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The Role of Biofilms Produced by *Streptococcus Pneumoniae* Isolated from Patients with Upper Respiratory Infections on Avoiding Innate Immunity

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Abstract:

Phagocytosis has been recognized as an important mechanism of the human innate immune response against infections .The aim of this experiment is to test the effect of biofilms produced by *Streptococcus pneumoniae* isolates as a cause agent of serious infections isolated from children in some Iraqi patients on avoiding some innate immunity aspects *in vivo*.

Twenty five isolates obtained from sputum of children diagnosed with upper respiratory infections. Two methods were used to detect the ability of biofilms formation, Congo red agar and Tissue Culture Plate (CRA,TCP) on the other hand the capacity to resist some innate immunity mechanisms was evaluated by testing the Percentage of killing, Opsonization factor and phagocytic index.

The results revealed different ability of isolates to form biofilms. 18(72%) producer by CRA, 16(64%) by TCP while 5(20%) weak by CRA and 9(36%) weak by TCP which reflected the different ability to affect by innate immunity as showed in the statistical analysis findings (P-value 0.05) that some isolates appeared strong ability to produce biofilms resisted the innate immunity mechanisms such as isolate numbered 22 showed 32% Bactericidal assay, 20% opsonization and 28% phagocytic index, while some others was sensitive and killed by innate immunity cells.

From the results of this study it can be concluded that the *Streptococcus pneumoniae* isolates differed in their ability to form biofilms, the important factor in the avoiding some innate immunity aspects and high resistant.

Keywords: *Streptococcus pneumoniae, Biofilms, Innate immunity.*

دورالأغشية الحيوية المنتجة من المكورات المسبحية الرئوية المعزولة من مرضى إصابات الجهاز التنفسي العلوي في تجنب المناعة المتأصلة

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الخلاصة:

عرفت عملية البلعمة كآلية مهمة للإستجابة المناعية المتأصلة ضد الإصابات. الهدف من هذه الدراسة هو إختبار تأثير الأغشية الحيوية المنتجة من قبل عزلات المكورات المسبحية الرئوية كعامل مسبب لأمراض خطرة.

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تم عزل هذة البكتريا من الأطفال لدراسة تأثير الأغشية الحيوية من التملص من بعض مظاهر الإستجابة المناعية المتأصلة خارج الجسم الحي. عشرون عزلة هو ما تم الحصول عليه من قشع أطفال شخصوا كمصابين بأصابات الجهاز التنفسي العلوي. أستخدمت طريقتان في هذه الدراسة لتحديد القابلية لتكوين الأغشية الحيوية هما طريقة أكار الكونغو الأحمر وطريقة الزرع على الأطباق.

من ناحية أخرى قيمت قابلية مقاومة بعض آليات الإستجابة المناعية المتأصلة من خلال قياس النسبة المؤية لعامل القتل وعامل الطهي وكذلك معامل البلعمة.

أظهرت النتائج قابلية مختلفة لإنتاج الأغشية الحيوية من قبل العزلات تمثلت بعدد ونسبة مؤية مقدارها 18 (72%) بطريقة الكونغو، بلغت 64 (64%) بطريقة الزرع كعزلات منتجة. بينما كانت5 (20%) بطريقة الكونغو و9(36%) كعزلات ضعيفة في إنتاج الأغشية الحيوية والتي عكست قابلية مختلفة للتملص من الإستجابة المناعة المتأصلة وكما ظهر في نتائج التحليل الإحصائي وبمستوى معنوية (0.05) أظهرت بعض العزلات ذات الانتاجية العالية للأغشية الخلوية مقاومة كبيرة لبعض آليات الاستجابة المتأصلة مثل العزلة المرقمة (22) والتي أظهرت مقاومة للقتل تمثل بعامل قتل بلغ 32% كما أظهرت مقاومة لعملية الأبسنة إذ بلغ 20% ومعامل بلعمة بلغ 28% بينما لوحظ أن بعض العزلات كانت حساسة للمقاييس السابقة وقتلت بواسطة خلايا المناعة المتأصلة.

الكلمات المفتاحية: المكورات المسبحية الرئوية، الأغشية الحيوية، المناعة المتأصلة.

Introduction:

Streptococcus pneumoniae is an important human respiratory pathogen that causes a variety of serious diseases such as community-acquired pneumonia, meningitis and sepsis [1]. It is also the main causal agent of otitis media in children[2].

Pneumococcal biofilms have recently detected on the surface of adenoid and mucosal epithelial tissues in children with recurrent middle-ear infections and otitis media with effusion [3,4].

The dispersal and growth of microbes, whether environmental or pathogenic, commonly involve the production of biofilms, that the primary mode of pneumococcal growth during colonization, recurrent otitis media, and the early stages of invasive disease [5,6].

Biofilms are defined as structured bacterial communities enclosed in a self-produced exopolysccharide matrix and adherent to abiotic orbiological surfaces [7]. Biofilm is characterized by the cells that are irreversibly attached to a substratum or to each other. Bacteria seem to initiate biofilm in response to specific some environmental conditions such as nutrient and oxygen availability [8].

Biofilm has an importance of protecting and saving the bacteria from host immune system and antibiotic treatment, creating a source of toxic metabolites and persistent infection, as well as facilitate the exchange of antibiotic-resistant genetic material[9].

Intact mucosal surfaces as well as mucosal immune responses are the first line of defense against *S. pneumoniae* infections[10]. Additional components of the first nonspecific barrier are antibacterial peptides, ciliated cells and the simultaneous action of mucus[11]. The secretory IgA is the most important factor preventing pneumococcal carriage. This process of mucosal immunity is relatively immature in young children[10].

Pneumococci are autolytic bacteria releasing DNA containing unmethylated CpG motifs which has shown to be recognized by toll like receptor-9 within endosomes [12,13].

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The aim of this study is to determine the ability of *S. pneumoniae* formed Biofilm to avoid some innate immune system aspects.

Materials and Methods:

Bacterial isolates:

This study included thirty isolates obtained from some Baghdad hospitals. The isolates were isolated from sputum of children diagnosed with upper respiratory infections by physician *S. pneumoniae* isolates were routinely on blood agar plates cultured.

Animals:

Sixty of BALB/c male mice, age (6-8) week, weight (20-25) gm used in this study. The animals were obtained from Drug Control Center and kept in animal house in the Science Collage of Al-Mustansiriyah University, Baghdad, Iraq.

Biofilms formation detection:

Congo red agar method (CRA):

Plates were inoculated by pure single isolated colony and incubated aerobically for 24-48 hr at 37°C, Positive result was indicated by black colonies with a dry crystalline consistency. The weak slime producers usually remained pink, though an occasional darkening at the centers of the colonies was observed. A darkening of the colonies, with the absence of a dry crystalline colonial morphology, indicated an indeterminate result [14].

Tissue Culture Plate Method (TCP):

The assay was performed in triplicate using 96-well flat-bottomed cell culture plates (Nunc, New York, NY, USA) as described in[15]. 10 ml of Trypticase soy broth with 1% glucose was inoculated with a loopful of tested organism from overnight culture on nutrient agar. The broth was incubated at 37°C for 24 hours. The culture was further diluted 1:100 with fresh medium. 96 flat bottom wells tissue culture plates were filled with 0.2 ml of diluted cultures individually. Only sterile broth was served as blank. Similarly control organisms were also diluted and incubated. All three controls and blanks were put in the tissue culture plates.

The culture plates were incubated at 37°C for 24 hours. After incubation, gentle tapping of the plates was done. The wells were washed with 0.2 ml of phosphate buffer saline (pH 7.2) four times to remove free floating bacteria. Biofilms which remained adherent to the walls and the bottoms of the wells were fixed with 2% sodium acetate and stained with 0.1% crystal violet. Excess stain was washed with deionized water and plates were dried properly.

Optical densities (OD) of stained adherent biofilms were obtained with a micro ELISA auto reader at wave length 570 nm. Experiment was performed in triplicate and repeated thrice. Average of OD values of sterile medium were calculated and subtracted from all test values.

Animal Infection Experiments:

Experiment of animal infection was as follows:

Mice were divided into 25group according to the results of biofilms production obtained of *S. pneumoniae* isolates. Each group contains 2 mice, In addition to 10 mice were injected intraperitonial as a control, the isolates was activated in brain heart infusion broth and incubated at 37°C for 24 hr, the activated isolates were diluted and compared with the turbidity of Macfarland solution to obtained the concentration of cells 1.5 X 108 cell per ml, each mouse was administrated with 0.2 ml of bacteria. Three days after infection all animals were sacrificed by cervical dislocation to obtain the blood by heart puncture, the serum was isolated.

Opsonization assay:

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Detection of opsonic antibodies was done by measuring opsonization activity *in vitro* according to [15].

A mixture of 0.05 ml suspension of activated bacteria, 0.05 ml serum from animals under study and 0,2 ml blood of non-immune human in sterile tube, incubited for 45 min at 37 °C in shaking incubator. After that made a slides stained with Giemsa stain, calculate 50 cells of polymorph nuclear cells (PMNs) to extracted percentage for opsonization and compared to control using this formula:

Opsonization factor = [No. of phagocytic PMNs/No. of phagocytic and nonphagocytic PMNs] X 100%.

Bactericidal assay:

Bactericidal assay was done as following:

0.5 ml of overnight culture of bacteria was added to 0.1 ml serum from animals under study and 0.35 ml blood from human then incubate the mixture for 3 hr. with shaking in shaker incubator. 0.1ml of mixture was placed on sterile dish, and then blood agar was added; incubate in 37°C for 24 hr. The experiment was repeated with non-immunized serum as a control[16].

Percentage of killing= [No. of micro-organism in dish contain ml of non-immune serum – No. of microorganism in dish contain ml of serum under study/No. of microorganism in dish contain ml of non-immune serum X 100%[16].

Phagocytosis:

Phagocytes were performed according to [17] with some modification. The blood should be used in (1-2) hr. after collection to ensure the activity of phagocytic cell and kept in anticoagulant tube. The isolates were inoculated in brain heart infusion broth for 24 hr in 37 ℃ after that the isolates were inoculated in microtiter plate to study phagocytosis on biofilm once and remain the same isolates in brain heart infusion broth for planktonic cell. Blood was centrifugation in 3000 rpm for 5 min, (take the supernatant plasma) to complete the experiment. (1-3) ml of plasma was mixed with 1ml of bacterial suspension (1*108) cell/ml in normal saline for planktonic, and 1ml on microtiter plate and the mixture placed on shaking incubator at 37 °C for 30 min. After incubation, one drop was taken and placed on clean glass slide, stained with Giemsa stain and calculate the number of phagocytic cell using this formula:

Phagocytic index = [No. of phagocytic cell/ No. of 100 cell of phagocytic and non-phagocytic cell] X 100 %.

Statistical analysis:

Statistical analyses were performed using a two-tailed Student's t-test. Values were determined tobe statistically significant if the $P \leq 0.05$.

Results and Discussion:

Biofilms formation detection:

Streptococcus pneumoniae is the leading cause of death in children worldwide and forms highly organized biofilms in the nasopharynx, lungs, and middle ear mucosa[18]. The capability of producing biofilms in vitro of all the *S. pneumoniae* strains investigated so far whether they are invasive isolates or normal-flora strains [19]. Two methods were used to evaluate biofilms formation (CRA and TCP) as shown in table-1.

The results of CRA showed that 72% of isolates were positive of biofilms formation, 20% negative production and 8% were non-producers. While the TCP raveled that 64% was determined as strong and 36% as weak for biofilms formation as shown in table-2.

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Table-1: Ability of biofilms production of *Streptococcus pneumoniae* isolates.

Isolate No.	CRA Method	TCP Method
1	Positive	Strong
2	Positive	Strong
3	Positive	Strong
4	Positive	strong
5	negative	weak
6	Positive	Strong
7	negative	weak
8	negative	weak
9	Positive	Strong
10	Positive	Strong
11	Positive	Strong
12	Non-identified	Weak
13	Positive	Weak
14	Positive	Strong
15	Positive	Strong
16	Positive	Strong
17	Positive	Strong
18	negative	Weak
19	Positive	Strong
20	Positive	Weak
21	negative	Strong
22	Positive	Strong
23	Positive	Strong
24	Non-identified	Weak
25	Positive	Weak

Table-2: Number and percentage of *Streptococcus pneumonia* isolates formed biofilms (prevalence).

Biofilms production	CRA Method		TCP Method	
	No. of isolates	Percentage of isolates	No. of isolates	Percentage of isolates
producer	18	72%	16	64%
weak	5	20%	9	36%
Non-producer	2	8%	-	-

TCP method can be represented as general procedure for biofilms detection comparing with CRA method due to more quantitative, most reliable and easy method[20]. The mechanism by which *S. pneumonia* isolates colonize and persist in the nasopharynx is still incompletely understood[18]. Molecular factors, including virulence factors, have been implicated in biofilm formation of *S. pneumoniae*.

Moscoso *et al*.[21] found that the amidases LytA, LytC, and LytB and adhesins such as CbpA, PcpA, and PspA play some role in *S. pneumoniae* biofilms. The process by which biofilm formation are initiated is a complex then attach to a surface of microorganisms or interface that is embedded in an extracellular matrix composed of various polymeric substances[22]. Biofilm structure protect the bacterium from environmental diversity and contribute in the resistance to antimicrobial agents and the immune response of the host[23].

Innate immunity parameters (Opsonization assay, Phagocytosis and Bactericidal activity):

Three parameters (Opsonization assay, Phagocytosis and Bactericidal activity) were tested to evaluate the effect of biofilms formed by different isolates of *streptococcus pneumoniae* on innate immunity *in vivo* and the relationship between the capacity to avoid and evasion of these isolates and different ability (producers, weak and non-identified) of formation biofilms as shown in table-3.

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Table-3: Biofilms production and innate immunity parameters (Opsonization assay, Phagocytosis and Bactericidal activity)

No. of isolates	Biofilm production CRA method	Biofilm production TCP method	No. of animal	bactericidal activity	Opsonization	Phagocytosis
1	P	S	1	50%	41%	20%
			2	48%	33%	22%
2	P	S	1	61%	29%	18%
			2	63%	35%	24%
3	P	S	1	53%	48%	29%
			2	47%	32%	23%
4	P	S	1	44%	21%	18%
			2	55%	32%	20%
5	N	W	1	90%	65%	55%
			2	72%	71%	49%
6	P	S	1	61%	41%	33%
			2	56%	38%	22%
7	N	W	1	60%	22%	13%
			2	43%	27%	15%
8	N	W	1	90%	70%	17%
			2	78%	71%	12%
9	P	S	1	75%	65%	50%
			2	66%	33%	46%
10	P	S	1	66%	45%	49%
			2	44%	48%	43%
11	P	S	1	33%	37%	62%
			2	45%	21%	66%
12	Non	W	1	80%	63%	50%
			2	78%	57%	41%
13	P	W	1	67%	52%	54%
			2	65%	61%	63%
14	P	S	1	40%	28%	22%
			2	37%	18%	17%
15	P	S	1	48%	33%	27%
			2	52%	40%	33%
16	P	S	1	69%	51%	35%
			2	70%	43%	34%
17	P	S	1	33%	31%	18%
			2	44%	35%	14%
18	N	W	1	76%	59%	53%
			2	82%	64%	46%
19	P	S	1	44%	47%	39%
			2	50%	43%	33%
20	P	W	1	90%	66%	60%
			2	83%	62%	66%
21	N	S	1	77%	58%	47%
			2	69%	62%	45%
22	P	S	1	35%	22%	23%
			2	29%	18%	33%
23	P	S	1	50%	39%	29%
			2	45%	38%	23%
24	N	W	1	78%	73%	60%
			2	88%	77%	55%
25	Non	W	1	69%	66%	61%
			2	80%	72%	55%

P=Producer, N=negative, S=Strong, Non=Non-identified, W=Weak

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As was showed in the tables-1 and 2, the isolates of *streptococcus pneumoniae* isolates appeared different ability of biofilms formation.

The findings of innate immunity parameters revealed different results, most negative, weak and non-identified isolates showed higher percentage of bactericidal activity in compared with strong or producer isolates. Similarly to bactericidal activity the results of Opsonization and phagocytosis obtained showed that the biofilms formed isolates have the ability to resist killing effect of innate immunity comparing with weak and non-producer isolates in the current experiment.

Depending on the results of immune response parameters tested, the isolates divided into two groups, the first which include weak and negative biofilms producer isolates appeared low resistance to innate immunity (5, 7, 8, 12, 13, 18, 20, 24, 25) as it noticed from the percentage of bacteri-cidal activity, opsonization and phago-cytosis. The rest of isolates showed resistance to the three immune response parameters tested which is represented the second group that include strong biofilms formation isolates.

To find out if there is a significant difference between the first groups of isolates, statistical analysis was done, in general no significant differences. On the other hand the differences among isolates of the second group showed slightly differences.

The isolate numbered 20 showed the higher percentage of bactericidal activity among the first group isolates, so it was compared with all isolates, the finding obtained that there was a significant difference with all isolates of the second group (table-4).

Table-4: Statistical analysis of comparing isolate numbered (20) with all isolates (bactericidal activity).

Isolates	N	Mean (%)	Std.	P-value (0.05)
20	2	86.5	4.94	
1	2	49	1.41	0.009
2	2	62	1.41	0.02
3	2	50	4.24	0.01
4	2	49.5	7.77	0.02
5	2	81	12.72	0.6
6	2	58.5	3.53	0.02
7	2	51.5	12.02	0.009
8	2	84	8.48	0.7
9	2	70.5	6.36	0.1
10	2	55	15.55	0.009
11	2	39	8.48	0.02
12	2	79	1.41	0.17
13	2	66	1.41	0.02
14	2	38.5	2.12	0.006
15	2	50	2.82	0.01
16	2	69.5	0.7	0.04
17	2	38.5	7.77	0.01
18	2	79	4.24	0.24
19	2	47	4.24	0.01
21	2	73	5.65	0.12
22	2	32	4.24	0.007
23	2	47.5	47.5	0.01
24	2	83	7.07	0.6
25	2	74.5	7.77	0.2

N=2

Regarding of Opsonization assay, the results revealed that the isolate numbered 24 showed (member of first group andnon-identified by CRA, weak by TCP of biofilms) a high percentage of opsonization factor, so it was represented as a standard to compare with all other isolates, table-5 showed that there are a no significant differences in comparing with isolates of first group, while the differences were not significant comparing with the second group.

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Table-5: Statistical analysis of comparing isolate numbered (24) with all isolates (opsonization).

Isolates	N	Mean (%)	Std.	P-value
24	2	75	2.82	
1	2	37	5.65	0.01
2	2	32	4.24	0.006
3	2	40	11.31	0.05
4	2	26.5	7.77	0.01
5	2	68	4.24	0.19
6	2	39.5	2.12	0.004
7	2	24.5	3.53	0.003
8	2	70.5	0.7	0.15
9	2	49	22.62	0.2
10	2	46.5	2.12	0.007
11	2	29	11.31	0.03
12	2	60	4.24	0.05
13	2	56.5	6.36	0.06
14	2	23	7.07	0.01
15	2	36.5	4.94	0.01
16	2	47	5.65	0.02
17	2	33	2.82	0.004
18	2	61.5	3.53	0.05
19	2	45	2.82	0.008
20		2	64	0.05
21	2	60	2.82	0.03
22	2	20	2.82	0.002
23	2	38.5	0.7	0.003
25	2	69	4.24	0.2

N=2

Table-6: Statistical analysis of comparing isolate numbered (24) with all isolates (phagocytosis).

Isolates	N	Mean (%)	Std.	P-value
13	2	58.5	6.36	
1	2	21	1.41	0.01
2	2	21	4.24	0.02
3	2	26	4.24	0.02
4	2	19	1.41	0.01
5	2	52	4.24	0.3
6	2	52.5	7.77	0.4
7	2	14	1.41	0.01
8	2	14.5	3.53	0.01
9	2	48	2.82	0.1
10	2	46	4.24	0.1
11	2	64	2.82	0.3
12	2	45.5	6.36	0.1
13	2	19.5	3.53	0.01
14	2	30	4.24	0.03
15	2	34.5	0.7	0.03
16	2	16	2.82	0.01
17	2	49.5	4.94	0.2
18	2	36	4.24	0.05
19	2	63	4.24	0.4
20	2	46	1.41	0.1
21	2	28	7.07	0.04
22	2	26	4.24	0.02
23	2	57.5	3.53	0.8
25	2	58	4.24	0.9

N=2

The statistical analysis was done on the results of phagocytosis to discover if the effect of differences of biofilms production of isolates on phagocytosis. The isolate 13 regarded as standard isolate to compare with other isolates number significant effect was observed in the phagocytic index between the isolate 13 and the isolates of first group , while the results all was significant when compared with isolates of second group (Table-6).

Opsonization is the second step of phagocytosis, it is activated by antigen-antibody complex .The enhancement of phagocytosis occur by opsonization. It is well known that the complement system represents the first lines of defense against invading pathogens such as *S. pneumoniae* and plays a vital role in both innate and acquired immunity[24].

Phagocytosis and complement activation avoidance is a common immune evasion strategy used by pathogens to allow long-term colonization.

The importance of biofilm formation done by some micro-organisms in the evasion of the host immune response is reported [25]. The formation of biofilms on a surface depends on multiple factors including attachment, intercellular interactions, chemotaxis, carbon sensing, and stress response[26].The fact that over half of all bacterial infections are thought to involve the biofilms[27].

Streptococcus pneumoniae is a cause of bacterial pneumonia, meningitis, and sepsis in children resulting in rates of morbidity and mortality worldwide[28]. Pneumococcal disease prevention required efficient recognition and clearance of the invading by the professional phagocytes and complement system [29].

Phagocytosis means the engulfment of invading microorganisms by monocytes, macrophages, dendrite cells and fibroblast[30]. En Opsonization makes an antigen more susceptible to phagocytic cells, particleattaches to protein to increase phago-cytosis is called opsonin[31].

The Gram-positive species *S. pneumoniae* are still caused of a global public health care problem[28,32].

The bacterial growth on blood agar revealed that bacteria resisted the phagocytosis.The importance of biofilm formation has been reported by several studies that showed theevasionof microorganisms from the host immune-response[33]. The novel role of the innate immunity has been recognized by phagocytic cells. The first line function of phagocytes such as neutrophils and macrophages in host is the innate immune defense which has been understood to reflect a variety of potent against intracellular microbicidal mechanisms[34]. Biofilm growth might promote the evolution of cooperative resistance mechanisms, such as extracellular enzymes that degrade antibiotics, which are not stable in planktonic cultures[35].

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