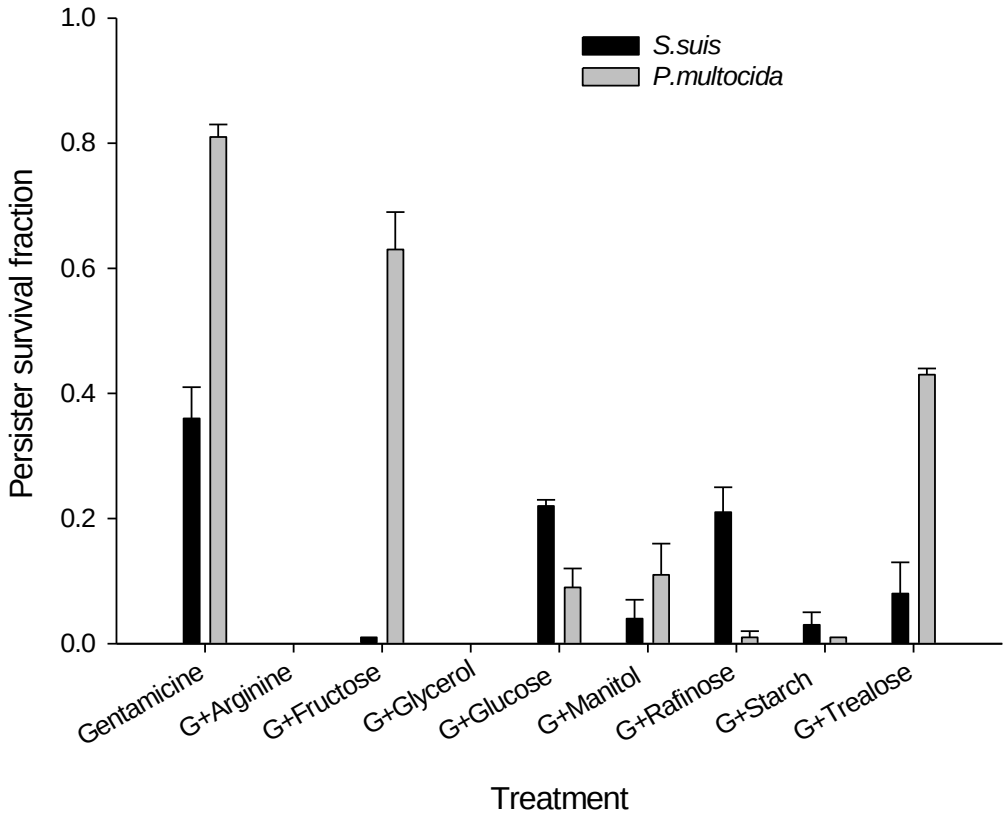

FIGURE 4. Aminoglycoside potentiating assay for persistent cells. Stationary-phase cultures were treated with ß-lactamic for 5 hours, and cells were incubated in M9 minimal medium with Gentamicin (G) and different carbon sources (Arginine, Fructose, Glycerol, Glucose, Manytol, Rafinose, Starch, and Trealose). For controls, G 100 µg/ml was used. The survival fraction was determined based on CFU. Error bars indicate the standard errors of the means./ Ensayo de potenciación con aminoglucósidos para células persistentes. Cultivos en fase estacionaria se trataron con antibiótico ß-lactámicos por 5 horas, las células se incubaron en el medio mínimo M9 con Gentamicina (G) y diferentes fuentes de carbono (Arginina, Fructosa, Glicerol, Glucosa, Manitol, Rafinosa, Almidón, Trealosa). Las células persistentes tratadas solamente con gentamicina (100 µg/ml) se utilizaron como control. La fracción de células sobrevivientes se determinó mediante conteo de UFC. Las barras indican la desviación estándar de las medias.

for an efficient pathogenesis (47,48).

The persistence of bacteria that can produce recalcitrant infections requires attention. This study has been focused on testing the formation of persistent cells in *S. suis* and *P. multocida* strains. In the case *S. suis*, its ability to tolerate antibiotics through the formation of persistent cells had already been informed (12). Although it has been considered that all bacteria have this ability, to our knowledge, prior to this study the formation of persistent cells for *P. multocida* had not been described.

S. suis and *P. multocida* strains formed persistent cells, but varying intensities, this phenotype could be wide distributed among *S. suis* and *P. multocida* isolates. Persistence understanding may contribute to improve the strategies aiming at the control of recurrent infections. Persistent cells should be taken into account when *S. suis* or *P. multocida* infections are produced in pigs and are not solved with an antimicrobial treatment.

It has been postulated that persistence is an important contributor to resistance emergence because it produces a continuous reservoir of viable cells in the presence of antibiotics (19). These results broaden the evidences about pathogens in veterinary medicine producing persistent cells, corroborating the observations on the differences in persistent levels among species or even among strains of the same species. Finally, it has been demonstrated that glycerol and arginine potentiated the effect of AG gentamicine in the eradication of persistent cells.

REFERENCES

1. Verraes C, Sigrid V B, Meervenne, V ECoillie E V, Butaye P, Catry B, Schaetzen MA, Van Huffel X, Imberechts H, Dierick K, Daube G, Saegerman Jan De Block C, Dewulf J and Herman L. Antimicrobial Resistance in the Food Chain: A Review Int. J. Environ. Res. Public Health.2013;10:643-2669.
2. Corona F and Martinez JL. Phenotypic Resistance to Antibiotics Antibiotics.2013;2:237-255.
3. Gang Z, Shi Q, Huang X and Xie XB. . The Three Bacterial Lines of Defense against Antimicrobial Agents. Int J Mol Sci. 2015;16:21711-21733.
4. Merle E, Olson HC, Douglas WM, Buret AG and Ronald R. Read biofilm bacteria: formation and comparative susceptibility to antibiotics. Can J Vet Res. 2012;66:86-92.
5. Lewis K. Persister cells, dormancy and infectious disease. Nat Rev Microbiol. 2007;5(1):48-56.
6. Keren I, Shah D, Spoering A, Kaldalu N, Lewis K. Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. J Bacteriol. 2004;186(24):8172-8180.
7. Portis E, Lindeman C, Johansen K. Antimicrobial susceptibility of porcine *Pasteurella multocida*, *Streptococcus suis*, *Actinobacillus pleuroneumoniae* from the United States and Canada, 2001 to 2010. J Swine Health Prod. 2013;21(1):30-41.
8. Moreno AM, Baccaro MR, Ferreira AJand Pestana de Castro AF. Use of Single-Enzyme Amplified Fragment Length Polymorphism for Typing *Pasteurellamultocida*subsp. *Multocida*Isolates from Pigs. J Clin Microbiol. 2003;41(4):1743-1746.
9. Gottschalk M, Lacouture S, Bonifait L, David R, Nahuel F, Daniel G. Characterization of *Streptococcus suis* isolates recovered between 2008 and 2011 from diseased pigs in Quebec, Canada. Vet Microbiol. 2013;162(2-4):819-25.
10. Thomas KW. Strategies for combating persister cell and biofilm infections Microbial Biotechnology John Wiley & Sons Ltd and Society for Applied Microbiology. 2017.
11. Allison KR, Brynildsen MP, Collins JJ. Metabolite-enabled eradication of bacterial persisters by aminoglycosides. Nature .2011; 473:216–220.
12. Willenborg J, Willms D, Bertram R, Goethe R and Valentin-Weigand P. Characterization of multi-drug tolerant persister cells in *Streptococcus suis*. BMC Microbiol. 2014;14:120.
13. Espinosa I, Báez M, Corona B, Chong D, Lobo E, Martínez S. Molecular typing of *Streptococcus suis* from pigs in Cuba. Biotecnología Aplicada. 2013;30(1):39-44.
14. Espinosa I, Báez M, Vichi J, Martínez S. Antimicrobial resistance and genes associated to the host-microbe interaction

of *Pasteurella multocida* isolates from swine in western Cuba. Rev Salud Anim. 2012;34(3):151-158

15. Espinosa I, Báez M, Percedo MI, Lobo E, Martínez S, Gottschalk M. Serotyping of porcine S*treptococcus suis*recovered from diseased pigs in the western region of Cuba. Rev Salud Anim. 2013;36(3):196-200.
16. Báez M, Espinosa I, Vichi J, Martínez S. Estudio de la sensibilidad *in vitro* frente a diferentes antimicrobianos en cepas de *S. suis*asociados a neumonía porcina. Rev Salud Anim. 2012;34(1):57-62
17. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals; Approved Standard, third ed., vol. CLSI document M31–A3. Clinical and Laboratory Standards Institute, Wayne, PA.2016.
18. Andrews JM. Determination of minimum inhibitory concentrations. J Antimicrob Chemother. 2001;48:5-16.
19. Mehmet AO, Wendy WK, MokM, Brynildsen P. Aminoglycoside-Enabled Elucidation of Bacterial Persister Metabolism. Curr Protoc Microbiol. 2006;36: 17.
20. Mehmet A, Brynildsen P. Establishment of a Method To Rapidly Assay Bacterial Persister Metabolism Antimicrobial Agents and Chemotherapy. 2013;57(9):4398-4409.
21. InfoStat (2002). *InfoStat versión 1.1*. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina.
22. Elie J, Van den Bergh MB, Verstraeten N, Michiels J. Molecular mechanisms and clinical implications of bacterialpersistence.Drug Resistance Updates. 2016.
23. Escudero JA , San Millan A ,Catalan A, De la Campa AG , Rivero E, Lopez G, Dominguez L, Moreno MA and Gonzalez-Zorn B. First characterization of Fluoroquinolone Resistance in *Streptococcus suis.* Antimicrobial Agents and Chemotherapy. 2007:51(2)777-782.
24. Chunping Z, Zhongqiu Z, Song L,Fang X andNing Y. Antimicrobial Resistance Profile and Genotypic Characteristics of *Streptococcus suis*Capsular Type 2 Isolated from Clinical Carrier Sows and Diseased Pigs in China. BioMed Res Int. 2015;Volume 2015.
25. European Committee on antimicrobial susceptibility testing. Breakpoints table for interpretation of MICs and zone diameters version 6.0.2016. http://www.eucast.org)
26. Bassetti M, Merelli M, TemperoniCh and Astilean A. New antibiotics for bad bugs: where are we? Annals of Clinical Microbiology and Antimicrobials. 2013;12:22.
27. Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S. Bacterial persistence as a phenotypic switch. Science. 2004;305(5690):1622-1625.
28. Keren I, Kaldalu N, Spoering A, Wang Y, Lewis K.Persister cells and tolerance to antimicrobials. FEMS MicrobiolLett. 2004;230:13-18.
29. Wiuff C, Zappala RM, Regoes RR, Garner KN, Baquero F, Levin BR.Phenotypic tolerance: antibiotic enrichment of noninherited resistance in bacterial populations. Antimicrob Agents Chemother. 2005; 49:1483–1494.
30. Hofsteenge N, Nimwegen V, Silander E. Quantitative analysis of persister fractions

suggests different mechanisms of formation among environmental isolates of *E. coli*. BMC Microbiol. 2013;13(25).

31. Barth VC, Rodriguez BÁ, Bonatto GD, Gallo SW, Pagnussatti VE, Ferreira CA, Oliveira S .Heterogeneous persister cells formation in *Acinetobacterbaumannii.* PLoS One 2013;8(12):e84361.
32. Manuel J, Zhanel G, Gand De Kievit T. Cadaverine suppresses persistence to carboxypenicillins in *Pseudomonas aeruginosa* PAO1. Antimicrob Agents Chemother. 2010;54:5173-5179.
33. Dorr T, Lewis K, Vulic M. SOS response induces persistence to fluoroquinolones in *Escherichia coli*. PLoS Genet 2009;5(12):e1000760.
34. Stewart B, Rozen D. Genetic variation for antibiotic persistence in *Escherichia coli*. Evolution. 2012;66(3):933-939.
35. Luidalepp H, Joers A, Kaldalu N, Tenson T. Age of inoculum strongly influences persister frequency and can mask effects of mutations implicated in altered persistence. J Bacteriol. 2011;193(14):3598-3605.
36. Lewis K. Multidrug tolerance of biofilms and persister cells. Curr Top Microbiol Immunol. 2008;322:107-131.
37. Jayaraman R. Bacterial persistence: some new insights into an old phenomenon. J Biosci. 2008;33:795-805.
38. Schuster CF, Bertram R. Toxin-antitoxin systems are ubiquitous and versatile modulators of prokaryotic cell fate. FEMS Microbiol Lett. 2013;340:73-85.
39. Melderen V L, Saavedra De B M. Bacterial toxin-antitoxin systems: more than selfish entities? PLoS Genet. 2009;5(3):e100437.
40. Dalebroux ZD, Swanson MS. ppGpp: magic beyond RNA polymerase. Nat Rev Microbiol. 2012;10:203-212.
41. Thomas K, Wood AB, Stephen J, Knabel C, Brian W. Bacterial Persister Cell Formation and Dormancy. Applied and Environmental Microbiology. 2013;23:7116-7121.
42. Lewis K. Persister cells. Annu. Rev. 950 Microbiol. 2010;64:357-372.
43. Cristo V, Barth VJ, Belisa A, Rodriguez V, Bonatt GD, Gallo SW, Pagnussatti VE, Sanchez F CA, Dias de Oliveira S. Heterogeneous Persister Cells Formation in *Acinetobacter baumannii*. PLOS ONE. 2013;8(12):e84361.
44. Van den Bergh B, FauvarM, Michiels J. Formation, physiology, ecology, evolution and clinical importance of bacterial persisters. FEMS Microbiol Rev. 2017;41(3):219-251.
45. Allison KR, Brynildsen MP, Collins JJ. Metabolite-enabled eradication of bacterial persisters by aminoglycosides. Nature. 2011;473:216-220.
46. Mehmet AO, Mark PB. Establishment of a Method To Rapidly Assay Bacterial Persister Metabolism. Antimicrobial Agents and Chemotherapy. 2013;57(9):4398-4409.
47. Gogoi M, Datey A, Keith T W and Chakravortty D. Dual Role of Arginine Metabolism in Establishing Pathogenesis. Curr Opin Microbiol. 2016;29:43-48.
48. Allison KR, Brynildsen MP, Collins JJ. Metabolite-enabled eradication of bacterial persisters by aminoglycosides. Nature .2011;473:216-220.