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Effects of Oxygen on In-vitro Biofilm Formation and Antimicrobial Resistance of Pseudomonas aeruginosae

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ABSTRACT

Background: Pseudomonas aeruginosa is an opportunistic human pathogen. This organism is thought to persist by forming biofilm within human infections. Bacteria growing in biofilm exhibits increased resistance to antimicrobial agents. In this study, we examined the effect of oxygen on the development of biofilm by P. aeruginosa and on the level of its resistance to the antibiotics. ***Methods:*** The P. aeruginosa control strain group and 45 clinical isolates were cultured and antibiotic susceptibility testing was performed by disk diffusion agar testing. Biofilm formations were examined by glass tube assay and were incubated at 37°C under normoxia (21% oxygen) and hypoxia (1% oxygen). ***Results:*** We observed that hypoxia affects the biofilm formation under hypoxic conditions as compared to normoxia. Among the antibiotics tested, penicillin, cephalosporin and carbapnem, all demonstrated decreased susceptibility values under hypoxia compared to normoxia. ***Conclusion:*** We established that hypoxia induced biofilm formation; this suggests that decreased oxygen may be a critical factor in the bacterial virulence. Moreover, we confirmed a strong positive correlation between hypoxia and antimicrobial resistance of P. aeruginosa.

Introduction

Pseudomonas aeruginosa (P. aeruginosa) is a motile, aerobic, Gram-negative bacterium. This bacterium is having minimal nutritional requirements and hence surviving under diverse environmental conditions.[1] P. aeruginosa is as an opportunistic human pathogen, a common cause of nosocomial infections and is responsible for infections in immunocompromised individuals, burn wounds, septicemia and cystic fibrosis[1,2]. Infections caused by this organism are likely due to a suite of well-regulated virulence factors and defense mechanisms such as multidrug resistance pumps (4) and biofilm formation.[2,3] This bacterium also is commonly isolated from infections thought to involve biofilm formation, including those associated with burn wounds, keratitis, urinary catheters, otitis media, and pneumonia in patients with cystic fibrosis.

Beta-Lactams (as, penicillins, cephalosporins, carbapenems and monobactams), aminoglycosids, and quinolons are commonly used in the treatment of P. aeruginosa infections. Resistance to these agents is in increasing and today, in many hospitals, multidrug-resistant P. aeruginosa pose the greatest therapeutic challenge. The increase in the incidence of multidrug resistance of P. aeruginosa has been attributed to a combination of factors. This increasing problem requires a novel comprehensive strategy that contains compliance with infection control principles, rational use of current antibiotics, and the development of new active agents.[4, 5]

The classical meaning of a biofilm is microbial growth on any layer, this mode distinction with the planktonic form, which is characterized by bacterial replication when not attached to surfaces. This organism is thought to persist by forming biofilms within the hypoxic mucus of the lung and skin injury infections. Bacteria growing in biofilms exhibit increased resistance to antimicrobials and the host immune response. Once established, biofilm welling bacteria are almost impossible to eradicate with current therapies.[3] Hypoxia and inflammation are coincidental events in human chronic infectious diseases due to P. aeruginosa.[6] Moreover, P. aeruginosa is capable of luxuriant growth under both aerobic and anaerobic conditions.[7]

The aim of study was to determine effect of hypoxia on In vitro antibiotic resistance and biofilm formation of P. aeruginosae.

Materials & Methods

Bacterial strains and antimicrobial susceptibility testing: The P. aeruginosa were identified by oxidase test, pigments production, fruity odor, biochemical tests and culture on selective media. The P. aeruginosa control strain (ATCC 27853) and clinical strains were cultured in Mueller-Hinton broth (MHB) (Merck, German). Antibiotic susceptibility testing was performed by disk diffusion agar assay. Antibiotics tested included: ceftazidim, cefepime, ticarcillin-clavulanic acid, amikacin, imipnem, ciprofloxacin, and

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tetracycline (Mast, UK). Bacteria were incubated at 37°C for antimicrobial resistance under normoxia (21% oxygen) and hypoxia (1% oxygen).

Biofilm assays: Biofilm formation was examined in a glass tube, as described previously.[8,9] Briefly, overnight P. aeruginosa cultures were diluted 1: 100 into Trypticase soy broth (Merck, German) with 1% glucose. Aliquots of each culture dilution were dispensed into three glass tubes (300 ml) and incubated (at 37 °C, 24 h). After incubation, tubes were decanted and washed with phosphate buffer saline (pH 7.3) and dried. Excess broth was removed, and biofilm were washed and stained with 1% (w/v) crystal violet for 15 min at room temperature. Excess stain was washed with deionized water, and tubes were dried in inverted position. Bacteria also were incubated at 37°C for biofilm formation under normoxia (21% oxygen) or hypoxia (1% oxygen) in a chamber.

Statistical analysis: The SPSS software ver,13 was used and the significance of the results was determined using the q^2 and, a P-value of less than 0.05 was considered significant.

Results

From 2010 to 2011, 45 P. aeruginosa isolates (non-duplicate) from different clinical specimens were recovered from 50 patients at Hospitals of Tabriz University of Medical Sciences in Azerbaijan, Iran. In this study, 79% clinical P. aeruginosa strains had produced biofilm.

Antimicrobial resistance of P. aeruginosa isolates to different antibiotics as determined by disk-diffusion agar is presented in Table 1. Thirty of 45(66.6) and 25 of 45 (55.5%) showed triple (resistant to 3 drugs) and quadruple (resistant to 4 drugs) resistance, respectively. As it is evident, P. aeruginosa showed highest resistance against tetracycline, ceftazidim, and amikacin, respectively.

Also, P. aeruginosa had moderate drug resistance to ciprofloxacin, ticarcilin-clavlunic acid, cefipim and imipenem, respectively.

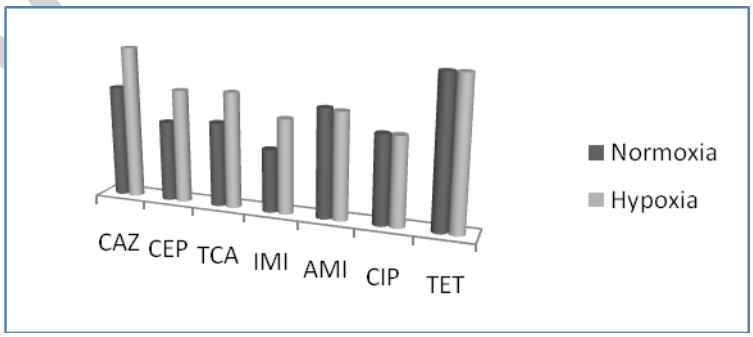
We have observed that hypoxia affects biofilm formation. We also investigated the susceptibility of P. aeruginosa to antibiotics under hypoxia compared to normal oxygen tension (Fig 1). In this study, diameters of inhibition zones for 7 antibiotics following exposure to either normoxia or hypoxia were determined. There was statistically significant difference between antibiotic resistance and oxygen pressure (Pv less than 0.05). Among antibiotics tested, penicillin, cephalosporin and carbapnem antibiotics demonstrated decreased inhibition zone values under hypoxia compared to normoxia. Other antibiotics tested showed similar inhibition zone values in normoxic and hypoxic cultures. Therefore, we proved whether hypoxia-induced biofilm production may contribute to changes in antibiotic susceptibility.

Table 1. Resistance pattern of P. aeruginosa isolates to different antibiotics by disk-diffusion agar method

	CAZ† (%)	CEP‡ (%)	TCA§ (%)	AMI¶ (%)	IMI†† (%)	CIPœ (%)	TET# (%)
Susceptible	19(42.2)	26 (57.8)	25(55)	20(45)	30(66.7)	25(55)	11(24.5)
Non-susceptible	26(57.8)	19(42.2)	20(45)	25(55)	15(33.3)	20(45)	34(75.5)

† Ceftazidim, ‡ Cefipim, §Ticarcilin-clavlunic acid, ¶amikacin,Imipenem††, Ciprofloxacin œ, ¶¶ Ceftazidim, #Tetracycline

Figure 1. Antibiotic resistance in P. aeruginosa under hypoxic and normoxic conditions. AMI, amikacin; TAZ, ceftazidime; CEP, cefepime; TCA, ticarcillin-clavulanic acid; IMI, imipenem; CIP, ciprofloxacin; TET and tetracyclin.



Discussion

Failure of therapy for P. aeruginosa infection often has been related to poor compliance with the treatment regimen and development of antibiotic resistance. The accelerating increase of bacterial resistance and resultant problematic treatment are directly responsible for the existing increase in morbidity and mortality associated with P. aeruginosa infections.[5]

Several studies have explained the useful role of oxygen in the treatment of various human diseases either alone or in combination with other therapies.[10]Although the impact of decreased oxygen tension on bacterial virulence has been studied for some pathogens, little is known about its effects on drug resistance in P. aeruginosa. Several studies have demonstrated that microaerobic to anaerobic conditions dominate in P. aeruginosa biofilms.[11,13] In this study, P. aeruginosa had visible monolayers biofilm on plates during overnight incubation under low oxygen tension.

Sabra reported that P. aeruginosa prefers microaerobic

conditions for growth and can create these environmental conditions itself through at least two mechanisms: blockage of oxygen transfer and formation of a polysaccharide layer.[11] However, hypoxia plays an important role in influencing gene expression in host cells and invading pathogens alike and can have a significant impact on the development of both infection and inflammation.[6,7] Growth in anaerobic or hypoxic area is possible using an inorganic terminal electron receptor or very slow growth by substrate level phosphorylation using arginine. Anaerobic respiration requires the presence of nitrous oxide (NO) as alternative terminal electron.[7] In other ways; P. aeruginosa is capable of a cell-density dependent form of cell-to-cell communication known as quorum sensing. Quorum sensing is an important component of virulence factor of this bacterium, also critical for biofilm formation and is essential for anaerobic growth.[7]

In chronic infections, P. aeruginosa produces alginate and grows in dense bacterial populations, forming mucoid biofilms.[6] Although decreased oxygen tension have been demonstrated in mucous layers in the lung, the effects of hypoxia on antibiotic resistance have not been investigated independently of biofilm formation. The antibiotic resistance of P. aeruginosa in response to hypoxia may be important for the treatment of infections.

Examination of biofilms has revealed that the oxygen concentration may be high at the surface but low in the centre of the biofilm where anaerobic conditions may be present. Similarly, growth and metabolic activity is stratified in biofilms, i.e. a high level of activity at the surface and a low level and slow or no growth in the centre and this is one of the reasons for the reduced susceptibility of biofilms to antibiotics.[14] As discussed previously, oxygen limitation and the metabolic rate are probably more important factors contributing to the tolerance of biofilms to aminoglycosides and ciprofloxacin.[15] Recently, it has been demonstrated that administration of DNase and alginate lyase enhanced the activity of tobramycin in biofilms by dissolving the biofilm matrix.[16]

To investigate the existence of a link between antibiotic resistance and biofilm activity in hypoxia, we used antimicrobial susceptibility test in different oxygen tension. This led us to hypothesize that change in oxygen pressure contributed to increased antibiotic resistance in hypoxia with betalactam drugs. The zone inhibition diameter values in normoxia remained unchanged. For the treating, many antibiotics are unable to eradicate biofilms, and therefore much work is required to devise ways to stop them from the host. Previously was employed a systems biology approach to identify candidate drug targets for biofilm-associated bacteria by imitating specific microenvironments found in microbial communities associated with biofilm formation. Taken together, this study demonstrates that metabolic modeling of human pathogens can be used to identify oxygen-sensitive drug targets and thus, that this systems biology approach represents a powerful tool to identify novel candidate antibiotic targets.[17] These In vitro observations support the hypothesis that oxygen therapy may be a new strategy that could be used against P. aeruginosa infections. Considering the increasing resistance, oxygen therapy appears to be useful in order to eradicate P. aeruginosa infectious, biofilm formation and to optimized the regime in case of treatment failure.

Conclusion

Biofilm formation has been shown to be an important virulence factor of the P. aeruginosa. Our data show that exposure to hypoxia induces selective antibiotic resistance in P. aeruginosa. Taken overall, the results show that oxygen therapy may be a particularly effective anti-P. aeruginosa strategy.

Acknowledgments

We have no conflicts of interest to declare.

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