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RESEARCH ARTICLE

Staphylococcus aureus Enterotoxin as a Major Risk Factor for Multiple Sclerosis Severity

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Abstract

Background: *Staphylococcus aureus* produce enterotoxins that function as super antigens which activate auto reactive CD4+ T-cells potentially target the basic myelin protein into the CNS. Objective: This study investigates the correlation between the colonization of *S. aureus* harbouring enterotoxins with multiple sclerosis (MS) exacerbation. Methods: A total of 200 nasal swabs were collected from three study groups: Healthy controls as a Non-MS Subjects (n=100), relapsing remitting MS or exacerbated (n=50) and newly diagnosed MS. (n=50). *S. aureus* was isolated from the anterior nares of these groups following standard operating procedures. Antibiotic susceptibility test against 19 antibiotics using disk diffusion method were done. Staphylococcal super Antigen SeA, *seB*, *seC* genes were amplified using standard conventional polymerase chain reaction (PCR) technique and resolved by agarose gel electrophoresis. Results: In this study, Out of 100 MS patients and 100 healthy control groups, there were 36% and 46% males and 64% and 54% females respectively. A total of 81(81%) were colonized with *S. aureus* includes, 31(38.2%) newly diagnosed MS and 50(61.8%) exacerbated MS while only 12(12%) isolated from control non-MS group. All *S. aureus* isolates were resistant to Methicillin (100%) and sensitive to Impenem (100%). PCR results showed that, the frequency of enterotoxins (*sea, seb and sec*) in MS patients was (42.4%) including (40.8%) were from newly diagnosed and (43.3%) were from relapsing remitting while there is no enterotoxins was detected in control group. the prevalence of *se A* was significantly higher ($p<0.00$) in the MS exacerbation (72%) than in newly diagnosed group (64.5%). Conclusions: The frequency of *S.aureus* isolates and its enterotoxin *seA* gene is high in the MS patients and this gene *serve* as an important marker in the severity of MS disease. Also *S.aureus* isolates were sensitive to imipenem which is considered as a better choice for nasal decolonization of *S.aureus* in the MS patients.

Keywords: *Multiple sclerosis (MS), Staphylococcus aureus, Super antigens, Enterotoxin A, B, C.*

Introduction

Multiple sclerosis (MS) is an autoimmune-inflammatory disease that occurs in the central nervous system (CNS) causing demyelination. The demyelination operation is related to infiltrating T-cells particular for major myelin proteins of the CNS, this disease has a peak start between 20 and 40 years old and influences women almost two times as often as men[1]. The most common form of disease is the relapsing-remitting MS (RR-MS), including around 85 - 90% of all cases. Although the etiology of MS is unknown; genetic and environmental factors play a role. Infectious pathogens are the possible environmental factors contributed to the MS development or severity [2]. Pathogens related to the severity or development of MS involve bacteria; such as *Chlamydia pneumoniae* , *Mycoplasma pneumonia* and *Staphylococcus aureus* which produced enterotoxins that function as superantigens [3]. It has been proposed that *S. aureus in nasal* carriage is related to several autoimmune diseases involving systemic lupus erythematosus, rheumatoid arthritis and Wegener's granulomatosis syndrome through superantigens such as toxic shock syndrome toxin-1 {tsst-1} or *staphylococcal* enterotoxins {SEs} [4].

Superantigens are proteins produced mostly by bacteria and some viruses that potently activate autoreactive CD4+ T cells, inducing massive cell proliferation and cytokine production, predominantly IL-2 and interferon (IFN)-γ [5].*Staphylococcus aureus* superantigens are capable of activating T cells due to their ability to bind to both major histocompatibility complex (MHC) class II molecules and specific Vβ regions of the T cell receptor. Furthermore, this leads to the activation of both antigen-presenting cells and T lymphocytes. Ultimately this mechanism results in the over-production of inflammatory cytokines and T cells followed by the presentation of autoimmune activated clinical symptoms [6].

The most important superantigens were the *Staphylococcus aureus* enterotoxins (A, B, C, D and E) and toxic shock syndrome toxin that participate in T cell activation [6]. More than 20 superantigens have been identified in *S. aureus* strains and at least 80% of the clinical strains of *S. aureus* harbor a minimum of one superantigen [7].A study in multiple sclerosis(MS) patients examining the association of bacterial superantigens with MS exacerbation has been recently carried out [8], therefore, colonization of the nose with enterotoxin A-producing *Staphylococcus aureus* may play a role in triggering autoreactive CD4+ T cell activation and Multiple Sclerosis exacerbations[9]. The role of *S. aureus* superantigens in the etiology of MS is currently still unknown, but Staphylococcal Enterotoxin A (*sea*), Staphylococcal Enterotoxin B (*seb*), Staphylococcal Enterotoxin C (*sec*) and Toxic Shock Syndrome Toxin-1 (*tsst-1*) have been demonstrated to be involved in the reactivation of a mouse model for MS ,known as experimental autoimmune encephalomyelitis (EAE) [10].

Furthermore, a previous study demonstrated that *S. aureus* superantigens, used at a nanogram range, were effective in inducing γδT cell activation in a panel of 16 gamma delta T cell clones isolated from MS patients and controls [11].A correlation between superantigens and patients with MS could lead to a better understanding of the etiology of this disease and possibly lead to MS treatment by antibiotic targeting the organisms that produce superantigens [9].The purpose of this study is to determine the prevalence of *S. aureus* colonization in non-MS and MS patients, and examine whether there is any correlation between MS patients and *S. aureus* harbouring the superantigen *sea, seb, and sec* genes.

Materials and Methods

Sample Collection

A total of 200 samples were enrolled in this study. A 100 nasal swab samples were collected from multiple sclerosis patients and 100 from healthy individuals during a period between November 2018 to January 2019 from Baghdad Teaching Hospital / Multiple Sclerosis Clinic in Medical City.

Bacterial Isolation

All specimens were cultured on 5% sheep blood agar and mannitol salt agar, incubated at 37^{O}C for 24 hours. *Staphylococcus aureus* colonies were identified using phenotypic and biochemical tests like morphology of colony, catalase and coagulase tests [12].

Antimicrobial Sensitivity Testing

Antibiotic susceptibility test were perfumed for each *S. aureus* isolate by disc diffusion method according to the clinical and laboratory standards institute(CLSI) guidelines [13] against Methicillin (10 µg), Vancomycin (30 µg), Azithromycin(15 µg), Erythromycin (10µg), Tetracycline (10µg), Amikacin (10µg) ,Rifampin (5µg), Penicillin-G(10µg), Meropenem (10µg), Doxycycline (30µg), Sulfamethoxazole/trimethoprim (25 µg), Ciprofloxacin (10 µg), Clindamycin (10 µg), Imipenem (10 µg), Cefotaxime (30 µg),Gentamicin(10µg), Ceftriaxone (10µg), Ampicillin (25 µg), Chloramphenicol (10 µg).All antibiotic disks were provided from Bioanalyse Group Ltd, UK.

DNA Extraction

The DNA was extracted from pure isolates of *S. aureus* after inoculation in Luria-Bertani broth by Promega genomic purification kit USA according to the company instructions.

PCR for Detection of Enterotoxin Genes

Polymerase chain reaction (PCR) was used for amplification and detection of (*sea, seb & sec)* genes of *S.aureus* with primers specific to each one were designed and synthesized in Alpha DNA (Canada) (Table 1) and master mix ready to be used obtained from Bioneer, Korea company.

Table 1: Sequences and products of *Staphylococcus aureus sea, seb and sec* genes

SE gene		Nucleotide sequences (5' → 3')	Products *bp*	References
sea	F	GGGAACAGCTTTAGGCAATC	564	[14]
	R	ATTTGAATACTGTCCTTGAGC		
seb	F	CCAGATCCTAAACCAGATGAG	599	[14]
	R	TGCAGGCATCATGTCATACC		
sec	F	GACATAAAAGCTAGGAATTT	257	[15]
	R	AAATCGGATTAACATTATCC		

PCR amplification of enterotoxin genes was performed in the (Eppendorf, UK) Thermal Cycler under following program: 1 cycle with 94 °C 5 min. for initial denaturation, 30 cycles for denaturation, annealing and extension with 94 °C 2 min, 50 °C 2 min. & 72 °C 1min respectively and finally 72 °C 5 min for final extension [16].The PCR products were analyzed by (1%) agarose gel electrophoresis [17].

Statistical Analysis

The statistical data were analyzed by statistical package (SPSS) ver. (22.0) , Chi-square exact test was used to measure significant associations between the frequency of *Staphylococcus aureus* in nasal carriage of MS patients and healthy nasal carriers with enterotoxin genes distribution. *P* values less than 0.05 were considered statistically significant.

Results

Staphylococcus aureus Colonization and Multiple Sclerosis

Out of 100 nasal swab samples collected from MS patients, 81 (81%) were positive for *S.aureus* whereas from 100 nasal swab samples collected from healthy controls, there were only 12(12%) positive for *S.aureus*. Consequently MS patients were most commonly colonized group in this study and there was a statistically significant differences between *S. aureus* colonization in healthy carriers and MS disease at (p = 0.00).On the other hand the relapsing remitting MS patients were most commonly colonized with *S.aureus* 50(61.8%) than newly diagnosed MS group 31(38.2%) with a p-value of (0.092), statistically non significant association between them (Table 2).

Table 2: distribution of *Staphylococcus aureus* in study groups

Study groups	Samples NO.	*S.aureus* positive			*P*-value
		NO.	%		
Non-MS group	100	12	12		0.00 (HS)*
Total MS patients	100	81	81		
New diagnosed MS group	50	31	38.2		0.092 (NS)**
MS exacerbated group	50	50	61.8	100	
Total groups	200	93	_		

* Highly significant; ** non significant

Demographical Distribution and *S.aureus* Nasal Carriage

According to gender distribution, the ratio of male to female was (1:1.7) includes 64(64%) of patients were female and 36 (36%) were male while in non-MS group there were 54(54%) female and 46(46%) male. In MS patients, a total of *S.aurus* positive was 81(81%) including 52(52%) female and 29(29%) male, while in control non-MS group, out of 12(12%) *S.aureus* positive there were 7(7%) female and 5(5%) male, statistically highly significant differences was observed between *S.aureus* colonization and gender distribution (*P* value= 0.00) as shown in (Table 3).

Table 3: Distribution *S.aureus* according to gender

Gender		Multiple sclerosis group		Non-MS control group		
		S.aureus		*S.aureus*		
		(+ve)	(-ve)	(+ve)	(-ve)	
Male	No (%)	29(29%)	7(7%)	5(5%)	41(41%)	0.0 (HS) *
Female	No (%)	52(52%)	12(12%)	7(7%)	47(47%)	0.0 (HS)

Total	**No (%)**	**81(81%)**	**19(19%)**	**12(12%)**	**88(88%)**	**0.0 (HS)**
		100(100%)		**100(100%)**		

* Highly significant

Antimicrobial Sensitivity Pattern

A total of 19 types of antibiotics were tested against *S.aureus* isolates in all study groups according to CLSI. MS patient groups were more resistant to antibiotics (53.3%) than control group (22.8%).Furthermore, within MS group, the exacerbated MS showed more resistance (60.6%) than newly diagnosed patients (46.3%). In all study groups there is complete resistance to Methicillin (100%) where as the lowest resistance (3%) were reported against Meropenem and Imipenem as shown in (Table 4).

Table 4: Antibiotic resistance pattern of *S.aureus* isolates.

ANTIBIOTICS	Multiple sclerosis patients			Non-MS
	New Dx. MS	R.R. MS.	Total	
	No.(%)	No.(%)	No.(%)	No.(%)
Methicillin ME	31(100%)	50(100%)	81(100%)	12(100%)
Vancomycin [VA]	3(9.6%)	5(10%)	8(10%)	0
Azithromycin[AZM]	18(58%)	43(86%)	61(75.3%)	0
Erythromycin [E]	22(71%)	45(90%)	67(82.7%)	8(66.6%)
Tetracycline [TE]	18(58%)	41(82%)	59(72.8%)	4(33.4%)
Amikacin [AK]	17(54.8%)	33(66%)	50(61.8%)	0
Rifampin[RA]	9(29%)	20(40%)	29(35.8%)	0
Penicillin-G [P]	21(67.7%)	47(94%)	68(84%)	12(100%)
Meropenem [MEM]	1(3.3%)	3(6%)	4(5%)	0
Sulfa/Trimthoprim [SXT]	20(64.5%)	45(90%)	65(80.2%)	0
Doxycycline [DOX]	8(25.8%)	26(52%)	34(42%)	0
Ciprofloxacin [CIP]	8(25.8%)	19(38%)	27(33.4%)	0
Clindamycin [DA]	9(29%)	24(48%)	33(40.8%)	0
Imipenem [IPM]	1(3.3%)	2(4%)	3(3.7%)	0
Cefotaxime [CTX]	10(32.3%)	24(48%)	34(42%)	0
Gentamicin [CN]	18(58%)	28(56%)	46(56.8%)	0
Ceftriaxone [CRO]	27(87%)	49(98%)	76(93.8%)	8(66.6%)
Ampicillin [AM]	23(74.2%)	48(96%)	71(87.7%)	8(66.6%)
Chloramphenicol [C]	9(29%)	24(48%)	33(40.8%)	0
Total	**46.3%**	**60.6%**		
	53.3%		**53.3%**	**22.8%**

Conventional PCR Screening for Enterotoxin Genes

By PCR essay, the amplification of sequences of *seA*, *seB* and *seC* genes were produced an amplicon of size 564 bp, 599 bp and 257 bp respectively.Figure (1, 2 and 3).

Figure 1: Gel electrophoresis of amplified PCR product (564 bp) for *sea* gene. Lane 1: 100 bp ladder. Lane 2: Negative control; Lanes 3– 10: Clinical isolates showing positive result. (1 % agarose, 7 v/cm², 1 hr)

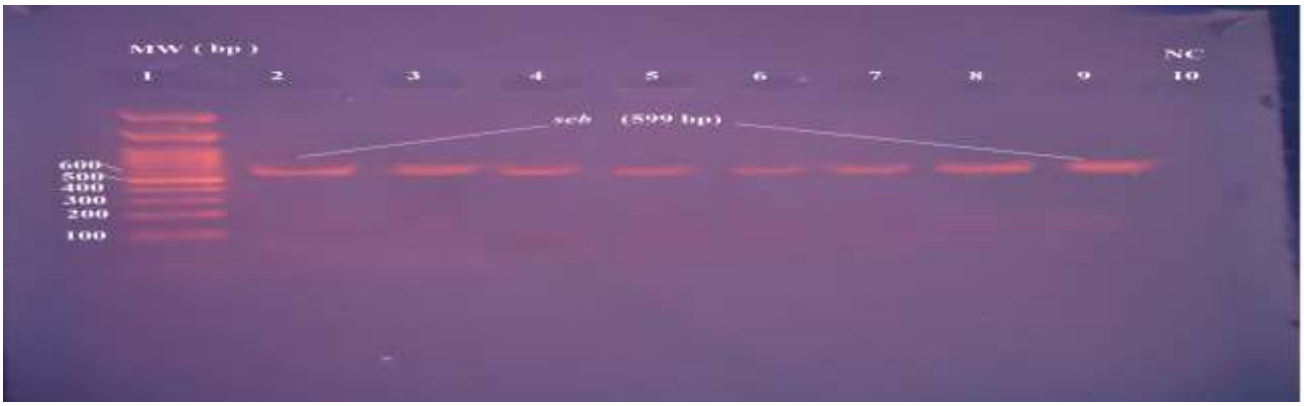

Figure 2: Gel electrophoresis of amplified PCR product (599bp) for *seb* gene. Lane 1: 100 bp ladder. Lanes 2 – 9 : Clinical isolates showing positive result. Lane 10: Negative control, (1% agarose, 7 v/cm², 1 hr)

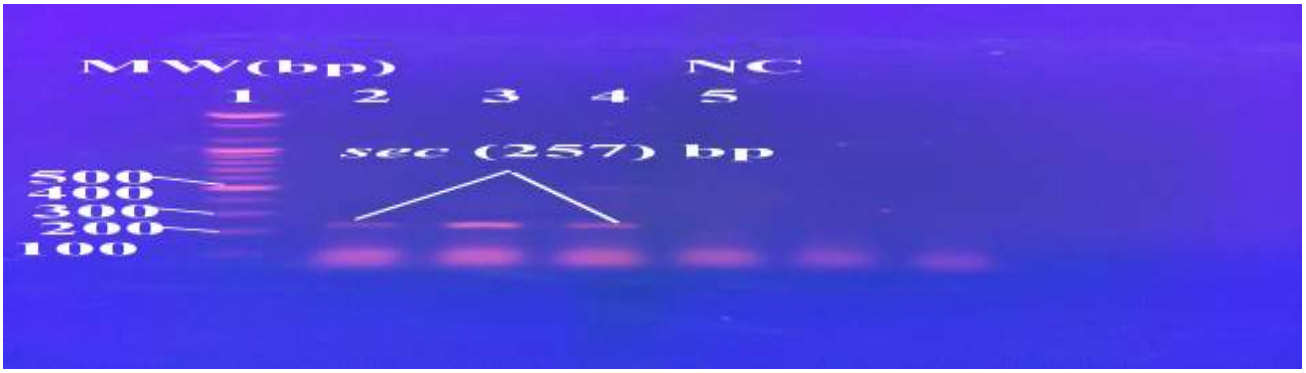

Figure 3: Gel electrophoresis of amplified PCR product (257 bp) for *sec* gene. Lane 1: 100 bp ladder. Lanes 2 – 4 : Clinical isolates showing positive result. Lane 5: Negative control (1 % agarose, 7 v/cm², 1 hr)

The current study reported that, among MS patients the most commonly detected enterotoxin genes were *seA* 56(69.1%) followed by *seB* 40(49.4%) while the least prevalent enterotoxin was *seC* 7(8.6%). Statistically, there was a highly significant differences between the distribution of *sea* and *seb* (P=0.00) in MS patient compared with healthy control but non-significant distribution were found in *seC* (P = 0.138).From overall *S.aureus* positive in MS groups the distribution of enterotoxins were 36(72%) of *se* A, 25(50%) of *se* B & only 4(8%) of *se* C in relapsing remitting group compared with newly diagnosed MS group that showed 20(64.5%), 15(48.4%) and only 3(9.7%) of *se* A, *se* B and *se* C respectively, whereas no enterotoxin genes were detected in non-MS control group . Significant differences were seen in the distribution of *se*A , *se* B and *se* C gene between MS groups (P=0.00002) compared with controls, (Table 5).

Table 5: Distribution of *S.aureus* enterotoxin genes

Study groups	*S.aureus* positive	Gene distribution				P-value
		sea	*seb*	*sec*	Total	
	No.(%)	No.(%)	No.(%)	No.(%)	No.(%)	
Control	12(12)	0(0)	0(0)	0(0)	0(0)	0.00002 (HS)*
Newly MS	31(38.2)	20(64.5)	15(48.4)	3(9.7)	38(40.8)	
R.R. MS	50(61.8)	36(72)	25(50)	4(8)	65(43.3)	
Total	93	56(69.1)	40(49.4)	7(8.6)	103(42.4)	
P-value		0.0 (HS)	0.0 (HS)	0.138 (NS)**	0.00002 (HS)	

* Highly significant; ** non significant

Discussion

Multiple sclerosis is a multifactorial disease with unknown origin but environmental agents like bacterial and viral infections more participate. *Staphylococcus aureus* superantigens which is considered the most triggering agent of autoimmunity and included in several autoimmune diseases like multiple sclerosis and rheumatoid arthritis [18]. In multiple sclerosis the presence of *S.aureus* enterotoxins in nasal carriage mostly (*sea,seb&sec*) considered a potent

factors for induction of autoreactive CD4+ T cells particularly in the myelin protein of CNS hence activation large number of cytokines that finally have a major role in MS severity and development [6,9]. In the present study the frequency of *S.aureus* nasal colonization in MS patients (81%), particularly in MS exacerbated group was high (61.8%) compared with non-MS healthy carriers which was only(12%) with highly significant differences (P=0.00), this may be due to more contact of these patients with healthcare settings although all MS patients were taken immune activation drugs that enhance immunity which indicates that the nasal bacterial colonization with enterotoxigenic *S. aureus* in MS patients was much more virulent and pathogenic than in non-MS group [8].

The current result in accordance with other study in Iran by *Pakbas et al* who reported that the nasal carriage rate of *S.aureus* in MS patiens & control group was (42%) and (23.3%) respectively [14]. Alternatively a study was conducted in Canada by *Mulvey et al*. revealed a lower prevalence of *S.aureus* in nasal carriage of MS patients (27%) and relatively higher in non-MS group (30%) [9].Regarding gender, this study observed that the frequency of *S.aureus* isolates in MS patients were 29% in males and 52% in females while in control group the percentage of *S.aureus* was 5% males and 7% females.

Concerning MS patients the result of this study coincide with *Sadeghi et al.,*2019,who reported that the frequency of *S.aureus* isolates in MS patients were 23% in males and 76% in females but this result disagreed with the same study [8] that reported the colonization of *S.aureus* in healthy nasal carriers was 56% males and 43% females. Globally *S.aureus* shows high resistance rate to several antibiotics.

The present study revealed that the *S.aureus* isolates in MS patients was (53.3%) multi-drug resistance especially in MS exacerbated group (60.6%) than newly diagnosed (46.3%) and control group (22.8%) with highly significant differences between MS patients & non-MS group regarded to resistance pattern. In this study *S.aureus* isolates in all groups were resistant to methicillin (ME)(100%) indicates that MRSA strains were also distributed in nasal carriage of healthy control [19], as well as there is a high percentage of resistance to ceftriaxone (93.8%), ampicillin (87.7%), penicillin-G (84%), erythromycin (82.7 %), sulphamethoxazole/ trimethoprim (80.2 %) and azithromycin (75.3%), while in control group in addition to methicillin resistance, *S.aureus* isolates showed high resistance rate only to penicillin-G(100%), indicating that all strains of *S.aureus* isolates in MS patients were resistant strains and responsible for multiple drug resistance (MDR) which leads to increase severity of multiple sclerosis(MS), while the *S.aureus* strain was different in control group in term of limited antibiotic resistance results and so non virulent. This result agreed with *Mehrabi et al* (2015) [16].

Who reported that resistant *S.aureus* strains in MS patients was considered as a risk factor for MS exacerbation with high level of resistance,against,tetracycline(80%),ampicillin(72.22%),methicillin(66.66%),erythromycin(66.66%),oxacillin(63.33%)and,sulphamethoxazole/trimethoprim(61.11%), while more than (90 %) of isolates in all study groups were susceptible to vancomycin , meropenem & imipenem which is provided a good regimen for decolonization of *S .aureus* in nasal carriage of MS patients in order to prevent severity or exacerbation [20].

To the best of our knowledge, it is believed that, it is the first study in Iraq concerning three enterotoxins of *S.aureus (sea, seb &sec)* identified in MS patients. The prevalence of *s.aureus* enterotoxin genes *(sea, seb, sec)* was higher in MS patients compared with control group that present no any enterotoxin genes. Statistically significant differences was observed between *S.aureus* harboring enterotoxins in MS patients and Non-MS Carriers (P= 0.00).

Among MS patients the most common prevalent enterotoxins were *sea* (69.1%) particularly *sea* in the relapsing remitting group was (72%) and in newly diagnosed MS was (64.5%). In addition the second most common prevalent enterotoxin in MS patients was *seb* (49.4%) with (48.4%) & (50%) in newly diagnosed & R.R. MS patients respectively ,while the least common enterotoxins distributed in MS patients was *sec* (8.6%) with (9.7%) & (8%) in newly diagnosed & R.R. MS patients respectively.

This indicates a statistically significant correlation between *S.aureus* harboring enterotoxin *(sea &seb)* and MS exacerbation (P= 0.00), however there was no statistically

significant correlation between *sec* & MS exacerbation (P= 0.138). Similarly other study results by *Sadeghi et al.* reported that *sea* was the most common frequent (40%) enterotoxins in MS patients than in nasal carriers (23%) [8], all these results indicate that healthy individuals was carrying nonpathogenic strains of *S.aureus* in nasal passages that considered as a normal commensal flora fight by competing against pathogens , in contrast to *S.aureus* strains in MS patients were have many virulence factors like enterotoxin genes, more MDR particularly in a stage of relapsing remitting or exacerbated MS that responsible for causing increase sequel, severity, development and pathogenesis of this autoimmune disease.

Conclusion

The nasal carriage of *S.aureus* in MS patients particularly in relapsing remitting group was very high compared with healthy individuals that mean the potential risk of *S.aureus* colonization in MS patients was high. High frequency of *S.aureus* harboring enterotoxin genes particularly (*se*A) support the evidence that *se*A play a very significant role in the development and severity of MS. Decolonization regimen of *S.aureus* in nasal carriage of MS. Patients with more effective antibiotics like imipenem to prevent severity or exacerbation of the MS disease.

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