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The Detection of Methicillin and Multiple Antibiotic-Resistant *Staphylococcus aureus* in Dairy Products as Sold in Parts of Kaduna State, Nigeria

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Abstract

Dairy products sold through unregulated channels are potential sources of a variety of microorganisms that are involved in food poisoning. Staphylococcus aureus is a known example of bacteria causing food-borne diseases. Methicillin resistance is a prominent index in food hygiene studies. In this study, multiple antibiotics and methicillin-resistant Staphylococcus aureus in dairy products were studied. A total of 320 dairy product samples, comprising 80 each of fresh milk, nono (cultured skimmed, defatted milk), manshanu (milk fat), and kindrimo (full-fat or partially skimmed cultured milk), were examined for possible contamination by Staphylococcus aureus. The antibiogram and the presence of antibiotic-resistant phenotypes of MRSA were determined using the agar disk diffusion method. The Polymerase chain reaction (PCR) technique was employed to detect the mec A gene. A total of 28 (8.75%) of S. aureus was detected in the current study. There was a significant difference at p ≤ 0.05 in the proportion of S. aureus contamination among the dairy samples. Multiple antibiotic resistance index (MAR > 0.2) was 19 (67.86%). The cumulative occurrence of MRSA for this study was 20 (71.43%), with 3.75%, 3.75%, 10.0%, and 7.5% occurrence in Nono, Kindrimo, Manshanu, and Fresh milk, respectively. The highest resistance to Ceforxitin was seen in Manshanu (77.8%), while the lowest was seen in Nono (50%). There was no significant difference at p≤0.05 in the proportion of Ceforxitin positive samples among the four dairy products. A total of 11 (39.29%) of the isolates haboured the Mec A gene. Antibiotic-resistant bacteria are at risk of being transferred to humans via milk. For safe and healthy milk consumption, new hygiene policies and management practices should be considered to increase food safety.

Key Words: Dairy Products, Methicillin Resistance, Mec A gene, Polymerase chain reaction (PCR).

INTRODUCTION

Milk is a source of many essential nutrients, including proteins, lipids, carbohydrates, vitamins, and minerals. It is extensively consumed in various forms all over the world and represents a vital part of the human diet (Alegbeleye *et al.*, 2018). The rise in demand for milk and milk products by the growing human population has led to a growing interest and concern for the quality and safety of milk products (Suh, 2022).

Foodborne outbreaks caused by milk and dairy products have led to hospitalizations and deaths for humans beings (Painter *et al.*, 2013). Foods of animal origin are considered an important source of antibiotic-resistant bacteria (ARB) and antibiotic resistance gene (ARG) dissemination, along the food chain (EFSA, 2009). ARBs and ARGs may be transmitted to humans either by the consumption of raw and processed foods, including milk, meat, and aquatic animal products (Gebreyes et al., 2017) or by animal contact (Oniciuc *et al.*, 2017). Milk and dairy products are often contaminated with antibiotic-resistant bacteria, including *S. aureus* (Zhang *et al.*, 2022). Also, methicillin-resistant *S. aureus* (MRSA) is an emerging problem in food-producing animals (Keyvan *et. al.*, 2020). *Staphylococcus* spp. is a bacterial pathogen that quickly obtains antibiotic resistance (Kot *et al.*, 2020). The appearance and spread of antimicrobial resistance has usually been ascribed to the misuse or indiscriminate use of antibiotics in human and animal health (Vidovic and Vidovic, 2020; Titouche *et al.*, 2022). This has become a major public health problem all over the world owing to the massive use of

antibiotics in feed to stimulate growth in both agriculture and livestock animals (Oniciuc *et al.*, 2017). Methicillin-resistant *S. aureus* (MRSA) is an emerging pathogen in livestock animals that can infect humans and has become a growing concern for public health. MRSA has been isolated as a mastitis pathogen in bulk tank milk (Doulgeraki *et al.*, 2017). Many researchers have reported the occurrence of MRSA from livestock animals and foods of animal origin; however, the effect of MRSA in food-related problems is still very rare. There are some concerns about foodborne MRSA infections (Herrera *et al.*, 2016). This study was therefore aimed at appraising the prevalence of *S. aureus*, its antibiotic resistance profiles, and related *mecA* genes among isolates of *S. aureus* from some local dairy products.

MATERIALS AND METHODS

Study area

The study area included four (4) local government areas in Kaduna state, proximal to Zaria. These included: Giwa (11° 16′ 51″ North, 7° 25′ 3″ East), Kaduna North (**10:35 North and Longitudes 7:25 East)**, Soba (10° 59' 2.7348" N and 8° 3' 36.5688" E), and Chikun (10°18'54.00" N 7°16'26.40" E).

Sample collection

A total of 320 samples, comprising 80 samples each (20 from each local government area) of Fresh milk, *nono* (cultured skimmed, defatted milk), *manshanu* (milk fat), and *kindrimo* (full-fat or partially skimmed cultured milk), were obtained from motor parks and markets. Samples were collected in sterile containers and placed in ice-packed coolers and taken to the laboratory. Samples were analyzed at the Department of Microbiology laboratory, Ahmadu Bello University, Zaria, within 6 hours of collection.

Isolation of *Staphylococcus aureus*

The isolation of *Staphylococcus aureus* was according to the procedure described by Imanifooladi *et al.* (2010). Dairy product samples, *Freshmilk, Nono, and Kindrimo,* were diluted in the ratio 1:100 in normal saline. From each solution produced, 10 ml was transferred to 90 ml of cooked meat media culture with 9% NaCl. For *Manshanu* samples, each was placed in a water bath set at 45 °C, and 10ml of each melted sample was also transferred to 90 mL of cooked meat media culture with 9% NaCl. All were incubated at 37 °C for 48 h. In the second phase, 1ml from each previously cultured medium was then transferred to Baird-Parker agar (BPA) and incubated for 24 hours. Black colonies with a transparent zone on Baird-Parker agar were considered presumptive *Staphylococcus* species. They were picked and stored on nutrient agar slants for further confirmation tests.

Biochemical characterization of isolates

Presumptive *Staphylococcus* species that were gram-positive cocci in clusters were subjected to some biochemical tests as described by Cheesbrough (2012). These included: coagulase, catalase, β-hemolysis, fermentation of glucose, and Mannitol. These were further confirmed using MicrogenTM STAPH- identification system (Microgen Bioproducts, United Kingdom).

Antibiogram Testing of *Staphylococcus aureus*

Antibiotic susceptibility tests for *Staphylococcus aureus* isolates were performed according to the Kirby-Bauer method as described by Keyvan et al. (2020) and the evaluation methods of the Clinical and Laboratory Standards Institute (CLSI) (CLSI,2014). Isolates grown on nutrient agar overnight were suspended in 2ml sterile normal saline (0.9% sodium chloride solution). A turbidity equivalent was prepared by comparing with a 0.5 MacFarland standard. Bacterial suspensions of 0.1ml were spread on plates of sterile Mueller-Hinton agar with the help of a sterile cotton swab to form a smooth bacterial lawn. The inocula were allowed to dry for 5 minutes. Commercially prepared standard susceptibility test discs impregnated with known agents and strengths were then dispensed on the agar surface. Within 15 minutes of application of the disc, plates were incubated overnight at 37 ^{0}C. *S.aureus* ATCC 6538 was used as a quality control organism in the antimicrobial susceptibility determination. Characterization of strains as susceptible, intermediate, or resistant was based on the size of the inhibition zone around the disc compared with the interpretation standards provided by the manufacturers. The following antibiotic disks were used: Tetracycline (30µg), Trimethoprim/Sulfamethoxazole (25µg), chloramphenicol (30µg), Erythromycin (15µg), Gentamicin (10µg), Amoxicillin/Clavulanic acid

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(30µg), Cefoxitin (30µg), Ciprofloxacin (5µg), and Vancomycin (30µg).

Detection of methicillin-resistant *Staphylococcus* spp.(MRS).

This was done using the evaluation methods of the Clinical and Laboratory Standards Institute (CLSI) CLSI, (2014). Isolates that were cefoxitin resistant were regarded as MRS isolates (Moglad and Attayb,2022).

Genotypic Screening for the *Mec*A Genes.

Table 1: Primers, Nucleotide Sequences, and Expected amplicon sizes of target genes of the bacterial isolates:

Primer	Nucleotide Sequence (5'-3')	Expected Amplicon Size(bp)	Reference
Mec-A2	AGTTCTGCAGTACCGGATTTGC	533	Lee (2003)
Mec-A1	AAAATCGATGGTAAAGGTTGGC		

Key: *MecA*= Methicillin-resistance component (oxacillin/methicillin resistance gene).

Bacterial Cell Preparation of *Staphylococcus aureus*

The preparation of the bacteria cell was carried out using the method described by Dubey (2009). Single colonies were picked from freshly streaked isolates on manitol salt agar and inoculated into 5ml Luria and Bertani (LB)* broth medium. These were incubated overnight at 37°C for 18h. Bacterial cells were then harvested by centrifugation at 4°C, 8,000rpm (6800 x g) in a refrigerated microcentrifuge for 30 seconds in Eppendorf tubes. The supernatants were decanted, and cells were harvested.

*Luria and Bertani broth media was prepared as follows (1 Litre);

Peptone (10g), NaCl (5g), IN NaOH (10ml), Yeast extract (5g), Distilled water (1 litre)

PH 7.0 adjusted with NaOH solution and sterilized at 121 °C for 15minutes.

Genomic DNA Extraction of *Staphylococcus aureus*:

Genomic DNA extraction was carried out with zuppy™ Genomic DNA extraction Kit (Inqaba Biotech, South Africa) using the protocol as described by the manufacturer.

To ascertain that the DNA was actually extracted, the eluent was subjected to agarose gel electrophoresis.

Multiple Antibiotic Resistance (MAR) and Multiple Antibiotic Resistance Index (MARI)

Multiple antibiotic resistance (MAR) for this study is defined as resistance of an isolate to three or more antibiotics (Osundiya *et al.*, 2013). Multiple antibiotic resistance index (MARI) was calculated according to Furtula *et al.* (2010) as the ratio of the number of resistance antibiotics to which an organism is resistant, to the total number of antibiotics to which an organism is exposed to.

PCR Amplification of Target Genes of *Staphylococcus aureus*

PCR amplification was done using the Gene AMP PCR system 9700 (Applied Biosystem), following standard conditions. This was followed by Agarose gel electrophoresis of the PCR products. Photographs of the band were taken using a gel documenting machine (Enduro™ GDS; Labnet). The sizes were assessed and estimated from the molecular sizes of the DNA ladder against their migration distance.

Statistical analysis

Pearson chi-square test was used to ascertain the significant differences at ($p \leq 0.05$) for positive outcomes using SPSS 23 version.

RESULTS

The recovery frequency of *Staphylococcus aureus* from Fresh milk, *Nono, Kindrimo,* and *Manshanu* in various sampling locations is presented in Table 1. Out of the total of 320 samples examined, 28 samples contained *S.aureus*. *Manshanu* had a higher frequency of occurrence of 11.25 % of *S. aureus*, while *Kindrimo* had the lowest frequency of 5(6.25%). Pearson Chi-square analysis of the proportions of *S.aureus* contamination among the four types of dairy products indicated a statistical significant difference at ($p \leq 0.05$).

The antibiotic susceptibility profile of *S.aureus* isolates from the samples is displayed in Table

2. Isolates showed 100% susceptibility to Gentamicin (10µg), Ciprofloxacin (5µg), and Chloramphenicol (30µg). However, 71.4 % of the isolates were resistant to cefoxitin.

The multiple antibiotic resistance (MAR) profile of isolates is displayed in Table 3. A MAR index of greater than 0.3 was observed in nineteen (19) of the isolates. The isolates from *Manshanu,* closely followed by those obtained from fresh milkshowed the high frequency of resistance to cefoxitin (Table 4). Meanwhile, the lowest resistance to cefoxitin was seen in Nono. There was no statistical significant difference, where p = 0.657>0.05 in the proportion of cefoxitin positive samples among the four dairy products. The image of the *Mec A* gene detection revealing the genotypic methicillin resistance is shown in Figure 1, where Manshanu had the highest occurrence. The gene was amplified in the following lanes: L2,L4,L5,L9,L11,L13,L14,L15,L16,L18. Lastly, the distribution of the *Mec A* gene among isolates from the various dairy products is displayed in Figure 2. The gene was isolated most from isolates obtained from *Manshanu*while the least percentage detection was from fresh milk.

Table 1: Frequency of occurrence of *S.aureus* in dairy samples.

Dairy Sample	No. examined	No. positive (%)	No. negative (%)	X^2	p-value
Nono	80	6 (7.5)	74 (92.5)		
Kindrimo	80	5 (6.25)	75 (93.75)		
Manshanu	80	9 (11.25)	71 (88.75)	18.836[a]	0.030
Fresh milk	80	8 (10.0)	72 (90)		
Total	320	28 (8.75)	62 (91.25)		

N = 320.

Table 2: Antimicrobial susceptibility pattern of *Staphylococcus aureus* isolates.

Antibiotics	Resistantn(%)	Sensitive n(%)
Amoxicillin/Clavulanic acid (Aug) (30µg).	14 (50)	14 (50)
Cefoxitin (FOX)(30µg)	20 (71.4)	8 (28.6)
Gentamicin(CN)(10µg).	0	28 (100)
Tetracycline (TE) (30µg)	18 (64.3)	6 (21.4)
Ciprofloxacin(CIP) ((5µg)	0	28 (100)
Trimethoprim/Sulfamethoxazole (SXT) ((30µg)	7 (25)	21 (75)
Chloramphenicol (C)((30µg)	0(0)	27 (96.4)
Vancomycin(VA)((30µg)	17 (60.7)	11 (39.3)
Erythromycin (E)((30µg)	17(60.7)	11(39.3)

Key: n = number of isolates resistant/sensitive out of 28

Table 3: MAR index Profile of isolates

N	MAR index	n (%)
1	0.0	0 (0)
2	0.1	2 (7.14)
3	0.2	7 (25)
4	0.3	7 (25)
5	0.4	6 (21)
6	0.5	0 (0)
7	0.6	2 (7.14)
8	0.7	4 (14.29)
9	0.8	0 (0)

Key
N- Number of antibiotics to which resistance is observed
MAR- Multiple antibiotic resistance
n = number of isolates out of 28 with MAR index

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Table 4: Cefoxitin resistance among isolates from various dairy products

Dairy Product	No. of isolates examined	No. of resistant isolates (%)	No. of sensitive isolates (%)	X^2	P- Value
Nono	6	3 (50)	3 (50)		
Kindrimo	5	3 (60)	3 (60)	1.612	0.657
Manshanu	9	7 (78)	2 (22)		
Freshmilk	8	6 (75)	2 (25)		
Total	**28**	**19 (68)**	**9 (32)**		

Total number of isolates(n) = 28.

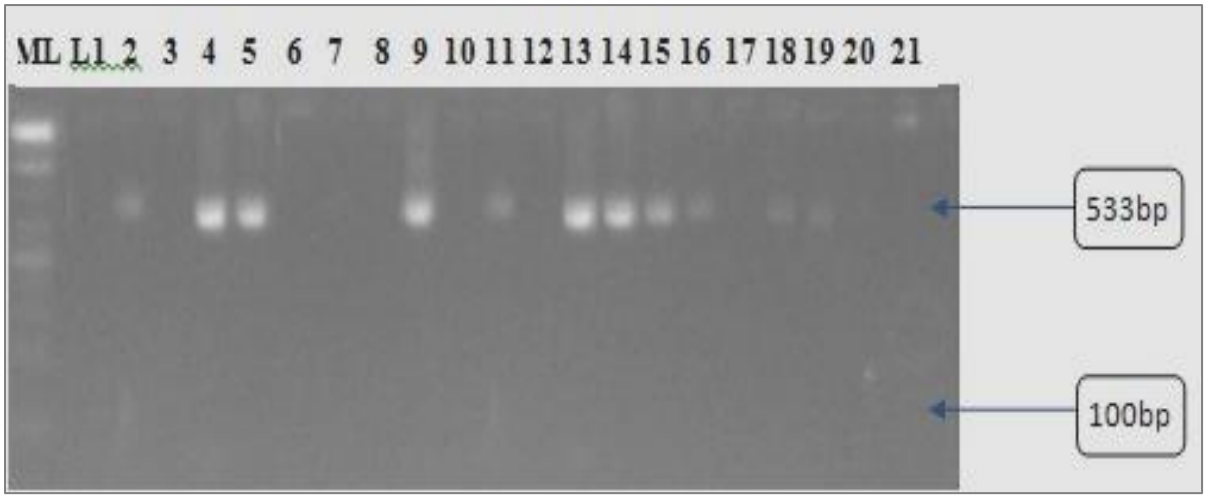

Figure 1: PCR amplification of the *Mec A* gene.
ML = Molecular Ladder, L = Lane , L1 = Negative control (PCR product with sterile water);
L2- L21= test organisms (*S.aureus* isolates) L2,L4,L5,L9,L11,L13,L14,L15,L16,L18 and L19=Positive amplification of 533bp for *Mec*Agene.

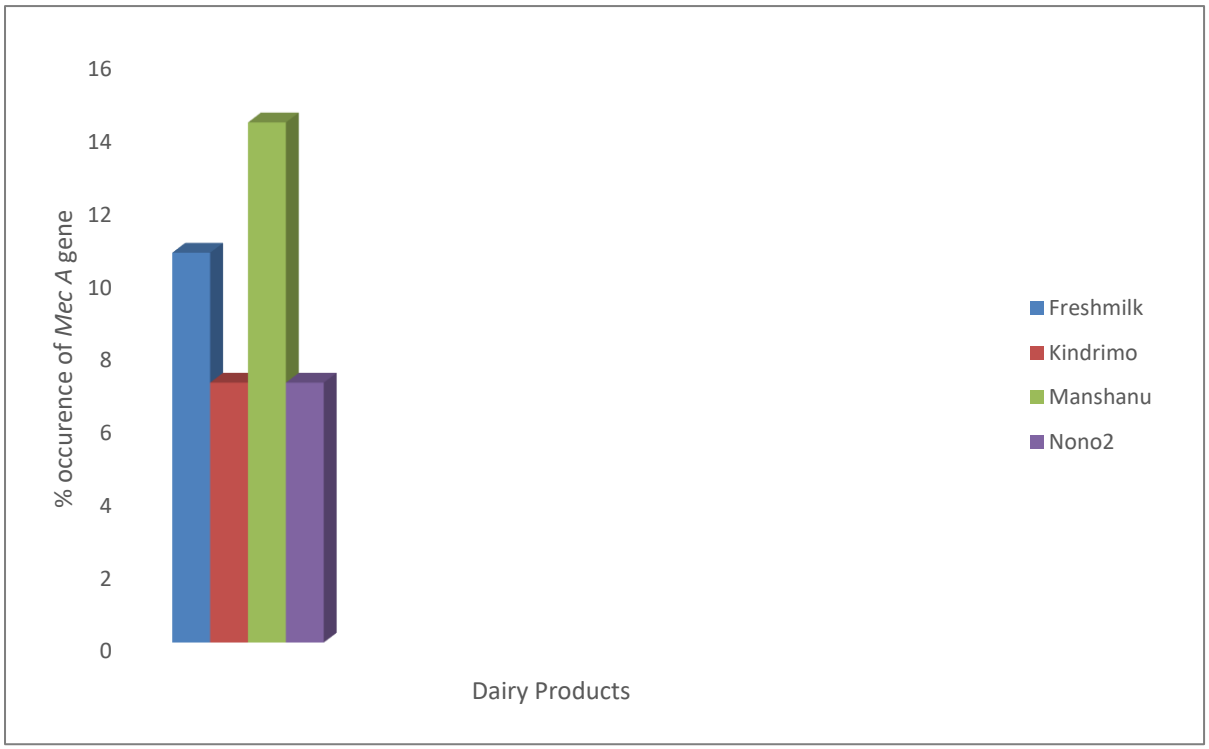

Figure 2: Percentage distribution of the *Mec A gene* among isolates from various dairy products

DISCUSSION

In this study, *S. aureus* was detected in 28 (8.75%) of the samples. The Pearson Chi – square with p = 0.016 < 0.05 indicates that there exists significant difference among the four dairy products in the proportion of positive occurrence of*S.aureus*, confirming these proportions to be unlikely due to chance. *S. aureus* is an opportunistic pathogen and has enterotoxigenic strains which are recognised to cause grave food borne diseases (Akinyele *et al.*,2013). It has been reported that consumption of the thermostable enterotoxins, rather than the bacterium itself, is responsible for foodborne illness (Mead *et al.*, 1999). The presence of *S. aureus* in this study was lower than that obtained from similar works by other researchers (Mehmeti *et al.*, 2017, Keyvan *et al*, 2020). This shows that the milking conditions and the hygienic quality of the milk and dairy products may cause differences between levels of *S. aureus* isolates in diverse countries.

The MAR index ranged from 0.3 – 0.7. MAR index values greater than 0.2 indicate a high-risk source of contamination where antibiotics are often used (Furtula *et al.*, 2010). Multiple antibiotic resistance (MAR) in bacteria is most commonly associated with the presence of plasmids, which contain one or more resistance genes, each encoding a single antibiotic resistance phenotype (Dainie*t al.*, 2005). As previously reported in the literature, *S. aureus* develops resistance to several antibiotics by gaining elements by horizontal transfer of mobile genetic material, modifying the drug-binding sites on molecular targets by mutations, and expressing endogenous efflux pumps (Foster, 2017). Transmission of MRSA to humans through the consumption of dairy products with direct contact with dairy cows may generate serious risks to food safety and public health.

The results (as shown in Table 4) revealed that 19 (67.86%) of the 28 *S.aureus* isolates were resistant to cefoxitin, an indication of methicillin resistance. A Pearson chi-square test of independence showed no statistically significant difference at (p≤ 0.05) in the proportion of ceforxitin positive samples among the four dairy product types. However, the high overall resistance rate (67.9%)suggests that ceforxitin resistance is a concern across all the dairy product types studied. The contamination of milk with MRSA could be caused by the direct transfer of the bacterial pathogen through mastitis infection of the udder, an unhygienic milking process, or a contaminated farm environment (Titouche et al.,2022).

In this study*mecA*gene was detected in 39.29% of the *S. aureus* isolates (11 out of 28). The overuse of ß-lactam group antibiotics for prophylactic and mastitis treatment in dairy cows may also be the basis of MRSA in milk and dairy products (Keyvan *et al.*, 2020). Similar results were obtained by Shanehbandi *et al.* (2014) while working with dairy products in North West Iran. The high prevalence of resistance genes should be considered as a potential health risk for humans and livestock. However, necessary precautions should be taken by concerned agencies and individuals to prevent the further spread of MRSA. Hygiene promotion and avoiding the unsupervised use of antibiotics may be some elementary steps in this regard.

CONCLUSION

In summary, the high incidence of multiple antibiotic-resistant (MAR) *S.aureus* in the dairy products poses a risk of being transferred to humans via milk. Therefore, for safe and healthy milk consumption, the uncontrolled use of antibiotics in dairy cows should be avoided. Additionally, new hygiene policies and management practices should be considered to increase food safety.

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