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Prevalence and Antibiogram of Methicilin-Susceptible *Staphylococcus aureus* (MSSA) Isolated from Raw Milk of Asymptomatic Cows In Abeokuta, Nigeria

Olufemi F.O., Akinduti P.A., Keinde O.B., Odunfa O.A.
Department of Veterinary Microbiology & Parasitology, College of Veterinary Medicine, Federal University of Agriculture, Abeokuta, Nigeria

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***Correspondence to:**

woleolufemi84@gmail.com

ABSTRACT

Most *S. aureus* show resistance to methicillin and are non-beta-lactam sensitive (MSSA) but are also resistant to other antibiotics and have been associated with mastitis, nosocomial infections, food poisoning, septicemia and other diseases in animals and man. No study has identified *S. aureus* in milk of apparently healthy cows in Abeokuta and its environment. In this study, 7 of 9 isolates *S. aureus* (77.8%) were MSSA and they all showed good susceptibility to all the routinely used antibiotics in Abeokuta viz: Augmentin, Cefuroxime, Gentamycin, Cotrimoxazole, Ofloxacin Tetracycline and Perfloxcin. The *S. aureus* isolates also show a low MIC values of 2-4 ug/ml to all the antibiotics used, except cotrimoxazole where a value of 8ug/ml was recorded indicating an emergence of resistance to the cotrimazole antimicrobial agents

Rapidly emerging multi-drug resistant strains of MSSA pose a threat to public health and make treatment failure quite imminent, therefore African countries, including Nigeria should consider having efficient control over misuse of antibiotics.

1. INTRODUCTION

Staphylococcus aureus has long been globally seen as normal colonizers of the skin and nares of animals such as pigs, cattle, horses, camels, turkeys, chickens, small ruminants and man. Infections due to staphylococci are of major importance to veterinary and human medicine. *S. aureus* is one of the most significant pathogens causing mastitis in cattle and ovine species (Koreen et al 2004), nosocomial infections and a wide range of human diseases, including endocarditis, food poisoning, toxic shock syndrome, septicemia, skin infections, soft tissue and bone infections (Zadoks et al 2000). The pathogenicity of *S. aureus* has been associated with extracellular products and virulence factors (Jarraud et al., 2002. Francis et al, 2005; El-Jakee et al., 2008*). S. aureus* was virtually susceptible to all antibiotics when antibiotics were newly introduced but by 1940's, a few years after antibiotics were introduced, diverse antibiotics-resistant strains of the microbe emerged (Chamber and DeLeo, 2009). The various emerging strains can be differentiated by antibiotic susceptibility, phenotypic determinants like virulence factors and genotyping (Isigidi et al, 1990; Stephan, et al., 2001; Quinne et al 2002., El-Jakee et al 2008., Jayawera et al 2017). The epidemiology of this microorganism in animals has gained interest in the last years, not only because of their importance in veterinary medicine due to the increase of infectious processes caused by this pathogen (Golding *et al* 2010., Mediavilla *et al* 2013), but because of their increasing evidenced zoonotic potentials in people that are in direct contact with these animals (farmers, veterinarians, and slaughterhouse workers) as well as in their relations (teachers, nurses, bankers, Insurance staff, Chief Executives of companies etc) that have no history of direct animal contacts (Benito *et al* 2014., Lozano *et al* 2016). The number of studies that focused

on the antibiotic resistance problem of *S. aureus* has grown globally, in the last decade and it is suggested that this problem is increasing especially in Africa.

In general, some *S. aureus* have been demonstrated to show resistance not only to commonly used antibiotics but also methicillin-derived and other non-beta-lactam agents (MRSA). The other major group of *S. aureus* is the group that are methicillin and non-beta-lactam sensitive (MSSA) (Akobi *et al* 2012, Chairat *et al*, 2015); but are as resistant to other antibiotics like, penicillin, tetracycline, erythromycin or clindamycin as the MRSA (Schaumburg *et al* 2013., Schaumburg *et al.*, 2015).

Nevertheless in most cases, penicillin resistance is higher among MSSA isolates in the African region than in other parts of the world (Ghasa *et al*, 2015; Chairat *et al*, 2015). The real extent of the burden of antimicrobial resistance of *S.aureus* is currently unknown since surveillance of drug resistance is only carried out in a few countries (WHO, 2015). Kimang'a (2012) associated extensive and efficient development of *S. aureus* antibacterial resistance in Africa to misuse of antibiotics brought about by poor control policies in the African countries.

A high number of researches has focused on the microbiological analyses of food products such as milk, meat, ready-to-eat, fish and eggs, with the objective of analyzing the presence of different pathogens and determine the rate of contamination of the tested food and food products (Kiyaria *et al*, 2007., (Elhag *et al*, 2014) while some studies have also demonstrated the presence of *S. aureus* in milk and the involvement of this pathogen in clinical and subclinical mastitis of dairy cow s (Abera *et al.*, 2010; Kadja *et al* 2010) . Recently some studies have been done on *S.aureus* in foods and food products like meat, dairy, eggs, chickens in different parts of Nigeria (Sokari *et al* 1991., Isura *et al*, 2010; Mai-siyama *et al* 2014; Ndahi, *et al.*, 2015) however, there is a dearth of information on MSSA in milk from apparently healthy cows in Abeokuta, Ogun State, Nigeria.
The objective of this study is to examine the prevalence of MSSA (*S. aureus*) isolates from 64 milk samples of apparently healthy cow obtained from Abeokuta, Ogun State. The *S. aureus* isolates will be characterized using methods like Gram staining, coagulase, catalase and other conventional tests. Also the resistance of the isolates to a wide range of antibiotics commonly used in the Southern part of Nigeria will be determined.

2. MATERIALS AND METHODS:

2.1 Sampling of milk: Sixty four raw cow milk samples were randomly collected from boarded and free ranged cattle found in different herds in Abeokuta, Nigeria. The udder of the cow was thoroughly sterilized with sterile swab soaked in methylated spirit and was gently pressed to obtain the milk which was collected into a sterile wide mouthed bottle. The milk samples collected were transported to the laboratory for analysis in cold chain.

2.2 Isolation and colonial morphology: one milliliter of the well mixed milk sample was thoroughly mixed with10 ml glucose peptone water and incubated at 37^0C for 24 hours. It was sub-cultured onto Nutrient agar, MacConkey and Mannitol Salt agar (Oxoid, UK) and further incubated overnight at 37^oC. The bacteria isolates obtained were purified and examined by their colonial morphology while Gram staining described by Cowan and Steel (1975), Baron and Finegold (1990) were used to characterize its cellular morphology.

2.3 Biochemical characterization: Each bacteria isolates obtained was characterized using API staphylococcus kit E234 and were appropriately identified.

2.4 *Staphylococcus aureus* characterization: identification of the suspected *S. aureus* was performed according to the Gold standard described by Adeniyi et al 2004. Lazano et al., 2016, Jayaweera, et al., 2017). Tube Coagulase test was done to identify *S. aureus* which produces the enzyme coagulase by adding 0.1ml overnight broth culture of suspected isolates in 5ml 1/5 dilute plasma. After 6 hours incubation, clump was observed that indicate a positive reaction.

2.5 Oxidase test: A pure colony of the test isolate was smeared into a piece of oxidase filter paper (Oxoid, UK) and color change to purple indicates a positive oxidase test. This test was done to rule out presence of Micrococcus species or other staphylococcus specie than *S. aureus.*

2.6 Antimicrobial susceptibility testing: The susceptibility pattern of each *S. aureus* isolate was tested against commonly used antibiotics by the disk diffusion method on Mueller Hinton agar according to

Bauer *et al* (1996).The following antibiotic disks were used: ampicillin (10 µg), amoxicillin/clavulanic acid (20 µg/10 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefuroxime (30 µg), gentamicin (10 µg), and ciprofloxacin (5 µg). Pure isolates of 0.5 McFarland was spread on Mueller Hinton agar and the antibiotic disks were incubated at 37°C for 18–24 hours.

The inhibition zones were measured and interpreted as sensitive, intermediate, or resistant according to Clinical and Laboratory Standards Institute guidelines (2012).

2.7 Methycillin susceptibility assay: Each identified *Staphylococcus aureus* isolate was tested against methicillin or methicillin-derivative (Cloxacilin) to determine their susceptibility pattern using modified standard micro-tube dilution spectro-photometeric bio-assay in 96-well microtitreplate (Shanmugapriya *et al.*,2012). Briefly, 100ul of 0.5MacFarland pure broth of *S. aureus* was added to serially diluted methicillin in 10% glucose peptone and incubated at 37^0C for 24 hours. Absorbance of the turbidity of each well before and after incubation was measured in microtitre plate at 590 nm wavelength and inhibition rate for MIC was determined for each antibiotics.

2.8 Data analysis: the significant ($p<0.05$) of the mullti-antibiotic *S. aureus* found was determined using Chi- square at 95% confidence interval while correlation coefficients of the *S. aureus* from the sources was determined.

3. RESULTS

Isolation and identification of *S. aureus* were performed in sixty-four milk samples obtained from apparently healthy dairy cow. Nine *S. aureus* isolates were obtained from 64 samples giving a prevalence of 14.0%. All the isolates were tested for their susceptibility to 9 different common antimicrobial drugs, including Cloxacillin. The 9 isolates were 100% susceptible to Gentamycin, Ofloxacin, Ciprofloxacin, Tetracyclin and Perflocin (Table 1), while 8 or 88.9% of the isolates were susceptible to Augmentin but 6 isolates (66.7%) were susceptible to Cefuroxime. Only 1 isolate (11%) was resistant to Cloxacilin, and 7 (77.8%) were susceptible to Cloxacilin. These 7 (77.8%) isolates were referred to as Methicillin-susceptible *S. aureus* (MSSA).

Table 1; antimicrobial susceptibility of *Staphylococcus aureus* isolates (N=9)

Antibiotics (µg/disc)	Antimicrobial susceptibility test		
	S	I	R
	Number	Number	Number
Augmentin (1/5)	8	1	0
Cefuroxime (30)	6	2	1
Gentamycin (10)	9	0	0
Cotrimoxazole (5/25)	7	1	0
Ofloxacin (10)	9	0	0
Cloxacillin (10)	7	1	1
Ciprofloxacin (10)	9	0	0
Tetracycline (30)	9	0	0
Perfloxcin (10)	9	0	0

The prevalence of *Staphylococcus aureus* among the samples and their MIC is shown in Figure 2

Table 2 Minimum inhibitory concentration of antibiotics tested against *Staphylococcus aureus* isolates

Antibiotics (µg/disc)	MIC (µg/mL)*	Number (n)
Augmentin (1/5)	2	7
Cefuroxime (30)	4	6
Gentamycin (10)	4	9
Cotrimoxazole (5/25)	8	7
Ofloxacin (10)	2	8
Cloxacillin (10)	2	6
Ciprofloxacin (10)	2	6
Tetracycline (30)	4	8
Perfloxcin (10)	2	9

*According to CLSI (2012)

4. DISCUSSION

In this study, 9 isolates of *S. aureus* were made from 64 milk samples taken from 64 apparently healthy cows that had no clinical mastitis. This gives a high prevalence rate of 14% *S. aureus* in the fresh milk samples. This rate compares well with Rodríguez-Lázaro *et al* (2017) that had isolation rate of 15.7% *S. aureus* (MSSA) from 868 diverse food samples taken from guinea pig, pork, rodents, turkey, antelope, duck, eggs and dairy products including cow milk. The result from this study also compares well with the work of Patchanee *et al* (2014) that has MSSA prevalence of 20% in Thailand. This high MSSA prevalence has given rise to great concern in terms of the zoonotic infections caused by this pathogen among livestock and humans regardless of whether the humans have contact with animals or not (Benito *et al.,* 2013).

Most studies on MSSA in the African continent are focused on human clinical isolates. However there are some baseline information from the African countries of Côte d'Ivoire, Democratic Republic of Congo, Egypt, Gabon, Madagascar, Nigeria, Senegal, Sudan, South Africa, Tunisia, Uganda, and Zambia (Lazano *et al.,* 2016). This result of this study thus provides some information on the prevalence of *S. aureus* in Abeokuta, Nigeria.

Some researchers have put MSSA prevalence in Africa at a highly variable rate of between 3% and 58% and that some clonal lineages of the pathogen seem better adapted to some animal (livestock, companion and wild) species (Lazano *et a*l., 2016). For instance in Nigeria, the following MSSA isolation rates were recorded: Bats, 14%; Cow, 15.0%; Camels, 15.7%; and Sheep, 3.5% (Akobi *et al* 2012. Mai-siyama *et al* 2014. Lazano et al 2016). This is different from what obtains in Tunisia as prevalence rates are as follows: cows, 1.3%; Camels, 41%; Sheep, 41%, and Goats, 41.7% (Lazano *et al* 2016).

While the prevalence of MSSA in fresh milk of apparently healthy animal may vary between 6.5% and 100% (Daka, *et al.,* 2012), the results in this study provides a baseline information on fresh milk in Abeokuta, Ogun State. Therefore *S. aureus* prevalence in fresh healthy cow milk may vary from this result in different parts of Nigeria.

In this study, 7 of 9 isolates, (77.8%) Table 1, were susceptible to Cloxacillin, a methicillin-derivative, hence were termed MSSA (Shanmugapriya *et al*., 2012). Only one isolate out of 9 (11%) was methicillin (cloxacillin) -resistant (MRSA). Also the 7 isolates showed good susceptibility to all the routinely used antibiotics in Abeokuta viz: Augmentin, Cefuroxime, Gentamycin, Cotrimoxazole, Ofloxacin Tetracycline and Perfloxcin. The MIC breakpoints of 2-4 ug/ml were obtained for most of the antibiotics. This study therefore supports the work of other researchers (Adesiyun *et al.,* 1992. Nagel *et al*, 2013. Mai-siyama et al 2014) that found *S. aureus* to be susceptible to most commonly used antibiotics.

In this study, *S. aureus* isolates have low MIC values of 2-4 ug/ml to all the antibiotics used, except cotrimoxazole where a value of 8ug/ml was recorded (Table 2). This result is again in line with a study from Gabon, Central Africa, where there was a high resistance to co-trimoxazole (Alabi *et al.,* 2013). The

frequent prescription of (amino) penicillins and co-trimoxazole both in Nigeria and Gabon could explain the high rates of the resistance obtained to these antimicrobial agents. This observation may mean a gradual but significant emergence of new strains of *S. aureus* due to continued and uncontrolled or indiscriminate use of antibiotics on the field.

5. CONCLUSION and RECOMMENDATIONS

This study attempted to provide baseline information for the prevalence and antibiotics resistance of *S. aureus* in fresh milk from apparently healthy cows in Abeokuta, Nigeria. The results obtained in this study compare well with those obtained from other parts of the world. Rapidly emerging multi-drug resistant strains of MSSA pose a threat to public health and make treatment failure quite imminent, therefore African countries, including Nigeria, should consider having efficient control over misuse of antibiotics. The use of antibiotics in animal husbandry as growth promoters must be discouraged as this enhances antibiotics-resistance among *S. aureus.*

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