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The efficacy of anti-virulence strategies in inhibiting quorum sensing system in *P. aeruginosa*

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Abstract

with the significant increase of bacterial resistance, many studies have been done to improve strategies to overcome this resistance, many strategies such as down regulating the virulence factors and controlling the behavior of the bacteria. the virulence of *P. aeruginosa* relies on a density-dependent mechanism mediated by diffusible molecules called auto-inducers this system known as quorum sensing system. therefore This system constitutes an important target for anti-virulence therapies, during the colonization *P. aeruginosa* mutants (less virulence type) exploits the public goods from wild type *P. aeruginosa* (more virulence type), what was done in this study is that the QS system inhibited in the wild type *P. aeruginosa* this inhibition decreases the selective advantages for *P. aeruginosa* mutants. this trail was done in randomized, placebo-controlled, double blind study, in which the anti-virulent drug was used is *azithromycin* macrolide antibiotic that has no bactericidal effect on *P. aeruginosa*, this drug used with intubated patients that infected with *P. aeruginosa.* With patients that did not receive *azithromycin* the levels of the less virulent type (lasR QS-mutants) have raised with time, but in presence of azithromycin the advantages of the less virulent type have decreased by inhibiting the growth of wild-type isolates.

inroduction

Pseudomonas aeruginosa is gram negative rod shaped bacteria, and a drug resistant bacterium, gives blue green pigment and fruity smell[(1)], also associated with numbers of hospital-acquired pneumonia infections, and accounts for some nosocomial urinary tract infections, and surgical wound infection cases, also of the infections in th e bloodstream, and is especially concerned with people with immunodeficiency where pneumonia and sever systemic infections can be caused.[(2)] the virulence factors of *p.aerugenosa* acting by density depending mechanism known as quorum sensing system. [(3)]

Quorum sensing system is the mechanism by which the bacteria communicate depending on their densities, this mechanism regulates bacterial communities to work as one unite , these communities communicate together by releasing molecules known as auto-inducers, these molecules control different functions in the bacteria such as biofilm formation, sporulation, symbiosis, and others.[3]

The system works by three mechanisms firstly the action of auto-inducers depends on bacterial concentrations, secondly bacteria has cytoplasmic and membranous receptors that responsible for sensing the auto-inducers and lastly identifying auto-inducers be the expressed receptors, thereby detecting the virulence of the bacteria.[3]

QS system was first discovered in a marine gram negative bacteria *vibrio fischeri,* Since studies have shown that this system controls phenotype bioluminescence.[3]

Lately several researches have been working on how to down regulate this system in bacteria, lately anti-virulence therapies such as *azythromycin* are the proposed solution in a world full on anti-biotic resistance growing issues, *Azithromycin* is a commonly used antibiotic belongs to macrolide family, it is used for *P. aeruginosa* the drug has no major bactericidal effect on *P. aeruginosa*. [4]

Lately many studies have been done to detect that the drug interacts with QS system of this pathogen and down regulates the production of it is virulence factors, in vivo and in vitro trials suggest that mutants (QS-cheats) especially the once that have mutation on the QS receptor lasR might have a selective advantage when QS-wild types are present by benefiting from the public goods of the wild type bacteria, when QS is blocked in wild bacteria this advantage is blocked too, So in presence of *azithromycin* the selection of less virulent QS-mutants is decreased, and the dominance of the more virulent QS-wild type bacteria is maintained.[4]

Aim of study

This study was done to detect the efficacy of anti-virulence strategies on *p. aeruginosa* in intubated patients.

Materials and Methods

Patients and clinical collection

This randomized, placebo-controlled, double-blind trial, was designed to test the effectiveness of *azithromycin* as a quorum-sensing agent in preventing *P. aeruginosa* pneumonia from occurring in ventilated patients with reported colonization. The trial was attended by twenty-one European centers, eight in France, four in Spain, three in Belgium, three in Poland, two in Serbia and one in Switzerland .the mechanically-ventilated patients had been screened every 48 hours for *P. aeruginosa* pneumonia colonization in the respiratory tract, some patients were not eligible in this study like patients who are receiving immunosuppressive drugs and patients who are having neutropenia, patients who are infected with *P. aeruginosa* and under antibiotic or macrolides treatment for the last 14 days of the experiment have been excluded too.(4)

patients with *P. aeruginosa* infection were chosen randomly and given either placebo or (300mg) iv of azithromycin daily for up to 20 days Only antibiotics that are inactive to *P. aeruginosa* were permitted during the trial. Tracheal aspirates were collected (0.3 to 5 ml) and one isolate of *P. aeruginoa*, (Period of collections was 3 to 20 days) 24 hours a day, The samples were frozen on-site at −80 ° C within 15 minutes and sent to the University Hospital of Geneva reference research laboratory on dry ice. The ratio of patients whose LasR mutant was isolated was logistically analyzed with duration (covariable) time, medication (placebo or *azithromycin*) and interaction in Genstat v10.(4)

In patient gene expression analysis

Total genomic DNA and total RNA had been extracted from prospectively collected tracheal aspirates. In 98 percent of aspirates, *P. Aeruginosa* DNA was detected (> 104 genomic copies / g aspirates), Confirming this organism is colonization. With the RNA extraction, the expression of the rpsL housekeeping gene was detected (> 5 per 104 copies / g aspirate) in 80% of the aspirates, This indicates that the quality of sample handling and the extractions of RNA is adequate to detect the expression of the bacterial genes in most tracheal aspirates. As a second quality control of RNA extracts from clinical samples, amounts of bacterium P. aeruginosa were plotted as

described by the qRT-PCR (quantitative revers transcription polymerase chain reaction) of genomic extractions of DNA against rpsL housekeeping expression.[4]

Determination of bacterial loads

qRT-PCR genomical DNA preparations determined the amount of *P. aeruginosa* in aspirates, Aliquots of genomic DNA preparations were diluted 10 times in H2O and 3 µl of this dilution was added to the PCR (polymerase chain reaction) mix containing 1×Quantitect Sybr Green Master Mix and 600 nM primers in a total volume of 15 µl. Atypical curve was obtained by applying 10-fold dilutions of a culture of *P. aeruginosa* to an aspirate collected from a non-colonized patient. As described above, DNA was then isolated and quantified by qRT-PCR 104 CFU (colony-forming unit) / g aspiration was observed under these conditions. During the 3-months analysis standard curves provided reproducible values. In aspirates, *P. aeruginosa* was present at amounts ranging from 4104–1.8108 CFU / g.[4]

In vitro experiments

In a 200µl minimal salt medium, *P. aeruginosa* strain PAO1 competed against a rare invading isogenic lasR knockout mutant, supplemented with 1% BSA in 96-well plates, shaken at 200 rpm at 37°C with or without *azithromycin* (5 and 10 mg / l) for 72 hours, Six wells had been inoculated with 107 overnight culture cells grown at 37 ° C in the LB (Lysogeny broth) medium. The selection coefficient of zero indicates that the strains are of equal fitness. The selection coefficients were decreased against the concentration of *azithromycin*.[4]

Results

In vitro

in a total lack of *erythromycin*, the growth ratio of the lasR mutant in monoculture was decreased in about 50 compared to wildtype, by adding *azithromycin* both phenotype densities decreased, the decrease for the wild type is much higher than the lasR mutant, which means that *azithromycin* inhibits the growth of wild type phenotype more than that of the lasR mutant.[4]

QS-inhibition in patient

The tracheal aspirates were followed to record the inhibition of QS by *azithromycin*, QS-circuit lasI and QS-target rhlA genes expression were reduced by *azithromycin*, however, QS-independent gene expression trpD. *Azithromycin*, of course, may have blocked the expression of some other genes that are not QS-related, Between those genes that their expression was strongly affected by *azithromycin* were QS-regulated genes, the measurement of the synthesis of elastase regulated by the lasR QS system was done to assess the development of *P. aeruginosa* QS in patients (from 650 isolates were collected).[(4)]

Elastase action differences associated with lasR mutations among independent wild type and mutant lasR alleles, lasR mutations among independent wild type and mutant lasR alleles associated with Elastase behavior differences LasR mutations thus lowered elastase activity, as well as some other lasR-regulated genes, thus in the 31 control group patients the ratio of lasR mutants markedly raised. In 30 patients who received *azithromycin*, the ratio of lasR mutants decreased slightly, whereas Placebo isolates showed declining in average elastase levels in vitro, and *azithromycin*-treated patients isolates showed an increase over time, as shown in figure.1. [(4)]

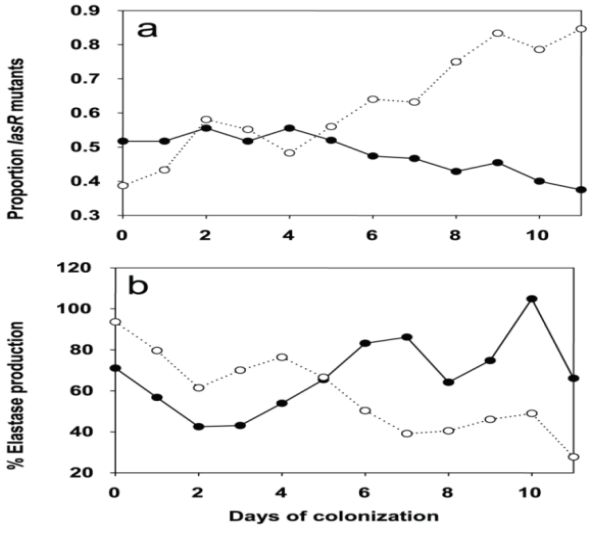

Figure.1. progrestion of lasR mutants the production of elastase in presence and absence of azithromycin. Solid lines show patients treated with azithromycin, and dotted lines show placebo group. [(4)]

the data that observed proved that the treatment with *azithromycin* blocked the activity of QS system in the wildtype *P. aeruginosa*, with placebo, bacterial densities were two times as high as with *azithromycin*, the decrease in bacterial densities by *azithromycin* indicates the decrease in bacterial growth ratio, also it could lower the selection of lasR mutants because if the public goods synthesis mediated by QS is also reduced, also the drug can block the growth of the wildtype bacteria less than the lasR mutants, in addition to that *azithromycin* affected the composition of the bacterial normal flora which may affect the pathogenicity of *P. aeruginosa*.[4]

Discussion

a multicenter placebo-controlled trial of 92 patients colonized by *p.aerginosa* (intubated patients) to detect the effect of anti-virulence strategy in preventing infections. today with the huge increase in bacterial resistant anti-virulence strategies are needed to avoid this resistance. *azithromycin* that is neither bactericidal nor bacteriostatic on *p.aerugenosa*, but this macrolide downregulates QS-system in *p.aerugenosa*.[5]

the initial study is started with 92 intubated patients that are colonized with *p.aerugenosa* (randomized patients), 45 of them received a placebo and 47 of them received azithromycin, some of the patients were excluded any patient had received antibiotics active against *p.aerugenosa* or if they had received macrolides. only 23 of the patients met the criteria for inpatient gene expression monitoring.[4]

in this study *Azithromycin* therapy has been shown to prevent lasR mutant selection (less virulent type), Therefore, the proportion of wild *P. aeruginosa* (more virulent type) in colonized patients increases, The main reason for this is that *azithromycin* blocks QS. Blocking QS prevents lasR mutants from manipulating the goods and decreases any possible QS-related costs, as the production of the extracellular product, in both vitro and in patient data of clinical trial indicate a key role of QS-dependent virulence in infection development and Support the consensus of animal model studies showing that the expression of QS and the production of public goods Associated to increased virulence, Consequently, *azithromycin* is likely to be of

therapeutic benefit to a patient being treated by inhibiting the QS-dependent virulence during treatment, Nevertheless, the patient is at risk of being colonized by extremely virulent bacteria with the potential for late-onset infections when treatment is stopped, in addition, the broad use of such ant-virulence treatments can also increase the prevalence of extremely virulent QS-wild isolates in the hospital, More widely, any intervention which reduces bacterial densities may also lead to a decreased selective advantage for less virulent mutants who do not produce public goods,The study shows both the short and long term outcomes of anti-virulence intervention as well as other treatments for pathogen virulence need to be considered carefully.(4)

another study shows that the natural cooperative selection is reduced toward QS-deficient with the treatment of azithromycin, which means that treated patients (with *azithromycin*) in this study are colonized with highly virulent bacteria (wild type *p. aeruginosa*), therefore if the prophylaxis was stopped this may raise the risk of infection. Anti-virulence therapies should be used with special care and caution.(5)

Conclusion

in this randomized, placebo-controlled, double-blind study it was observed that *azithromycin* reduces the selection of the QS system in type *p. aeruginosa* in patients that colonized by type *p. aeruginosa*. in this study QS system is inhibited in the wild type *P. aeruginosa* thereby the selective advantages for *P. aeruginosa* mutants are decreased .

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