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Short Communication **Open Access**

Effects of rinsing on *Staphylococcus aureus* load in frozen meats and fish obtained from open markets in Benin City, Nigeria

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Abstract:

Background: *Staphylococcus aureus* is a ubiquitous bacterium present in the environment and one of the leading causes of superficial and deep infections. In the food industry, it is acclaimed to be globally responsible for several food-borne diseases. This study was designed to isolate methicillin-resistant *S. aureus* (MRSA) and determine the effect of rinsing on MRSA load in frozen meat and fish obtained from open market in Benin City.
Methodology: Forty frozen meat samples (15 beef, 10 fish and 15 chickens) were randomly obtained from five markets in Benin City. The samples were analysed before and after rinsing using standard culture-based techniques to determine heterotrophic bacterial count, isolation of *S. aureus,* MRSA, and antibiotic susceptibility testing. Data were analysed using SPSS version 21 and Microsoft excel 2016, and association between variables were measured using Student's *t*-test with a probability level of < 0.05.
Results: The natural logarithm (LN) of heterotrophic bacterial count (CFU/g) before rinsing were 11.53±1.25 (beef), 11.16±0.95 (fish) and 11.42±1.58 (chicken), while the counts after rinsing were 2.70±0.45 (beef), 2.68±0.25 (fish) and 2.79±0.49 (chicken) (p<0.05). Sixteen of the 40 (40%) were positive for *S. aureus*, of which 4 (10%) were MRSA. Amongst the frozen meat evaluated in the study, beef had the highest frequency of *S. aureus* contamination (46.7%) followed by chicken (40.0%) and fish (30.0%). The profile of antibiotic resistance of *S. aureus* showed that they were least resistant to ciprofloxacin (6%) but showed high resistance to erythromycin (94%), amoxicillin/clavulanic acid (87.5%) and trimethoprim-sulfamethoxazole (81%). Multiple antibiotic resistance index of *S. aureus* was calculated to be 0.63.
Conclusion: The findings in this study revealed that frozen foods could act as a vehicle for the dissemination of antibiotic-resistant bacteria (ARB) and potential health risks for consumers.

Keywords: *Staphylococcus aureus*; antibiotic-resistant bacteria; MRSA; frozen meat; rinsing

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Effets du rinçage sur les charge de *Staphylococcus aureus* dans les viandes congelées et les poissons obtenus sur les marchés ouverts de Benin City, Nigéria

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Abstrait:

Contexte: *Staphylococcus aureus* est une bactérie ubiquitaire présente dans l'environnement et l'une des principales causes d'infections superficielles et profondes. Dans l'industrie alimentaire, il est reconnu pour être globalement responsable de plusieurs maladies d'origine alimentaire. Cette étude a été conçue pour isoler *S. aureus* résistant à la méthicilline (SARM) et déterminer l'effet du rinçage sur la charge de SARM dans la viande et le poisson congelés obtenus sur le marché libre de Benin City.
Méthodologie: Quarante échantillons de viande congelée (15 bœuf, 10 poissons et 15 poulets) ont été obtenus au hasard sur cinq marchés de Benin City. Les échantillons ont été analysés avant et après le rinçage en utilisant des techniques de culture standard pour déterminer le nombre de bactéries hétérotrophes, l'isolement de *S. aureus*, le SARM et les tests de sensibilité aux antibiotiques. Les données ont été analysées à l'aide de SPSS version 21 et de Microsoft Excel 2016, et l'association entre les variables a été mesurée à l'aide du test t de Student avec un niveau de probabilité <0,05.
Résultats: Le logarithme naturel (LN) du nombre de bactéries hétérotrophes (UFC/g) avant rinçage était de 11,53±1,25 (bœuf), 11,16±0,95 (poisson) et 11,42±1,58 (poulet), tandis que les comptages après rinçage étaient de 2,70±0,45 (bœuf), 2,68±0,25 (poisson) et 2,79±0,49 (poulet) ($p<0{,}05$). Seize des 40 (40%) étaient positifs pour *S. aureus*, dont 4 (10%) étaient SARM. Parmi les viandes congelées évaluées dans l'étude, le bœuf présentait la fréquence la plus élevée de contamination par *S. aureus* (46,7%), suivi du poulet (40,0%) et du poisson (30,0%). Le profil de résistance aux antibiotiques de *S. aureus* a montré qu'ils étaient les moins résistants à la ciprofloxacine (6%) mais présentaient une résistance élevée à l'érythromycine (94%), à l'amoxicilline/acide clavulanique (87,5%) et au triméthoprime-sulfaméthoxazole (81%). L'indice de résistance aux antibiotiques multiples de *S. aureus* a été calculé à 0,63.
Conclusion: Les résultats de cette étude ont révélé que les aliments surgelés pourraient servir de vecteur de dissémination de bactéries résistantes aux antibiotiques (ARA) et de risques potentiels pour la santé des consommateurs.

Mots clés: *Staphylococcus aureus*; bactéries résistantes aux antibiotiques; SARM; viande congelée; rinçage

Introduction:

Staphylococcus aureus is a ubiquitous bacterium in the environment as well as one of the leading causes of superficial infection in the hospital environment. In the food industry, it is acclaimed to be globally responsible for several food-borne diseases precisely ranked as the third most important foodborne pathogen (1). Frozen meats, fish or chicken are an important source of protein in diets such that day-to-day consumption of either of beef, fish or chicken is a common phenomenon across people of all social strata. It is worthy of note that none of the aforementioned frozen food is consumed directly without prior rinsing before cooking. Nonetheless, it is also on record that following poor storage conditions, unhygienic practices and when not properly cooked, frozen food might become a time-bomb for community infection by pathogenic bacteria. The above has been proven to be true because certain bacteria, particularly MRSA are capable of producing toxins which are heat stable (2).

Moreover, some strains of bacteria isolated from food-producing animals have been reported to be pathogenic, with potential public health implications in humans (3,4). Individuals with compromised immune system such as the elderly and children (with under-developed immune system) are majorly at high risk of infection with pathogenic *S. aureus*. Infections caused by the bacterium are either through active invasion of cells and tissues leading to abscess formation in the affected areas or through production and release of toxins that can kill cells and destroy tissues (5). Treatments of infections due to *S. aureus* have been a challenge especially because of multi-resistant strain such as the methicillin-resistant *S. aureus* (MRSA).

MRSA is a pathogen of increasing significance in the hospital and community (3). The strain of *S. aureus*, which is resistant to methicillin and related antibiotics are usually very difficult to treat and manage. Till date, several strains of MRSA have successfully been isolated from different sources just as Petinaki and Spiliopoulou (6) opined that they can be categorized according to their sources of isolation, which could either be in the hospital settings (hospital-associated MRSA, HA-MRSA, food animals or livestock (livestock-associated MRSA, LA-MRSA) or from the community (community-associated MRSA, CA-MRSA). The presence of MRSA or any bacteria of public health importance in frozen food products can pose a potential health hazard for consumers and by extension the entire community thereby resulting in serious economic loss, as well as low human productivity (2).

Most times, detection of staphylococci in meat is often connected to poor hygienic practices during slaughtering, transportation, chopping, storage and point of sale, by persons involved in the chain of production. Pathogenic bacteria from contaminated frozen foods can be transferred to other high-risk surfaces which could further serve as source of infection in the community. More so, stainless steel surfaces, polythene surfaces, and bags, can harbour large number of bacteria from contaminated frozen meats (7,8).

Increasing levels of antimicrobial resistance have been reported in staphylococci, which make this pathogen become an important challenge in disease control (3). Despite the enormous public health challenge posed by this pathogen, there are limited informations available on their occurrence and characteristics in frozen foods such as beef, fish and chicken in Benin City. In this study, we attempt to evaluate the presence of MRSA and the effect of rinsing on the bacterial burden of frozen animal food products sold in different markets within Benin metropolis to examine the public health significance.

Materials and method:

Study setting and sample collection

Random samples of poultry meat (n= 15), ice fish (n=10) and beef (n=15) were collected from five randomly selected open markets in Benin City: Oliha, Ogida, Useh, Oluku and Egor markets. The samples were transported in clean polythene bags in cooler boxes to the Research Laboratory of Applied Microbial Processes and Environmental Health, University of Benin. All samples were analyzed within 4 hours of collection.

Sample analysis

Samples were analyzed in triplicates both in unwashed (without rinsing) and washed (properly rinsed with distilled water) forms. The standard bacteriological technique was employed using a ten-fold serial dilution for the unwashed samples while a five-fold serial dilution was used for the washed samples. For the unwashed frozen meat samples, 30 g of the meat (generic) was weighed into a flask containing 270 ml of peptone water. For the washed or the rinsed sample, the same quantity of meat (30 g) was weighed into 120 ml of sterile peptone water. The formula employed for the demystification of dilution factor is given as; dilution factor = final volume/aliquot volume, where final volume = aliquot volume (sample volume) + diluent volume.

The stock solution was then serially diluted into a set of five tubes maintaining the dilution factors for unwashed meat samples. After serial dilution, 100 µl (for the unwashed samples) and 1000 µl (for the rinsed sample) of inoculum volumes from the third, fourth and fifth tubes of the sets were plated in triplicates on nutrient agar supplemented with fluconazole (to prevent fungal growth). The plates were incubated at room temperature 28±2ºC for 24 –48 hours. Bacterial colonies were counted using a colony counter and results recorded. Enumeration of the isolates was conducted using the formula by Willey et al., (5); $\frac{cfu}{g} = \frac{number\ of\ colonies\ x\ dilution\ factor}{volume\ of\ inoculum}$

Isolation of *Staphylococcus aureus* from frozen food samples

Following enumeration of heterotrophic bacteria (total bacterial load) from the meat samples, colonies were first sub-cultured on nutrient agar. Colonies presumptively identified as *S. aureus* from positive Gram stain, and catalase and coagulase tests positive, were sub-cultured on selective media; mannitol salt agar, Vogel Johnson, and oxacillin-resistant screening agar plates (Oxoid, Basingstoke, Hampshire, England) to phenotypically confirm their identity as *S. aureus* and methicillin-resistant *S. aureus* (MRSA).

Antibiotic susceptibility testing

Antibiotic susceptibility test (AST) was performed on confirmed isolates by the Bauer-Kirby disk diffusion method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guideline (9), using standard antibiotic disks (Oxoid, Basingstoke, England).

For the AST, bacterial cultures (18-24 hours) were standardized to 0.5 McFarland standards (~1.5×10^8 CFU/ml) and streaked on Mueller Hinton agar (MHA) using a sterile loop. Antibiotic disks were placed on the plates using a sterile forcep and incubated aerobically for 24 hours at 37ºC. The diameter of inhibition zone around each disk was measured using a meter rule and interpreted as resistant (R), intermediate resistant (I) and sensitive (S) according to the CLSI guideline (9).

The antibiotic disks used were ciprofloxacin CIP (5µg), amoxicillin/clavulanic acid AMC (20/10µg), erythromycin E (15µg), ceftriaxone CRO (30µg), trimethoprim/sulfamethoxazole (1.25/23.75µg), meropenem MEM (10µg) and gentamicin CN (10 µg).

Multiple antibiotic resistance (MAR) index

The MAR index was determined using the method described by Chitanand et al., (10), which defines MAR index as percentage of antibiotics isolate is resistant to divided by the sum of the percentages of total antibiotics tested against the isolate. According to Davis and Brown (11), MAR index of isolates ≥ 0.2 is indicative of high antibiotic use.

Statistical analysis of data

Data were entered into Microsoft Excel 2016 and analysed using SPSS version 21.0. Descriptive statistics (means, standard deviation and percentages) and Students' '*t*' test were used for comparative analysis of HBC of unwashed and rinsed frozen food samples using a probability level of < 0.05.

Results:

The mean (±SD) of logarithmic heterotrophic bacterial counts for beef, fish and chicken samples before rinsing were respectively 11.53±1.25 CFU/g, 11.16±0.95 CFU/g and 11.42±1.58 CFU/g compared to respective counts of 2.7±0.45, 2.68±0.25 and 2.79±0.45 after rinsing (Fig 1), which showed significant differences ($p<0.05$) in the bacterial burden of frozen meat samples before and after rinsing (Table 1).

The overall prevalence of *S. aureus* and MRSA in the frozen meat samples were 40% (16/40) and 10% (4/40) respectively. Of the frozen meat evaluated, beef had the highest frequency of *S. aureus* contamination (46.7%), followed by chicken (40.0%) and fish (30.0%). The antibiotic resistance profile of *S. aureus* showed high resistance to erythromycin (94%), amoxicillin/clavulanic acid (87.5%) and trimethoprim-sulfamethoxazole (81%). The MARI of *S. aureus* was 0.63, which is above the permissible value of 0.2.

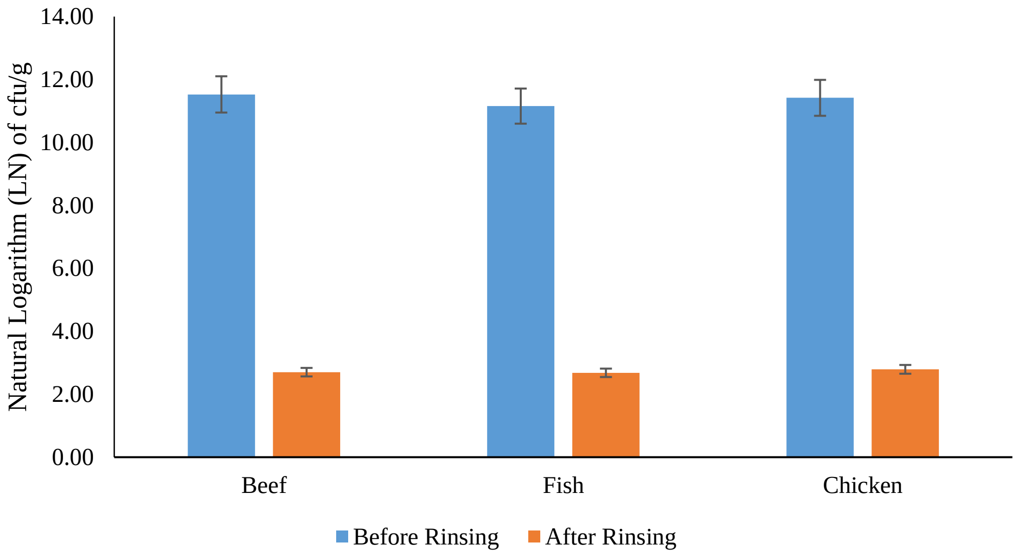

Fig 1: Mean logarithm heterotrophic bacterial count (CFU/g) of frozen food samples before and after rinsing

Table 1: Mean logarithm heterotrophic bacteria counts of frozen food samples before and after rinsing

Parameters	Before rinse	After rinse	*p* value	T. crit	T. cal
Beef	11.53±1.25	2.70±0.45	0.001	2.365	5.356
Fish	11.16±0.95	2.68±0.25	0.036	2.365	2.598
Chicken	11.42±1.58	2.79±0.49	0.013	3.182	5.303

Tcrit= T critical; Tcal= T calculated

Table 2: Prevalence of *S. aureus* and MRSA from frozen meat samples from markets in Benin City

Samples	**Total number of samples**	***Staphylococcus aureus* (%)**	**MRSA (%)**
Beef	15	7 (46.7)	2 (13.3)
Fish	10	3 (30.0)	0 (0)
Chicken	15	6 (40.0)	2 (13.3)
Total	40	16 (40.0)	4 (10.0)

Table 3: Antibiotic susceptibility of staphylococcal isolates from meat samples

Antibiotics	Susceptibility profile (n=16)	
	Sensitive (%)	Resistant (%)
Meropenem (10µg)	11 (69.0)	5 (31.0)
Erythromycin (15µg)	1 (6.0)	15 (94.0)
Amoxicillin/clavulanic acid (20/10µg)	2 (12.5)	14 (87.5)
Ceftriaxone (30µg)	9 (56.0)	7 (44.0)
Trimethoprim/sulfamethoxazole (1.25/23.75µg)	3 (19.0)	13 (81.0)
Gentamicin (10µg)	6 (37.5)	10 (62.5)
Ciprofloxacin (5µg)	15 (94.0)	1 (6.0)

Discussion:

The detection of *S. aureus* in frozen meat (beef, fish and chicken) samples is to pave way for identifying possible source of contamination in case there is an outbreak of infection due to improperly cooked frozen meats. It is also very necessary because pathogenic *S. aureus* has been known to produce several heat-stable toxins capable of causing harm in humans. The latter is such that the bacterium produces the toxin in the meat samples and while the bacterium could be eliminated by heat (high temperature), the heat-stable toxin would still be potent to cause injury after consumption of such contaminated products.

The heterotrophic bacterial counts of the frozen food samples (beef, fish & chicken) before rinsing were found to be above LN 11.10 CFU/g. The results from this study agree with that of Islam et al., (12) who reported bacterial counts of LN 11.5 CFU/g from frozen chicken samples. The values obtained in our study is also similar to the study by Al-Tarazi et al., (13) who reported mean bacterial count of meat samples in the range of LN 6.27 to LN 10.67. Also closely related to the counts obtained in our study, Fahim et al., (14) reported bacterial count of LN 11.33 CFU/g. The bacterial counts in these studies could be related to microbiology of the meat samples, especially with reference to the microbiome of the animals. Environmental factors as well as hygiene status of food handlers also have a role to play in reference to the heterotrophic count of bacteria in meat.

Technically speaking, the bacterial counts after rinsing was significantly different from the counts before rinsing. This emphasize the importance of rinsing of food materials prior to cooking and consumption as this practice reduce the number of potential microorganisms capable of causing infection in the host. Although there is no standard guide on how proper rinsing should be done, the mere act of rinsing can reduce microbial population and the risk of injury to humans.

In this study, a total of 40 % of frozen meats were contaminated with *S. aureus* while 10% were MRSA isolates. This finding is in agreement with several studies in literatures which had reported the presence of *S. aureus* in frozen meat samples. Wu et al., (15) reported that 35% of frozen meat samples and meat products in China were contaminated with *S. aureus* and a further 7.5% of the isolates being confirmed MRSA. Savariraj et al., (16) isolated the bacterium in 76.7% of frozen retail pork outlets in India. Higher prevalence of *S. aureus* has also been reported from frozen meat and fresh meat samples in Karbala province (Iraq) where 64% of the frozen meat samples were contaminated with this bacterium (17). In Nigeria, the situation is not different as Oranusi et al., (18) posited that while freezing is meant to preserve food by halting the growth of microbes, the reports of illness involving frozen food is definitely a function of microbial activity. Igbinosa et al., (3) also made similar observations with regards to *S. aureus* and strains of MRSA where it was reported that 20% and 28% of chicken and beef samples respectively were contaminated with MRSA. Moreover, Igbinosa et al., (3) reported multi-drug resistance profile (which is described as resistance to antimicrobial agent in more than two antibiotic classes) for MRSA strains which were of public health importance. Our finding is similar to this, where *S. aureus* isolates were tested against antibiotics in eight different classes. One of the isolates was resistant to all eight antibiotic classes while majority of the isolates (>80%) were resistant to the following antibiotics; erythromycin (94%), amoxicillin/ clavulanic acid (87.5%), and trimethoprim-sulfamethoxazole (81%). Similar multi-drug resistance pattern was reported by Islam et al., (12) and Al-Tarazi et al., (12) in *S. aureus* isolates from frozen meats.

The multiple antibiotic resistance (MAR) index is used to evaluate usage of antibiotics in an environment. The MAR index of 0.63 for *S. aureus* isolates in our study is an indication of overuse of antibiotics in our environment, and health risks associated with transmission and spread of MDR bacteria pathogens. MDR raises a health concern because it limits treatment options available for microbial infections (3), which can lead to prolongation of illness, higher cost of therapy, and increased risks of death.

Conclusion:

The assessment of MRSA from frozen foods in Benin City shows that obvious frozen foods can be home or habitation for bacterial pathogens of public health importance. Proper rinsing, which can eliminate plethora of pathogenic bacteria isolates from frozen food before preparation should be emphasized. With the increase in prevalence of antibiotic-resistant bacteria in frozen foods, public health awareness is essential as a preventative measure.

Conflicts of interest:

Authors declare no conflict of interest

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