Original Article

Commensal Bacteria Anginosus Group of Streptococci (AGS) and Cystic Fibrosis Pathogen PS. Aeruginosa in a Biofilmcontent: An in Vitro Study

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ABSTRACT

OBJECTIVE: The objective of the study was to investigate the application on growth of anginosus group of Streptococci (AGS) by *Ps. aeruginosa* when grown together, and the effect on the virulence of *Ps. aeruginosa* when grown together with AGS on a biofilm model.

METHODOLOGY: In this study we used nitrocellulose filter paper as biofilm model representing the lung epithelium. The AGS strains used were AGS F (*S. constellatus* from bronchiectasis), AGS 3a (*S. anginosus* from cystic fibrosis exacerbation), AGS PC 4890 (*S. anginosus* from dental plaque), AGS 2227 (*S. Intermedius* from unknown source), AGS EF 52 (*S. contellatus* from supra gingival plaque), AGS AC 9612 (*S. anginosus* from supra gingival dental plaque), AGS AM 699 (*S. constellatus* from supra gingival dental plaque) and AGS 1a (*S. anginosus* from cystic fibrosis exacerbation). *Ps. aeruginosa* strain used was LES 1 (Liverpoolepidemic strain of *Ps. seruginosa*).

RESULTS: A concentration of AGS strain in monoculture ($1x10^5$cfu), Ps. aeruginosa monoculture ($1x10^5$cfu), AGS+Ps. aeruginosa mixed culture ($1x10^5$cfu of each) was used to inoculate the biofilm. NAS agar plate and PIA agar plates were used to count the growth of AGS and *Ps. Aeruginosa*respectively, and pyocyanin and elastase assays were done to check the virulence of the *Ps. aeruginosa*. It was observed that there was a 10 folds increase in the growth of all the AGS strains; when grown in co-culture with the *Ps. aeruginosa* strain as compared to when grown in mono culture; both in the 24 and 48 hours aerobically incubated biofilms. When grown in an anaerobic environment AGS in monoculture had the same growth as when grown aerobically with *Ps. aeruginosa*in co culture. There was no effect in the growth of *Ps. aeruginosa* in mono or co culture with AGS in 24 and 48 hours biofilm.

CONCLUSION: The data supported the hypothesis that anginosus group of Streptococci grows better in presence of Ps. Aeruginosain a co-culture, as compared to when grown alone in an anaerobic environment, whereas the virulence factors (Pyocyanin and Elsastase production) of Ps. aeruginosa increases when grown in co-culture as compared to when grown alone in aerobic conditions.

KEYWORDS: Anginosus group of Streptococci (AGS), Cystic fibrosis, Commensals,Biofilm content.

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INTRODUCTION

Biofilms may be define as the group of microbial cells; stick to each other; adhere superficially and embedded in a forge of material that is self-formed extracellular polymeric substance matrix. The other materials, like crystals made up of minerals, particles that are formed by erosion or from mud, or different segment of the blood comes under the category of non-cellular type of materials. These material are environmental depending by which various biofilms are formed that may found in their matrix also.These biofilms form various types of surfaces such as the surface of living tissues, medical devices, industrial system particularly water piping. Microcolony is the basic structural unit to form the biofilms.If the cells are closed in microcolony than it provides the best environment for some materials such as nutrients, genes exchanges and sensing of quorum. Further redox reaction can takes place in micro colonies due to composing of various species[1]. In the attachment or detachment of cells in biofilms two important factors were involved i.e. signaling from one cell to another cell and sensing of quorum[2].

Cystic fibrosis is a polymicrobial disease that includes *Streptococci* which are usually avirulent commensal organisms and the *Ps. Aeruginosa*in a biofilm. This is due to the ability of the *anginosus group of Streptococci* whichhas an ability to modulate the pathogenicity of *Ps. aeruginosa* by enhancing its virulence factors[3,4].

Pseudomonas aeruginosa

These are motile oxidase positive strictly aerobic bacteria. They secrete range of exotoxins which contribute to the pathogenesis of the disease. *Ps.aeruginosa* is ubiquitous and inhabits human and animal gut, water, soil, sewage and moist surfaces. It contaminates hospital environments and many hospitals have multiresistant strains which can be transmitted between patients and the hospitals. It is a major opportunistic pathogen of the immunocompromised causing a wide range of hospital acquired infections[5].

Pseudomonas aeruginosa and anaerobes in cystic fibrosis

Chronic lung infections which lead to irreversible reduction in lung function are the main cause of death and morbidity of cystic fibrosis patients[6]. *Pseudomonas aeruginosa;* inside the hypoxic mucus; produces anaerobic environment that occur mostly in the patient of cystic fibrosis with persistent respiratory infection[7, 8]. Lungs infection in patients of cystic fibrosis in this anaerobic environment is often polymicrobial, although anaerobic bacteria present, but are undetectable by aerobic culture techniques[9-11]. The anaerobes in such patients resemble those found in other anaerobic pulmonary infections for example nosocomial empyema[12], lung abscesses and empyema[13] where aerobes and anaerobes are found together in a polymicrobial infection.

Rationale

This study carried out to show that AGS bacteria is a normal commensal of the oral cavity whereas Ps. aeruginosa is a normal commensal of the drainage system, pipe lines, dental chairs and the hospital environment. If the sewerage system is not kept under hygienic conditions and the oral hygiene is not maintained appropriately than the virulence factor of Ps. aeruginosa can increase with consequentdetrimental effects in lung diseases associated with cystic fibrosis; the number of anginosus group of bacteria may increase and can cause caries and other oral infections.

METHODOLOGY

Culture and maintenance of bacterial strains

Streptococci group i.e. Anginosus: They were stored frozen on beads at -70°C. Unless otherwise stated incubation was in some special type of environment particularly in an anaerobic type of atmosphere that consist of nitrogen 80% ,hydrogen 10% and carbon dioxide 10%. Routine subculture done on Blood Agar Base No. 2 (Oxoid Ltd, Hampshire, UK) supplemented with 6% (vol/vol) defibrinated horse blood (TSC Biosciences Ltd, Buckingham, England).Liquid culture was carried out in Broth of Todd with 0.5% yeast extract (THY BROTH) (BBL Becton Dickson & Co. Sparks, USA).Counts for AGS from mono and mixed biofilms and liquid cultures were obtained on NAS plates.

NAS agar

Lab M 12 Sensitivity Test Agar STA (Lab M Ltd, Lancashire, UK) 40g/L
Sulphamethazine (Sigma Aldrich, St. Louis, USA) 1.0g/L
Nalidixic acid (Sigma Aldrich, St. Louis, USA) 37.5mg/L
Dispense 40g STA in 1litre bottle and dissolve in 976mls distilled water. Autoclave and when cooled add dissolved antibiotics followed by 6% (vol/vol) defibrinated horse blood. Pour plates.

Pseudomonas aeruginosa strain: Strains were stored at -70°C . A total of 10% of the CO_2 used in the incubation of plates that made up of agar at recommended temperature of 37°C.Routine subculture was on Pseudomonas Isolation Agar (PIA) from Becton Dickson Microbiology Systems Sparks MD 21152 USA; supplemented with (20mls) glycerol. Liquid culture of *Ps.aeruginosa* was in THY broth.Counts of *Pseudomonas aeruginosa* strains from mono and mixedbiofilms and liquid cultures were *obtained* on PIA plates.

Biofilm growth

For cultures of mono (AGS or *Ps. aeruginosa* alone) and mixed (AGS + *Ps. aeruginosa*) biofilms on filters, Todd Hewitt Yeast Extract Agar (THY + 1.5% Technical Agar No. 3 OxoidLtd, Basingstoke, Hampshire England + 0.5% yeast extract) (THY Agar) was used. Biofilm growth was allowed to continue for 24 hrs and/or 48hrs as required in specific experiments.
The detailed method of setting up and growning biofilms is given below:
Cultures of AGS on (BAB) and *Ps. aeruginosa* (on PIA) were inoculated into 15mls of THY broth and incubated overnight at 37°C. The AGS strain was incubated anaerobically and *Ps. Aeruginosa*aerobically in a shaking incubator.AGS strain and Ps. aeruginosa (approximately $1x10^5$cfu in 100ul of THY) were applied alone or in combination to almost the entire surface of a filter disc (Millipore Nitrocellulose Membrane Filter Type 0.22um GSWP04700) previously placed aseptically onto THY agar, taking care not to inoculate the agar surface directly. For a typical biofilm experi-

ment the following filters were prepared:-

AGS strain in monoculture ($1x10^5$cfuapplied)

Ps. aeruginosa monoculture ($1x10^5$cfuapplied)

AGS+Ps. aeruginosa mixed culture ($1x10^5$cfu of each applied)

Duplicate filters for each of the above were prepared and incubated for 24hrs (2 filters) and 48hrs (2 filters). Until otherwise stated all biofilms were incubated at 37°C aerobically.For each experiment the initial viable count of the inoculae for AGS and Ps. aeruginosa alone and AGS + Ps.aeruginosa together were determined by 10-fold serial dilution in phosphate buffered saline and plating (10µl) onto NAS agar (for AGS in monoculture and for the AGS component of the mixed inoculum) and onto PIA (for Ps. aeruginosa in monoculture and for the Ps. aeruginosa component of the mixed inoculae). All NAS plates were incubated anaerobically and all PIA plates were incubated aerobically as described above.After incubation of the biofilms for 24 and 48hours filters were removed from the THY agar, placed into 10mls of PBS and vortexed for 1 minute to dislodge the bacteria from the filter surface and disperse them. Following vortexing the cell suspension was serially diluted in PBS (10-fold dilution from neat to 10^{-6}) and plated onto NAS and PIA agar for AGS and LES counts respectively; NAS plates were incubated anaerobically and PIA plates incubated aerobically.

RESULTS

Biofilm growth of AGS 3a (CF) under aerobic and anaerobic conditions at 24 hrs and 48 hrs of incubation were investigated. The result of the bacterial counts for each strain alone and in combination is shown in table 2p=<0.05 t-test result for AGS 3a mono aerobic versus AGS 3a in co culture.P=<0.05 t-test result for AGS 3a mono anaerobic versus AGS 3a in co culture. *Ps. aeruginosa* strain LES 1 showed no significant difference in cell numbers between mono and co- culture biofilm. Whereas AGS 3a showed an approximately 10-fold increase in numbers when grown together with *Ps. aeruginosa* over when growing alone. In addition AGS 3a numbers in co-culture (aerobic) were greater than when growing alone aerobically or anaerobically.AGS F (*S. constellatus* from bronchiectasis) and LES 1 were grown alone and in co-culture for 24 and 48 hrs of aerobic incubation, AGS F were also incubated anaerobically for comparison. The resulting biofilm counts are shown in Table III where p=<0.01 t-test result for AGS F aerobic mono versus AGS F in co-culture.As observed for *S. anginosus* strain 3a, *S. constellatus* (AGS F) from bronchiectasis in anaerobic monoculture showed 10 fold increase in growth compared to when grown aerobically. Similarly when in co-culture with *Pseudomonas aeruginosa* strain LES 1 an approximately 10 fold increase in cell numbers was observed compared to mono culture biofilms. No difference in growth of LES 1 strain either alone or in co culture with AGS F at 24 and 48 hrs aerobic incubation period was observed. *Pseudomonasaeruginosa* CF strain LES 1 was grown in biofilms with *S. anginosus* CF strain AGS 3a and *S. contellatus* no-CF bronchiectasis strain AGS F. biofilms were assayed for pyocyanin and elastase production. The results for the biofilm growth at 48 hrs incubation AGS 3a and LES 1 aerobically at 48 hrs and the virulence assays (pyocyanin and elastase) on culture supernatant as shown in table IV. The results of pyocyanin and elastase activities are shown in Table V.The results for the biofilm growth at 48 hrs incubation AGS F and LES 1 aerobically and the virulence assays (pyocyanin and elastase) on culture supernatant at 48 hrs are shown in Table IV. The results of pyocyanin and elastase activities are shown in Table VI. The biofilm cell counts in mono and co culture show the same pattern as obtained previously with no increase in *Ps. aeruginosa* strain LES 1. However there was significant increase observed in both virulence factors (pyocyanin and elastase) produced by LES 1 when it was grown in co culture with AGS 3a compared with the levels expressed in mono culture. The same trend explained above was also observed for the co culture AGS F with LES 1.

TABLE I: STRAINS USED IN THE EXPERIMENT

Anginosus Group of Streptococci (AGS)	
DWW2	Mucoid strain of Ps.aeruginosa from cystic fibrosis exacerbation
AGS F	S. constellatus from bronchiectasis
AGS 3a	S. anginosus from cystic fibrosis exacerbation
AGS PC 4890	S. anginosus from dental plaque
AGS 2227	S. intermidius (Unknown)
AGS 1a	S. anginosus from cystic fibrosis exacerbation.
AGS EF 52	S. contellatus from supra gingival plaque.
AGS AC 9612	S. contellatus from supra gingival plaque.
AGS AM 699	S. contellatus from supra gingival plaque.
Ps. Aeruginosa	
PA01	Ps. aeruginosa from a burn wound
Midlands 1	Cystic fibrosis strain of Ps. aeruginosa
LES 1	Lverpool epidemic strain of Ps. aeruginosa

TABLE II: AVERAGE GROWTH OF AGS 3A ALONE AND IN CO CULTURE WITH LES 1 BOTH UNDER AEROBIC AND ANEROBIC CONDITIONS

Strains	24hrs Ave CFU/FILTER	SD	48 hrsAve CFU/FILTER	SD
AGS 3a O_2 (mono)	3.43E+07	9.40E+06	7.75E+07	8.41E+07
LES 1 (mono)	4.20E+10	6.65E+09	3.71E+10	4.56E+10
AGS 3a (Co)	8.50E+08	1.65E+08	8.49E+08	6.81E+08
LES 1 (Co)	4.25E+10	3.54E+09	2.75E+10	2.90E+10
AGS 3a ANO2 (mono)	1.65E+08	4.53E+07	6.47E+07	8.25E+07

TABLE III: AVERAGE GROWTH OF AGS F ALONE IN CO CULTURE WITH LES 1 BOTH UNDER AEROBIC AND ANEROBIC CONDITIONS

Strains	24 hrs Ave CFU/FILTER	SD	48 hrs Ave CFU/FILTER	SD
AGS F (mono)	3.54E+05	3.49E+05	1.69E+08	2.12E+06
LES 1 (mono)	3.30E+10	9.48E+09	6.22E+10	7.07E+08
AGS F (Co)	1.00E+08	1.41E+07	5.03E+09	
LES 1 (Co)	5.10E+10	3.25E+09	5.93E+10	
AGS F ANO2 (mono)	1.35E+08	3.11E+07	1.07E+09	2.85E+08

TABLE IV: AVERAGE GROWTH OF AGS F ALONE AND IN CO CULTURE WITH LES 1 UNDER AEROBIC CONDITIONS

Strains	48hrs Ave CFU/FILTER	SD
AGS 3a(mono)	3.04E+08	2.28E+08
LES 1 (mono)	6.93E+10	6.15E+09
AGS 3a(Co)	2.51E+09	2.59E+08
LES 1 (Co)	6.65E+10	5.64E+09

TABLE V: AVERAGE ABSORPTION OF PYOCYANIN PIGMENT AT 520nm

Strains	48 hrs Ave CFU/FILTER	SD
AGS F(mono)	1.99E+08	8.45E+07
LES 1(mono)	8.02E+10	6.26E+09
AGS F(Co)	5.74E+09	6.74E+08
LES 1(Co)	8.26E+10	5.01E+09

TABLE VI: AVERAGE ABSORPTION OF PYOCYANIN PIGMENT AT 520nm

Strains	Ave abs@520nm	SD
AGS 3a	0	0
LES 1	0.0095	0.001153
Co culture	0.0383	0.014809

TABLE VII: AVERAGE ABSORPTION OF ELASTASE ACTIVITY AT 495nm ELASTASE ASSAY ON 48 HRS BIOFILMS CULTURE SUPERNATANT

Strains	Ave abs@495nm	SD
AGS 3a	0.005533	0.008208
LES 1	0.005367	0.00215
Co culture	0.0355	0.002858

TABLE VIII: AVERAGE ABSORPTION OF ELASTASE ACTIVITY AT 495NM ELASTASE ASSAY ON 48 HRS BIOFILMS CULTURE SUPERNATANT

Strains	Ave abs@495nm	Stdev
AGS F	0	0
LES 1	0	0
Co culture	0.0247	0.015324

DISCUSSION

Anginosus group of Streptococcihave the potential to boost up the exacerbations of the pulmonary area. It was also evidenced by the authors that the dominancy with respect to numerical with the onset of cystic fibrosis exacerbations but this was not detected by sputum standard culture method. Further the antimicrobial therapy for the AGS may be more effective than anti-*Pseudomonal* therapy and significant reduction in the number of AGS[14]. These are important findings and

open an avenue of potentially fruitful research that may result in significant improvement in the quality of life for CF patients and perhaps patients suffering from other chronic respiratory infections such as non-CF bronchiectasis and chronic obstructive pulmonary disease in which AGS have also been observed in significant numbers from sputa.

Bacterial diseases are often an outcome of the complex interactions between the pathogens and the host and more recent evidence is building to indicate interplay between pathogens and the indigenous, generally avirulent commensal microflora of the host[15]. In the context of this project the overtly pathogenic bacteria may alter the "behavior" of the host microflora including the AGS of the oropharynx or *vice versa*.Viridans group streptococci commensal organisms of the oropharynx and coagulase-negative staphylococci have a potential to change the expression of gene of a main pathogen and its name was *Pseudomonasaeruginosa,* enhancing expression of many important virulence factors including pyocyanin and elastase has been reported as well as an enhancement of lung inflammation[3]. In the present study *Ps. aeruginosa*was shown to increase the growth of anginosus group of streptococci strains growing in a biofilm to approx 10 fold greater than when the latter were growing alone. At the same time inceased numbers of anginosus group of streptococci caused up-regulation or expression of *Ps aeruginosa* virulence factors; this was especially clear when the numbers ofanginosus group of streptococci present in pyocyanin-producing areas of co-culture with mucoid strain DWW2 were compared to the numbers growing in non-pyocyanin producing areas of the biofilm. It has been suggested that *Ps. aeruginosa* may reduce the redox potential to create hypoxic conditions in the biofilm producing an anaerobic environment for anginosus group of streptococci to thrive in[7,16]. However, this possibility was not addressed in the present study and remains to be investigated. Significantly, the microbial diversity present in the sputum of CF patients, including species normally found in the mouth, has been shown not to be a consequence of oral contamination and the potentially central role of oral bacteria in the etiology of CRIs, is becoming increasingly recognized[16,17].

CONCLUSION

The presence of AGS in a biofilm resulted in elevated expression of *Ps. aeruginosa* virulence factors (pyocyanin and elastase) and confirms previously published data.Similar behavior occurred between all 3 AGS species (*S. anginosus*, *S. constellatus* and *S. intermedius*), including AGS strains from dental plaque as well as from chronic respiratory infections, and *Ps aeruginosa* strains from CF infections; however, preliminary evidence obtained from co-culture with environmental *Ps. aeruginosa* strain PAO1 indicated that this relationship may not extend to *Ps aeruginosa* strains with faster growth rates (*i.e* from burns, wounds and the environment).

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