

Co-infection with *Pseudomonas aeruginosa* impacts virulence of *Staphylococcus aureus* and intensifies the severity of infection

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Abstract: Multi-specie infections display diverse interactions among pathogens that influence the severity of disease. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the two most important opportunistic, nosocomial and drug-resistant pathogens. Poly-infections due to *S. aureus* and *P. aeruginosa* are more destructive and result in worse patient outcome than mono-infection. The two organisms are commonly isolated from cystic fibrosis respiratory cultures. Studies demonstrated that *S. aureus* pre-colonization among cystic fibrosis patients is a hazardous for beginning *P. aeruginosa* aviation route infection. This work meant to explore the impact of *P. aeruginosa* on the destructiveness of *S. aureus* and the level of disease's seriousness by utilizing *in-vitro* co-culture and host cell model. The outcomes showed that *P. aeruginosa* outcompetes and suppresses the growth of *S. aureus* when co-cultured. The host factors expression profile indicated elevated expression of TNF α , IL-6 and IL-12, recommending the unique mechanism of host cell healing inhibition by multispecies. Co-infection resulted in significant increase in IL-8 together with the 10-fold induction of iNOS expression when contrast with *S. aureus* mono-infection. This indicates that the presence of *P. aeruginosa* heads the infection towards more severity and complications and delays cell healing process.

Keywords: *P. aeruginosa*; *S. aureus*, co-infection, inter-specie interactions.

INTRODUCTION

Most of the infections occur due to multi-specie colonization by pathogens and within such multi-species infections, pathogens unveil distinctive communications that affect the severity of disease. In spite of the fact that this reality has been acknowledged since the time of Pasteur, vast majority of investigations have been centered around a mono-bacterial species grown in isolation (Pasteur and Joubert, 1877). A little is known about interspecies interactions in poly-microbial infections.

S. aureus and *P. aeruginosa* are the two most important opportunistic, nosocomial and antibiotic resistant pathogens. Research literature proposes that *S. aureus* and *P. aeruginosa* poly-infections are more destructive and/or result in worse patient outcomes than mono-infections (Pastar *et al.*, 2013; Rosenbluth *et al.*, 2004; Hendricks *et al.*, 2001). *P. aeruginosa* is a Gram-negative bacterium with high level of adaptability and the bacterium is unsusceptible to many antimicrobials (Pearson *et al.*, 2000). *P. aeruginosa* is an opportunistic bacterium that out-competes other species such as *S. aureus* in cystic fibrosis lung, becoming dominant and difficult to eradicate. *S. aureus* is one of the most important Gram positive opportunists, causes a range of infections from skin to food-borne illnesses and other frightful infections (Lowy, 1998). From 1995 to 2005, the infection prevalence of *S. aureus* in children with cystic fibrosis increased from 39.5% to 63% (Razvi *et al.*, 2009). *S.*

aureus infections are mostly acquired during childhood, initiate changes in the lungs of CF patients that allow *P. aeruginosa* to finally infect these patients which all add to a worse patient outcome (Sagel *et al.*, 2009; Hauser *et al.*, 2011; Cystic Fibrosis Foundation, 2014).

Negative relationship between *P. aeruginosa* and *S. aureus* during adolescent years and young adulthood have brought into light several studies with respect to inter-microbial interactions of these two life forms (Ruger *et al.*, 2014; Baldan *et al.*, 2014; Fugere *et al.*, 2014; Park *et al.*, 2012). These pathogens are ordinarily isolated from respiratory cultures of cystic fibrosis and it has been shown that risk factors for introductory *P. aeruginosa* respiratory infection in patients with cystic fibrosis include pre-colonization of *S. aureus* (Hoffman *et al.*, 2006; Maselli *et al.*, 2003).

Herein we concentrated on inter-specie interactions of two medically important human pathogenic bacteria by utilizing an *in-vitro* host cell model. Present study is aimed to explore the impact of *P. aeruginosa* on the growth and virulence of *S. aureus* and the level of infection's severity. Our data suggests that *S. aureus* is negatively impacted by the presence of *P. aeruginosa* and co-infection by these pathogens make the condition more frightful.

MATERIALS AND METHODS

Microorganisms and culture growth conditions

S. aureus ATCC 25923 and *P. aeruginosa* ATCC 27853 were obtained from American type culture collection. They were cultured in LB broth and incubated at 37°C.

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Table 1: Primers used in the study

Gene	Sequence (5' - 3')
TNF- α	ATGAGCACTGAAAGCATGATCCGG
	GCAATGATCCCAAAGTAGACCTGCCC
IL-10	TTGCCAAGCCTTGTCTGAGAT
	TTCTCCCCCAGGGAGTTCAC
IL-6	GTCTCCTCATTGAATCCAGATTGG
	AGCTCAGCTATGAACTCCTTCTC
IL-12	TGGAGCACTCCCCATTCTT
	TGCGCTGGATTCGAACAA
iNOS	TGGAATTCACCTCAGCTGTGC
	GATGTTGTAGCGCTGGACG
β -actin	CCAAGGCCAACC GCGAGAAGATGAC
	AGGGTACATGGTGGTGCCGCCAGAC

Table 2: Growth of *S. aureus* in the presence of *P. aeruginosa*

Culture	Mono-culture	Co-culture with <i>P. aeruginosa</i>
<i>S. aureus</i>	2.8×10^3 CFU/ml	6.4×10^2 CFU/ml

Preparation of cell-free supernatant

Overnight bacterial culture containing 1.5×10^8 CFU/mL was centrifuged at 10,000xg for 10 min at 4°C. The supernatant was harvested and this cell free supernatant was filtered via 0.2 μ m filter and was utilized in bacterial viability staining.

***P. aeruginosa* S. aureus Co-culture study**

We investigated the interactions between *S. aureus* and *P. aeruginosa* in pure mono culture as well as in co-culture by comparing the growth kinetics of the two organisms. Briefly, bacteria were grown as pure culture or in co-culture in LB broth followed by quantification of total cfu/mL of the organisms by differential isolation of *P. aeruginosa* and *S. aureus* on cetrinide agar and mannitol salt agar (Oxoid), respectively.

Activity of *P. aeruginosa* against *S. aureus*

Anti *S. aureus* activity of *P. aeruginosa* was identified using well diffusion assay as described by (Athanasiadis *et al.*, 2009). Live/dead BacLight bacterial viability kit (Invitrogen) was utilized to observe the viability of *S. aureus* cells in the presence or absence of *P. aeruginosa* derived cell free supernatant (CFS) according to protocol detailed by the manufacturer.

Cell culturing

A549 human type II alveolar epithelial cell line (CCL-185) was used in this study. The cells were maintained in DMEM medium (Thermofisher Scientific) supplemented with 10% heat inactivated fetal bovine serum and 100 μ g/ml of penicillin-streptomycin (Invitrogen) to 75% confluency at 37°C in 5% CO₂. Cells were washed three times by PBS and transferred into the culture medium without serum and antibiotic for 2 hours' prior infection and then used for adhesion experiments.

Alveolar epithelial cells infection

A549 cells were mono-infected and co-infected in 24 well polystyrene plate for 24 hours. Trypan blue dye was used to obtain the multiplicity of infection of 1:10. Following incubation, unattached cells were washed with phosphate buffer saline and then adherent cells were detached by using trypsin-EDTA solution (0.5% porcine trypsin and 0.2% EDTA in PBS, Sigma, USA) along with 0.2% Triton X-100. This mixture of cells was diluted and plated onto cetrinide agar and mannitol salt agar plates (Hawdon *et al.*, 2010).

Determination of mRNA expression

Total RNA was extracted from A549 cells by using RNAiso plus. Nano drop was used to determine the concentration of RNA and then cDNA was synthesized by using cDNA kit (Transgene) as per manufacturer protocol. The mRNA expression level of TNF- α , IL-6, IL-10, IL-12 and iNOS was determined by RT-PCR using beta actin as house keeping gene (Schnupf and Sansonetti, 2012). Table 1 displays sequence of the primers used in this study.

STATISTICAL ANALYSIS

The obtained results were statistically analyzed by using Student's *t*-test, on the SPSS v.11.5 software platform (SPSS Inc., Chicago, IL, USA). *P* < 0.05 was taken as the level of significance.

RESULTS***P. aeruginosa* and *S. aureus* co-culture study**

Antibacterial activity of *P. aeruginosa* was seen against *S. aureus*. *P. aeruginosa* not just repressed the growth of *S.*

aureus on agar plate yet its cell free extract also has got anti- *S. aureus* metabolites as depicted in fig. 1 and Table 2. This observation was further confirmed by co-culture study where we found that strain of *P. aeruginosa* outcompetes and arrests the growth of *S. aureus*. Following bacterial viability staining, we observed that the population of *S. aureus* was adversely affected by the presence of *P. aeruginosa* derived CFS (fig. 2). Taken together, these outcomes demonstrate that the strain of *P. aeruginosa* is capable of killing of planktonic growth of *S. aureus*, showing negative relationship between two life forms.

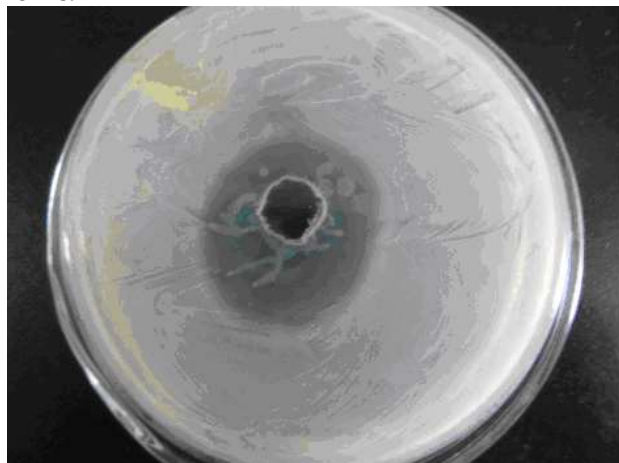


Fig. 1: Antibacterial activity *P. aeruginosa* against *S. aureus*.

Effect on alveolar cells gene expression

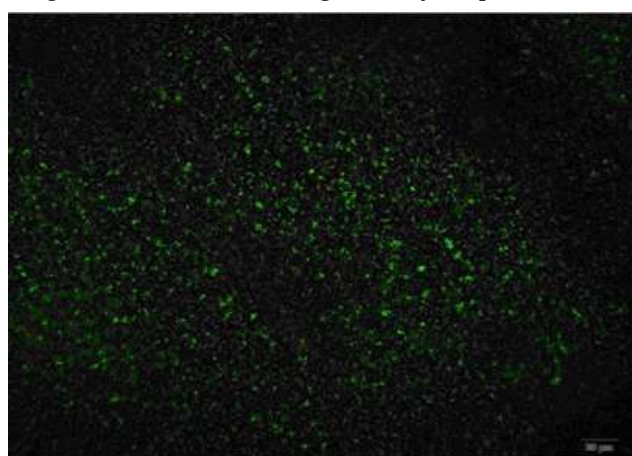
The mRNA expression investigations performed here firmly exhibit modulation in host gene expression profile during co-infection. The host factors expression profile indicated elevated expression of TNF α , IL-6 and IL-12, recommending the unique mechanism of host cell healing inhibition by multispecies. Dual infection with *P. aeruginosa* and *S. aureus* significantly amplified iNOS

expression to 10-fold contributing to the severity of infection. Co-infection with *P. aeruginosa* differentially regulates host cell gene expression as depicted in Figure 3. Unfortunately, we have not identified clear picture in case of IL-6.

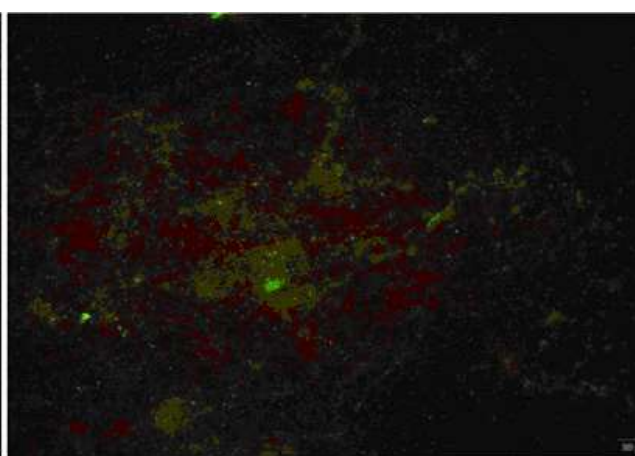
DISCUSSION

Studying interactions among species of different niches and adaptive processes prompting co- or poly-microbial infections is now evidently important. Poly-microbial infection is associated with extensive need for antibiotics compared with patients with mono-infection suggesting that an interaction may happen between microbial species (Hubert *et al.*, 2013). It has been proposed that *S. aureus* could set up the respiratory tract epithelia of cystic fibrosis patients to promote subsequent colonization of *P. aeruginosa* (Lyczak *et al.*, 2002). The objective of this investigation was to examine the influence of *P. aeruginosa* on interaction with *S. aureus* in dual culture infection, keeping in mind the end goal to increase imperative knowledge on the transaction happening between these stubborn human pathogens, which is still to a great extent indistinct. To our knowledge, few scientific studies have assessed the impact of co-infection of *S. aureus* with *P. aeruginosa* on the host immunity.

Herein we have shown that in poly-microbial growth, *P. aeruginosa* strain was able to out-compete *S. aureus* in planktonic forms and this competition is thought to be linked with worse patient outcomes. Our obtained results emphasize the significance of bacterial interactions in poly-microbial infections and this is in agreement of previous study which reported that *P. aeruginosa* restrained the growth of *S. aureus* in vitro (Dalton *et al.*, 2011). One of the studies evident that *P. aeruginosa* can able to lyse the cells *S. aureus* to secure iron discharged for its own growth (Mashburn *et al.*, 2005). In addition, it



S. aureus (control)



S. aureus in the presence of *P. aeruginosa* CFS

Fig. 2: Cells of *S. aureus* in the presence of *P. aeruginosa* cell free supernatant (CFS) - Bar indicates 20 μ m

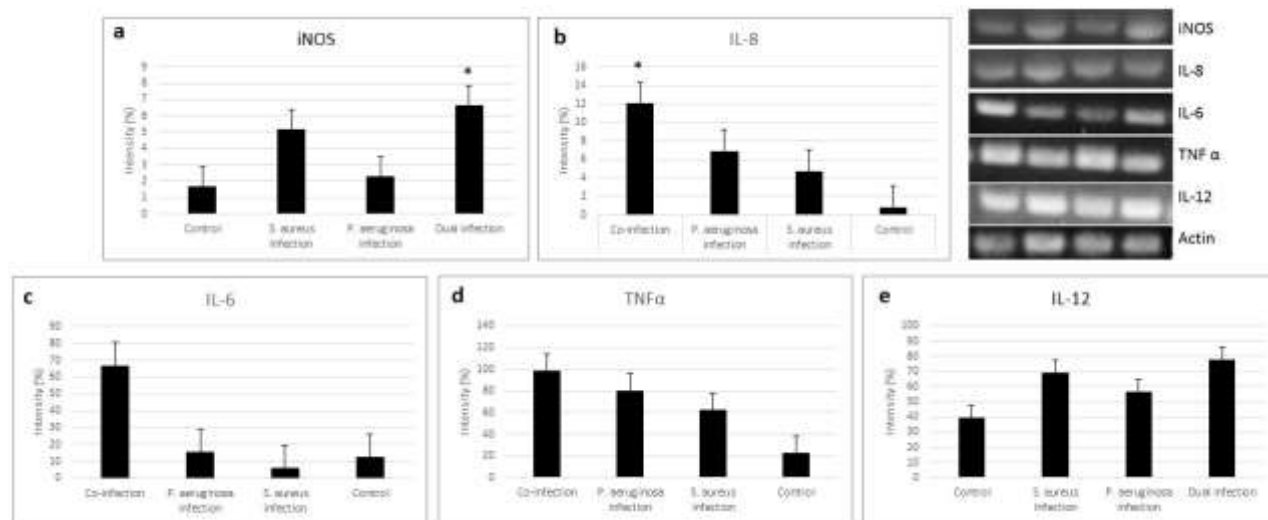


Fig. 3: A549 alveolar cells mRNA expression profile

Co-Infection of *S. aureus* together with *P. aeruginosa* exacerbates the severity of infection by elevating inflammatory cytokine immune responses (a: INOS, b: IL-8, c: IL-6, d: TNFα and e: IL-12) of the host.

*P<0.05 was taken as the level of significance.

has been reported that the growth and metabolism of *S. aureus* are arrested and highly affected due to respiratory inhibitors released by *P. aeruginosa* (Maselli *et al.*, 2003). Although, several mechanisms have been described by which *Pseudomonas* virulence factors and metabolites exert a negative influence on *S. aureus* growth (Biswas *et al.*, 2009; Yang *et al.*, 2011; Qin *et al.*, 2009). However, the exact mechanism of *S. aureus* cell death in the presence of *P. aeruginosa* remains uncertain. In many in vitro model, *P. aeruginosa* strongly reduces *S. aureus* during co-culture (Baldan *et al.*, 2014; DeLeon *et al.*, 2014). However, in previous studies where *P. aeruginosa* and *S. aureus* have been co-isolated, both pathogens exacerbates disease severity by contributing independently and additively compared to infection with a mono specie (Sagel *et al.*, 2009; Hauser *et al.*, 2011).

Co-infection with *P. aeruginosa* differentially regulates host cell gene expression. The mRNA expression profile of TNF α, a major cause of tissue necrosis, IL-12 and IL-6, major inflammatory cytokines and IL-8, a chemokine and iNOS were induced in case of multi specie infection contributing to the severity of infection. In one of the rabbit ear-wound models, co-infection with *S. aureus* and *P. aeruginosa* caused an elevated expression of IL-1b and TNF-α, demonstrating a higher pro-inflammatory response contrasted with mono species infection (Seth *et al.*, 2012).

CONCLUSION

This study concludes that infection of *S. aureus* together with *P. aeruginosa* exacerbates the severity of host pathogenesis by elevating inflammatory cytokine immune responses of the host.

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