Advanced Journal of Microbiology Research Vol. 2016
Available online at http://internationalscholarsjournals.org/journal/ajmr

Full Length Research Paper

A comparative study on biofilm formation of non-typeable *Haemophilus influenzae and Pseudomonas aeruginosa* under single culture or co-culture

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Accepted 3 December, 2015

Individual bacteria can accumulate to form biofilm which can help bacteria escape from host immune defense and many antimicrobial agents. Therefore biofilm involves a large number of bacteria which can induce chronic diseases. Here, we compared the differences of biofilm formation among Nontypeable *Haemophilus influenzae* (NTHi) single culture condition, *Pseudomonas aeruginosa (P. aeruginosa)* single culture condition and NTHi- *P. aeruginosa* co -culture condition. Biofilm clone formation unit (CFU) counting revealed that the number of clinical isolated *P. aeruginosa* was larger than clinical isolated NTHi 3 days after incubation. In co-culture condition, the CFU was significantly higher than both types of single culture condition. Consistent with this result, measurement of crystal violet staining of bacteria attached on the plate well showed that, when co-cultured, the absorbance at 570 nm enhanced 2 - 3 times compared with this two types of single culture condition. Our results were confirmed under scanning electron microscope (SEM). 3 days after inoculation, the co-cultured bacteria stacked more densely to form channels and mushroom-like structure. Whereas both single cultured groups exhibited a more or less sparse, flat-net shape. Intriguingly, both clinical isolated NTHi and *P. aeruginosa* showed weaker capability in the case of biofilm formation compared with their sibling strains NTHi49247 and *P. aeruginosa* 27853, respectively. Because all the clinical strains were isolated from children whose immuno-system had not yet developed mature or strong enough, bacteria that had less infectious capability were opportunistically invasive. Our results indicate that NTHi and *P. aeruginosa* can form biofilm *in vitro* synergistically.

Key words: Nontypeable haemophilus influenzae, *Pseudomonas aeruginosa*, dynamic observation, biofilm formation.

INTRODUCTION

Bacteria can live in one of the two phenotypes: sessile or planktonic. When free living bacteria attach to surface, they begin to proliferate themselves, aggregate and secrete exopolysaccharide (EPS) that helps to sequester nutrients and planktonic cells. The aggregated cell-EPS complex will finally mature and form a water channel, containing mushroom-like three layer structure, termed biofilm. Bacteria inside the biofilm showed properties that are dramatically different from their free-living style. Biofilm structure enhances bacteria communication, nutrient exchange and metabolic efficiency of the community. Thereby this structure confers the bacteria more resistant to antibiotics and host immuno-attack. Floating bacteria that in some case are released from mature biofilms will aggravate infections or result in acute attack to host and eventfully lead to repeated infections (Bauer et al., 2002; Slinger et al., 2006). It was reported that at least 65% of bacteria related diseases, especially chronic bacterial infectious diseases, such as cystic fibrosis (CF), diffuse

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bronchiolitis, valve endocarditis, periodontitis, otitis media, etc. are directly associated with biofilm formation (Jia, 2003).

P. aeruginosa and Nontypeable *H. influenzae* are two common pathogens isolated from patients with chronic respiratory system diseases following bacteria infections. Previous work revealed that children with *P. aeruginosa* infection had prior infection with NTHi (Abman et al., 1991). Single-pathogen infection with NTHi or *P. aeruginosa* showed no statistically significant differences in airway inflammatory markers (Muhlebach et al., 1999). Reports imply that NTHi may cooperate with or synergize *P. aeruginosa* during infections. *P. aeruginosa* under biofilm state exhibits more than 1,000-fold increase in antibiotic resistance and is more able to evade the host defense responses (Bjarnsholt et al., 2007; Jesaitis et al., 2003). Wang et al found that NTHi was capable to generate bacterial biofilm *in vitro* (Wang et al., 2008). However, the effect of biofilm formation in mixed infection by the two bacteria is poorly understood.

In this paper, we compared the speed and compact degree of biofilm formation by *H. influenzae* and *P. aeruginosa.* .We found that, in *in vitro* co-culture condition, the biofilm formed more prosperous in density and thickness than each single culture condition. Our data imply that, NTHi and *P. aeruginosa* can form biofilm in a synergistic manner when co-infected.

MATERIALS AND METHODS

Bacterial strains

NTHi strain ATCC 49247 and *P. aeruginosa* strain ATCC 27853 were obtained from National Center for Clinical Laboratory. 40 clinical strains of NTHi and 39 clinical strains of *P. aeruginosa* form Children's Hospital of Chongqing Medical University.

Culture conditions

All strains were reconstituted from frozen glycerol stock cultures. Nontypeable *H. influenzae* propagated on chocolate agar plates(PangTong, Chongqing, China) overnight at 37°C with 7% CO_2, and *P. aeruginosa* propagated on blood agar plates (PangTong, Chongqing, China) overnight at 37°C. To establish the equal multiplicity of infection (MOI) by these two strains, 3.6×10^7 CFU/ml bacteria suspensions were prepared with saline, respectively.

Biofilm CFU counting assay

For single type of bacteria culture, 100 ul of 3.6×10^7 CFU/ml bacteria suspension was inoculated into 1 ml broth medium in polystyrene tube, respectively. The culture medium was fresh brain heart infusion (BHI) broth for NTHi, and Luria-Bertani(LB) broth for *P. aeruginosa.* In co-culture condition, 50 ul of 3.6×10^7 CFU/ml NTHi and 50 ul 3.6×10^7 CFU/ml *P. aeruginosa* were added together into 1 ml BHI. All samples were cultured at 37°C with 7% CO_2 till the require time, and then discarded the suspension. The tube was washed three times with distilled water, followed by sonicate 10 min at 250 w to release the biofilm bacteria and vortex.

Crystal violet measurement

10 ul, 3.6×10^7 CFU/ml NTHi was diluted in 1 ml of BHI, and 10 ul, 3.6×10^7 CFU/ml *P. aeruginosa* was diluted in 1 ml of LB. For co-culture, 5 ul of each bacteria were added into 1 ml of BHI, Then, 200 ul of the diluted suspension was inoculated into non-tissue culture treated polystyrene flat-bottomed 96-well microtiter plates, respectively. Cells were cultured at 37°C with 7% CO_2 till setting time, followed by pouring off the suspension and washing three times with distilled water. The method of crystal violet stain was described previously (Favre-Bonté et al., 2003) and measured at 570 nm.

Morphological observations of biofilm formation under SEM

100 ul microliters bacteria suspensions were inoculated into 3 ml broth medium in 6-well plates, respectively, and a cement disc was added in each well. The cement disc was pick out at require time, fixed and dehydrated. Photograph was carried out as previously described (Starner et al., 2006, Gallaher et al., 2006)

Statistical analysis

All analyses of statistical significance were performed with repeated measures variance analysis using SPSS 14.0 Software. One tail T-test was applied and P value < 0.05 was considered statistically significant.

RESULTS

Clone formation counting assay during biofilm formation process

To quantify the dynamic process of bacteria biofilm formation, we applied clone formation counting assay. On day 2, there was no obvious difference between single culture and co- culture groups. Whereas, on day 3, the biofilm CFU/ml in co- culture groups was averagely 1.6 times of that in clinical isolated *P. aeruginosa* (clinical Pa) single culture groups and 2.2 times of that in clinical isolated NTHi(clinical NTHi) single culture groups (Figure 1 green, dark blue and yellow curves). In co-culture situation, Curve trend between clinical isolated and ATCC standard strains (27853*P. aeruginosa* and 49247NTHi) was almost the same (Figure1, brown and green curves). Interestingly, we found that there was significantly more bacteria ($1.72 \pm 0.30 \times 10^8$/ml day3) in 49247NTHi biofilms than that in biofilm of clinical isolated NTHi groups [($1.09 \pm 0.03 \times 10^8$ /ml day3) ($P < 0.05$) Figure 1. light blue and yellow curves]. Further more, the biofilm CFU of ATCC 27853 *P. aeruginosa* showed a similar manner when compared with their clinical isolated sibling *P. aeruginosa*. On day 5, the co-culture group also showed significantly high CFU compared with single culture groups in both clinical isolated and ATCC standard strains. We also observed a very astonishing phenomenon that clinical isolated *P. aeruginosa* strains had higher CFU($1.49 \pm 0.14 \times 10^8$ /ml day3) than clinical isolated NTHi CFU($1.09 \pm 0.03 \times 10^8$ /ml day3). But this distinctness did not exist between standard 27853 *P.*

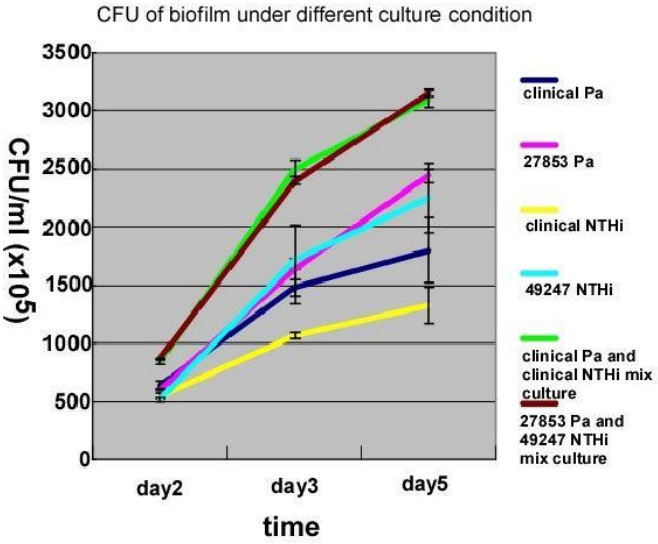

Figure 1. Clone formation unit /ml of biofilm under different culture conditions. On day 3 and 5. 49247NTHi (light blue)> clinical NTHi (yellow); clinical *P. aeruginosa* (clinical pa) and clinical NTHi mix-culture (green curve)>clinical Pa (dark blue curve)>clinical NTHi (yellow curve) ($P < 0.05$).

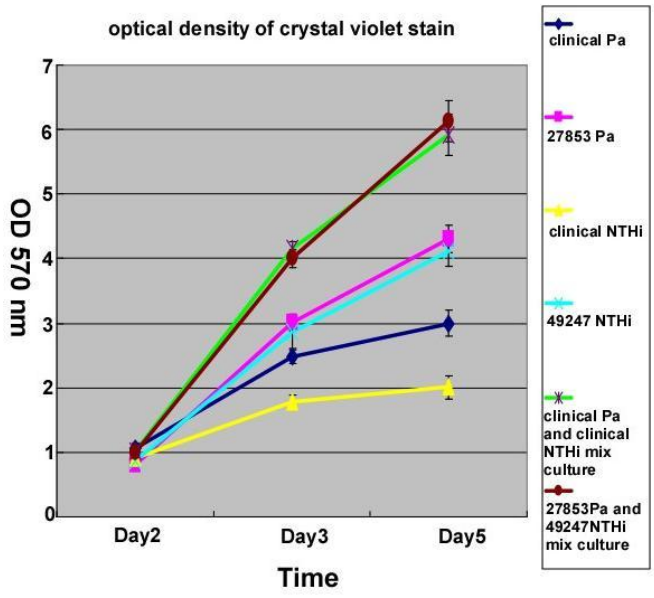

Figure 2. Optical density of crystal violet stain under different culture conditions. On day 3 and 5, 49247NTHi (light blue)> clinical NTHi (yellow); clinical *P. aeruginosa* (clinical pa) and clinical NTHi mix-culture (green curve)>clinical Pa (dark blue curve)>clinical NTHi (yellow curve) ($P < 0.05$).

aeruginosa and 49247 NTHi strains.

In vitro crystal violet staining measurement

To confirm the results from CFU counting, we applied crystal violet stain and detected the absorbance at 570 nm. On day 3 and 5, the co- cultured groups showed most significantly higher optical density (OD) than single culture groups ($P < 0.01$) . In addition, the OD curve on day 3 and 5 merge well between clinical isolated and ATCC standard stains when co-cultured [Figure 2. brown and green curves]. Nevertheless, the biofilms formed by 49247NTHi were always more dense than our clinical isolated NTHi strains as determined with crystal violet staining [Figure 2. light blue and yellow curves]. Finally, we found that clinical isolated *P. aeruginosa* formed biofilm better than clinical isolated NTHi, which was identical to our observation of CFU counting.

Scanning electronic microscopy (SEM) of the biofilm

Using scanning electronic microscope, we observed direct evidence of biofilm formation under co-culture and single culture conditions. Consistent with CFU counting and crystal violet staining data, SEM revealed that in co-cultured groups, the biofilms formed significantly on day 3, with greater biofilm mass than either type of bacteria single culture condition. The co-culture biofilms seemed more matured with thickened mushroom-like structure, and stacked more densely (Figure 3 C and C'). In single cultured groups, either bacteria biofilms attached to plate surface and exhibited extensive biofilm structure but not as dense as the co culture situation as identified in Figure 3.

DISCUSSION

P. aeruginosa and Nontypeable *H. influenzae* are two common opportunistic pathogens isolated from respiratory system (Sethi, 2000; Hancock et al., 1983; Bilton et al., 1995; Doig et al., 1988; West et al., 2002). Both types of bacteria can form biofilm and induce series of chronic respiratory diseases such as cystic fibrosis lungs (West et al., 2002; Singh et al., 2000; Starner et al., 2006) and COPD(Eldika and Sethi, 2006). Single bacteria spices of biofilm formation has been studied extensively in various aspects previously.(Starner et al., 2006; Gallaher et al., 2006; Whiteley et al., 2001; Klausen et al., 2003). However, co-infection of these two bacteria to respiratory systems seems more common in clinical condition. Till now, the dynamic process of biofilm formation under co-infectious situation is poorly understood. In this paper, we first compared the biofilm growth parameters between single culture and co-culture situation of *P. aeruginosa* and Nontypeable *H. influenza*.

The results showed that under co-culture condition, the biofilm formed more densely and more prosperously than either single culture of *P. aeruginosa* or *H. influenza* at the same time. On another hand, we found that in single culture condition, clinical isolated NTHi strains had less capability to form biofilm than standard 49247NTHi. Given that all our clinical strains were isolated from

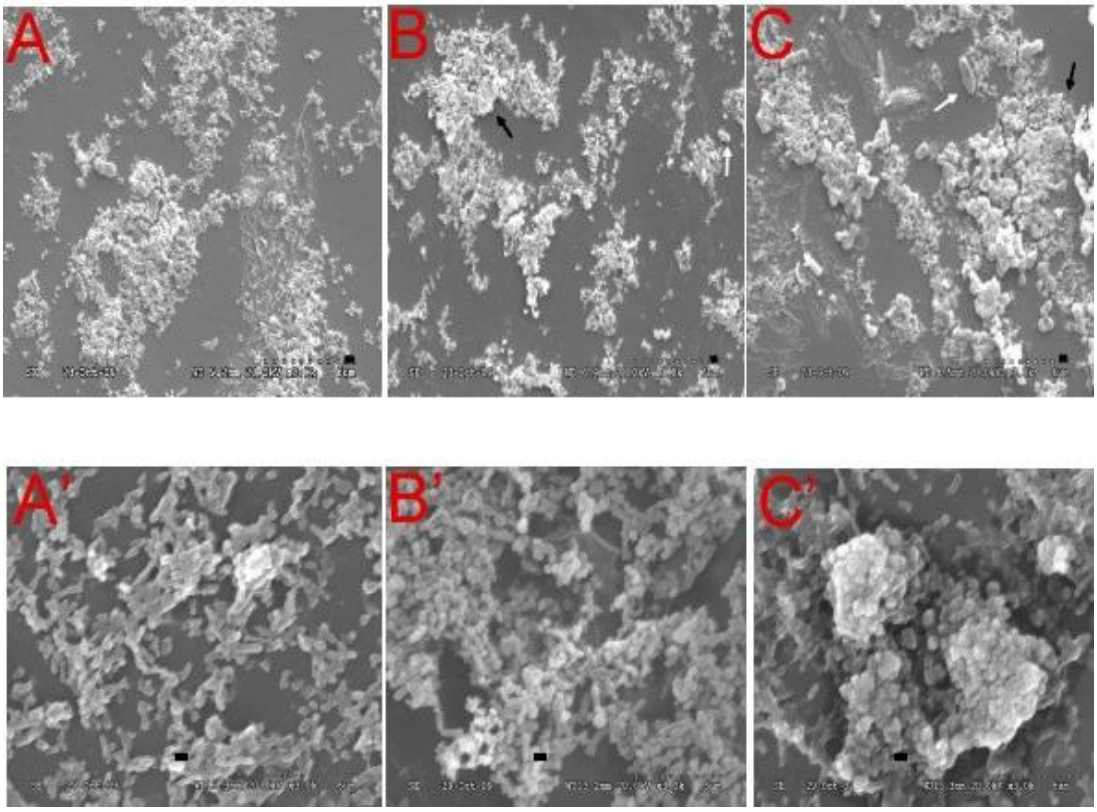

Figure 3. Scanning electronic microscopy (SEM) of biofilm formation on day 3. A and A', *P. aeruginosa* single culture; B and B', Nontypeable *Haemophilus influenzae* single culture*;* C and C', *P. aeruginosa* and Nontypeable *Haemophilus influenzae* co-culture condition. Scale bars indicate 6 um.

children whose immuno-system had not yet developed mature and strong enough, bacteria that had less infectious capability were opportunistically invasive. In co-culture condition, the curve of clinic isolated and standard strains merged very well in both CFU and crystal violet staining assays. These results suggest that in co- culture condition, *P. aeruginosa* or *H. influenza* have some functional redundancy to compensate each other`s shortcoming during biofilm formation process. Therefore, Nontypeable *Haemophilus influenzae* and *P. aeruginosa* can form biofilm synergistically *in vitro*.

ACKNOWLEDGMENTS

We are grateful to staff of our study group for their work in study design and manuscript preparation, and we also greatly appreciate Mr. Qianglin Duan for English usage and paper revision.

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