MOLECULAR DETECTION OF ENTEROTOXIGENIC *Staphylococcus aureus* IN READY-TO-EAT COWHIDE [*PONMO*] COLLECTED FROM LAGOS METROPOLIS, NIGERIA

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* becomes problems due to post cooking cross-contamination through mishandling of raw materials and/or final product. This study aimed to investigate the incidence of *S. aureus* in ready-to-eat processed cowhide locally called *ponmo,* followed by molecular detection of enterotoxigenic.

Methodology: *S. aureus* isolates and its enterotoxin genes in 60 RTE cowhide products from different locations in Lagos, Nigeria. Samples of RTE *ponmo* were processed microbiologically and the result showed that 25 of the total 60 samples [41.67%] harbored **c**oagulase-positive *S. aureus*; 20 bacterial isolates [33.50%] that were other bacteria different from *S. aureus*, and 15 of the tested *ponmo* samples [24.83%] 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.

Results: 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. It can be concluded that RTE *ponmo* vended in the study sites is of low hygienic quality and may be risky to consumer's health except if there is improvement in the processing hygiene practice. 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 the highest [95%; 19/20].

Conclusion: High level hygiene practice and good manufacturing practices are required during the production, distribution and marketing of *ponmo*.

Keywords: *Ponmo*; cowhide; *Staphylococcus aureus*; Enterotoxins; Ready-to-eat food; PCR; Antimicrobial susceptibility testing.

1.0 INTRODUCTION

Ponmo is an important traditional meat delicacy otherwise loosely referred to as cowskin, consumed by the majority of Nigerians including almost all ethnic nationals [1]. It is safe to estimate its consumption by at least 70 % of the population, excluding babies and infants. Although consumed more by low-income earners because of its cheaper price compared with other meat forms, it is consumed by all without discrimination [1]. *Ponmo* consumer population in Nigeria may be safely put at above 80 million. It is also popular in other West African countries like Cameroon, Ghana, Benin, Togo and others, hence of economic significance. *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] remain one of the greatest concerns in public health and food safety; they are caused by many pathogens that contaminate food and food products [2]. 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* have been reported in animal skin used for *ponmo* production [4, 5]. Most of these microorganisms are of public health concern when capable of toxin production.

Staphylococcus being detected in 100% of pigs and cows, 90% of humans and horses, 77% of laboratory mice, and 40% of dogs [6]. 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□], 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 support the growth of *Staphylococcus aureus* and are ideal for enterotoxin production including milk, meat, meat products, dairy products, and RTE food [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], reason been that these toxic proteins can withstand temperatures of up to 100°C for several minutes, improperly cooked 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], because some strains of *Staphylococcus aureus* can withstand heat and drying, they can easily infect foods. This contamination could be caused by food handlers or the environment, where bacteria thrive and release toxins in undercooked or insufficiently cooked foods, especially if they are left out in the open. 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.

2.0 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 aerobicaly on shaker water bath at 37^{o}C overnight. Inoculum was taken from the broth culture onto Mannitol Sallt 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. Briefly, a reaction volume of 10 μL including 2 μL of Mastermix PreMix, 0.1 μL each of primer pair, 2 μL of template DNA, and 5.8 μL of double distilled water was mixed. The cycling parameters included, 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].

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 μL reaction mixture, which included 2 μL of Mastermix PreMix, 0.1 uL of primer pair, 2μL of template DNA, and 5.8μL 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.

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

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.

3.0 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%] of the 60 samples, while 15 [24.83%] of the samples did not grow [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].

In Egypt [28], examined that the prevalence of coagulase positive *S. aureus* in their examined samples was 20, 20, 12, 32 and 28% in the minced beef, sausage, beef burger, hand and nasal swabs, respectively, while the prevalence of coagulase negative *S. aureus* was 28, 36, 24, 44 and 36% of the examined samples respectively. The absence of *S. aureus* in up to 24.83% of the *ponmo* samples analyzed is of major concern. Although, the long periods of subjection to heat at high temperature during singling is enough to eliminate indigenous bacteria in the samples, the fact that the samples are not totally fresh and have been handled by the vendors and exposure to the non-sterile environment or cross-contamination of the market and the restaurant from where they are purchased makes no bacteria growth worrisome. [29] reported that cooked meat products may be loaded by many foodborne pathogens *S. aureus* due to post-cooking cross-contamination through mishandling and/or getting in touch with raw materials. A good explanation for the absence of bacteria in 24.83% samples could be 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 [unpublished reports]. It is also not unlikely the nutrient medium used and the incubation conditions did not support the growth of certain bacterial contaminants on the *ponmo*.

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

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

#Distribution based on phenotypic or biochemical features.

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 [from total of 60 samples collected] have *S*. *aureus* while 78.33% of *ponmo* samples are 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 detected enterotoxin gene, in which nine out of the thirteen examined isolates [69.2%] were 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 [Figure 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].

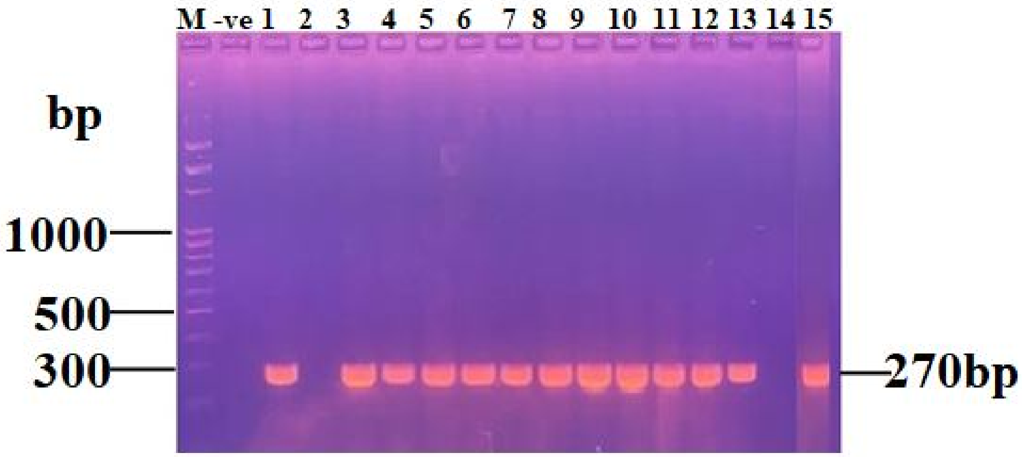

Figure 1: PCR amplification of *nuc* gene of *S. aureus* on 1.5% agarose gel electrophoresis. Lane M: 100 bp DNA ladder, Lane -ve: Negative control. Lanes 1, 3, 4-13 and 15: *nuc* gene.

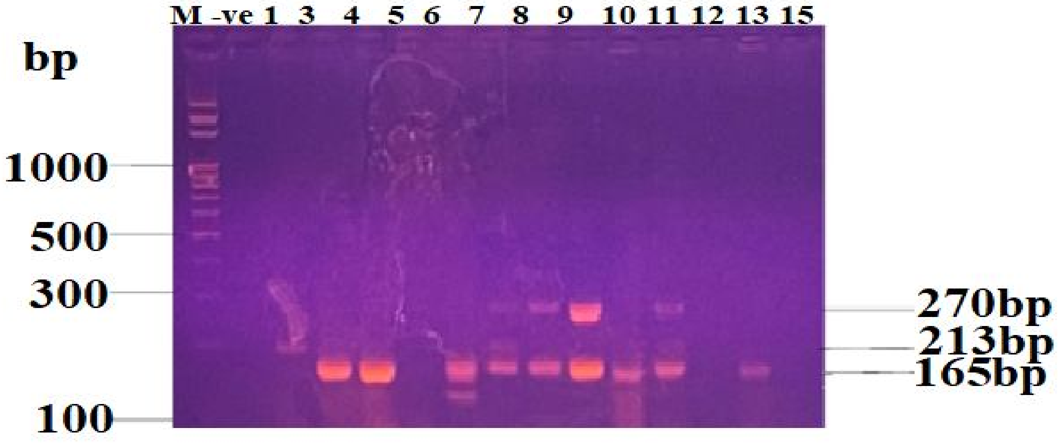

Figure 2: Agarose gel electrophoresis of multiplex PCR of SA-A [270 bp], SA-B [165 bp], ENT-C [102 bp], SA-D [303bp] and SA-E [213 bp] enterotoxin genes for characterization of *S. aureus*. Lane M: 100 bp DNA ladder. Lane -ve: Negative control.

3.3 Antibiotics Sensitivity Pattern of the *Staphylococci* Isolates

Twenty [20] isolates were randomly selected from the 25 suspected *Staphylococci* isolates for this and they 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 the highest [95%; 19/20]. The percentage of antimicrobial resistance of *S. aureus* isolates are shown in Figure 3

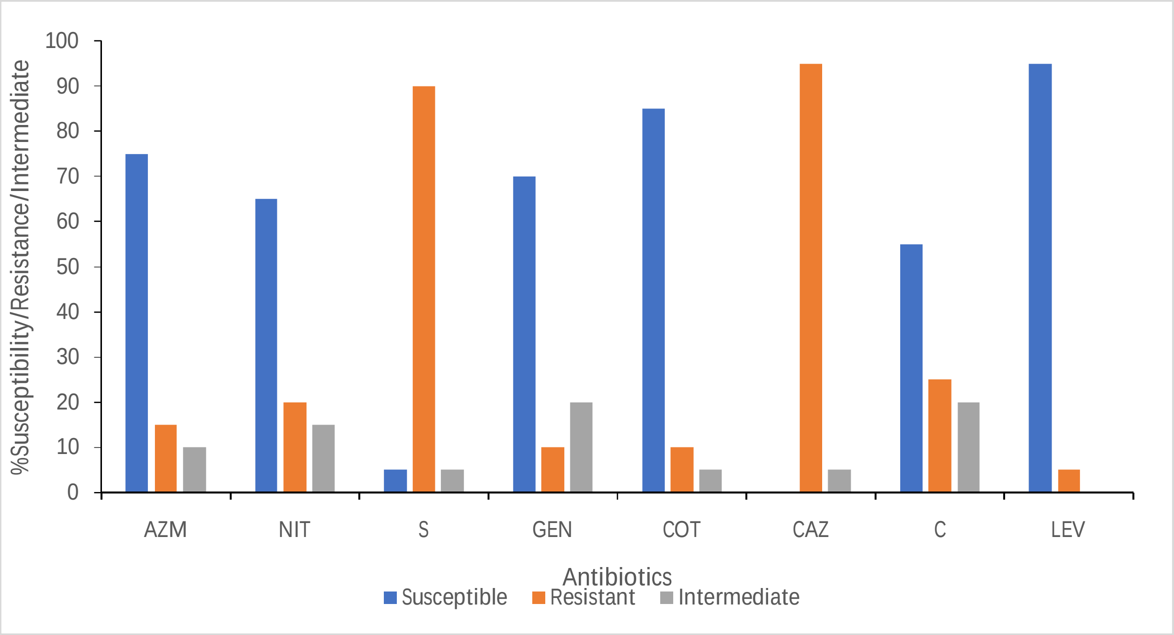

Figure 3: The percentage of antimicrobial resistance profiles of *S. aureus* isolates. AZM = azithromycin, NIT = nitrofurantoin, S = streptomycin, GEN = gentamicin, COT = co-trimoxazole, CAZ = ceftazidime, C = chloramphenicol, LEV = levofloxacin.

From this study results of molecular detection of enterotoxigenic *S. aureus* showed that SA-B gene was the most prevalent detected, in which nine out of the thirteen examined isolates where positive for SA-B, in addition, out of the thirteen examined isolates, four carried SA-A gene, two carried SA-E gene, one carried both SA-A and SA-D genes, one also carried both SA-B and SA-E genes, and two carried SA-A, SA-B and SA-E genes [Figure 2]. This is in agreement with the reports of [30, 31, 32, 33], who recorded detection of entero-toxigenic *S. aureus* carrying different enterotoxin genes from ready to eat meat products using multiplex PCR. Also, from this study it could be concluded that the *nuc* gene is standard for the identification of *S*. *aureus* as most samples isolated were positive towards that particular gene.

4.0 CONCLUSION AND RECOMMENDATION

The ready-to-eat *ponmo* vended in the study sites were demonstrated to harbor enterotoxigenic *S. aureus* with type B being the most prevalent, followed by A and E. 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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