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High Prevalence of Staphylococcal Enterotoxins (SEs) Virulence Genes Among Methicillin-Resistant *Staphylococcus aureus* Isolated from Patients with Different Clinical Manifestations in Khartoum State, Sudan

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ABSTRACT

Background: Staphylococcal enterotoxins (SEs) are family members of more than 20 different staphylococcal and streptococcal exotoxins. SEA and SEB are the most common toxins in staphylococcus-related food poisoning. **Objectives** The present study aimed to estimate the prevalence of SEs Methicillin-Resistant *Staphylococcus aureus* (MRSA) isolated from different clinical samples. **Methods:** A cross-sectional laboratory-based study was conducted from November 2020 to January 2021. To isolate and identify *S. aureus*, the conventional method and protein A latex test were used. MRSA was detected using methicillin (MET 5µg) by using the Kirby-Baur disk diffusion method. Extracted DNA was amplified for the specific Staphylococcal enterotoxins (SE) gene to confirm the SEA, SEB and SEC. **Result:** The SEA gene was observed among 120 (100%) clinical isolates. The combined staphylococcal enterotoxin genes A, B and C were found in 33.3% of the isolates. Respiratory tract infection (RTI) shows the highest percentage of SEA 28.3%. **Conclusion:** The present study showed a high prevalence of SEA in clinical settings at Khartoum State. A high prevalence of the combination of more than one gene occur in SEA with SEC, which was represented 82.5%. The present study showed a strong relationship between MRSA and SEA that could be due to a specific strain of MRSA carrying the SEA gene. Further studies should be done about Staphylococcal enterotoxins.

INTRODUCTION

Staphylococcal enterotoxins (SEs) are family members of more than 20 different staphylococcal and streptococcal exotoxins (Pinchuk *et al.*, 2010) and they are the causative agents of staphylococcal food poisonings (Bhatia *et al.*, 2007). They share a common phylogenetic relationship, structure, function, and sequence homology (Wu *et al.*, 2016). The *S. aureus* enterotoxins (SEs) are potent gastrointestinal exotoxins synthesized by *S. aureus* throughout the logarithmic growth phase or during the transition from the exponential to the stationary phase (Argudín *et al.*, 2010). Staphylococcal enterotoxins are basic proteins made up of approximately 220–240 amino acids and have similar molecular weights of 25–30 kDa (Wu *et al.*, 2016). They are active in high nanogram to low microgram quantities. They are resistant to extreme conditions (heat treatment, low pH) that easily destroy the bacteria that produce them and to proteolytic enzymes, hence retaining their activity in the digestive tract after ingestion (Argudín *et al.*, 2010). Most genes coding for SEs are located on mobile elements, including plasmids, prophages, *S. aureus* pathogenicity islands, genomic island, or next to the staphylococcal cassette chromosome elements. (Pinchuk *et al.*, 2010, Argudín *et al.*, 2010). Most of these are mobile genetic elements, and their spread among *S. aureus* isolates can modify their ability to cause disease and contribute to the evolution of this important pathogen (Argudín *et al.*, 2010). These bacterial proteins are pyrogenic and are connected to significant human diseases that include food poisoning and toxic shock syndrome (Pinchuk *et al.*, 2010). SEA and SEB are the most common toxins in staphylococcus-related food poisoning; SEB is also a potential biological weapon of war and terrorism (Pinchuk *et al.*, 2010, Wu *et al.*,

2016). Presently, 23 enterotoxins have been identified as distinct serological entities, including SEA, SEB, SEC, SED, and SEE. Recently, molecular biological methods for the detection of enterotoxin including nucleic acid hybridization and polymerase chain reaction (PCR) have been evolved (Wu *et al.*, 2016). The present study was aimed to estimate the prevalence of SEs among MRSA isolated from different clinical samples, including Blood, Sputum & Tracheal Aspirate, Skin & Wound Swab, Genital Swab, Body Fluids, Urine, etc.

MATERIALS AND METHODS

Study Design:

A cross-sectional laboratory-based study was conducted during the period from November 2020 to January 2021 at Khartoum State. A total of one hundred and twenty *S. aureus* clinical isolates (n, 120) were collected from patients with different clinical signs attending different hospitals at Khartoum State, Sudan.

Sample Collection, Phenotypic Characterization of *S. aureus* and detection of MRSA:

The clinical specimens enrolled in the study, as shown in Table 1, were collected in sterile containers under aseptic conditions according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (Clsi, 2009). These clinical specimens were related to Infectious diseases, as shown in Table 1. Standard scheme for identifying all staph. Isolates was used according to (Cheesbrough, 2005). These include colony morphology on blood agar, mannitol salt agar, MacConkey agar, Gram-stain, catalase, coagulase tests, and protein A latex test. MRSA was detected according to CLSI, using a methicillin (MET 5µg) disc on the Mueller-Hinton agar plate (CLSI, 2009).

Table 1: shows the clinical specimens and infectious disease

Clinical specimens	Infectious disease
Blood	Septicemia
Sputum & Tracheal Aspirate	RTI
Eye swab	Eye infection
Wound Swab	Skin & Wound Infection
Genital Swab	Genital infection
Urine	UTI
E.N.T Swabs	Ear infection
Tissue	Empyema
Body Fluids	p. sepsis
	ARDS
	Others

PCR Amplification of SE (A, B and C) Genes:

DNA was extracted by boiling method according to the method described by (Ahmed *et al.*, 2014), as follows: two colonies of bacteria were suspended in molecular biology-grade water and were centrifuged at $12,000 \times g$ for 5 min. The supernatant was discarded and the cell pellets were re-suspended in 100 μ l of water then

subjected to boiling at 100°C in a water bath for 10 min, cooled on ice for 15 min and centrifuged at $12,000 \times g$ for 5 min. The supernatant was then transferred into a new Eppendorf tube and stored at -20°C until use. The primers used for the PCR amplification were those described by (Ahmed, 2020). The primers used had the following sequences (Table 2).

Table 2: The sequences, band size and temperature of SEA, SEB and SEC

Primer	Sequence	Band Size	Temp	Target
SEA-1	TTGGAACGGTTAAAACGAA	120	50	SEA
SEA-2	GAACCTTCCCATCAAAAACA			
SEB-1	TCGCATCAAACACTGACAAACG	478	50	SEB
SEB-2	GCAGGTACTCTATAAGTGCC			
SEC-1	GACATAAAAGCTAGGAATTT	257	50	SEC
SEC-2	AAATCGGATTAACATTATCC			

The master mix was prepared as follows: Multiplex PCR protocol was adopted in 20 μ L volume which contains 4 μ l Hot Firepol Multiplex Mix 5X ready to load (Solis BioDyne, Tartu, Estonia), 1 μ l from each (SEA, SEB and SEC) forward and reverse primer, 5 μ l DW and then add 5 μ l of template DNA (Bangratz *et al.*, 2020). The thermal protocol was performed by using gradient PCR Thermal Cycler K960 as follows: initial denaturation step (one cycle at 94°C for 12 minutes) followed by 30 repeating cycles of denaturation (30 seconds at 95°C), annealing (40 seconds at 62°C), and extension (40 seconds at 72°C), extension for 1:5 minutes at 72°C followed by final

extension step at 72°C for 10 min (Yu *et al.*, 2018). PCR amplicon was visualized on 1.5% agarose gel and bands sizes were compared with DNA ladder (100 bp). Positive results will produce a band of 120, 478, 257 bp for SEA, SEB and SEC respectively.

RESULTS

Bacterial Isolates:

Identification of the isolates was carried out by using colony morphology on MSA and MacConkey agar, Gram's Stain and different biochemical tests as shown in Figure 1. Also, PCR was used to confirm the presence of SEA, SEB and SEC genes as illustrated in Figure 2.

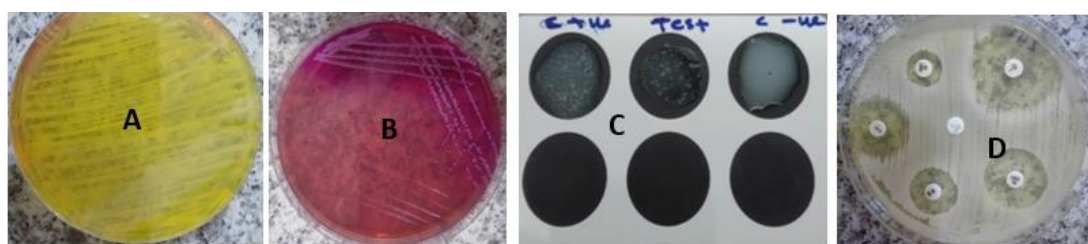


Fig. 1: A: 24 hr old culture of isolated bacteria on A: MSA (Producing golden –yellow color) and B: on MacConkey showing lactose fermenter colonies; C: protein A Latex test showing positive results and D: DST against methicillin antibiotics showing resistance to it.

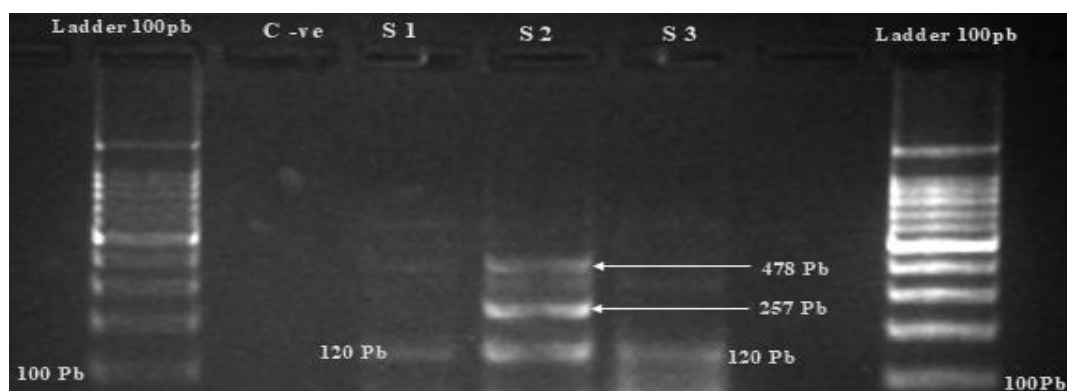


Fig. 2: 1.5 % Agarose gel electrophoresis of PCR products. Lane M: 100 bp molecular weight marker, Lane 2: negative control, S1, S2, S3, are tested isolates showing positive results for SEA (S1, S2, S3), SEB (S1, S2) and SEC (S2).

Demographic Data:

Among the 120 study subjects, 66 (55.0%) were Males, while 54 (45.0%) were females. The ages of patients were classified into four categories: the highest frequency

was shown in the age group more than 45 years old 56 (46.7%), and the lowest frequency was shown in the age group 17- 30 years old 13 (10.8%) as shown in Table 3.

Table 3 shows the demographic data of a patient, including age and gender

Demographic data					
Gender			Age groups		
Gender	Frequency	Percent	Age groups	Frequency	Percent
Male	66	55.0	0-16	22	18.3
			17-30	13	10.8
Female	54	45.0	31-45	29	24.2
			>45	56	46.7
Total	120	100.0	Total	120	100.0

Of the total 120 bacterial isolates, the result showed a high frequency of SEA and SEC among MRSA isolates 120 (100%) and 99 (82.5) respectively while the low

frequency was identified among SEB 45(37.5%), The result also showed a high frequency of SEA among community infection 98 (81.7%), as shown in Table 4.

Table 4: shows the frequency of Enterotoxins, including on community and hospital

Enterotoxin	Positive/120		Community		Hospital	
	frequency	%	frequency	%	frequency	%
A	120	100	98	81.7	22	18.3
B	45	37.5	35	29	10	8.5
C	99	82.5	82	68.5	17	14

A combination of SEA with SEC genes was detected in 99 (82.5%) out of the total 120 MRSA isolates. In comparison, the combination of three enterotoxin genes SEA,

SEB and SEC was detected in 40 (33.3%), High frequency of combined SE genes in community infection was found among SE (A+C) genes 82(68.3%) as shown in Figure 3.

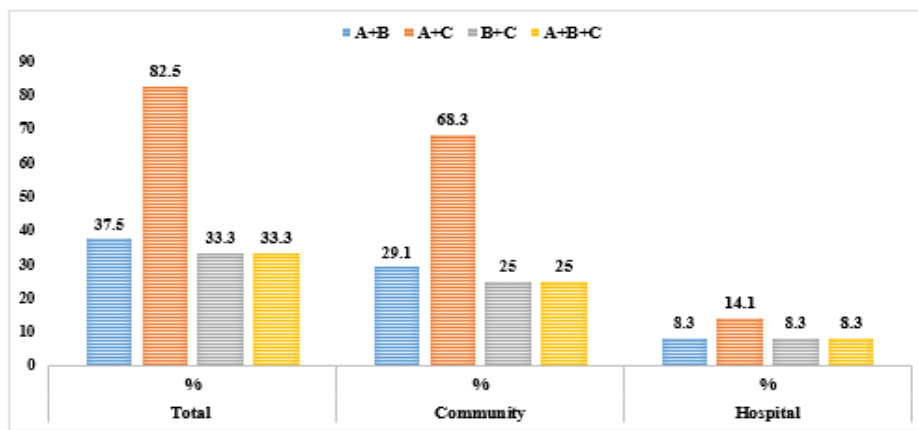


Fig. 3: Frequency of the combination SE genes among MRSA isolate.

The results in Figure 4 show the percentage of SE among infectious diseases among the 120 MRSA isolates, RTI shows the highest percentage of SEA (28.3%) followed by septicemia (22.5%), UTI (12.5%) and Skin & Wound Infection (11.7%). Regarding SEB,

RTI was 10%, Septicemia 9.1%, UTI 5% and Skin & Wound Infection 4.1% and SEC was found among RTI (22.5%), Septicemia (20.8%), UTI (9.1%) and Skin & Wound Infection (9.1%).

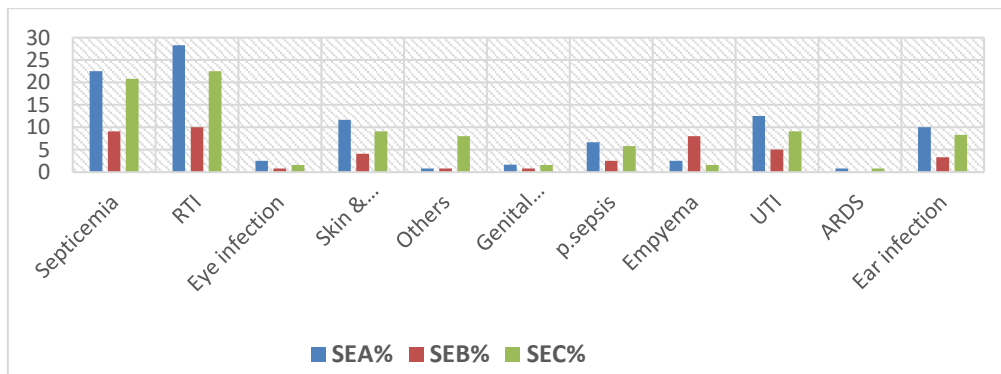


Fig. 4: Correlation between SE and infectious disease types

DISCUSSION

Staphylococcal enterotoxins (SE) are a large family of toxins that cause gastroenteritis and are potent superantigens that cause T-cell stimulation and the most important of these is SEA which is the most common SE associated with food-borne illness in humans (Mahmoud, 2017). The present study investigated the distribution of SE genes among MRSA isolated from a different clinical sample. The result showed that 100% of MRSA isolates possess SEA, no previous data reported result similar to our result. But the study conducted in Iran by (Kamarehei *et al.*, 2013) showed SEA among 60.6%, also (Fooladvand *et al.*, 2019) in Iran reported SEA was 23.5% and finally (Ahmed, 2020), which enrolled the study in Sudan observed SEA was 19.4% among different MRSA isolates. In contrast (Jiménez *et al.*, 2011) in Colombia did not detect SEA in any MRSA isolates. The present study showed the presence of combinations of more than one enterotoxin gene; the most frequent combined genes are SEA and SEC, which were detected among 82.5% of the total MRSA isolate, (Fooladvand *et al.*, 2019) found 40.8% of this combination, and a low result of a combination of SEA with SEC reported by (Ahmed, 2020) which was 4.3%. In contrast (Jiménez *et al.*, 2011) reported 18.8% combination of SEA with SEB. Furthermore, the study revealed that the combination of the three enterotoxin genes SEA, SEB and SEC was detected in 33.3%. Another result of a combination of three enterotoxin genes was 14.7% reported by (Fooladvand *et al.*, 2019). In the correlation of infectious disease against SE, the present study showed that RTI had 28.3% SEA. In contrast, (Kamarehei *et al.*, 2013) observed the frequency of SEA positive isolates in blood specimens was 72.7%. The study represented in Iran by (Pourmand *et al.*, 2009) reported that SEA was 43.7% in UTI.

Conclusion

Staphylococcal enterotoxins are the main *S. aureus* virulence factor that causes serious clinical conditions for public health. The present study showed a high prevalence

of SEA in clinical settings at Khartoum State. The most frequent gene combination occurs for SEA and SEC, which were detected among 82.5%. The present study showed a strong relationship between MRSA and SEA. That could be due to a specific strain of MRSA carrying the SEA gene. Further studies are needed and the enterotoxin test is recommended to be done routinely.

Ethical Clearance:

This study was approved by the Ethical Committee, University of Gezira, Sudan. Written consent was obtained from every participant before he/she was enrolled in the study.

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