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Prevalence of Methicillin Resistant *Staphylococcus aureus* (MRSA) in Cattle Milk from dairy Herds in Oyo State, Nigeria

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ABSTRACT

Methicillin-resistant Staphylococcus aureus (MRSA) has received a lot of attention in recent years as a zoonotic organism of global concern. Contaminated milk, especially those from mastitic cows, serve as reservoirs for humans in the epidemiology of antibiotic resistant MRSA. This study was designed to determine the level of contamination of bulk fresh milk from dairy cattle herds with MRSA in Ibarapa, Oyo and Oke-Ogun areas of Oyo State and the antibiotic resistance profile of the isolates. One hundred and sixty-five (165) milk samples were obtained from the study areas and used for the study. Staphylococcus aureus was isolated from the samples using bacterial culture and biochemical tests. Methicillin-resistant Staphylococcus aureus was identified using cefoxitin disk diffusion method. All the S. aureus isolates were subjected to microbial susceptibility test. Ninety (54.5%) milk samples were positive for Staphylococcus spp. out of which 52 (31.5%) were Staphylococcus aureus and 13 (7.9%) yielded MRSA. Antibiogram of S. aureus indicated highest resistance to Cloxacillin (88.5%) followed by (Augmentin 67.3%) and Ceftrazidine (67.3%). Ten out of the 13 MRSA isolates were multidrug resistance while all the isolates were 100% susceptible to ofloxacin. The results of this study showed that milk produced from dairy cattle in Oyo State was contaminated with MRSA. This portends serious food safety and public health risk among the consumers of such milk especially in raw or improperly pasteurized form. Proper dairy herd health management and prudent use of antibiotics and hygienic milking procedures are hereby recommended to prevent contamination of milk and subsequent spread of the organism to humans.

Keywords: Methicillin-resistance, *Staphylococcus aureus* (MRSA), Fresh cattle milk, Antimicrobial resistance.

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INTRODUCTION

Milk is one of the most nutritious food known to man and provides the first basic nutrients for all mammals [1]. Dairy products in form of milk cream, butter, yoghurt, sour milk are satisfactory diet at any stage of human life [2]. Nigeria is the largest producer of dairy in West Africa and the third largest producer of cow milk in Africa [3]. The production and consumption of milk in the country are not adequately documented, but the consumption was estimated at 22 g per person per day and average annual growth of 7.95 % [4].

A greater proportion of dairy cattle production in Nigeria are reared by traditional nomadic, sedentary or pastoralist Fulanis mostly at subsistence level with milkmaids processing bulk of the milk into local cheese and yoghurt [5]. The traditional hand milking employed is mostly insanitary, using unsterilized containers for processing, with the cow udders not usually washed and disinfected before milking while flies fly into the milk containers which aid bacterial contamination of the milk [3]. Despite unhygienic milking practices, raw milk and milk products are relished by herders' households as well as rural and urban dwellers in the country [6].

Milk is a good substrate for *S. aureus* growth and dairy products are common sources of staphylococcal food poisoning [7]. *Staphylococcus aureus* is a gram positive, ubiquitous bacterium, widely isolated from many environmental sites (like dust, water, air, faeces), it is also part of normal bacterial flora of mammals [8]. The organism has also been isolated from milkers' hands and milking equipment [9]. It is an important food borne pathogen causing food poisoning resulting from ingestion of food containing one or more preformed enterotoxins produced by *S. aureus* [10]. The symptoms of Staphylococcal food poisoning have a rapid onset and include nausea, vomiting and diarrhoea [11]. Contamination of milk with *S. aureus* results from skin and mucosae of cows, mastitis or environmental sources during handling and processing of milk [7]. Staphylococcal food poisoning ranks third among reported food borne diseases in the world [12]. The presence of *Staphylococcus aureus* on the skin and mucosae of food producing animals and frequent association of the pathogen with mastitis often lead to contamination of milk [13]. Contamination of milk can also occur, from humans and environmental sources during handling and processing [14]. *Staphylococcus aureus* and *Streptococcus agalactiae* are the major incriminated pathogens causing intramammary infections in dairy animals [15]. In India, about 56.9% of sub-clinical mastitis was reportedly caused by *S. aureus* [16]. The emergence of Livestock associated methicillin resistant *S. aureus* (LA-MRSA) further made *S. aureus* of great zoonotic importance that can be contracted through the consumption of bovine milk [17]. This is further exacerbated by indiscriminate use of antibiotics by cattle producers. There are many points of contamination with MRSA during milking, handling and processing of bovine milk [18]. Methicillin resistant *S. aureus* isolates are usually resistant to beta-lactam antibiotics like penicillin, cloxacillin, and amoxicillin and are often multidrug resistant [13, 19]. Tetracycline resistant MRSA have also been reported in cattle [20, 21].

Effort to industrialize the traditional herds has resulted in government introduction of public private partnership for cooperative milk collection schemes from herdsmen into designated milk collection centres across the country [22, 23]. In view of possibilities for MRSA contamination of bovine milk during milking, handling and processing as well as high incidence of staphylococcal food poisoning with high fatality rate, there is need for research to aid monitoring and control along the food chain [24]. This study determined the level of contamination of bulk fresh milk from dairy cattle herds with *S. aureus* and MRSA in Ibarapa, Oyo and Oke-Ogun areas of Oyo State by the isolation of the organism andthe determination of their antibiotic resistance profiles.

MATERIALS AND METHODS

Study Area

Oyo State is an inland state in south-western Nigeria, the capital of the state is Ibadan. Oyo State covers approximately an area of 28,454

square kilometres [25]. Savannah agroecological vegetation abounds in the northern part of the state where several sedentary and nomadic pastoralists rear large heads of dairy cattle mostly White Fulani breed. Milk from the herds were collected and pooled together at three milk collection centres in Ibarapa, Oyo and Oke-Ogun towns.

Sample Size and Sampling

Fresh raw milk from each herd was pooled together in milking vessels. The portions to be sold to the milk collection centres were poured into the milk tank supplied by the centres. Sample size of 165 fresh bulk milk samples was calculated from the herd population using a prevalence of 52.5% [26].Fresh bulk milk samples were collected from dairy herds in Ibarapa, Oyo and Oke-Ogun over a period of 4 weeks using simple random sampling method; About 10 ml fresh milk from bulk tank of each herd were collected into labelled sterile sample bottle and transported in an insulated container to the Food and Meat Hygiene Laboratory of the Department of Veterinary Public Health and Preventive Medicine, University of Ibadan.

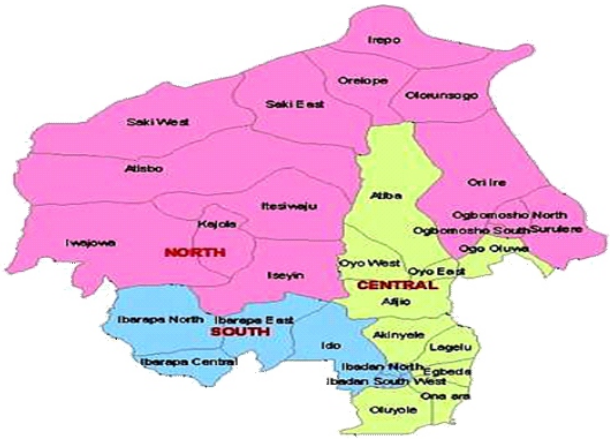

Figure 1: *The map of Oyo State showing the senatorial districts from which samples were collected*

Key: *Yellow- Oyo Central, Blue- Oyo South, Lilac- Oyo North*

Microbial Isolation and Identification of *Staphyloccocus aureus*

Bacteriological culture was performed following standard microbiological technique and in accordance to the Clinical and Laboratory Standard Institute [27]. A loopful of milk sample was streaked on 5% sheep blood agar (Oxoid, UK) using inoculating loop. The plates were incubated aerobically at 37 °C and examined after 24–48 hours of incubation. Gram's staining reactions of suspected colonies, morphology and their haemolytic patterns on sheep blood agar were determined and recorded. Colonies that were positive for Gram's staining appearing as typical grape-like structures under the microscope (100 x and 400 x) were further sub-cultured on nutrient agar plates (Oxoid, UK) and incubated at 37 °C for 24 hours. Obtained pure colonies were stored and maintained on nutrient slants for further characterization.

Presumptive isolates were further biochemically characterized using catalase, coagulase, mannitol salt agar and purple agar base tests. Isolates that were catalase and coagulase positive and showed fermentation of mannitol and maltose (strong yellow discoloration of both media) were considered positive for *S. aureus*. For the isolation of MRSA, antibiotic sensitivity discs which contain cefoxitin was placed on the surface of each plate by means of antibiotic disc dispenser and incubated at 37 °C for 18-24 hours. Isolates that did not produce zones of inhibition (resistant) were taken as MRSA.

Antimicrobial Susceptibility Test

Disk diffusion method of antimicrobial susceptibility method was used according to Clinical Laboratory and Standard Institute, CLSI (2019) [27]. Mueller Hinton agar was prepared according to manufacturer's instructions, single colony of each *S. aureus* isolate and MRSA was selected and emulsified in 3 ml sterile normal saline solution in a sterile

test tube. The turbidity of the suspension was then adjusted to the density of barium chloride standard (0.5 McFarland) standardize the inoculum size. Sterile cotton swab was then dipped into the standardized bacterial culture suspension and squeezed against the sides of the test tube to remove the excess fluid. The swab was used to inoculate unto Mueller Hinton agar. Nine antimicrobial discs comprising; ceftazidime (30 µg), cefuroxime (25 µg), gentamicin (10 µg), Oxacillin (10 µg), ceftriaxone (30 µg), erythromycin (10 µg), cloxacillin (25 µg), ofloxacin (10 µg) and augmentin (30 µg) were placed on the agar using the antibiotic disc dispenser. The plates were then allowed to stand for 30 minutes for diffusion of active antimicrobial agents. Plates were inverted and incubated at 35-37 °C for 24 hours. After incubation the plates were examined for zones of inhibition around the discs. These zones were measured to the nearest millimetre using transparent ruler. The measurements were interpreted as 'resistant', 'intermediate,' and 'sensitive' according to the recommendation of CLSI (2019) [27], *Staphylococcus aureus* ATCC25923 (ATCC, USA) was used as control organism.

RESULTS

Staphylococcus species appeared on defribrinated sheep blood agaras large white to golden round, convex of about 1-4 mm in diameter with sharp border colonies. On blood agar plates, colonies of *S. aureus* were surrounded by lightened yellow transparent zones of clear beta haemolysis. Results revealed that 90 (54.5%) of bovine milk samples gave typical staphylococcal spp. colonies on blood agar. Fifty-two (31.5%) coagulase positive *S. aureus* were obtained from 7 (13.46%), 23 (44.23%) and 22 (42.31%) milk samples from Ibarapa, Oyo and Oke-Ogun respectively (Table 1). The MRSA isolates, resistant to cefoxitin obtained from the 52 coagulase positive *S. aureus* were thirteen (13) implying contamination of 13 bulk fresh milk by MRSA in the study area and overall prevalence of 7.9% for the study. Samples from Oke-Ogun had the highest prevalence (3.64%) while Oyo and Ibarapa had a prevalence of 3.03% and 1.21% respectively (Table 1).

Antimicrobial Susceptibility Profile of MRSA Isolates to Antibiotics

Three of the isolated *S. aureus* were resistant to all the 9 antibiotics tested while over 30 were resistant to 5 antibiotics (Table 2). The highest rate of resistance was recorded against cloxacillin (76.9%) and oxacillin (69.23%). Gentamicin recorded 23.1% resistance while no (0%) resistance was recorded to ofloxacin (Table2). Ten out of the 13 MRSA isolated in this study were resistant to at least 2 antimicrobial agents while 7 were resistant to at least 3 antibiotics. Five antibiotic resistant profiles were recorded among MRSA in this study, they are; GEN-CTR-AUG-ERY-CAZ-CRX-CXC-OXC, CTR-AUG-ERY-CAZ-CRX-CXC-OXC, CAZ-CRX-CXC-OXC, CRX-CXC-OXC, and CXC-OXC(Table 3).

Table 1: Prevalence of *Staphylococcus* spp., *S. aureus* and MRSA isolated from dairy cattle at Ibarapa, Oyo and Oke Ogun areas of Oyo state

Serial number	Study area	Number (%) of samples collected	Number (%) *Staphylococcus* spp. identified (N= 165)	Number (%) *S. aureus* identified (N= 165)	Number (%) MRSA identified (N= 165)
1	Ibarapa	51	22 (13.3)	7 (4.2)	2 (1.12)
2	Oyo	56	33 (20)	23 (13.9)	5 (3.03)
3	Oke Ogun	58	35 (21.2)	22 (13.3)	6 (3.64)
	Total	165	90 (54.54)	52 (31.52)	13 (7.9)

Table 2: Number of *S. aureus* and MRSA isolated from Bovine milk in Ibarapa, Oyo and Oke Ogun areas of Oyo Statethatwereresistant to tested antibiotics

Serial Number	Antibiotics	Number of r esistant *S. aureus* n=52 (%)	Number (%) of r esistant MRSA (n=13)
1	CAZ	35 (67.3)	6 (46.2)
2	CRX	34 (65.4)	7 (53.8)
3	GEN	13 (25.0)	3 (23.1)
4	CTR	27 (51.9)	5 (38.5)
5	ERY	32 (61.5)	5 (38.5)
6	CXC	46 (88.5)	10 (76.9)
7	AUG	35 (67.3)	5 (38.46)
8	OXC	13 (25.0)	9 (69.23)
9	OFL	3 (5.8)	0 (0)

MRSA- Methicillin resistant *Staphylococcus aureus,*CAZ- Ceftrazidine, CRX- Cefuroxine, GEN- Gentamicin, CTR- Ceftriazone, ERY- Erythromycin, CXC- Cloxacillin, AUG- Augmentin, OXC- Oxacillin, OFL- Ofloxacin

Table 3: Antibiotics resistant profile of MRSA isolated from Bovine milk in Ibarapa, Oyo and Oke Ogun areas of Oyo State

Serial number	Antibiotics							
1	GEN	CTR	AUG	ERY	CAZ	CRX	CXC	OXC
2	GEN	CTR	AUG	ERY	CAZ	CRX	CXC	OXC
3	GEN	CTR	AUG	ERY	CAZ	CRX	CXC	OXC
4		CTR	AUG	ERY	CAZ	CRX	CXC	OXC
5		CTR	AUG	ERY	CAZ	CRX	CXC	OXC
6					CAZ	CRX	CXC	OXC
7						CRX	CXC	OXC
8							CXC	OXC
9							CXC	OXC
10							CXC	OXC

DISCUSSION

The prevalence recorded in this study could have arisen from poor herd hygiene, contaminated water and unhygienic milking practices. Milking of cows with subclinical mastitis is another major possible source of milk-borne *S. aureus* and MRSA. Contamination of teat and udder tissue with increased bacteria concentrations that invade and infect the udder could lead to the development of mastitis, elevated somatic cell counts and bacteria milk contamination. Livestock Associated Methicillin-resistant *S. aureus* (LA-MRSA) is considered to be an important zoonosis because of its capacity to colonize a wide range of hosts including man [28]. The prevalence of MRSA obtained in this study is higher than the prevalence rate of 4.8% reported by Gali*et al*., (2013) [26] but similar to the prevalence rate (6.3%) reported by Lim *et al*., (2013) [29] in Korea.

Methicillin resistant *Staphylococcus aureus* infected cattle act as reservoirs and later transmit the bacteria to other animals and humans [30, 31]. Colonization of cattle by MRSA may be an occupational risk to the people in close contact with the animals. Methicillin resistant *Staphylococcus aureus* remains a major intrinsic and extrinsic ubiquitous milk contaminant from housing materials, fodders, equipment, air, skin, non-bovine animals and human sources [28, 32].Indiscriminate use/injection of antibiotics in humans and cattle could enhance intra mammary infection with LA-MRSA and human-to-bovine transmission of MRSA [33].

The preponderance of multidrug resistant MRSA in bulk milk in this study could be due to the unrestricted, uncontrolled and irrational use of antibiotics by cattle producers without veterinary supervision. *Staphylococcus aureus* isolates that were resistant to cloxacillin, a β-lactam antibiotics, in this study may have resulted from indiscriminate use and abuse of cloxacillin in the management of mastitis in the dairy cattle. Methicillin resistant *Staphylococcus aureus* isolates which are resistant to beta-lactam antibiotics like penicillin, cloxacillin and amoxicillin have been reported to often be multidrug resistant [19]. The indiscriminate use of antibiotics which could have led to the development of resistance to tested antibiotics in the isolated MRSA in this study may not be unconnected with the fact that the tested antibiotics are relatively inexpensive and are generally available from distributors/retailers. They can be purchased easily from most vendors without prescription in within the study area. The widespread use of antibiotics may have enhanced the development of virulence in*S. aureus* through acquisition of resistance genes which facilitates its ability to survive most categories of antibiotics [34]. Most of the coagulase positive *S. aureus* isolates in this study were multidrug resistant because they showed resistance to cloxacillin, augmentin, ceftrazidine, erythromycin and cefuroxime and are of clinical and public health concern. The sub-therapeutic use of these antimicrobial agents either as prophylactics or therapeutics as well as imprecise dosages given to sick or healthy animals may have led to the level of resistance of *S. aureus* isolates to these antibiotics. These drugs are administered to treat mastitis by unqualified personnel without performing antibiotic sensitivity test.

The misuse of antibiotics could also be due to poor drug use control and ineffective implementation of legislations concerning drug use in the study area [35, 36].

This finding of multidrug resistance in *S. aureus* and MRSA in this study is in tandem with the report of Sekhan *et al.,* (2011) [37] who found ofloxacin most sensitive (91.97%) in *S.aureus* isolated from their study. The

susceptibility of MRSA isolates in this study to ofloxacin may be suggestive of the fact that ofloxacin are less abused in the areas of study and may be recommended for the treatment of staphylococcal infection.

Staphylococcus foodborne disease is one of the most common foodborne disease and is of major concern in public health programs worldwide [38]. This is due to the production of one or more toxins by the bacteria during growth at permissive temperatures [39]. The emergence of LA-MRSA is now a major public health issues in the current global awareness and mitigations for AMR. Bovine milk and its products are considered as one of the main sources of spreading MRSA in humans and has contributed to the epidemiology of antimicrobial resistance [38].

The dairy industry should be concerned about food safety as outbreaks of diseases in humans have been traced to the consumption of raw unpasteurized milk as well as contaminated pasteurized milk. Entry of foodborne pathogens like *S. aureus* and MRSA through contaminated raw milk into dairy food processing plants can lead to the persistence of these pathogens in the production lines and subsequent contamination of processed dairy products which may result in morbidity and mortality in man[39].

Conclusions and Recommendations

This study revealed that some of the milk supplied from local dairy cows in the study areas were contaminated with multidrug resistant *S. aureus* andsubset belonging to MRSA strain. There is need to improve milk quality through proper animal management, prompt diagnosis/treatment of mastitis, prudent use and better enforcement of the regulation of the sales and use of antibioticsby the National Agency for Food and Drugs Administration and Control (NAFDAC)/Veterinary Council of Nigeria (VCN), milk hygiene practices and effective storage/pasteurization to ensure the safety of milk. Enlightenment of herdsmen and milk maidson proper handling of milk, good personal hygiene,adequate cleaning of surfaces and equipment like the milking machine can reduce the spread of *S. aureus* and MRSA throughout the milk/product processing line.To achieve food safety and maintain good public health in milk/product, holistic approach should be engaged to ensure that milk and its products are wholesome, free from pathogens and fit for human consumption.

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