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Biofilm Formation of Staphylococcus Aureus in Multiple Sclerosis Patients and its Essential Role in the Pathogenicity of the Disease

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Abstract

Background: Multiple sclerosis (MS) is a chronic inflammatory, autoimmune neurological disease of the central nervous system (CNS). One of the most important factors that lead to increased bacterial resistance to an antibacterial agent and also increase the severity of multiple sclerosis, is the capability of producing enterotoxins and biofilm.
Aim: To investigate the biofilm-forming Staphylococcus aureus and its correlation with multiple sclerosis severity.
Methods: one hundred nasal swab segments from "multiple" "sclerosis" patients and 100 from controls. Staphylococcus aureus was cultured on blood agar, nutrient agar, mannitol salt agar, biochemical tests, and biofilm assay tests were done.
Results: A case-control study including (81%) of MS patients were colonized with Staphylococcus aureus in the nasal cavity while only (12%) were colonized in controls, By using the crystal violet microtiter plate method, all Staphylococcus aureus isolates in multiple sclerosis patients can produce biofilm depending on cutoff point (0.171) that measured by ELISA technique, out of 81% isolates from multiple sclerosis patients there were 97.5%, strong biofilm producers, while only 2.5% of isolates were weak biofilm producers.
Conclusions: All isolates produce biofilm in high density correlated with increased antibiotic resistance of isolates and increase disease severity.

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Keywords: Multiple sclerosis Staphylococcus aureus; Biofilm formation.

1. Introduction

Multiple sclerosis (MS) is a disorder that impacts the central nervous system leading to a broad range of possible symptoms such as visual problems, leg and arm movements, balance, and sensations. It's an enduring disorder that may occasionally result in severe disabilities, although it may sometimes become mild, in MS the immune system pounds the protecting sheath (myelin) which surrounds nerves fibers with transmission issues generated between the brain and other parts of the body. Ultimately, this disorder may induce permanent injury or nerve degenerations [1]. The major clinical manifestation of MS is uncertain that includes any parts of the body but the primary symptoms are considered the most frequent and include sensory disturbance, walking difficulty, vision problems, urinary system dysfunctions, cognitive with emotional impairments as well as sexual disorders. An accurate MS diagnosis depends on medical history with neurological assessment by the use of imaging examinations e.g., magnetic resonance imaging (MRI), lumbar puncture (LP) to analyze the cerebrospinal fluid (CSF) as well as blood examination [2]. It was suggested that Staphylococcus aureus in the nasal canal is due to different autoimmune disorders including rheumatoid arthritis, systemic lupus erythematous and Wegener's granulomatosis syndrome via super antigens like toxic shocks syndrome toxin-1[tsst-1] and several staphylococcus enterotoxins [SE] [3,4]. Staphylococcus aureus possesses a system of virulence factors. Such factors allow the microorganism to succeed in causing a broad range of animal and human illnesses. The virulence factors aid in attaching to the cells of the host and break down the host's immune shields, tissue attack leading to sepsis formation and eliciting toxin-mediated syndrome. This is the principle of continuous infections by staphylococcus without strong responses by the host's immune system. Founded on their mechanism of actions and roles in pathogenicity [5,6]. The microorganism can enter the blood stream and disseminate systemically to other organs resulting in sepsis. Such hematogenous spread can lead to osteomyelitis, endocarditis, kidney carbuncles, epidural abscesses, and septic arthritis. Without bloodstream infections, specific syndromes may happen because of the extracellular staphylococcal toxins. These syndromes include toxic shock syndromes, scalded skin syndromes as well as foot-borne gastroenteritis [7]. Staphylococcus aureus can cause a multilayered biofilm implanted within a slime layer or glycocalyx with heterogeneous protein expression throughout. Preliminary investigations indicated the solid part of the glycocalyx is mostly comprised of Staphylococcal, teichoic acids (80%), and host proteins [8]. The capability of Staphylococcus aureus for biofilm formation may promote the organism's persistence in contaminated surfaces or infection zones.

Nomenclature			
Agr	accessory gene regulator	MDR	Multiple drug resistance
CLSI	Clinical and Laboratory Standards Institutes	MRI	magnetic resonance imaging
CNS	central nervous system	MRSA	Methicillin-resistant Staphylococcus aureus
CSF	cerebrospinal fluid	MS	Multiple sclerosis
DDT	disk diffusion test	O.D	optical density
ELISA	enzyme-linked immunosorbent assay	R.R.MS	Relapsing-Remitting Multiple Sclerosis
HS	Highly Significant	SEs	staphylococcal enterotoxins
LP	lumbar punctures	tsst-1 toxic	shock syndrome toxin-1

Within the biofilms, the bacteria exhibit high tolerance to phagocytosis, antibiotics, and disinfectants and permit the capture of nutrients like phosphate, nitrogen, and carbon, which are important for the cell [9]. Detachment of Staphylococcus aureus biofilm is controlled via the quorum-sensing system accessory gene regulator (Agr).

2. Materials and methods

2.1. Specimen collection

One hundred nasal swabs specimens from patients with multiple sclerosis as well as 100 controls were obtained in a period from December 2021 to February 2022 from the Multiple Sclerosis" Clinic Baghdad teaching hospital In Medical City.

2.1.1. Culture media of samples

The preparation of all culture media was done as specified by the guidelines of the manufacturer; the autoclave at 121^{o}C (15 Ib/In2) for 15 minutes was used to sterilize the media, which was then incubated at 37°C for 24 hours to confirm sterility, then placed at (4°C) until use including 1-Blood agar medium, 2-Muller-Hinton Agar, 3-Nutrient broth, 4- Mannitol salt agar, 5-Luria-Bertani broth.

2.1.2. Biochemical tests

The catalase test was done on all isolates to differentiate between staphylococci and streptococci genus staphylococci were positive and streptococci were negative. Coagulase tests were done to exclude staphylococcus species other than S.aureus and all species were coagulase-negative except S.aureus [9].

2.1.3. Biofilm formation detection

The assay of a quantitative microtiter plate for biofilms formation with slight modifications was carried out as demonstrated by Shanmugaraj Gowrishankar [10].

2.1.4. Antimicrobial susceptibility tests

Staphylococcus aureus isolates tested to different antibiotics were determined by disk diffusion test (DDT) by the Clinical and Laboratory Standards Institutes (CLSI), resistance pattern of isolates was very high in multiple sclerosis patients.

2.2. Statistical analysis

The data of this study were analyzed by using the SPSS version (22.0) to analyze and assess the results of the study for the determination of correlations between biofilm formation, antibiotic resistance, and progression of multiple sclerosis courses. P-values equal to or less than 0.05 were regarded as statistically significant.
Inclusion criteria were 100 patients diagnosed with multiple sclerosis, and 100 as randomly healthy controls, while exclusion criteria included immunocompromized patients, pregnant women, having chronic diseases like diabetes, and taking immunosuppressive drugs.

3. Results

3.1. Duration of multiple sclerosis disease

Results of this study showed that patients of newly diagnosed multiple sclerosis (MS) stage were focused on the first and second intervals (< 1 year & 1-5 years), they are accounted for (60%) and (40%) respectively, while the result of relapsing-remitting (MS) stage was focused on the second and third intervals (1-5 years and >5 years) and they are accounted (48%) and (46%) respectively,
as well as results of both stages were recorded with highly significant differences at P<0.01value, indicating that duration of disease has a strong relationship with the stage of disease, Table 1.

Table 1 Distribution of MS disease duration between stages of the disease

Disease Duration	No. %	Disease stage		Total	P-value
		Newly Diagnosed Ms	R.R. Ms		
< 1 yr.	No. %	30(60%)	3(6%)	33(33%)	P=0.000
1 - 5	No. %	20(40%)	24(48%)	44(44%)	
> 5 yrs	No. %	0(0%)	23(46%)	23(23%)	
Total	No. %	50(100%)	50(100%)	100(100%)	

(*) HS: Highly Sig. at P<0.01; Testing based on Contingency Coefficient test.

3.2. Implication of S.aureus

The results revealed that *S.aureus* colonization in the nasal carriage of patients with multiple sclerosis was 81% while the ratio of *S.aureus* in the control group was only 12% demonstrating statistically meaningful differences in the commonness of colonization between them (P= 0.00), as displayed in a Table 2.

Table 2 Frequency of Staphylococcus aureus in all study groups

All study groups	No. of collected samples	S. aureus positive(No %)	P-value
Control	100	12(12%)	P=0.0 (HS)
MS group	100	81(81%)	

(*) HS: Highly Sig. at P<0.01; S: Sig. at P<0.05; Testing based on Contingency Coefficient test.

3.3. Biofilm formation of S.aureus isolates

3.3.1. Biofilm measurement

In the present study, concerning the measurement of optical density (o.d.) of biofilm by ELISA reader, the results showed that the estimated cutoff point was equal to (0.171) nanometer at a highly significant *P*=<0.01, Table 3.

Table 3 Biofilm measurement optical density in ELISA reader

Cutoff Point	Maximum control O.D.	Maximum MS O.D.
0.171	0.576	0.171

3.3.2. Frequency of biofilm production in all study groups

Regarding the cutoff point of biofilm measurement (0.171) was estimated by ELISA reader at optical density OD was 595, any OD value less than 0.171 the isolate was supposed weak biofilm producer whereas any OD equal or higher than cutoff value was considered strong biofilm producers, as a consequence, there were only 2(2.5%) isolates from overall 81(100%) *S.aureus* positive of MS patients were considered weak biofilm producers while a total of 12 (12%) of controls were weak biofilm producers indicating highly significant differences in biofilm production between *S.aureus* isolated from those MS patients and healthy controls at (*P*=0.00) as shown in Table 4.

Table 4 Frequency of *S.aureus* isolates producing biofilm in MS patients and controls.

biofilm	Patients			controls
	Newly MS(No.%)	R.R. MS(No.%)	Total (No.%)	
Strong +ve	30 (96.8)	49(98)	79(97.5)	0
Weak +ve	1(3.2)	1(2)	2(2.5)	12(12)
Total	31(100)	50(100)	81(100)	12(12)
P-value		*P*= 0.594 (NS)		*P*= 0.00(HS)

(*) HS: Highly Sig. at P<0.01; S: Sig. at *P*<0.05; Testing based on Contingency Coefficient test.

3.3.4. The correlation between antibiotic resistance and biofilm formation

The result of the current study revealed a strong association between resistance to 19 kinds of antibiotics and production of biofilm in *S.aureus* isolated from newly diagnosed, relapsing-remitting MS patients compared with healthy controls. Statistically, there is a highly significant correlation between antibiotic resistance and biofilm production at (*p*=0.00), Table 5.

Table 5 The correlation between antibiotic resistance and biofilm formation

Antibiotics	Biofilm						*P* value
	Newly MS		R.R. MS		control		
	+ve	Weak +ve	+ve	Weak +ve	+ve	Weak +ve	
ME (R/S)	30/0	1/0	49/0	1/0	0/0	12/0	
VA (R/S)	3/27	0/1	5/44	0/1	0/0	0/12	
AZM (R/S)	17/13	1/0	421/7	1/0	0/0	0/12	
E (R/S)	21/9	1/0	14/5	1/0	0/0	8/4	
TE (R/S)	17/13	1/0	40/9	1/0	0/0	4/8	
AK (R/S)	17/13	0/1	33/16	0/1	0/0	0/12	
RA (R/S)	8/22	0/1	19/30	1/0	0/0	0/12	
P (R/S)	20/10	1/0	46/3	1/0	0/0	12/0	
MEM (R/S)	1/29	0/1	3/46	0/1	0/0	0/12	
SXT (R/S)	11/19	1/0	5/44	1/0	0/0	0/12	
DOX (R/S)	7/23	1/0	26/23	0/1	0/0	0/12	
CIP (R/S)	8/22	0/1	19/30	0/1	0/0	0/12	
DA (R/S)	9/21	0/1	23/26	1/0	0/0	0/12	
IPM (R/S)	1/29	0/1	2/47	0/1	0/0	0/12	
CTX (R/S)	9/21	1/0	24/25	0/1	0/0	0/12	
CN (R/S)	17/13	1/0	28/21	0/1	0/0	0/12	

CRO (R/S)	26/4	1/0	48/1	1/0	0/0	8/4
AM (R/S)	22/8	1/0	47/2	1/0	0/0	8/4
C (R/S)	9/21	0/1	23/26	1/0	0/0	0/12

(*) HS: Highly Sig. at P<0.01; S: Sig. at *P*<0.05; Testing based on Contingency Coefficient test.

3.3.5. Correlation between S.aureus colonization, average of biofilm production & antibiotic resistance.

The analysis results showed a highly significant difference at $p<0.01$ concerning *S.aureus* colonization with average antagonism to several antibiotics and biofilm production between stages of MS disease corresponded with healthy controls as indicated in Table 6.

Table 6 Correlation between S.aureus colonization, biofilm production & antibiotic resistance

Parameters	New Dx. MS(No.%)	R.R. MS. No. (%)	Control No. (%)	Total(No.%)
S. areus result	31(38.2%)	50(61.8%)	12 (12%)	93(37.3)
Antibiotic resistance average	46.3%	60.6%	22.8%	-
Weak biofilm No. (%)	1(3.2%)	1(2%)	12(12%)	-
Strong biofilm No. (%)	30(96.8%)	49(98%)	0(0%)	-

P-value, chi-square test: P=0.000 (HS)
Pair wised comparisons: [New Dx. MS & R.R. MS.] X Control
Recorded highly sig. different at P<0.000
And no sig. at P>0.05 between diseased groups

4. Discussion

This study revealed that the duration of MS disease was focused on (<1 – 1-5 years) in the newly diagnosed stage whereas in the relapsing-remitting stage the disease duration was focused on (1-5 and >5 years), the newly diagnosed MS patients in this study focused on these periods because of the samples collection status, signs, and symptoms of some patients were diagnosed lately after progression in clinical presentation accompanied with more than one-year duration while depending on the appearance of signs and symptoms in relapsing-remitting MS patients there was a long duration of disease. This investigation's outcomes coordinated with the study performed in Iran by [11], who conveyed that all stages of MS disease were focused on (1-5 and 6-10) years duration. Our study revealed that *S.aureus* colonization in nasal carriage of healthy controls was only 12 % , while in MS patients, the frequency of *S.aureus* was 81% , this result agreed with study was conducted by [12], who stated that the *S.aureus* colonization frequency in patients with MS was high (68.33%) in MS exacerbated group and (50%) in MS stable group compared with low in healthy individuals or non-MS group (23.75%), the interpretation of this result depends on the complex etiology of MS disease that includes environmental agents like bacterial superantigens frequency in *S.aureus* isolates accompanied with increase nasal colonization of those patients more than healthy carriers indicates that the colonization rate of *S.aureus* within the nasal carriage of patients with MS plays an essential role in infection development with establishment of this autoimmune disease, whereas the present results of the study were in disagreement with a study performed in Canada by [13], who reported that *S.aureus* colonization in healthy peoples was high (30%) compared with MS patients (24.2%). In the current study, the biofilms of *S.aureus* isolates were determined by the crystal violet microtiter plate method. The cutoff value (0.171) according to an optical density (O.D.) was measured by an ELISA reader.

Concerning the biofilm cutoff point the current data revealed that out of 81 MS patients (100%) colonized with *S. aureus*, there is 79 (97.5%) *S.aureus* isolates can form biofilm or strong biofilm producers while only 2(2.5%) isolates were weak biofilm producer, regarding control group all isolates were weak biofilm producers. In the present analysis, the powerful biofilm producer of *S.aureus* isolates in MS patients was (97.5%), while in the control group all these isolates can produce weak biofilm and only (12%), this study coordinated with a study published in Turkey by [14], who reported that the percentage of slim adhesion genes of *S.aureus* isolates in patients with MS was (81.6%) higher than healthy individuals (58.3%), these results indicate that the resistant *S.aureus* isolates in patients with MS can produce slime and biofilm to become more virulent and pathogenic that leading to more serious consequences of MS patients whereas less biofilm, fewer virulence factors and low level of antibiotic resistance of *S.aureus* isolates were detected in healthy non-MS carriers.

In this analysis, there is a highly powerful correlation between *S.aureus* colonization frequency in the nasal carriage of MS patients 81% corresponded with healthy controls 12%, an average of antibiotic resistance to different antibiotics were 53.3% in MS patients, 22.8% in controls and capacity to produce biofilm was 97.5% with strong ability, 2.5% only with weak ability in MS patients, 12% of all S.aureus isolates have weak capacity for biofilm production in the control group, this study is comparable to a study performed by [15], who reported that (97.5%) MRSA of all S.aureus isolates have the higher rate of antibiotic resistance to multiple antibiotics including amikacin, ceftriaxone, ciprofloxacin, erythromycin, gentamicin, mupirocin, rifampin, tetracycline, and tobramycin was 64.1%, 76.92%, 51.28%,87.18%, 71.8%, 10.26%, 5.13%, 89.74%, and 61.54%, respectively with biofilm results ranged from non-biofilm producers was (2.5%), weak biofilm producers were (17.5%), moderate biofilm producers were (62.5%) and strong biofilm producers were (17.5%), indicating more interprets about MRSA isolates in MS patients are virulent and have many virulence factors responsible for more resistance and more biofilm production particularly increasing in relapsing-remitting MS stage than different MRSA isolates of non-MS carriers which are normally colonized that have limited ability to resistance and biofilm. The existing analysis conveyed a highly significant correlation between strong biofilm formation and resistance to several antibiotics of S.aureus isolates in MS groups corresponded with a healthy control group including weak biofilm production attended with less antibiotic resistance, this study overlaps with the study of Klowak et al., 2011 [16], was performed in Nepal and said that among 76(20.9%) of S.aureus isolates 45 (59.2%) were MDR, 36 (47.4%) were MRSA and 35 (46.1%) were biofilm producers, whereas the existing study conflicted with the study performed in India by Slany et al., 2017 [17], who documented that there was no significant correlation between biofilm production and antibiotic resistance, also India study results revealed that (80.69%) of S.aureus isolates were considered as MRSA that showed complete antibiotic resistance (100%) against variable types of antibiotics and only (16.76%) of isolated MRSA were considered strong biofilm producer.

5. Conclusions

A High frequency of *Staphylococcus aureus* in the nasal carriage of multiple sclerosis patients mainly in the established relapsing-remitting (R.R.MS) group corresponded with low frequency in healthy nasal carriers. The duration of multiple sclerosis disease in more patients was about >5 years along with more *staphylococcus aureus* isolates. All isolates produce biofilm but the density is high in established relapsing-remitting MS and newly diagnosed MS patients compared with very low in controls. A highly significant correlation was found between biofilm formations and antibiotic resistance in MS patients.

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