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Prevalence and antibiotic resistance patterns of methicillin-resistant *Staphylococcus aureus* in raw milk and soft cheese (*wara*) sold in Abeokuta, Nigeria

EO Omoshaba*, OE Ojo, O Sofela & OI Onifade

Department of Veterinary Microbiology and Parasitology, College of Veterinary Medicine. Federal University of Agriculture, Abeokuta, Ogun State, Nigeria

*Correspondence: Tel.: +2347035019122; E-mail: omoshabaeo@funaab.edu.ng

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Abstract

The emergence of antibiotic-resistant bacteria in farm animals, their environment and food of animal origins is of significant potential public health importance. Methicillin resistant *Staphylococcus aureus* (MRSA) is an important opportunistic pathogen both in humans and in cattle. A total of 200 samples comprising of 100 each of raw milk and *wara* were collected from five different locations in Abeokuta, Ogun State. The samples were examined using standard bacteriological methods for the isolation and identification of *Staphylococcus* species including MRSA. Latex agglutination test of Penicillin-binding protein 2a (PBP2a) was used to further confirmed MRSA isolates. The susceptibility to antibiotics was determined by Kirby Bauer diffusion method. In all, *S. aureus* was detected in 52 (26%) of 200 samples of which 50 were confirmed as MRSA and two were Methicillin-sensitive *Staphylococcus aureus* (MSSA). Out of 50 MRSA isolates, 15 (15%) were from 100 raw milk and 35 (35%) were from 100 *wara* samples. The two MSSA isolates were from raw milk. The *Staphylococcus aureus* isolates from raw milk showed high resistance to ceftazidime 17 (100%), ampicillin 16 (94.1%), doxycycline 11 (64.7%), tetracycline 17 (100%), oxacillin 15 (88.2%), augmentin 17 (100%), gentamycin 15 (88.2%), colistin 15 (88.2%), and sulphamethoxazole 16 (94.1%). Isolates from *wara* were resistant to ceftazidime 35 (100.0%), ampicillin 35 (100%), doxycycline 15 (42.9%), tetracycline 23 (65.7%), oxacillin 35 (100.0%), streptomycin 20 (57.1%), augmentin 33 (94.3%), gentamycin 17 (48.6%), colistin 35 (100%), and sulphamethoxazole 27 (77.1%). The presence of MRSA in the raw cow milk and *wara* sold in Abeokuta may be due to overdependence on antibiotics in cattle production. Contamination along processing and marketing chain of *wara* due to unhygienic practices could also contribute to the presence of MRSA in the product. This constitutes a potential public health risk to consumers of milk and milk products in Abeokuta.

Keywords: Antibiotics, Milk, MRSA, Resistance, Soft cheese, Unhygienic

Introduction

Staphylococcus aureus is a gram positive, non-motile, non-spore forming, catalase positive, coagulase positive, facultative anaerobe causing several diseases including boils, pustules, impetigo,

osteomyelitis, mastitis, septicaemia, meningitis, pneumonia and toxic shock syndrome (Abebe *et al.*, 2013). *Staphylococcus aureus* is a leading cause of gastroenteritis resulting from consumption of contaminated food. The organism can be found in water, dust and air but food handlers are the main sources of contamination of milk and milk products (Peles *et al*., 2007).

Methicillin resistant *Staphylococcus aureus* has caused serious infections in humans and animals in recent years (Peton & Le Loir, 2014). It has become a public health issue owing to its ability to not only cause pathology but also resist most of the potent antibiotics available for treatment. Methicillin resistant *Staphylococcus aureus* (MRSA) was first identified in 1961 and, over the years, it has spread from human to human, human to animals and vice versa (Umaru *et al.,* 2013). Based on epidemiological findings, there are different strains of MRSA that have been documented; the hospital-associated MRSA (HA-MRSA), community-associated MRSA (CA-MRSA) and livestock-associated MRSA (LA-MRSA). The hospital-associated MRSA was thought to be the only strain found in patients that were admitted into the hospitals but it was discovered that there were other strains identified in communities affecting people within the communities outside the hospital settings (Deleo *et al*., 2010). More recently, livestock-associated MRSA was isolated from pigs and was found to be very pathogenic not to pigs alone but to humans and other animal species (Loeffler & Lloyd, 2010). Cases of LA-MRSA have been reported in humans but little or no work in cattle has been documented in Nigeria (Umaru *et al.,* 2013; Mai-siyama *et al.* 2014).

Methicillin-resistance *Staphylococcal aureus* (MRSA) is a problematic pathogen in human medicine and appears to be an emerging problem in veterinary medicine (van Duijkeren *et al*., 2010). Infections and colonization have been widely reported in animals such as horses, dogs, cats, birds and cattle (Muhammad *et al.,* 2014; Strastkova *et al*., 2009; Rich & Roberts, 2004). There are limited reports on MRSA from African countries (Carmen *et al.,* 2016; Mai-siyama *et al.,* 2014; Ghebremedhin *et al*., 2009: Okon *et al*., 2009). Between 1996 and 1997, the prevalence of MRSA, determined in eight African countries, was relatively high in Nigeria, Kenya and Cameroon and low in Tunisia and Algeria (Fadeyi *et al.*, 2010; Nwankwo *et al*., 2010). In Nigeria, MRSA colonization and infection have been reported in humans with varying prevalence of between 20 and 60% (Olonitola *et al.,* 2007).

Even though, it has been documented that MRSA is one of the transmissible pathogens of zoonotic and anthroponotic importance, little is known about the vehicles for animal-to-human mode of transmission of MRSA. Several authors have reported bidirectional transmission of MRSA (Price *et al*., 2012; AVMA, 2014). Animal to human transmission could be by direct contact, unhygienic environment as well as handling and consumption of infected animal products (Tassew *et al*., 2016), whereas human to animal transmission is still not clear (Weese, 2010).

It has been observed that very little is known about the possible role of milk and milk products as vehicles for the zoonotic transmission of MRSA from cattle to humans in the study area. *Staphylococcus aureus* is a common pathogen found in cow milk, but the presence of MRSA strains in milk and milk products meant for human consumption has not been well documented in the south western part of Nigeria. This study provides a preliminary report on the prevalence of MRSA in both fresh cow milk and locally produced *wara* in Abeokuta, Ogun State, Nigeria.

Materials and Methods

Samples and sample collection

Two hundred samples comprising of 100 fresh milk and 100 locally produced *wara* were collected from five different locations (Rounda, Opeji, Alabata, Kotopo, Oke-ata) in Abeokuta. Abeokuta is the capital city of Ogun State, South-West Nigeria. Forty samples including 20 fresh milk and 20 *wara* samples were collected from each location.

Five millilitres of fresh milk sample were collected directly from the udder of each lactating cow by manual expression after the udder, teat and adjacent flank areas have been thoroughly cleaned and disinfected with 70% alcohol. Milk samples were collected into sterile sample bottles.

Five *wara* were purchased from four *wara* vendors in each location and kept in sterile transparent polythene bags and transported in cooler with ice packs to the laboratory within one hour of collection. Both raw milk and *wara* samples were collected early in the mornings for two months.

Enumeration of Staphylococcus aureus

One millilitre of fresh milk and two grams of thoroughly homogenised cheese samples were transferred separately into test tube containing 9 ml of normal saline. This was subjected to tenfold one in ten serial dilution in ten test tubes. After the serial

dilution, 1 ml of 10^{-5} and 10^{-8} dilutions were spread on the surface of separate prepared plates of Mannitol salt agar. These were incubated at 37 °C aerobically for 24 hour (Cheesbrough 2006). The colonies on each plate were counted and total staphylococcal count was calculated and expressed in colony forming unit per millilitre (cfu/ml).

Isolation and identification of the Staphylococcus aureus in raw milk and wara samples

Typical yellow colonies of *Staphylococcus aureus* from bacterial count plates were subcultured on a freshly prepared Mannitol salt agar plates and incubated at 37^{o}C for 18 hours to obtain pure isolates. A single discrete colony from each sample was preserved on nutrient agar slopes and kept at 8^{o}C in the refrigerator for further tests. Identification of *S. aureus* was based on cultural, Gram staining/microscopy and biochemical characteristics including catalase test, oxidase test, haemolysis on sheep blood agar, DNAse test and coagulase test (slide and tube) (Cheesbrough, 2006).

DNase test

The DNAse medium was inoculated with a loopful of overnight growth of *Staphylococcus aureus* isolates on blood agar plate in a band. The inoculated plates were incubated for 18 hours aerobically at 37° C. After incubation, the plates were flooded with sufficient 1 N hydrochloric acid (HCl) and allowed to penetrate the whole medium surface for 2 min. DNase positive *Staphylococcus aureus* was surrounded by clear zones of depolymerized DNA while the medium further away from the inoculated band was opaque and whitish due to polymerized DNA. Colonies of DNase negative *S. aureus* did not show any clearing around the colonies. *Staphylococcus aureus* ATCC 29213 was used as positive control.

Antimicrobial sensitivity testing

The isolates were tested against a panel of eleven (11) antibiotics using the Kirby-Baue disk diffusion method (CLSI, 2014). Antimicrobial agents including tetracycline (30 µg), augmentin (30 µg), doxycycline (30 µg), neomycin (30 µg), sulphamethoxazole (30 µg), oxacillin (1 µg), cefoxitin (30 µg), ampicillin (1 µg), amoxicillin (25 µg) colistin (25 µg) and ceftazidime (30 µg) were selected for the antimicrobial sensitivity testing according to the recommendation of Clinical and Laboratory Standards Institute (CLSI, 2014).

For antimicrobial susceptibility testing, pure colonies of test isolates were grown on nutrient agar overnight. Bacterial colonies were emulsified in normal saline matched to 0.5 McFarland turbidity standard. The bacterial emulsions were inoculated onto Mueller-Hinton agar and antibiotic discs were placed at equidistance using the antibiotic disc dispenser (Oxoid, UK). The plates were incubated for 18 hour at 35 ^{O}C. The diameter of zone of inhibition was measured and interpreted according to CLSI (2014) guidance.

Penicillin-binding protein 2a (PBP2a) Latex agglutination test

Isolates of *S. aureus* that showed resistance to oxacillin by the antimicrobial disk diffusion test were further tested for the production of the altered penicillin binding protein (PBP2a) (responsible for methicillin resistance) using the PBP2´ latex agglutination test kit (Oxoid®, DR 0900). The latex agglutination was performed according to the manufacturer's instructions on 18hr-old cultures of *S. aureus* grown on 5% sheep blood agar plates. Three to five colonies of test organisms were picked from the plate; the colonies were suspended in 4 drops of the extraction reagent 1. The suspension was boiled for 3 minutes after which it was allowed (approximately three hundred million bacterial cells are in a drop of the suspension) to cool to room temperature before one drop (50 ul) of extraction reagent was added and thoroughly mixed. The mixture was centrifuged and 50 µl of the supernatant was reacted with test latex sensitised with the PBP2a-specific monoclonal antibody in a latex agglutination reaction. The supernatant was also tested against the control latex provided with the test kit. In each case, agglutination with the supernatant was assessed within 3 minutes of rocking. Any level of agglutination seen with the test latex but not the control latex particles was considered positive.

Data analysis

Programs Excel version 2003 (Microsoft ^{R} Office Excel 2003) was used for data collection, management and analysis of data. Chi-square test was used to compare the differences in the prevalence between raw milk and *wara*. Odds ratio and 95% CI were computed and the results were considered significant at $p<0.05$ while antibiotic resistance pattern was presented in percentages and tables.

Results

The mean staphylococcal count in milk samples from the five locations ranged from $1.4x10^7$ in Opeji to $4.5 x 10^8$ in Alabata (Table 1). There was no significant difference ($p>0.05$) in the staphylococcal count in samples from Kotopo, Alabata and Rounda. However, the staphylococcal counts of the milk samples from three locations (Kotopo, Alabata and Rounda) were significantly higher ($p<0.05$) than those from Oke-Ata and Opeji. There was heavy presence of staphylococci in the locally produced *wara* sold for human consumption in all the locations with counts ranging from $6.0×10^6$ in Oke-ata to $6.2×10^7$ in Kotopo and Opeji (Table 1). In all the sampling locations except Opeji, the mean staphylococcal counts in raw milk samples were significantly higher than in *wara* samples (Table 1). Overall, *S. aureus* was isolated from 52 (26.0%) out of 200 samples (Table 2). The rate of isolation was significantly higher ($p<0.05$) in *wara* (35%) than in raw milk (17%) samples (Table 2). Haemolysis of the 52 *Staphylococcus aureus* isolates on 5% sheep blood agar revealed that 28 (53.9%) showed complete haemolysis, 15 (28.9%) partial haemolysis and 9 (17.3%) no haemolysis. DNAse result showed that 20 (38.5%) of the *Staphylococcus aureus* isolates were positive while 32 (61.5%) were negative. 40 (76.9%) of the *S. aureus* isolates were positive for slide coagulase while all the 52 (100%) of the isolates were positive for tube coagulase (Plate 1).

Table 1: The mean staphylococcal count of raw cow milk and *wara* from five locations in Abeokuta, Nigeria

Locations	Total staphylococcal count (colony forming units/millilitre)	
	Raw milk	*wara*
Kotopo	$3.2 x 10^8$	$6.2×10^7$
Alabata	$4.5 x 10^8$	$3.7×10^7$
Rounda	$4.2 x 10^8$	$1.6×10^7$
Oke-ata	$4.1 x 10^7$	$6.0×10^6$
Opeji	$1.4 x 10^7$	$6.2×10^7$

Table 2: Detection of *Staphylococcus aureus* in raw cow milk and *wara* from five different locations in Abeokuta, Nigeria

Locations	Number (%) of positive samples		
	Raw milk	*wara*	Total
Rounda	3 (15.0)	12 (60.0)	15 (37.5)
Oke-ata	4 (20.0)	10 (50.0)	14 (35)
Kotopo	2 (10.0)	7 (35.0)	9 (22.5)
Opeji	3 (15.0)	4 (20.0)	7 (17.5)
Alabata	5 (25.0)	2 (10.0)	7 (17.5)
Total	17 (17)	35 (35)	52 (26)

Table 3: Antimicrobial resistance rates of *Staphylococcus aureus* isolated from raw cow milk and *wara* in Abeokuta, Nigeria

Antimicrobial agents (disc concentration)	Number (%) of resistant isolates by sample type		Total
	Raw milk	*Wara*	
Ceftazidime (25 µg)	17 (100)	35 (100.0)	52 (100)
Ampicillin (25 µg)	16 (94.1)	35 (100)	51 (98.1)
Doxycycline (5 µg)	11 (64.7)	15 (43)	26 (50)
Tetracycline (30 µg)	17 (100)	23 (65.7)	40 (76.9)
Oxacillin (1 µg)	15 (88.2)	35 (100.0)	50 (96.2)
Streptomycin (10 µg)	0 (0)	20 (57.1)	20 (38.5)
Augmentin (25 µg)	17 (100)	33 (94.3)	50 (96.2)
Gentamycin (10 µg)	15 (88.2)	17 (48.7)	32 (61.5)
Colistin (25 µg)	15 (70.6)	35 (100)	50 (96.2)
Sulphamethoxazole (300 µg)	16 (94.1)	27 (77.1)	43 (82.7)
Cefoxitin (30 µg)	15 (88.2)	35 (100.0)	50 (96.2)

Out of the 17 coagulase positive *S. aureus* in the *wara* samples, 15 isolates were confirmed as MRSA based on their resistance to oxacillin and cefoxitin as well as the presence of penicillin-binding protein 2a (PBP2a) while the 35 coagulase positive *Staphylococcus aureus* in the raw milk were MRSA. In all, a total of 50 (25%) methicillin resistant *Staphylococcus aureus* (MRSA) isolates were recovered from the 200 samples examined.

The rate of antimicrobial resistance among the 52 isolates of *Staphylococcus aureus* is as follows: ceftazidime (100%), ampicillin (98.1%), augmentin (96.2%), cefoxitin (96.2%) colistin (96.2%), oxacillin (96.2%), sulphamethoxazole (82.7%), tetracycline (76.9%), gentamycin (61.5%), doxycycline (50%) and streptomycin (38.5%) (Table 3). Fifteen (88.2%) out of 17 *S. aureus* isolates from fresh milk and all the 35 (100%) isolates from *wara* showed resistance to oxacillin and cefoxitin suggesting methicillin-resistance attribute. The 50 oxacillin- and cefoxitin-resistant isolates tested positive to PBP2a production using PBP2´ latex agglutination test. Thus, they were confirmed as MRSA while the two Methicillin-sensitive *Staphylococcus aureus* (MSSA) were negative to PBP2a production.

Plate 1: (A) DNAse test; *Staphylococcus aureus* isolate(positive control) topmost; two test *S aureus* isolates showed clear zones indicating depolymerization of DNA. (B) Tube coagulase test; the upper tube showed no gelling (negative control) and lower tube showed gelling due to coagulase production by the *S. aureus* , (C) 5% sheep blood agar for haemolysis test; *S. aureus* isolates showed complete haemolysis. (D) Slide coagulase test; no clumping (negative control) of the isolate on the left but there is clumping (+ve) of the isolate on the right side of the glass slide

Discussion

The microbial safety of any food product is determined by its level of microbial loads. The Staphylococcal loads of both raw milk and processed milk product (*wara*) were higher than the acceptable level of 10^5 cfu/ml (Oluwafemi & Lawal, 2015). The presence of *S. aureus* tends to reduce the quality of the milk and milk products through their metabolic activities and could precipitate food poisoning due to elaboration of toxins that could lead to illnesses when consumed by humans. In this study, mean bacterial counts of *S. aureus* were significantly higher in raw milk than in *wara*. This is similar to the findings of higher staphylococcal count milk than in milk products reported in earlier studies (Makwin *et al.*, 2014; Oluwafemi & Lawal, 2015). The high staphylococcal counts in milk could be due to the colonization of the mammary glands of the lactating cow by *S. aureus. Staphylococcus aureus* is a major cause of clinical and subclinical mastitis in cow leading to the presence of the organism in milk. *Staphylococcus aureus* from environmental source can easily colonize the udder of cows. Moreover, unhygienic method of milking cows especially hand-milking and the use of contaminated utensils could lead to milk contamination by *S. aureus* from extraneous sources.

The processing of raw milk to *wara* involves heating which is expected to eliminate or greatly reduce the level of bacteria present in the milk. This could be the reason for the higher level of staphylococcal count in raw milk than in the *wara*. However, the presence of *S. aureus* in the *wara* suggests that the processing methods were inadequate to completely eliminate bacteria from the finished ready-to-eat product. The presence of *S. aureus* in *wara* could also be due to contamination of the *wara* during post-production handling such as during marketing and distribution. One major observable problem is ignorance due to lack of awareness and the poor level of education among cattle herders, milk processors and vendors. Consequently, this has negative impact on adherence to hygiene practices in handling food during processing and marketing (Omemu *et al*., 2014). Ogbolu *et al*. (2014) reported that unclean hands of workers, poor quality of materials and water for processing, temperature fluctuations during hawking as well as reselling of leftovers from previous day could accelerate bacterial contamination thereby posing a significant health risk to consumers.

In this study, the overall prevalence of MRSA was 25%. This was higher than the 4.8% to 7.5% prevalence reported in milk and related products in Kaduna and Plateau States of Nigeria (Suleiman *et al*., 2012; Umaru *et al.,* 2013). However, Mai-siyama *et al.* (2014) reported 37.9% MRSA prevalence in ruminants slaughtered in Maiduguri while Lee (2003) reported a prevalence of 29.3% in raw milk in Korea higher than the 25% detection rate observed in the present investigation. The presence of antimicrobial resistant bacterial strains including MRSA in animals and in food of animal origins is of significant public health implications (Smith *et al*., 2002). In this present study MRSA could be transferred from cattle to humans via milk and milk products serving as vehicles for its transmission when such contaminated products as in this study are consumed.

Apart from resistance to the beta-lactam antibiotics (ampicillin, ceftazidime, augmentin, cefoxitin, oxacillin), the MRSA isolates also demonstrated to varying degrees, resistance to other antimicrobials including aminoglycosides (streptomycin, gentamicin), tetracyclines (tetracycline and doxycycline), colistin and sulphomethoxazole. Selection pressure due to indiscriminate antimicrobial usage in food-producing animals is a major factor contributing to the emergence and dissemination of antimicrobial resistant bacterial strains (Ojo *et al.*, 2016).

In conclusion, the presence of pathogenic organisms such as MRSA in raw milk and *wara* sold in Abeokuta poses major public health risk to the human populace. Therefore, there is a need for proper education of local milk producers, processors and marketers on the importance of strict adherence to hygiene practices in handling milk and milk products. There is also a need for public awareness on livestock-associated MRSA and the importance of acting appropriately to prevent the zoonotic transmission of MRSA through contaminated raw milk and milk products. Indiscriminate use of antibiotics should be discouraged among cattle rearers. Better knowledge on the distribution of MRSA and risk assessment in the food chain is required to formulate strategies for preventing the spread of the organism.

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