Effects of sub inhibitory levels of antibiotics biology essay

Science, Biology

Deenu, S. Romey, A. M. Jennifer, R. J, Pragasam, V. Renal Research Lab, School of Bio Sciences and Technology, VIT University, Vellore - 632014, TamilNadu, India, pragasam. v@vit. ac. in

Abstract

Regulation of gene expression based on the population density (Quorum Sensing, QS) plays a pivotal role in virulence of many opportunist pathogens like Pseudomonas aeruginosa. These microbial communications (QS) in a way co-ordinate the mechanisms that help a microbe to dominate in stress conditions, offered by the host body. Animal models that have been infected with QS deficient mutants fail to defend host immune system and cause less severe infections, compared to microbes with intact QS systems. The other aspect of the above said idea is that at sub lethal concentrations (SIC) the same antibiotics (natural as well as the synthetic) compounds can influence the expression of various genes codes for characters like virulence, pathogenicity, biofilm formation capacity, cyto-toxicity, motility and etc. It's been identified that the target genes as well as the mode of action (MOA) vary considerably at SIC unlikely to that in MIC. The variation in the rate of specific gene expression is mainly due to the alterations in transcription as well as in translation levels, whose analysis need complex micro-array experiments. When the SIC of an antibiotic triggers the expression of some genes, another SIC of the same antibiotic found to repress some other genes. Through our study we are investigating the effect of SIC levels of antibiotics on QS regulated behaviours of P. aeruginosa. Antibiotic treated and untreated P. aeruginosa PAO1 were analyzed for the expression of specific traits, which are under QS switch. Among the selected antibiotics 5

antibiotics at various SIC levels showed both repression and expression in QS regulated behaviours. Among the antibiotics, Cefuroxime (CXM) showed notable results, which is in par with the results obtained with other cepahalosporin antibiotics, detailed in other literatures. Apart from CXM other antibiotics which showed commendable results include; Nalidixic acid (NA), Amoxicillin (AM), Amplicillin (AMP) and Co-trimoxazole (COT). This study support the aspect of complex change in MOA of antibiotics at SIC. The mechanism of action for this observation need to be clarified with further studies,

INTRODUCTION

Low molecular weight molecules produced by the microbes are present in all environments and have wide spectrum of function. Only a small portion of these molecules are known for their therapeutic uses, as antibiotics. But it is been argued in detail that, their utility at present, might not be the exact function of these molecules. Since 1950s, detailed studies been done on the pharmacology, dosage, side effects and so on associated with the antibiotics. Development of new techniques such as gene expression profiling studies and proteome analysis etc try to correlate the mode of action of varying concentrations of antibiotics at whole cell responses or the cellular functions of the target cells. Based on these results it’s been confirmed that various antibiotics affects expression patterns of diverse genes, which are not included in the target function. So besides anti microbial functions, it is equally plausible that antibiotics have functional interactions with microbial cellular processes. Unlike, lethal concentrations of antibiotics to control

microbes, sub lethal concentrations also have relevance in environmental and clinical aspects. Evidence suggests that antibiotic induced reduction of bacterial fitness is not always the result upon antibiotic exposure. Detailed study upon this area revealed that many small molecule inhibitors shown to exhibit contrasting properties when tested at low concentrations, compared to higher concentrations; phenomenon of ' hormesis'. Hormesis was initially used to describe the effects of low levels of radiation. However, the term is used to describe biological responses to environmental signals or stresses which are characterized by biphasic dose dependant responses, exhibiting low-dose stimulation and high-dose inhibition or vice versa. This indirectly explains the unusual effect of sub inhibitory concentration of antibiotics upon metabolic and cellular responses. Through microarray technology, functional groups of genes which are influenced by the SICs of antibiotics have studied and documented. Interestingly same antibiotic at different SICs induced and reduced the expression of similar and/or different genes. The various phenotypes which are influenced by the SICs of antibiotics include biofilm formation, gene transfer (conjugation), bacterial adhesion, protein secretion, quorum sensing (virulence suppression), flagellin expression, toxin production etc. However, a set of signature genes which are universally affected by specific antibiotic has not been identified yet. Pseudomonas aeruginosa is an opportunistic pathogen which causes severe infections, particularly in immuno compromised patients. This bacterium is responsible for majority of the life threatening and nosocomial infections, hence high morbidity and mortality rates. P. aeruginosa forms biofilms during the infection process, which adds to the difficulties of eradicating infections by

antibiotic intervention, since bacterial cells living as biofilms are tolerant to antibiotics. Macrolides such as erythromycin, clarithromycin, and the erythromycin derivative azithromycin (AZM) have been shown to inhibit the enzymatic activity of guanosine diphosphomannose dehydrogenate in the alginate biosynthetic pathway of mucoid P. aeruginosa strains at SIC levels. It has been suggested that AZM treatment inhibits neutrophil recruitment to the lung by reducing the levels of expression of proinflammatory cytokines and inhibition of neutrophil migration, resulting in a significant reduction in airway specific inflammation. It's been suggested that inhibition of cell-cell communication is the mode of action by which AZM exerts its activity in P. aeruginosa and reduces the lung infections. In gram-negative bacteria, cell-cell communication, also known as quorum sensing (QS), relies upon small diffusible signal molecules (N-acyl L-homoserine lactones [AHLs]), which interact with transcriptional activators to couple gene expression with cell population density. In gram-negative bacteria, cell-cell communication, also known as quorum sensing (QS), relies upon small diffusible signal molecules (N-acyl L-homoserine lactones [AHLs]), which interact with transcriptional activators to couple gene expression with cell population density. The QS system of P. aeruginosa is organized hierarchically, with the RhlI-RhlR components being subordinate to the LasI-LasR components. LasI directs the synthesis of N-3-oxo-dodecanoyl-L-homoserine lactone (OdDHL), whereas RhlI synthesizes N-butanoyl-L-homoserine lactone (BHL). In addition to the AHL signal molecules BHL and OdDHL, a third intercellular signal, 2-heptyl-hydroxy-4-quinolone (designated the Pseudomonas quinolone signal [PQS]), has been found to be part of the QS regulon in P. aeruginosa. Combining the

above said aspects, the aim of this study is to analyze the influence of sub lethal concentrations of conventional antibiotics, used for urinary tract infection (UTI), upon QS regulated gene expression patterns of Pseudomonas aeruginosa PAO1. To attain our aim, we selected 11 antibiotics, used against UTIs and their sub lethal concentrations were identified using broth Microdilution method, as described by CLSI standards. By analyzing the SIC levels of the selected antibiotics, 5 of the antibiotics tested showed anti quorum sensing activity and used for further study. The antibiotics were dissolved and diluted using appropriate solvents and diluents, described by CLSI standards. Through literature survey we selected various behaviours in P. aeruginosa, which are QS regulated (Pyocyanin, Pyoverdin, EPS, Biofilm, motility, Protease, Rhamnolipid production and etc) and tested at SIC levels of antibiotics. The growth control served as positive control (100% expression) and the increase/decrease in the expression was represented in percentage of expression, compared with the growth control.

MATERIALS AND METHODS

Compound preparation: The antibiotics were purchased from Hi Media Laboratories, Mumbai, India. The stock solutions of antibiotics were prepared and diluted with the help of appropriate solvents and diluents as directed by Clinical Laboratory Standard Institute (CLSI) guidelines. The prepared stock solutions were stored at 40C. Further from the stock solution, working solution wa prepared in appropriate diluents. Bacterial strains and growth conditions: Pseudomonas aeruginosa PAO1was used as target pathogen and cultured aerobically in Leuria Bertani (LB) broth (pH-7. 2)

under 150 rev min-1 agitation in a rotary shaker at 370C for overnight.. For experimental analysis PAO1 was subcultured in the same medium to reach a final OD of 0. 4 at 600nm. Determination of sub inhibitory concentration (SIC) of antibioticsThe SIC of antibiotics against PAO1 was determined by micro dilution method as per clinical and laboratory standards institute (CLSI) guidelines. Briefly, 1% of the test organism (0. 4 OD at 600nm) added to the appropriate growth medium supplemented with twofold serial dilutions of antibiotics to attain final concentrations ranging from 1to 120µg/mL in a microtitre plate (MTP). the SIC was recorded as the concentrations that showed visible cell growth, comparable with the growth control (without antibiotic treatment). All the assays in the present study was performed using these SIC concentrations.. Pyocyanin quantification assay: Working solutions of antibiotics at sub inhibitory concentration was added in 5 ml of LB broth containing 1 % (50 µl) of PAO1 culture and incubated at 370C for a minimum of 18 h. After incubation, the cell-free supernatants of PAO1 cultivated in the presence and the absence of PAA were extracted with 3 ml of chloroform and then re-extracted into 1 ml of 0. 2 N HCl to get a pink to deep red solution. The absorbance of the solution was measured spectrophotometrically at OD 520. Pyoverdin quantification assay: The pyoverdin assay was adapted from the methods of Cox and Adams. The LB medium culture supernatant was diluted ten-fold in tris-HCL Buffer (pH-7. 4), and 3mL aliquots were added to a four sided quartz cuvette. The relative concentration of pyoverdin was based on the fluorescence of the supernatant at an excitation wavelength of 405 nm and emission wavelength of 465 nm with the help of a spectroflourimeter. Quantification of EPS: PAO1

cells were allowed to form biofilm in glass slides (1×1 cm) in the presence and absence of antibiotics at SIC levels in petridish, incubated at 370C. EPS quantification was carried out by total carbohydrate assay (Phenol-Sulphuric acid method). In brief, glass slides, after incubation, were washed in 0. 9% NaCl (0. 5ml) and incubated in an equal volume of 0. 5ml of 5% phenol and five volumes of concentrated H2SO4 and the absorbance was measured at 490nm. Effect of antibiotics upon biofilm formationThe effect of SIC levels of antibiotics upon bacterial biofilm development was determined by quantifying the biofilm biomass through MTP assay. Briefly, 1% of target bacterial cultures (OD adjusted to 0. 4 at 600 nm) were added into 1 ml of respective growth medium and cultivated in the presence and absence of varying concentrations of antibiotics (1-120µg/ml) without agitation for 16 h. After incubation, the planktonic cells in the wells of MTPs were removed and the wells rinsed twice with sterile water. The surface-adhered cells in the MTP wells were stained with 200µl of 0. 4% CV solution. After 2 min, excess solution was removed and CV in the stained cells was solubilised with 200µl of 95% ethanol. The biofilm biomass was quantised by measuring the intensity of CV in ethanol at OD650 using a UV–visible spectrophotometer. Swimming and swarming assays: In swimming assay 3 microliter overnight cultures of the PAO1 (0. 4 OD at 600nm) were point inoculated at the centre of the swimming agar medium consisting of 1% tryptone, 0. 5% NaCl and 0. 3% agar with different concentrations of antibiotics. For swarming assays, 5microlitre (0. 4 OD at 600nm) overnight culture of PAO1 were inoculated at centre of the swarming agar medium consisting of 1% peptone, 0. 5% NaCl, 0. 5% agar and 0. 5% of filter-sterilized D-glucose with different

concentrations of antibiotics . The plates were then incubated at 300C in upright position for 16 hours. The reduction in swimming and swarming migration was recorded by measuring swim and swarm zones of the bacterial cells after 16 hours. Growth curve studiesOvernight cultures from test pathogen (1%; 0. 4 OD at 600 nm) were inoculated in a 250-ml Erlenmeyer flask containing 50 ml of LB broth supplemented with various sub inhibitory concentrations of antibiotics. The flasks were incubated at the optimum temperature of respective pathogens under 180 rpm in a rotator shaker. The cell density was measured by UV–visible spectrophotometry at 24 h. Data analysisAll experiments were performed in triplicates and the obtained values were represented as mean values. The cell density was compared with the growth control in all the assays. Data is represented as percentage reduction or increase in expression calculated by the following equation. Percentage inhibition = 100-(OD of test*100)/OD of growth controlRESULTS: Effect on pyocyanin productionPyocyanin is one of the many toxins produced and secreted by Pseudomonas aeruginosa. Pyocyanin is a blue, secondary metabolite with the ability to oxidise and reduce other molecules and therefore can cause damage to mammalian cells during infection. Pyocyanin is a zwitterion at blood pH, it is easily able to cross the cell membrane. Due to its redox-active properties, pyocyanin generates reactive oxygen species. Table-1 represents the effect of antibiotics on characters which are under QS switch.

Table-1

Antibiotic (mcg)

OD at 520nm

% Reduction

Growth Ctrl0. 121 ± 0. 0030. 0CXM (20)0. 025 ± 0. 00179. 09AM (5)0. 081 ± 0. 00133. 07COT (3)0. 057 ± 0. 00452. 47NA (1)0. 031 ± 0. 00374. 88AMP(4)0. 087 ± 0. 00228. 33Effect on pyoverdin productionPyoverdin is a fluorescent siderophore produced by Pseudomonas aeruginosa. The effect of SIC levels of various antibiotics upon QS regulated behaviours are represented in Table-2.

Table-2

Antibiotic (mcg)

Emission at 465nm

% Reduction

Growth Ctrl304. 47 ± 0. 020. 0CXM (20)19. 18 ± . 0193. 7AM (5)193. 03 ± 0. 0235. 7COT (3)122. 4 ± 0. 0259. 8NA (1)77. 33 ± 0. 0174. 6AMP(4)205. 91 ± 0. 00232. 37Effect on biofilm productionBiofilm mode of growth is one of the most effective and active strategy which help microbes like P. aeruginosa to become metabolically active to thrive and proliferating in stress conditions which are prevailing in environments like lungs, urinary tract etc. The effect on biofilm formation by various SIC levels of antibiotics are represented in Table-3.

Table-3

Antibiotic (mcg)

OD at 490nm

% Reduction

Growth Ctrl1. 172 ± 0. 010. 0CXM (20)0. 718 ± 0. 0338. 75AM (5)0. 372 ± 0. 0668. 3COT (3)0. 701 ± 0. 0140. 2NA (1)0. 71 ± 0. 0239. 45AMP(4)0. 905 ± 0. 01722. 8Effect on EPS productionExtracellular polymeric substances, also known as exo-polysaccharide, or EPS, are high-molecular weight compounds secreted by P. aeruginosa into their environment. EPS establish the functional and structural integrity of biofilms, and are considered the fundamental component that determines the physiochemical properties of a biofilm. These compounds are important in biofilm formation and cells attachment to surfaces and constitute 50% to 90% of a biofilms' total organic matter. Table-4 represents the effects of SIC levels of antibiotics upon EPS production

Table-4

Antibiotic (mcg)

OD at 600nm

% Reduction

Growth Ctrl0. 6 ± 0. 0250. 0CXM (20)0. 17 ± 0. 01371. 83AM (5)0. 478 ± 0. 01520. 32COT (3)0. 294 ± 0. 0150. 92NA (1)0. 44 ± 0. 0326. 97AMP(4)0. 43 ± 0. 0128. 74