Study on the Persistence of Acinetobacter Baumannii against Antibiotics

Dr. R. Karthik*

Assistant Professor, Department of Biotechnology, Dhanalakshmi Srinivasan College of Arts and Science for Women, Perambalur, Tamil Nadu

research@dscollege.ac.in

ABSTRACT

Persisters are phenotypic variants of normal susceptible bacterial populations that survive prolonged exposure to high doses of antibiotics and are responsible for relapse of infection. Acinetobacter baumannii formed highest percentage of persister cells against rifampicin followed by amikacin and least against colistin. In the present study, three different anti-persister approaches were employed to target the persister cells. First, curcumin synergistically increased reactive oxygen species (ROS) production in combination with colistin, reducing significantly the survival of persister cells. Second, exogenous supplementation of metabolites viz. glucose, fructose or citrate or osmolytes increased proton- motive force (PMF) that facilitated amikacin uptake leading to decreased newlinepersister cells survival. Third, quorum-quenching enzyme lactonase effectively reduced persister cells against antibiotics in biofilm formed in its presence. These strategies can be effectively employed as treatment options with anti-persister potential for the control of chronic and relapsing A. baumannii infections.

Keywords – *Acinetobacter Baumannii, Microbiology, Biotechnology in Applied Microbiology*

INTRODUCTION

Infections caused by antibiotic-resistant pathogens have started posing a serious threat to humanity in the present-day global health care. Since the discovery of first antibiotic, penicillin, by Alexander Fleming in 1928, antibiotics have been the first line of attack for combating various infectious diseases. Their extensive use, however, has led to the development of antibiotic resistance, making it difficult to treat even the trivial infections (WHO, 2016). Acinetobacter baumannii is one of the six „superbugs‟ identified by the Infectious Diseases Society of America (IDSA) as “ESKAPE” group comprising of Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, A. baumannii, Pseudomonas aeruginosa and Enterobacter species (Rice, 2008). It is a Gram-negative, non-fermentative, coccobacillus belonging to the family Moraxellaceae and has in recent years gained increasing notoriety as a nosocomial pathogen. It figures in the “critical” category of World Health Organisation‟s (WHO) priority pathogens list for development of new antibiotics (WHO, 2017). A. baumannii has been implicated in a diverse range of infections including pneumonia, bacteremia, wound

and burn infection, urinary tract infection (UTI) and meningitis. It is conspicuously prevalent in intensive care units where numerous outbreaks have been extremely difficult to control (Gordon et al., 2010; Lee et al., 2017). The rapid emergence and global dissemination of drug resistant A. baumannii as a major nosocomial pathogen highlights its successful adaptation to the 21st century hospital and health-care ecosystem (Wong et al., 2017).

Antibiotic resistance has always been considered as the main culprit for the treatment failures. The current antibiotic resistance crisis stems from two discrete phenomena i.e. drug resistance and drug tolerance. Resistance mechanisms such as alteration or mutation of target proteins to avoid antibiotic binding/action and overexpression of efflux pumps allow the pathogens to grow even at elevated doses of antibiotics. Many pathogens which are susceptible during laboratory testing, are not amenable to antibiotic therapy due to antibiotic tolerance, an alternative strategy to resist high doses of antibiotics (van den Bergh et al., 2017). Antibiotic tolerance is the property of persister cells which are the isogenic phenotypic variants of normal susceptible bacterial populations (Lewis, 2015). These are transitory tolerant cells, either slowgrowing or growth arrested (Cabral et al., 2018; van den Bergh et al., 2017). Unlike resistant cells, persisters do not grow in the presence of antibiotics and arise without undergoing genetic changes (Michiels et al., 2016b). Upon re-inoculation in antibiotic free-medium, persisters produce a population genetically identical to the original culture, equally susceptible to antibiotic treatment (Maisonneuve and Gerdes, 2018; Wiuff and Andersson, 2017). Persister cells are found in all phases of cell growth with a frequency of 0.0001 to 0.001% of the population in the exponential phase and as high as 1% in bacterial biofilms and stationary phase of cultures (Lewis, 2018a).

Quorum sensing is one of the important mechanisms involved in persister cells formation. Quorum sensing is a cell to cell communication based signaling system that allows bacteria to share information via signaling molecules (autoinducers) that control/influence a variety of traits, such as virulence factors along with biofilm formation in pathogens (Bassler and Losick, 2016). There are reports suggesting the role of quorum sensing in increasing persistence of P. aeruginosa (Möker et al., 2017). It has also been reported that quorum sensing signaling molecules induce a protective mechanism in A. baumannii against oxidative stress and increase its persistence against amikacin and carbenicillin antibiotics (Bhargava et al., 2015). Since, quorum-sensing regulates biofilm formation in bacteria and majority of the cells in biofilms are persister cells (Trastoy et al., 2018; Wood et al., 2017), therefore, targeting quorum-sensing will be an effective strategy against persister cells in the biofilm.

OBJECTIVE OF THE STUDY

1. Role of quorum-sensing in persistence of biofilm cells using quorumquenching enzyme acyl-homoserine lactonase.

2. Effect of natural compounds with pro-oxidants and antioxidants properties on persistence, alone and in combination with antibiotics.

MATERIAL AND METHODS

Antibiotic discs for testing antibiotic sensitivity

Antibiotic discs for different classes of antibiotics (Penicillins, β-lactams, cephalosporins, carbapenems, aminoglycosides, tetracyclines and fluoroquinolones) were purchased from Himedia Laboratories Pvt. Ltd, India.

DYES

2',7'-dichlorofluorescin diacetate (DCFDA)

1 mM solution of 2', 7'-Dichlorofluorescin diacetate (Sigma) was prepared in DMSO and stored at -20 °C protected from light.

N-phenyl-1-napthylamine solution (NPN)

10 mM solution of N-phenyl-1-napthylamine (Himedia) was prepared in sterile distilled water and stored at -20 °C protected from light.

Ethidium Bromide (EtBr) for efflux and accumulation assays

2 mg/ml solution of Ethidium bromide (Himedia) was prepared in the sterile distilled water for efflux and accumulation assays and stored at 4 °C protected from light.

DNA AND PROTEIN MARKERS

DNA ladders

50 bp DNA ladder was obtained from GeneDireX Pvt. Ltd (India) and 100 bp DNA ladder was purchased from BIOCHEM life technologies.

Protein marker

PINK Plus Prestained protein ladder was purchased from GeneDireX Pvt. Ltd. (India). It contained proteins with molecular weights: 175, 130, 95, 70, 62, 51, 42, 29, 22 and 10.5 kDa.

SOLUTIONS AND BUFFERS

Phosphate buffered saline (PBS)	**(pH 7.4)**
Sodium chloride	7.650 g
Disodium phosphate, anhydrous	0.724 g
Dipotassium hydrogen phosphate	0.210 g

FORMATION OF PERSISTER CELLS BY A. BAUMANNII AGAINST ANTIBIOTICS

Exposure of A. baumannii cells at late exponential phase to 40X amikacin, 20X rifampicin and 10X colistin (on the basis of concentration-dependent persister assay) resulted in a biphasic killing pattern with rapid killing of bulk of the antibioticsensitive population followed by a plateau of the surviving persister cells with diminished killing rate of less than one log cycle. Maximum persisters (1.92%) were observed against rifampicin at 20X MIC, 0.10% against amikacin at 40X MIC and least (0.08%) against colistin (10X MIC) at 24 h in the time-dependent assay.

The percentage of persister cells also varied with growth-phase, as more persister cells were formed in stationary phase than the exponential phase. Exposure of cells at late stationary phase to 10X amikacin, 20X rifampicin and 20X colistin (on the basis of concentration-dependent persister assay) also resulted in a biphasic killing pattern. Maximum persisters (65.2%) were observed against rifampicin at 20X MIC, 42.6% against amikacin at 10X MIC and least (8.5%) against colistin (20X MIC) at 24 h in the time-dependent assay.

Growth curve of A. baumannii

A single colony of A. baumannii ATCC 17978 was inoculated to 20 ml LB broth and grown at 37 °C, 180 rpm for 16 h. 1% of the overnight grown culture was added into different flasks containing 20 ml LB broth and grown at 37 °C with shaking at 180 rpm. The bacterial growth was monitored upto 24 h by drawing the culture at different time intervals (0, 2, 4, 6, 8, 10, 12, 16 and 24 h) and measuring the optical density at 600 nm. Colony forming units per ml (CFU/ml) at the respective time intervals were calculated by serially diluting the culture in PBS. 100 µl of each dilution was spread plated on LB agar plates and incubated at 37 °C for 16 h to determine the number of viable colonies. Dilutions that yielded 30- 300 colonies were included for counting CFUs (Sieuwerts et al., 2018).

NATURAL HABITAT

A. baumannii exhibits ubiquitous existence in nature thriving abundantly in soil as well as in water. A. baumannii is most extensively found in the hospital environment, predominantly inhabiting intensive care units (ICUs) (Towner, 2019). Human skin and respiratory mucosa provide a strong fertile niche for its colonization but A. baumannii is remarkably notable for predominantly clustering in hospital healthcare settings (Michiels et al., 2016b). A. baumannii isolates have been reported from animals, lice, vegetables, soil and water also (Eveillard et al., 2017). However, this still remains to be ascertained whether these isolates are contaminants from the hospital reservoirs or from any other natural habitat of this species (Antunes et al., 2017). Isolation of A. baumannii strains from hospital settings weeks or even months after transmission from infected patients clearly indicates its resilience to withstand long term aridity as well as high temperatures (Eugenin, 2018). Commonest reservoirs of A. baumannii in a hospital setting include ventilators and their accessories, bed mattresses, intravenous devices, catheters, suction apparatus, central lines, sinks and taps (Giamarellou et al., 2018) and even the hands of hospital workers (Hanlon, 2015).

Bacteremia

Presence of central venous catheter or dissemination of A. baumannii due to extensive pneumonia results in the bloodstream infections. The other predisposing factors that cause bacteremia are intravenous lines, mechanical ventilation, operations, renal transplants, chest tubes, urinary catheterization, trauma, long hospitalization and antibiotic treatment (Kurcik-Trajkovska, 2019). It is very common in elderly immunecompromised individuals (Cisneros and Rodríguez-Baño, 2018). The overall mortality rate from ICU-acquired bacteremia (34.0 to 43.4%) was higher in comparison to nonICU wards (16.3%) (Cisneros and Rodríguez-Baño, 2016). Bloodstream infections due to A. baumannii were responsible for the highest mortality rate in the ICUs as compared to other pathogens of ESKAPE group (Leão et al., 2016).

Trauma and other wound infections

A. baumannii has been identified with trauma-related or post-surgical osteomyelitis, skin and soft tissue infections (Yun et al., 2018; De Carvalho et al., 2017). About 2.1% of ICU acquired skin and soft tissue infections are reported to be caused by A. baumannii. It is known as a causative pathogen behind the burn-related infections which are difficult to eradicate (Trottier et al., 2017). A. baumannii has emerged at the top of pyramid of antibiotic resistant pathogens responsible for high morbidity and mortality. A. baumannii is popularly known as "Iraqibacter" because of its dramatic emergence in the military hospitals during Iraq war. It was the most frequently isolated pathogen from the wounds of combat causalities in Iraq and Afghanistan. It was isolated from the battle victims (32.5% of cases) having open tibia fractures (Falagas et al., 2015; Petersen et al., 2017; Whitman, 2017). Similar A. baumannii associated skin, soft tissue and wound infections were reported to occur in the trauma victims of natural disasters like earthquakes (Wang et al., 2015; Zhang, 2017), floods (Apisarnthanarak and Warren, 2015), tsunami (Maegele et al., 2015) and also the bystanders in the areas involved in active military conflicts (Dallo and Weitao, 2018; Wong et al., 2017).

TREATMENT OF A. BAUMANNII INFECTIONS

A. baumannii is a notorious organism that has been reported to cause various drugresistant nosocomial infections. The antibiotics commonly used for the treatment of infections caused by A. baumannii.

Administration of broad spectrum parenteral antibiotics is the major treatment for A. baumannii infections (Doi et al., 2015). Although several controlled studies in patients with A. baumannii infections have shown that beta lactam antibiotic therapy is effective for susceptible strains but the ideal antibiotic therapy for A. baumannii infections is still a matter of debate given its penchant for acquiring resistance (Doi et al., 2015). Presence of protective outer membrane in A. baumannii easily stymies the entry of currently available antibiotics. Extended infusion of intravenous carbapenem is the preferred treatment because of the early onset of action and greater half life in serum. Given the challenge of resistance, antibiotics against carbapenem-resistant strains appears nearly depleted and consensus on ideal antimicrobial regimen for such infections is indeed lacking (Lee et al., 2017; Turner et al., 2016).

Another alternative includes the use of sulbactam (β-lactamase inhibitor), which has shown potent bactericidal activity against the isolates of A. baumannii. In A. baumannii, sulbactam has been reported to show great affinity for penicillinbinding proteins (Rafailidis et al., 2017). Clinical outcomes demonstrated higher effectiveness of sulbactam with ampicillin against bloodstream infections by A. baumannii (Temocin et al., 2015). Combination of sulbactam/ampicillin/ carbapenem has been reported to be effective against skin and soft tissue infections and bacteremia caused by carbapenem-resistant A. baumannii (Hiraki et al., 2017). In a murine model, tazobactam, another β-lactamase inhibitor has been reported to increase the activity of colistin and daptomycin against pneumonia (Sakoulas et al., 2017).

GENERAL STRESS RESPONSE

General stress response is an important mechanism that has role in virulence, biofilm formation and successful bacterial survival under stressful conditions (Harms et al., 2016). RpoS, which encodes sigma factor, is known as the master stress-response regulator and a central regulator of various stationary phase-inducible genes (Harms et al., 2016). RpoS is reportedly known to get accumulated in the cells as they enter stationary phase or when under stress conditions such as nutrient deprivation, pH and temperature changes, oxidative stress and biofilm formation (Battesti et al., 2015, 2016). In E. coli, the genes and structural proteins that assist in formation and degradation of biofilm in response to stress are regulated by RpoS (Sharma et al., 2016). RpoS mutants in P. aeruginosa were observed to have decreased survival on exposure to heat stress and imipenem, thus suggesting the importance of RpoS to manage stressful conditions (Colvin et al., 2014).

CONCLUSION

he continuous mis- and overuse of the antibiotics has sped up the evolution of extensive drug-resistant organisms, which has become a global threat in the healthcare settings in the absence of new and novel antibiotics (Martens and Demain, 2017; WHO, 2015). Another challenge is the recalcitrant nature of the chronic infections attributed to the persister cells, the phenotypic variants tolerant to antibiotics that can resume growth as soon as the antibiotic pressure drops (Fisher et al., 2017; Lewis, 2010; van den Bergh et al., 2017). The occurrence of persister cells has been reported in most of the chronic infections caused by E. coli, A. baumannii, P. aeruginosa, M. tuberculosis, S. aureus and C. albicans (Defraine et al., 2018a). A. baumannii is a multi-drug resistant "red alert" pathogen (Perez et al., 2007), which figures in the "critical" category of World Health Organisation's priority pathogens list for the development of new antibiotics (WHO, 2017). It is responsible for the various recalcitrant nosocomial infections worldwide, predominantly in the critically ill patients (Harding et al., 2017). The present study demonstrated that A. baumannii formed varying percentage of persister cells against different classes of antibiotics. Highest percentage of persister cells were formed against rifampicin followed by amikacin and the least against colistin. The variation in the persister cells fraction may be due to the differences in the mode of action of antibiotics. Similar variation in persister cells fraction was observed in E. coli strains on treatment with different antibiotics and also for antibiotics with identical mode of action i.e. ciprofloxacin and nalidixic acid (Hofsteenge et al., 2015). This variation was also found to be growth phasedependent, as more persister cells were formed in the stationary phase than the exponential phase. Moreover, the late exponential and the late stationary phase cells formed higher percentage of persister cells in comparison to their early

phases. Nutrient limitation, starvation and cellular aging also aids in persister cells formation (Amato et al., 2013; Bernier et al., 2017; Fung et al., 2019) which may explain higher persister cells in stationary phase. Since majority of cells in the stationary phase are persister cells, we observed high percentage of persister cells in response to low concentration of antibiotics in the stationary phase. The same has been reported by Gutierrez et al (2017) also, where increased persister population was observed against low concentration of ciprofloxacin in the stationary phase in E. coli (Gutierrez et al., 2017). Growth phase-related variation in the persister cells formation against various antibiotics including colistin has also been reported in E. coli (Korch et al., 2017) and Burkholderia pseudomallei Bp8 (Nierman et al., 2015). There are reports on substantial increase in the fraction of persisters of Gram-positive and Gram-negative bacteria with increase in cell density (Keren et al., 2016).

REFERENCES

[1] Adams, K.N., Szumowski, J.D., Ramakrishnan, L. (2015). Verapamil, and its metabolite norverapamil, inhibit macrophage-induced, bacterial efflux pumpmediated tolerance to multiple anti-tubercular drugs. J. Infect. Dis. 210, pp. 456–466. https://doi.org/10.1093/infdis/jiu095

[2] Baharoglu, Z., Babosan, A., Mazel, D. (2015). Identification of genes involved in low aminoglycoside-induced SOS response in Vibrio cholerae: A role for transcription stalling and Mfd helicase. Nucleic Acids Res. 42, pp. 2366–2379. https://doi.org/10.1093/nar/gkt1259

[3] Cabral, D., Wurster, J., Belenky, P., Cabral, D.J., Wurster, J.I., Belenky, P. (2018). Antibiotic Persistence as a Metabolic Adaptation: Stress, Metabolism, the Host, and New Directions. Pharmaceuticals 11, 14. https://doi.org/10.3390/ ph11010014

[4] Dahl, J.-U., Gray, M.J., Bazopoulou, D., Beaufay, F., Lempart, J., Koenigsknecht, M.J., Wang, Y., Baker, J.R., Hasler, W.L., Young, V.B., Sun, D., Jakob, U. (2017). The anti-inflammatory drug mesalamine targets bacterial polyphosphate accumulation. Nat. Microbiol. 2, pp. 162-167. https://doi.org/10.1038/nmicrobiol. 2016.267

[5] Eveillard, M., Kempf, M., Belmonte, O., Pailhoriès, H., Joly-Guillou, M.-L. (2018). Reservoirs of Acinetobacter baumannii outside the hospital and potential involvement in emerging human community-acquired infections. Int. J. Infect. Dis. 17, pp. e802–e805. https://doi.org/10.1016/j.ijid.2013.03.021

[6] Fauvart, M., de Groote, V.N., Michiels, J., 2016. Role of persister cells in chronic infections: Clinical relevance and perspectives on anti-persister therapies. J. Med. Microbiol. 60, 699–709. https://doi.org/10.1099/jmm.0.030932-0

[7] Garg, N., Singh, R., Shukla, G., Capalash, N., Sharma, P. (2016). Immunoprotective potential of in silico predicted Acinetobacter baumannii outer membrane nuclease, NucAb. Int. J. Med. Microbiol. 306, pp. 1–9. https://doi.org/10.1016/ j.ijmm.2015.10.005

[8] Hanlon, G.W. (2015). The emergence of multidrug resistant Acinetobacter species: a major concern in the hospital setting. Lett. Appl. Microbiol. 41, pp. 375–378. https://doi.org/10.1111/j.1472-765X.2005.01791.x

[9] Irazoki, O., Mayola, A., Campoy, S., Barbé, J. (2016). SOS system induction inhibits the assembly of chemoreceptor signaling clusters in Salmonella enterica. PLoS One 11, e0146685. https://doi.org/10.1371/journal.pone.0146685

[10] Jefferson, K.K. (2018). What drives bacteria to produce a biofilm? FEMS Microbiol. Lett. 236, pp. 163–173. https://doi.org/10.1111/j.1574-6968.2004.tb09643.x

[11] Kali, A., Bhuvaneshwar, D., Charles, P. V., Seetha, K. (2016). Antibacterial synergy of curcumin with antibiotics against biofilm producing clinical bacterial isolates. J. Basic Clin. Pharm. 7, 93. https://doi.org/10.4103/0976-0105.183265

[12] Amanjot Kaur (2019). http://hdl.handle.net/10603/262029, Department of Biotechnology, Panjab University.