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ROLE OF BACTERIAL BIOFILM IN RESISTANT CASES OF CHRONIC RHINOSINUSITIS AND OTITIS EXTERNA

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ABSTRACT

Bacterial biofilms consist of a complex, organized community of bacteria that anchor to both biotic and abiotic surfaces. They are composed of layers of embedded, live bacteria within extruded ex-polymeric matrix. This configuration allows for evasion of host defenses and decreased susceptibility to antibiotic therapy while maintaining the ability to deliberately release planktonic bacteria, resulting in recurrent acute infections. Thus, bacterial biofilms were hypothesized to contribute to the progression and persistence of chronic rhinosinusitis and otitis externa.

KEYWORDS: Biofilm, Chronic Rhinosinusitis, Otitis Externa.

Biofilm is a three-dimensionally structured, specialized community of adherent microorganisms surrounded by an extracellular polymeric substance (EPS). Biofilm communities in most environments, including human disease, tend to be polymicrobial. By including multiple bacterial and/or fungal species in a single community, biofilms obtain numerous advantages, such as passive resistance, metabolic cooperation, by-product influence, quorum sensing systems, an enlarged gene pool with more efficient DNA sharing, and many other synergies, which give them a competitive advantage. In general, the greater the diversity, the more robust the biofilm is in terms of its survivability (Wolcott et al; 2013).

One of the most important features of biofilms is the high resistance to antibiotics and to host immune mechanisms, such as less susceptibility to opsonization and phagocytosis (Vuong et al; 2004) (Leid et al; 2002).

In vitro tests have demonstrated that certain strains of bacteriain a biofilm state can be more than 1000 times more resistant to antibiotics compared to the minimum of the mechanisms of resistance is due to the physical barrier exerted by the polyssacharide matrix that blocks the diffusion of the compounds or inactivates the biocide activity of some agents. (Stewart et al; 2001) (Costerton et al; 2003).

A number of different techniques have been used to investigate and appreciate the complex nature of biofilms.

The classic approaches have included both scanning electron and transmission electron microscopy (SEM and TEM, respectively) microscopy.

These techniques provide detailed imaging of the intricate architecture, developmental stages, and polymicrobial nature of biofilms.

However, both of these techniques can be limited in clinical utility due to difficulty in fixation, the presence of artifacts in the fixation process, and difficulty to identify individual bacterial species. SEM also has difficulty differentiating between mucus, clot, and biofilm, whereas TEM only renders a two-dimensional section of the biofilm (Ferguson et al.;2005).

To overcome these obstacles and to streamline the process in biofilm identification, fluorescent in-situ hybridization (FISH) with confocal laser scanning microscopy (CLSM) has been used.

CLSM with FISH has the advantage of not only being able to render three-dimensional biofilm structures, but also speciate the bacteria visualized. Crystal violet

staining is an inexpensive assay to assess in-vitro biofilm growth; however, it does not reflect in-vivo biofilms

Chronic rhinosinusitis is one of the most common chronic disorder. This disease significantly reduces the quality-of-life of its sufferers and is a socioeconomic burden on the community.

Patients with recurrent or chronic rhinosinusitis report a deteriorating sense of general healthand vitality when compared to the general population.

CRS represents a spectrum of inflammatory and infectious processes concurrently affecting the nose and paranasal sinuses and is characterized by a minimum of two symptoms.

These include nasal congestion or nasal discharge (anterior/posterior nasal drip), facial pain, and a reduction in the sense of smell. In addition, the presence of polyps andmucosal edema is one of the main presentations at examination. The duration of the disease tends to exceed 12 weeks (Al-Mutairi et al; 2011).

Otitis externa, also called swimmer's ear, involves diffuse inflammation of the external ear canal that may extend distally to the pinna and proximally to the tympanic membrane (Rowlands et al; 2001).

Otitis externa lasting three months or longer, known as chronic otitis externa, is often the result of allergies, chronic dermatologic conditions, or inadequately treated acute otitis externa.

MATERIAL AND METHODS

Forty patients were included in this study. Culture of forty cases, twenty cases of otitis externa and twenty cases of chronic rhinosinusitis. Then all specimens inoculated on blood, MacConkey Agar. Plates incubated overnight at 37° C. after bacterial identification and storage. Quantitative determination of biofilm formation *(O'Toole et al,;1998).*

Exclusion criteria

-Patients receiving antibiotics 48hours prior to sample collection.
-Patients with clinical evidence of fungal infection.
-Patients with mixed infection i.e otitis media and otitis externa.

This method was done to all isolated bacterial pathogen. Overnight culture in tryptic Soya Broth (TSB) were dilute 1:100 with fresh TSB. Three wells of a sterile 96-well flat-bottomed plastic tissue culture plate with a lid (Cellstar, greiner bio-one) filled with 0.2 ml of each bacterial suspension. A negative control was used containing sterile broth without bacteria. (Perez and Barth, 2011).

The plates were covered and incubated aerobically for 24 hours at 37°C. After incubation, the content of the wells was aspirated and each well was washed 3 times with 0.25 ml sterile physiological saline. The plate was vigorously shaken to remove all non-adherent bacteria.

The remaining attached bacteria were fixed using 0.2 ml of absolute methanol per well for 15 minutes followed by methanol removal and air drying.

The plates were stained for 5 minutes with 0.2 ml of 0.2% Hucker crystal violet solution. Excess stain was rinsed with running tap water. The plates are allowed to air dry.

The dye bound to the adherent cells was resolubilized with 160 µl of 30% (v/v) glacial acetic acid. The optical density (OD) of each well was measured at 590 nm using automated plate reader.

The cut-off OD (ODc¬) were calculated as three standard deviations above the mean OD of the negative control wells. Results were interpreted as follow: (Stepanovic et al; 2007).

$OD < OD_c$	Non-adherent and no biofilm formation
$OD_c < OD < 2*OD_c$	Weakly adherent
$2*OD_c < OD < 4*OD_c$	Moderately adherent
$4*OD_c < OD$	Strong adherent

RESULTS

Table. (1): Distribution of the studied cases according to diagnosis (n=40).

Diagnosis	No.	%
CRS	20	50.0
OE	20	50.0

Table. (2): Distribution of the studied organisms for CRS cases (n=20).

CRS	No.	%
Organism		
Staph Aureus	3	15.0
MRSA	10	50.0
Citrobacter	1	5.0
Klebsiella	2	10.0
Strept	1	5.0
E-coli	1	5.0
Staph. Epidermidis	2	10.0

Table. (3): Distribution of the studied organisms according to level of biofilm for CRS cases (n=20).

CRS	No.	%
Biofilm		
Non	9	45.0
Weak	5	25.0
Moderate	4	20.0
Strong	2	10.0

Table. (4): Distribution of the studied organisms for OE cases (n=22).

OE	No.	%
Organism		
Pseudomonas	10	45.5
Proteus	6	27.3
Diphteroid	1	4.5
Staph .Aureus	4	18.2
MRSA	1	4.5

Table (5): Distribution of the studied organisms according to level of biofilm for OE cases (n=22).

OE	No.	%
Biofilm		
Non	3	13.6
Weak	12	54.5
Moderate	7	31.8

Table. (6): Relation between level of biofilm and organism for CRS cases (n = 20).

CRS	Biofilm								χ^2	^{MC}p
	No (n=9)		Weak (n=5)		Moderate (n=4)		Strong (n=2)			
	No.	%	No.	%	No.	%	No.	%		
Organism										
Staph Aurous	2	22.2	0	0.0	0	0.0	1	50.0	16.445	0.845
MRSA	4	44.4	3	60.0	2	50.0	1	50.0		
Citrobacter	0	0.0	1	20.0	0	0.0	0	0.0		
Klebsiella	0	0.0	0	0.0	2	50.0	0	0.0		
Strept	1	11.1	0	0.0	0	0.0	0	0.0		
E-coli	0	0.0	1	20.0	0	0.0	0	0.0		
Staph. Epidermidis	2	22.2	0	0.0	0	0.0	0	0.0		

Table. (7): Relation between level of biofilm and organism for OE cases (n = 22).

OE	Biofilm						χ^2	^{MC}p
	No (n=3)		Weak (n=12)		Moderate (n=7)			
	No.	%	No.	%	No.	%		
Organism								
Pseudomonas	0	0.0	6	50.0	4	57.1	9.230	0.286
Proteus	0	0.0	4	33.3	2	28.5		
Diphteroid	1	33.3	0	0.0	0	0.0		
Staph .Aureus	1	33.3	2	16.6	1	14.2		
MRSA	1	33.3	0	0.0	0	0.0		

χ^2, p: χ^2 and p values for **Chi square test.**
^{MC}p: p value for **Monte Carlo** for Chi square test.

DISCUSSION

Forty samples have been collected in karmouz health insurance hospital, collected between 11/2016 to 4/2017. Twenty cases of CRS and twenty cases of OE.

Results shows different organisms in CRS and different organisms in OE with two cases with mixed infection.

Most common isolates from CRS patients were MRSA and staphylococcus aureus representing 65% of isolates.

In our study biofilm forming bacteria in CRS were found in (11 of 20) about 55% using microtiter plate assay.

Another study, in the largest series to date, (Prince et al.; 2008) did not directly observe the presence of biofilms in patient samples but instead assessed the ability of bacteria recovered from CRS patients to grow biofilms in vitro using the Calgary biofilm assay. Of 157 samples obtained in a tertiary rhinology clinic, they noted a biofilm formation rate of 28.6%.

Speciation of the cultures showed that S. aureus was the most commonly isolated organism (33%), but that 20% of patients had either purely pseudomonal infections or poly-microbial infections containing P. aeruginosa.

In a prospective study by (Psaltis et al.; 2007) 40 patients undergoing FESS for CRS had mucosal samples taken intraoperatively and were analyzed for mucosal biofilms using CLSM showing 50% of their study population with evidence of biofilms (20/40).

In another study by (Sanderson et al.; 2006) used CLSM and FISH analysis to examine intraoperative samples taken from 18 patients with CRS and five controls undergoing septoplasty. The analysis found 78% (14/18) of patients with detectable bacteria in a biofilm matrix.

(Ferguson and Stolz ;2005) used TEM in conjunction with bacterial cultures to show biofilms on 50% (2/4) of patient samples taken intraoperatively in presumed CRS, both of which grew out P. aeruginosa.

The other 2 patients were discovered to have a nonbacterial etiology to the CRS. (Ramadan et al; 2005) obtained intraoperative samples from the ethmoid bullae from five patients undergoing functional endoscopic sinus surgery (FESS), all of which showed morphological criteria of biofilms on SEM. The mucosal surface of all specimens also showed various degrees of abnormality that ranged from disarrayed cilia to a complete absence of cilia and goblet cells.

A follow-up study by (Sanclement et al; 2005) observed biofilms in 80% (24/30) of patients.

The discrepancy in the above-mentioned results might actually exist, or could be a result of the different detection methods used and/or differences in the patient populations studied. Furthermore, this inconsistency of data could be due to the fact that the collection of small samples was not representative of the entire sino-nasal cavity, Regardless of these discrepancies, the consistent demonstration of biofilms on the sino-mucosal samples of patients with CRS suggests that these complex structures might play a role in either the pathogenesis or persistence of chronic rhinosinusitis.

On the other hand, we will discuss biofilm in Otitis Externa.Using microtiter plate assay, most common isolates from OE patients were Pseudomonas 45% and Proteus 27% and staphylococcus Aureus 22.7%.

In another study ,microbiological results for OE swabs shows that the incidences of Pseudomonas has varied from a low 12% to a high of 80% and the incidence of staphylococcus has been reported as low as 8.5% and as high as 29%. (Stroman et al; 2001) (Agius et al; 1992).

In our study biofilm were identified in about 19 of 22 isolates representing 86.3% of chronic otitis externa patients. Biofilms were identified in 23 of the 25 patients (92%) with chronic otitis externa. On the contrary, in the acute otitis externa group, biofilms were isolated only in three cases (20%). The difference in frequency was significant from a statistical point of view. This evidence suggests that biofilm is a determining factor in the development and maintenance of chronic external otitis. (Fusconi et al.:2011).

CONCLUSION

Bacterial biofilms are highly organized structures composed of communities of bacteria encased within a protective extracellular matrix. Bacterial biofilms represent one of many possible etiologies for the occurrence and persistence of inflammation in CRS and OE.

RECOMMENDATIONS

Ongoing investigation into biofilm treatment and management is needed. New treatments including surgery, topical antibiotics, surfactant therapy, and disruption of quorum sensing mechanism are being evaluated worldwide.

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