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Molecular Detection of Enterotoxigenic *Staphylococcus aureus* in Ready-to-eat Cowhide ('Ponmo') Collected from Lagos Metropolis, Nigeria

Teniola, Olakunle David [a], Abisoye, Fiyinfoluwa Solomon [a*], Folounso, Temitayo Veronica [b], Fowora, Adenike Muinah [c], Ikuesan, Adeleke Felix [a] and A. Bamidele, Tajudeen [c]

[a] Biological Sciences Department, Olusegun Agagu University of Science and Technology [OAUSTECH], PMB 535, Okitipupa, Ondo State, Nigeria.
[b] Microbiology Department, Adekunle Ajasin University [AAU], Akungba-Akoko, Ondo State, Nigeria.
[c] Biotechnology Department, Nigerian Institute of Medical Research [NIMR], Yaba, Lagos, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Ready-to-eat [RTE] animal products like *ponmo* are preferred by consumers due to its palatability and quality. However, foodborne pathogens particularly *Staphylococcus aureus* are sources of concern due to cross-contamination of raw and cooked cowhide. This study aimed to investigate the incidence of enterotoxigenic *S. aureus* in ready-to-eat locally processed cowhide.
Methodology: Sixty (60) RTE cowhide samples were collected from different locations in Lagos, Nigeria and analyzed using conventional microbiological and molecular techniques for the detection of toxigenic *S. aureus* contamination. Suspected *S. aureus* isolates were confirmed by the presence of thermostable endonuclease [*nuc*] gene in their genome.

**Corresponding author: Email: fiyinfoluwapraiz50@gmail.com;*

Results: Result showed that 25 (41.67%) and 20 (33.50%) samples harbored **c**oagulase-positive *S. aureus* and 20 other bacterial species different from *S. aureus*, respectively while 15 (24.83%) of the tested *ponmo* samples yielded no bacterial growth. Thirteen of the 15 randomly selected from the 25 suspected isolates were confirmed as *S. aureus* by the presence of thermostable endonuclease [*nuc*] gene in their genome. Enterotoxigenic genes were confirmed in all the 13 PCR detected *S. aureus*. Enterotoxin B gene is most prevalent in *ponmo*. Multiplex PCR detection of *S. aureus* enterotoxins [SE] genes revealed the molecular detection of different isolates carrying staphylococcal enterotoxin types A and B, mixed strain carrying both staphylococcal enterotoxins type A and type D. Antibiotic susceptibility of 20 *S. aureus* isolates revealed varying degrees of susceptibility patterns against the antimicrobial agents. Generally, gentamicin 70% (14/20), azithromycin 75% (15/20), co-trimoxazole 85% (17/20), levofloxacin 95% (19/20) were the most effective antibiotics to *S. aureus*. A low, ≥50% susceptibility was recorded to chloramphenicol 55% (11/20) and nitrofurantoin 65% (13/20). A higher resistance to streptomycin (90%; 18/20) and ceftazidime (95%; 19/20) was identified, with resistance to ceftazidime being the highest (95%; 19/20).

Conclusion: It can be concluded that RTE *ponmo* vended in the study sites is of low hygienic quality and may be of health risk to consumers. High level hygiene practice and good manufacturing practices are required during the production, distribution and marketing of *ponmo* to curb the potential health consequences of eating *ponmo.*

Keywords: Antimicrobial susceptibility testing; cowhide; enterotoxins; PCR; ponmo; Staphylococcus aureus; ready-to-eat food.

1. INTRODUCTION

Cowhide also called *Ponmo* in Lagos and other parts of Yoruba speaking ethnic nationality is an important traditional and favourite alternative to consumed by majority of Nigerians and other West African countries irrespective of their ethno-geopolitical background, hence its economic significance. Although, 'ponmo' consumed all without discrimination [1] but more by low-income earners because of its cheaper relative to other meat forms. *Ponmo* may be brown type described as directly processed dried cow hide stored over time and processed by careful singling and soaking to achieve a unique textural product that can be described as a ready-to-eat meat form. Alternatively, it is directly prepared as white *ponmo* by direct cooking of the cowskin after removing the hair. Nutritionally, it is poor, and unconfirmed reports adopt eating it as a form of weight control.

Foodborne diseases [FBD] caused by many pathogens that contaminate food and food products [1] remain one of the greatest concerns in public health and food safety. In 2010, FBD were estimated to cause 600 million illnesses, resulting in 420,000 deaths and 33 million disability-adjusted life years [DALYs], demonstrating that the global burden of FBD is of the same order as the major infectious diseases such as HIV/AIDS, malaria, and tuberculosis. It is also comparable to certain other risk factors such as dietary risk factors, unimproved water and sanitation, and air pollution [3].

Microorganisms such as *Micrococcus, Staphylococcus, Bacillus* and *Streptococcus* most of which are of public health concern due to their toxin production have been reported in animal skin used for *ponmo* production [4,5]. Nagase *et al* [6] reported the detection of *Staphylococcus* in 100% of pigs and cows, 90% of humans and horses, 77% of laboratory mice and 40% of dogs. Staphylococcal foodborne disease is caused by contamination of food during preparation or serving by preformed *S. aureus* enterotoxin [7]. Enterotoxins are chromosomally or plasmid-encoded exotoxin that enters the stomach and intestines through contaminated foods and water causing symptoms such as cramps, nausea, vomiting or diarrhea [8,9,10]. They are heat labile ($>60^0$), and are of low molecular weight and water-soluble. Enterotoxins are frequently cytotoxic and kill cells by altering the apical membrane permeability of the mucosal (epithelial) cells of the intestinal wall [11]. *Staphylococcus aureus* is a cluster-forming spherical-shaped Gram-positive bacterium known to cause foodborne intoxication. Contamination by toxigenic *S. aureus* in RTE food is a major public health issue in both developing and developed countries like the USA and Japan [12,13]. In 1997, approximately 185,000 people suffered from staphylococcal enterotoxin related food-poisoning including thousands of deaths. [14]. Due to two aggravating characteristics such as toxin production and a wide range of antibiotic

resistance, *Staphylococcus aureus* is regarded as the third most important cause of foodborne illness in the world [15].

A wide variety of foods including milk, meat, meat products, dairy products, and RTE food support the growth of *Staphylococcus aureus* and are ideal for enterotoxin production [16,17]. The five principal classical forms of staphylococcal enterotoxins [SEs] include SEA, SEB, SEC, SED, and SEE, as well as the toxic shock syndrome toxin [TSST-1] that causes toxic shock syndrome in humans [18].

Although *Staphylococcus aureus* can create a wide range of enterotoxins, classical enterotoxins A, B, C, D, and E are responsible for 95% of food poisoning outbreaks [19] because these toxic proteins can withstand temperatures of up to 100°C for several minutes. Improperly cooked food or undercooked food contaminated with bacteria or its preformed toxins in sufficient amounts can cause staphylococcal food poisoning in as little as a few hours, with symptoms such as nausea, vomiting, and diarrhea [20]. Immunodiagnostic approaches as well as molecular biology techniques such as the Polymerase Chain Reaction (PCR) can be used to detect the presence of staphylococcal enterotoxins in meals [21]. This study was designed to investigate the prevalence of enterotoxigenic *S. aureus* strains in RTE cowhide (*ponmo*) product from different areas in Lagos State, Nigeria.

2. MATERIALS AND METHODS

2.1 Collection of Samples

A total of 60 samples of RTE cowhide *ponmo* were collected from various restaurants and street sellers in different locations in Lagos State, Nigeria. Twenty *ponmo* samples each were purchased from different vendors at Yaba, Mushin and Oyingbo markets and transported aseptically to the laboratory for bacteriological analysis and molecular detection of toxigenic *S. aureus* contamination.

2.2 Coagulase-positive *Staphylococcus aureus* Isolation and Identification

This was performed according to standard procedures, briefly the *ponmo* was suspended in Nutrient Broth (Oxoid CM0067) and incubated aerobically on shaker water bath at 37^{o}C overnight. Inoculum was taken from the broth culture onto Mannitol Salt Agar (MSA, Oxoid 0085) and incubated as above for 24 hrs. All yellow, catalase, coagulase and DNase positive colonies presumed to be *S. aureus* were used for further assays [22].

2.3 DNA Extraction and PCR Detection of NUC Gene

The extraction of DNA was done on the pure colonies by boiling, following the methods of [23], while the *nuc* gene was detected by PCR using specific primers (Table 1) and standard methods. A reaction volume of 10 *μ*L including 2 *μ*L of Mastermix PreMix, 0.1 *μ*L each of primer pair, 2 *μ*L of template DNA, and 5.8 *μ*L of double distilled water was mixed. The cycling parameters were, initial denaturation at 94°C for 3 minutes; 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 40 seconds, and extension at 72°C for 45 seconds and final extension at 72°C for 10 minutes [24].

Picture of ready-to-eat *ponmo*

2.4 PCR Detection of *Staphylococcal enterotoxins*

The Biorad DNA Engine Dyad Peltier thermocycler was used to accomplish multiplex PCR amplification. The SolisBiodyne PCR Mastermix and specific primers specified in Table 1 were used to amplify the SE genes SEA, SEB, SEC, SED, and SEE from *Staphylococcus aureus*. The PCR assay was performed in a total volume of 10 *µ*L reaction mixture, which included 2 *µ*L of Mastermix PreMix, 0.1 uL of primer pair, 2*µ*L of template DNA, and 5.8*µ*L of double distilled water. The cycling programme was; initial denaturation at 94°C for 3 minutes; 35 cycles of denaturation at 95°C for 30 seconds, annealing at 52^{o}C for 40 seconds, and extension at 72°C for 45 seconds; and final extension at 72°C for 10 minutes.

2.5 Antimicrobial Susceptibility Testing

The PCR confirmed isolates were subjected to susceptibility testing using 8 antibiotics. This was performed by the Kirby-Bauer agar disk diffusion method on Mueller-Hinton according to [25]. The pure colonies were suspended in sterile normal saline, adjusted to 0.5 MacFarland standard before making a lawn on Mueller Hinton agar (Oxoid CM0337B) using sterile swab stick. The agar plates were left for few minutes to allow the surface dry before introduction of antibiotics. The antibiotics used were nitrofurantoin (NIT), gentamicin (GEN), streptomycin (S), co-trimoxazole (COT), ceftazidime [CAZ), chloramphenicol (C), levofloxacin (LEV), and azithromycin (AZM). The plates were incubated aerobically at 37^{o}C for 24 h, and the diameters of the zone of inhibition measured and results interpreted.

2.6 Quality Control

All samples were collected and analyzed aseptically, nuc gene generating *Staphylococcus aureus*, and a negative control, sterile distilled water, were included in the PCR run.

3. RESULTS AND DISCUSSION

3.1 Prevalence of *S. aureus* Isolates Found in RTE *ponmo* Samples

Using phenotypic or biochemical tests coagulase positive *Staphylococcus aureus* was found in 25 41.67%) of the 60 RTE *ponmo* samples taken from various locations. However, bacteria other than coagulase positive *S. aureus* were found in 20 (33.50%), while 15 samples (24.83%) of the samples did not exhibit bacterial growth (Table 2). *Staphylococci* are one of the most prevalent bacterial contaminants according to USFDA [2004] [25] reported that *S. aureus* is ubiquitous in nature and inhabits the mucous membranes and skin of most warm-blooded animals, including food animals and humans. The skin of the handlers may contain *Staphylococcus aureus*, a sign that processed goods can be contaminated [26]. Numerous episodes of food poisoning brought on by touch with hands are caused by *S. aureus* [27].

Table 1. Primers used for detection of *S. aureus* enterotoxins, and *nuc* genes

Primer	Sequence 5'- 3'	Product size [bp]
Enterotoxins gene primers		
SA-Ua-F	TGTATGTATGGAGGTGTAAC	-
SA-A-R	ATTAACCGAAGGTTCTGT	270
SA-B-R	ATAGTGACGAGTTAGGTA	165
ENT-C-R	AAGTACATTTTGTAAGTTCC	102
SA-D-R	TTCGGGAAAATCACCCTTAA	303
SA-E-R	GCCAAAGCTGTCTGAG	213
Nuc gene primers		
Nuc-F	GGGTTGATACGCCAGAAACG	270
Nuc-R	TGATGCTTCTTTGCCAAATGG	270
Ua, universal; f, forward; r, reverse		

Table 2. Distribution of bacteria isolated from RTE *ponmo* samples purchased from different locations in Lagos State, Nigeria

Isolate	Oyingbo	Yaba	Mushin	Total	Percentage [%]
S. aureus	8	10	7	25	41.67
Others bacteria	9	6	5	20	33.50
No growth	4	5	6	15	24.83
Total	21	21	18	60	100

#Distribution based on phenotypic or biochemical features

Nossair et al. [28] reported the prevalence of coagulase positive *S. aureus* were 20, 20, 12, 32 and 28% in the minced beef, sausage, beef burger, hand and nasal swabs, respectively examined In Egypt, while the prevalence of coagulase negative *S. aureus* were 28, 36, 24, 44 and 36% of the samples respectively. The absence of *S. aureus* in up to 24.83% of the *ponmo* samples analyzed is of major interest suggesting that certain food handlers are compliant with good hygiene practices thereby preventing contamination and cross-contamination of the ready- to -eat ponmo. However, [29] reported that cooked meat products may be loaded with many foodborne pathogens such as *S. aureus* due to post-cooking cross-contamination through mishandling and/or contact with raw materials. The absence of bacteria in 24.83% samples could also be attributed to material used for burning the hide which might be inhibitory to bacterial growth. Harmful and hazardous materials such as tires, different forms of toxic plastics dangerous to growth have been used in many localities.

3.2 Detection of Enterotoxin Genes among *S. aureus* Isolates

The *S. aureus* specific *nuc* gene was amplified in 13 (52%) of the suspected 25 isolates for *S. aureus* in this study. The *nuc* gene found in 13 isolates confirms that 21.67% of the RTE *ponmo* samples have *S. aureus* while 78.33% of *ponmo* samples were free from enterotoxin-producing *S. aureus*. When these chosen isolates were further investigated for enterotoxin genes type A through E using multiplex PCR, results confirmed the molecular detection of *S. aureus* isolates carrying enterotoxin genes. The SA-B gene was the most prevalent enterotoxin gene, with nine out of the thirteen isolates (69.2%) being positive for SA-B. In addition, out of thirteen examined isolates, four isolates [30.8%] carried SA-A gene, two isolates (15.3%) carried SA-E gene, one isolate (7.7%) carried both SA-A and SA-D genes, another one isolate [7.7.%] also carried both SA-B and SA-E genes, and two isolates (15.3%) carried SA-A, SA-B and SA-E genes were detected (Fig. 2). Results were comparable with [30,31,32,33], who recorded detection of entero-toxigenic *S. aureus* isolates carrying different enterotoxin genes from ready- to-eat meat products using multiplex PCR.

Staphylococcus aureus enterotoxins are the major virulence factor causing food poisoning by ingestion of foods contaminated with *S. aureus* heat-stable enterotoxins. The main SEs incriminated in SFP are staphylococcal enterotoxin A (SEA), staphylococcal enterotoxin B [SEB], staphylococcal enterotoxins C [SEC], and staphylococcal enterotoxins D (SED); *Staphylococcus aureus* enterotoxin type A is the most common cause of SFP worldwide, but the involvement of other classical SEs (SEB to SEE) have been also recorded which made PCR detection of enterotoxigenic *S. aureus* essential to identify staphylococcal food poisoning [34,35].

3.3 Antibiotics Sensitivity Pattern of the *Staphylococci* Isolates

Twenty (20) isolates were randomly selected from the 25 suspected *Staphylococci* isolates. Results showed varying degrees of susceptibility patterns against the antimicrobial agents as follows; gentamicin 70% (14/20), azithromycin 75% (15/20), co-trimoxazole 85% (17/20), levofloxacin 95% [19/20]. A low, ≥50% susceptibility was recorded to chloramphenicol 55% (11/20) and nitrofurantoin 65% (13/20). A higher resistance to streptomycin (90%; 18/20) and ceftazidime (95%; 19/20) was identified, with resistance to ceftazidime being 95% (19/20). The percentage of antimicrobial resistance of *S. aureus* isolates are shown in Fig. 3.

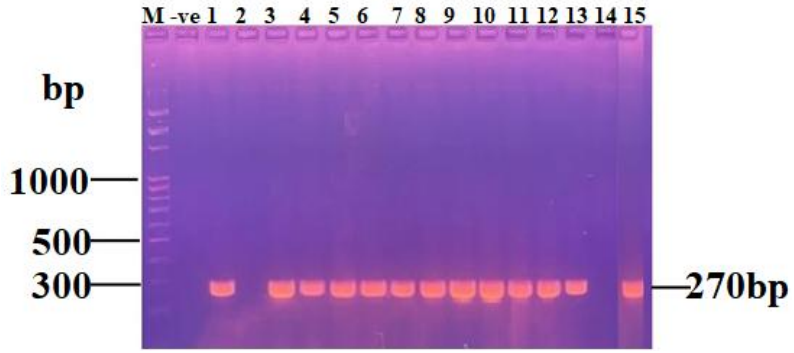

Fig. 1. PCR amplification of *nuc* gene of *S. aureus* on 1.5% agarose gel electrophoresis. Lane M: 100 bp DNA ladder, Lane -ve: Negative control. Lanes 1, 3, 4-13 and 15: *nuc* gene

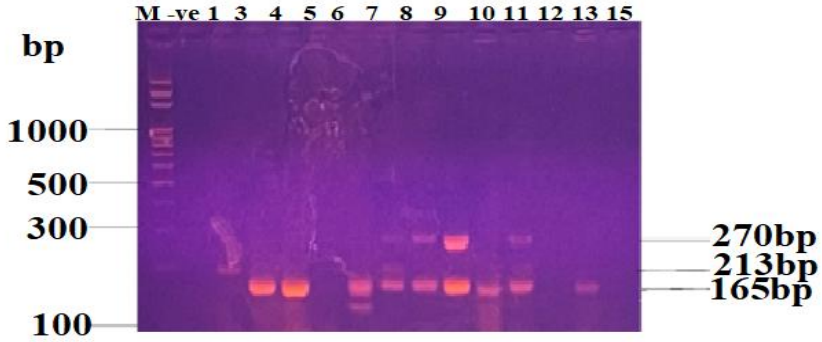

Fig. 2. Agarose gel electrophoresis of multiplex PCR of SA-A [270 bp], SA-B [165 bp], ENT-C [102 bp], SA-D [303bp] and SA-E [213 bp] enterotoxin genes for characterization of *S. aureus*. Lane M: 100 bp DNA ladder. Lane -ve: Negative control

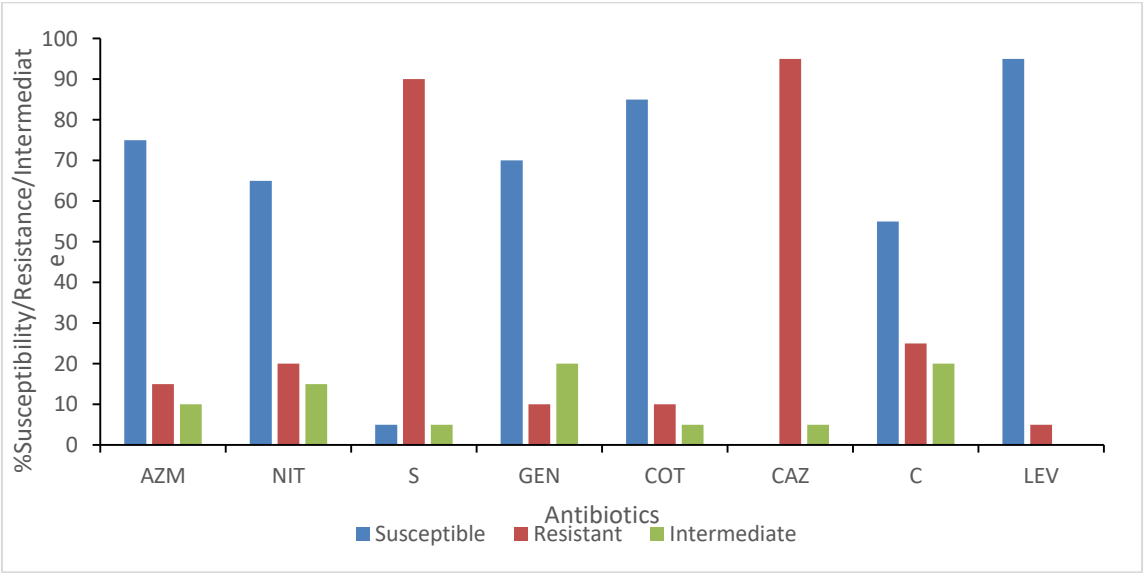

Fig. 3. The percentage of antimicrobial resistance profiles of *S. aureus* isolates. AZM = azithromycin, NIT = nitrofurantoin, S = streptomycin, GEN = gentamicin, COT = co-trimoxazole, CAZ = ceftazidime, C = chloramphenicol, LEV = levofloxacin

4. CONCLUSION AND RECOMMENDATION

The results obtained in this study suggests that enterotoxigenic *S. aureus* is common in ready – to – eat 'ponmo' in the locality with type B being the most prevalent, followed by A and E and some carried multiple toxin types. Most of the bacterium also showed resistance to streptomycin and ceftazidime. The prevalence of *S. aureus* among the tested samples, in the study sites, especially the enterotoxigenic strains highlights the necessity of enforcing hygienic practices within fast food and street vended foods kitchens. In the future, the molecular and ecological characterization of isolated toxigenic *S. aureus* strains must be performed to determine the origin of the contamination. Better knowledge of strict hygienic practices during the collection of raw materials, preparation and handling of food, storage, and serving must be passed on to food handlers.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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