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DETECTION OF THE CLASSICAL ENTEROTOXIN ENCODING GENES OF STAPHYLOCOCCUS AUREUS IN STREET VENDED FOODS AND FOOD HANDLERS IN PORT HARCOURT METROPOLIS, NIGERIA

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ABSTRACT
Staphylococcus aureus classical enterotoxins (SEA-SEE) have been reported to be highly implicated in Staphylococcal food poisoning (SFP) outbreaks with the conclusive diagnostic criteria based on the detection of staphylococcal enterotoxins in food. Studies also show that the hands of food handlers carry *S. aureus* strains that produce at least one staphylococcal enterotoxins that contaminate food items during food processing. In this study, the prevalence of *S. aureus* and its classical enterotoxins in 135 street vended foods and their handlers respectively was determined using polymerase chain reaction. SEA was detected by positive amplification of the sea gene in 4(26%) from food samples of Roasted meat ('Suya'), Fried meat, Burger and Doughnut. The sed gene was detected in 3(20%) from food handlers of Doughnut, Beef burger and Cupcake while see gene was detected in 1(6%) in food samples of Cupcake. The presence of enterotoxigenic *S. aureus* in street vended foods and handlers pose a public health threat to consumers because it enhances the spread of these virulent strains in the food chain, within the community and even in hospital settings. Strict enforcement of public health laws by environmental health officers should be employed and the microbiological guidelines such as hazard analysis and critical control points (HACCP) should be strictly implemented and adhered to because it will go a long way in preventing *S. aureus* contamination in Port Harcourt metropolis.

KEYWORDS: Street vended foods, Food handlers, Classical enterotoxins, Staphylococcal Food Poisoning (SFP), Polymerase Chain Reaction (PCR), Hazard analysis and critical control points (HACCP).

INTRODUCTION
Staphylococcus aureus which is a normal flora of the skin and mucous membranes of healthy persons especially the anterior nares has been reported to be the most pathogenic and most studied specie of the genus Staphylococcus because it exhibits a variety of virulent factors. Some examples are the Panton-Valentine leukocidin toxin (PVL), toxic shock syndrome toxin 1 (TSST-1), hemolysins, exfoliative toxins (ETs), and staphylococcal enterotoxins (SEs) with which it causes a wide range of clinical infections such as minor skin and soft tissue infections to life threatening conditions such as necrotizing pneumonia, septic arthritis, endocarditis, and osteomyelitis and food poisoning.[1] Food microbiologists have identified *S. aureus* as a food borne hazard regarding it as the third major cause of food borne diseases across the globe.[2] The expression of a diverse array of the enterotoxin types A,B,C,D,E,G,H,I,J,K,L,M,N,O,P,Q,R and U by *S. aureus* confers on the organism the ability to cause food poisoning.[3] However, only the enterotoxin types A, B, C, D and E otherwise called "classical enterotoxins" have been reported to be implicated in 95% of food poisoning outbreaks.[4] Staphylococcal food poisoning (SFP) occur as a result of the intake of food items that contain high levels of preformed staphylococcal classical enterotoxins.[5,6] Symptoms such as abdominal cramping, nausea, forceful vomiting associated with diarrhea or without diarrhea usually occur rapidly about 2 to 8 hours after consumption of contaminated food.[7] The infection usually resolves on its own after about 24 to 48 hours but can be severe such that hospitalization is required in young children, pregnant women, elderly persons and immunocompromised persons.[8] The non-fastidious characteristic of *S. aureus* allows the organism to grow on substrates with a wide range of temperature (7 - 48^{0}C), pH (4.2 to 9.3) and reduced water activity (a_w = 0.86).[9] It has been demonstrated that since the organism cannot compete favourably with the naturally occurring microbiota in raw foods, contamination is usually as a result of improper handling practices and storage conditions that encourage the multiplication of the

organism its enterotoxins.[10] Food handlers, especially those who carry enterotoxin-producing strains of the organism in their noses and on their hands have been suggested to be the major source of food contamination by virtue of the fact that *S. aureus* naturally colonise the skin and mucous membranes of apparently healthy persons.[11] This contamination may occur through direct contact or through secretions from their respiratory tracts.[12] Commonly incriminated foods in staphylococcal intoxication include products of meat, poultry, dairy and bakery especially creamy pastries, cakes and sandwich fillings. Salads and vegetables and salty foods such as hamburgers have also been implicated.[13] The rising occurrence of staphylococcal food poisoning has become a food safety issue of global concern.[14] Although there is a poor surveillance data on foodborne diseases in Africa, data on a number of outbreaks have however been recorded from epidemiological studies conducted.[15] Because of increasing urbanization and changes in lifestyle of consumers, there is a tremendous shift from eating home-prepared food to consumption of street vended foods in many developing countries. Phenotypic studies on the microbiological quality of street vended foods in Port Harcourt and other major cities in Nigeria by other authors have shown a high prevalence of *S. aureus*.[16,17] There is very little data on the characterization of *S. aureus* isolates from street foods and handlers using molecular methods for the detection of virulent determinants. It has therefore become necessary to employ molecular assays which has proven to be more accurate, sensitive and has a high discriminatory power to detect enterotoxigenic strains of *S. aureus*.[18] The aim of this study was to investigate the presence of *S. aureus* in street vended foods and their handlers in Port Harcourt, Nigeria, genetically confirm isolates as *S. aureus* by amplifying the 16s rRNA and nuc genes and to detect the enterotoxin genes sea, seb, sec, sed and see using polymerase chain reaction.

MATERIALS AND METHODS

Sample Collection

A total of 270 samples comprising 135 street vended food samples and 135 hand swab samples from food handlers after obtaining informed consents. The street vended food samples consisted of nine (9) different food types as follows: Meat pie, Roasted meat (‘Suya’), Fried meat, Salad dressing, African salad (‘Abacha’), Beef burger, Doughnut, Cupcake and Fish pie. These were purchased from the different selling points in the five major zones that make up Port Harcourt metropolis. Outlets such as street hawkers, kiosks and restaurants were utilized. The food samples were collected from the containers used by the food vendors to sell them using a sterile spatula and immediately transferred to sterile containers with ice packs and labelled appropriately. The hand swab samples were collected by swabbing the palms and in between the fingers of food handlers with sterile cotton wool swab sticks moistened in 0.1% peptone water. All samples were immediately transported to the laboratory for prompt analysis.

Isolation, Enumeration and Identification of *S. aureus*

Ten (10g) of a representative portion of each food sample was homogenized in 90ml of sterile peptone water (0.1%). A 10 fold serial dilution done and an aliquot of 0.1ml inoculated onto mannitol salt agar for total staphylococcal counts (TSC) by spread plating. The food and hand swabs of food handlers were inoculated onto mannitol salt agar for the presumptive identification of *S. aureus* using the streak plate method and incubated aerobically at 37^0C for 24hours as described by El-Jakee et al.[19] Further confirmatory tests such as Gram’s staining and biochemical tests were done according to standard methods by Cheesbrough.[20]

Molecular Characterization

DNA extraction and PCR amplification of the 16s rRNA, nuc and sea-see genes of fifteen (15) selected multidrug resistant (MDR) *S. aureus* isolates were carried out in accordance with the methods and protocols described by Brakstad, Klindworth et al and Chapaval et al.[21-23] using specific primers (Table 1). This was done in a thermal cycler (ABI 9700, Applied Biosystems) for 35 cycles at a final volume of 40 microlitres (40µl) with specific thermal cycling conditions as described by Rall et al.[24] (Table 2). The PCR mixture included: the 2X Dream Taq Mastermix (Taq polymerase, buffer, DNTPs, $MgCl_2$, 0.4M Primers) supplied by Inqaba, South Africa and the extracted DNA. The PCR products were resolved on a 1% agarose gel at 130V for 25 minutes and visualized on a blue light transilluminator.

Table 1: List of Primers for PCR Amplification.

Gene	Primer	Oligonucleotide sequence (5'-3')	Location within the gene	Expected amplicon size (bp)
16srRNA	F	AGAGTTTGATCCTGGCTCAG	27-1492	1500
	R	CGGTTACCTTGTTACGACTT		
nuc	NUC-1	GCGATTGATGGTGATAGGGTT	147-181	270
	NUC-2	AGCCAGCCTTGACGAACTAAAGC		
sea	SEA-1	TTGGAAACGGTTAAAACGAA	490-509	200
	SEA-2	GAACCTTCCCATCAAAAACA	591-610	
seb	SEB-1	TCGCATCAAACTGACAAACG	634-653	478
	SEB-2	GCAGGTACTCTATAAGTGCC	1091-1110	
sec	SEC-1	GACATAAAAGCTAGGAATTT	676-695	257
	SEC-2	AAATCGGATTAACATTATCC	913-932	
sed	SED-1	CTAGTTTGGTAATATCTCCT	354-373	278
	SED-2	TAATGCTATATCTTATAGGG	632-671	
see	SEE-1	TAGATAAAGTTAAAACAAGC	491-510	209
	SEE-2	TAACTTACCGTGGACCCTTC	640-659	

Source: Brakstad, Klindworth et al and Chapaval et al.[21-23]

Table 2: Thermal Cycling Conditions.

Gene	Initial Denaturation		Denaturation		Annealing		Extension		Cycles	Final Extension	
	Temp (^{0}C)	Time (min)	Temp (^{0}C)	Time (sec)	Temp (^{0}C)	Time (sec)	Temp (^{0}C)	Time (sec)		Temp (^{0}C)	Time (min)
16s rRNA	95	5	95	30	52	30	72	30	35	72	5
nuc	95	5	95	30	52	30	72	30		72	30s
sea-see	95	5	95	30	45	30	72	30		72	5

Source: Rall et al.[24]

RESULTS

The Total Staphylococcal Count (TSC) was highest in African Salad, Roasted meat ('Suya') and Salad dressing with values of 1.0 x 10^6, 3.0 x 10^5 and 3.0 x 10^5 cfu/g respectively. Cupcake, Meat pie, Beef burger and Fish pie had slightly lower values of 1.0 x 10^5, 4.0 x 10^4, 2.0 x 10^4 and 1.0 x 10^4 cfu/g respectively (Table 3).

Table 3: Total Staphylococcal Count (TSC) from Food Samples.

Food type	Mean Staphylococcal count (cfu/g)	Satisfactory ACC levels, CFS[25]
African Salad ('Abacha')	1.0 x 10^6	<20 - <10^4
Roasted meat ('Suya')	3.0 x 10^5	
Salad dressing	3.0 x 10^5	
Cupcake	1.0 x 10^5	
Meat Pie	4.0 x 10^4	
Beef burger	2.0 x 10^4	
Fish pie	1.0 x 10^4	
Doughnut	1.0 x 10^3	
Fried Meat	1.0 x 10^3	

Key: < = Less than, ACC = Aerobic colony count, CFS = Centre for Food Safety

Occurrence of *S. aureus* in Food and Food Handlers

A total of 233 *S. aureus* isolates were recovered from 270 street vended foods and food handlers sampled. Of these 233 isolates, 98 (72%) were recovered from food samples while 135 (100%) were recovered from hand swabs from food handlers representing an overall prevalence of 86%.

Detection of the 16s rRNA and Thermonuclease Genes

All *S. aureus* isolates were positive for the 16s rRNA and thermonuclease (nuc) genes. PCR products of the expected sizes were generated (Figure 1).

Detection of Staphylococcal Enterotoxin Genes

Of the fifteen (15) multi drug resistant (MDR) isolates subjected to PCR, sea gene was detected in 4(26%) from food samples of Roasted meat ('Suya'), Fried meat, Burger and Doughnut. sed gene was detected in 3(20%) from food handlers of Doughnut, Beef burger and Cupcake while see gene was detected in 1(6%) in food samples of Cupcake. All the amplicons produced bands of the expected sizes (Figure 2). seb and sec genes were neither detected in food nor food handlers in this study.

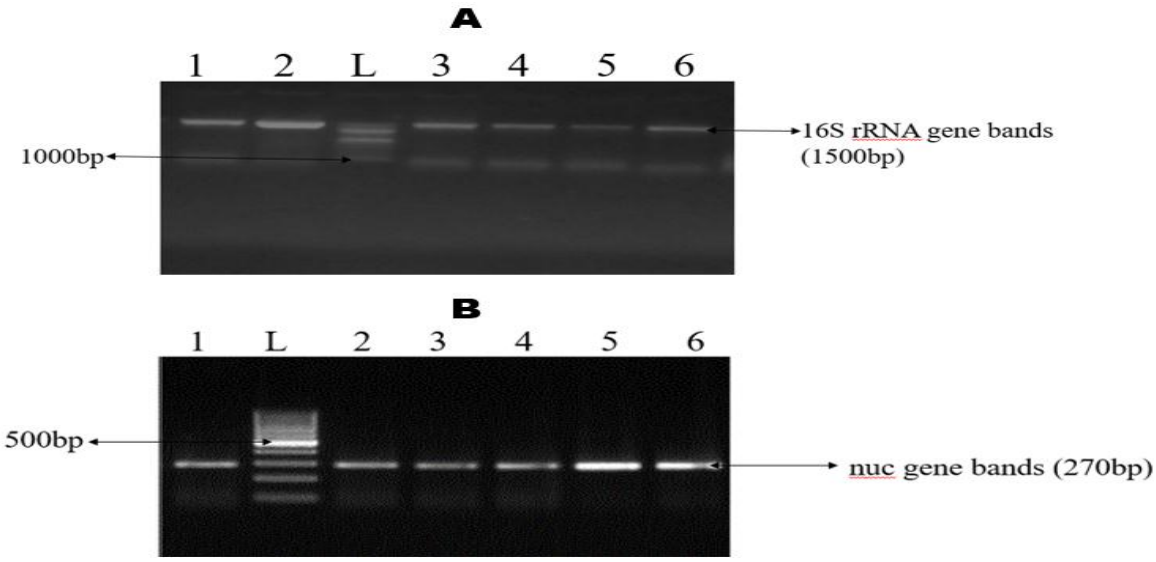

Fig. 1: Agarose gel picture showing the amplified 16S rRNA gene of 1500bp. Lanes 1-6 represents the is olates and Lane L represents the 100bp DNA ladder (molecular marker) highlighting the 1000bp mark. B: Amplification of the nuc gene of 270bp. Lanes 1-6 represents the isolates and Lane L represents the 100bp DNA ladder (molecular marker) highlighting the 500bp mark.

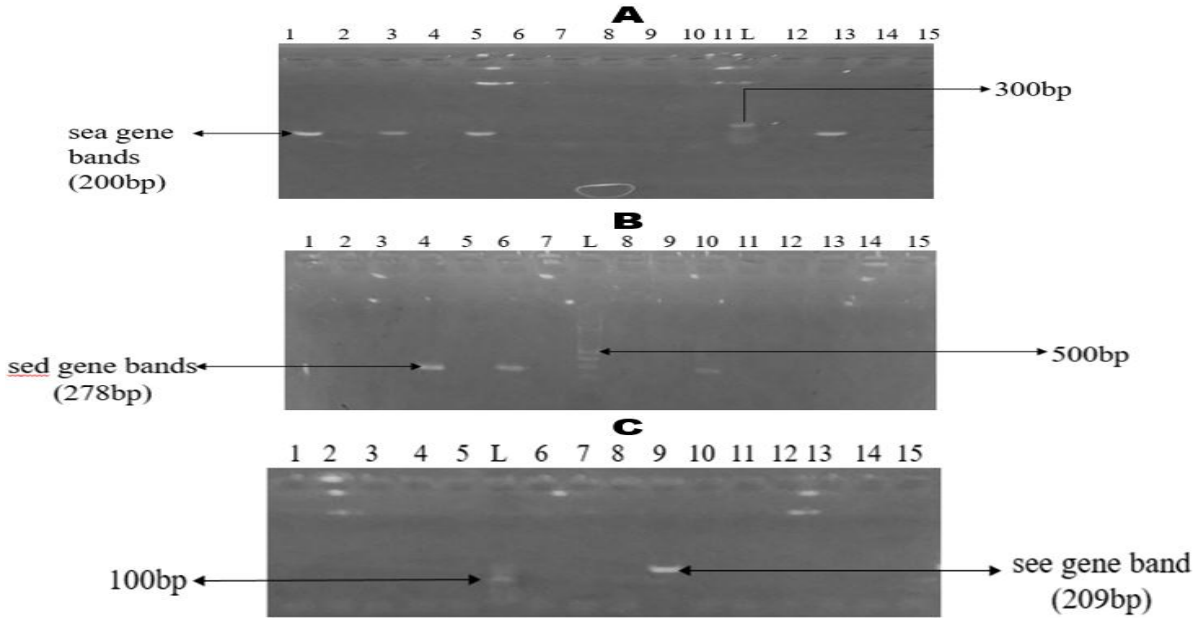

Fig. 2: Agarose gel picture showing the amplified genes. A: sea genes of 200bp in isolates 1, 3, 5 and 13, Lane L represents the DNA ladder at 300bp. B: sed genes of 278bp in isolates 4, 6, and 10, Lane L represents the DNA ladder at 500bp. C: see gene of 209bp in isolate 9, Lane L represents the DNA ladder at 100bp.

DISCUSSION

Comparative DNA sequence analysis of microbial genes have shown the 16S rRNA gene amplification as a good technique for detecting bacteria but quite insensitive for specie differentiation hence the detection of the nuc gene which is unique to *S. aureus* was amplified for further genetic confirmation of the identity of the organism. The high levels of contamination of street vended foods with *S. aureus* recorded in this study are most likely as a result of poor hygienic practices and frequent handling by the colonized food handlers during food preparation as reported by several studies stating that processed foods requiring frequent handling during preparation are more vulnerable to *S. aureus* contamination.[26,27] This is evident in the fact that most street vended foods in Port Harcourt city are often processed and served with bare hands. Errors in food processing such as insufficient time and temperature during initial cooking and reheating processes, prolonged exposure of foods at room or outdoor temperature accounting, preparing foods for extended periods of time prior to serving, insufficient cleaning of processing equipment or utensils and storage in contaminated environments are also responsible for high contamination levels recorded.[28] The conclusive diagnostic criteria of Staphylococcal food borne disease (SFD) are based upon the detection of staphylococcal enterotoxins in food[29] or recovery of at least 10^5 *S. aureus* from food remnants.[30] To this end, various phenotypic methods have been used to detect enterotoxin production in *S. aureus* in Port Harcourt but molecular methods that employ the use of the polymerase chain reaction (PCR has proven to be a more useful and reliable tool for detecting toxigenic genes.[31] and was thus utilized in this study. SEA was the most frequently encountered enterotoxin in food and not in food handlers with a percentage detection of 27% correlating with previous data in the United States where SEA was recovered from 77.8% of all SFD outbreaks followed by SED (37.5%) and SEB (10%). SEA was also reported to be the most commonly found enterotoxin among SFD outbreaks in Japan, France, and the United Kingdom.[32] and a probable reason for its high frequency in foods could be because of its high resistance to proteolytic enzymes. SEB was undetected in this study most likely due to its low prevalence. Reports also show that SEC although undetected in foods in this study are also implicated in SFD as seen in several outbreak reports such as gastrointestinal illnesses that occurred via contaminated coleslaw in the United States caused by SEC produced by methicillin-resistant *S. aureus* (MRSA) from an asymptomatic food handler.[7] In this study, only SED was detected in food handlers and none in food and is in line with previous studies that state that 12.5% of *S. aureus* from the hands of the food handlers produced one or combination of staphylococcal enterotoxins.[33] SEE which was detected in food correlates with results from other studies where SEE was detected in soft cheese made from unpasteurized milk and was responsible for Six SFD outbreaks that occurred in France in 2009.[29]

CONCLUSION

The presence of enterotoxigenic *S. aureus* in street vended foods and handlers pose a public health threat to consumers because it enhances the spread of these virulent strains in the food chain, within the community and even in hospital settings. Strict enforcement of public health laws by environmental health officers to check the operations of street food vendors and handlers should be employed and the microbiological guidelines such as hazard analysis and critical control points (HACCP), good hygienic practices (GHPs) and good manufacturing practice (GMPs) all instituted by the World Health Organization in conjunction with the United States Food and Drug Administration (FDA) should be strictly implemented and adhered to because it will go a long way in preventing *S. aureus* contamination in Port Harcourt metropolis.

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