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Full Length Research Paper

Prevalence and antibiotic resistance profiles of Methicillin resistant *Staphylococcus aureus* (MRSA) isolated from bovine mastitic milk in Plateau State, Nigeria

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Abstract

Mastitis is one of the major challenges of the dairy industry, culminating in the use of a lot of antibiotics which in most cases are often abused leading to resistance. One of the commonly resisted antibiotics is methicillin which is also referred to as Oxacillin. The prevalence of methicillin resistant *Staphylococcus aureus* (MRSA) and minimum inhibition concentration (MIC) of oxacillin 1µg/ml against, Oxacillin resistant *Staphylococcus aureus* (ORSA) from subclinical mastitic milk was investigated from 339 quarters of 85 cows, 105(30.9%) were found to be mastitic. One hundred and three (98%) Staphylococcus aureus were isolated from the mastitic quarters. Seventy three of the isolates tested against 12 antibiotics used in the study area, showed twenty six (35.6%) to be resistant to oxacillin and 10 other antibiotics. All the seventy three isolates were susceptible to vancomycin and resistant to penicillin, and also resistance to more than one antibiotic. The 26 ORSA were further confirmed by growth on ORSAB medium and the detection of the *23SrRNA* specie specific fragment of *S. aureus* using PCR. PCR was also used to detect the gene *mec*A in 2(7.6%) of the 26 ORSA, and the *blaZ* gene in all the 26 ORSA. The MIC of oxacillin 1µg/ml for the *mec*A positive isolates was 2.4µg/ml to ≥ 10µg/ml higher than the non – *mec*A isolates 1.2µg/ml-2.5µg/ml. There is a need for urgent measure(s) to tackle the problem of antibiotic resistance.

Keywords: Methicillin, Oxacillin, Antibiotics resistance, *Staphylococcus aureus.*

INTRODUCTION

Staphylococcus aureus is one of the important causative agent of mastitis all over the world (Cabral *et al*., 2004), causing both sub-clinical and clinical form of mastitis in cattle (Pradeep *et al*., 2003). Mastitis is one of the major causes of antibiotic use in dairy cows (Mitchell *et al*., 1998; DANMAP, 2003). Antimicrobial therapy plays a role in mastitis control by reducing the levels of herd infection and preventing new infections. However, bacteriological cure rate against *S. aureus* for antimicrobial therapy is relatively low due to pathogen characteristics such as the ability to survive inside the host cell, ability to adapt to different environmental conditions, presence of virulence factors and pathological changes induced in chronic infection (Rabello *et al*., 2005; Waldvogel, 2000). Resistance of mastitis pathogens to antimicrobial agents is well documented in dairy cows (Umoh *et al*., 1990; Pitkala *et al*., 2004). The use of antimicrobial agents is associated with the risk of inducing resistance to antimicrobial agents among bacteria, reduction of cure rates after treatment of clinical mastitis and transmission of resistance bacteria to humans via food chain (WHO, 1997; Sol *et al*., 2000; Ungenmuch, 1999). Increased antimicrobial resistance, stems from a multitude of

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factors that include the wide spread and sometimes inappropriate use of antimicrobial agents, the extensive use of these agents as growth enhancers in animal feed, the increase in regional and international travel, and the relative ease with which antimicrobial- resistant bacteria cross geographic barriers (Lowry, 2003).

Another interesting case of *S. aureus* resistance is the penicillinase - resistant penicillins referred to as methicillin (oxacillin)- resistant *S. aureus* (MRSA). MRSA produces a specific penicillin binding protein PBP2′ that possesses reduced affinities for binding to β-lactam. The PBS2′ is encoded by the *mec*A gene carried by a large mobile genetic element, i.e Staphylococcal cassette chromosome mec (SSC*mec*) (Kwon *et al*., 2005). Such organisms are frequently resistant to most of the commonly used antimicrobial agents, including the aminoglycosides, macrolides, chloramphenicol, tetracycline, flouroquinolones, lincosamides, trimethoprim-sulfamethaxazole and sulfonamides (Mandell *et al*., 1995; Feng *et al*., 2008). Some strains of MRSA have been designated epidemic strains with a higher prevalence between countries (Lee, 2003). In order to control the spread of the infections, the sources of contamination and mechanism of transmission must be identified. Presently the transmission of MRSA is thought to occur primarily from colonized persons (Murder *et al*., 1991), the environment (Udo *et al*., 1996), food products and cows with mastitis (Dervrise and Homes, 1975). In Nigeria the rate of MRSA among healthy carriers working in a critical unit of a hospital is 52% (Fadeyi *et al*., 2010). Other studies in Nigeria indicated MRSA to be common in the environment (Kesah *et al*., 2003; Adesida *et al*., 2005; Taiwo *et al*., 2005; Azeez-Akande *et al*., 2008; Nwankwo *et al*., 2010).There is dearth of information on MRSA from food products including milk in Nigeria. The purpose of this study was to determine the prevalence of MRSA, susceptibility of oxacillin resistance *S. aureus* against 12 antibiotics commonly used in the study area, determine the minimum inhibitory concentration for the MRSA and to assay for the presence of gene encoding methicillin resistance.

MATERIALS AND METHOD

Sample collection and analysis

Milk samples were collected from dairy cows in six Local Government Areas (Jos South, Jos North, Jos East, Barkin Ladi, Riyom and Bassa) in the Northern Part of Plateau State. Prior to samples collection, the udder, teats and adjacent flank areas were thoroughly washed and dried with single- service sanitary paper towel and the teats were disinfected with 70% alcohol. Fifteen milliliters of milk from each quarter was collected. The milk samples were transported in an ice box to the laboratory within 3 hours. A total of 339 quarter milk samples were collected between 2008 and 2009. Individual quarter milk samples were subjected to California Mastitis Test (CMT) and cows whose California mastitis test was +1 and above were considered to be mastitic and selected for bacteriological investigation according to Roberson *et al*. (1992).

Antibiotic susceptibility screening

The isolates were checked for viability and purity by subculturing on Brain Heart Infusion (BHI) agar. Two or three colonies from the agar plates were inoculated in tryptose soy broth (laboratories Britannia) and incubated at 37^{o}C for 6 to 8h. The cultures were adjusted to 0.5 Mcfarland standards. Antimicrobial susceptibility test was conducted on oxacillin (1µg) resistance strains, against 12 antimicrobial agents following the Kirby - Bauer disk diffusion method (Quinn *et al*., 1999). The antimicrobial agents used were erythromycin (5ug), amoxicillin (10ug), methicillin (5ug), cloxacillin (5ug), penicillin G (10 iu), clindamycin (2ug), lincomycin (5ug), gentamicin (10ug), trimethoprim-sulfamethoxazole (1.25ug), chloramphenical (30ug), vancomycin (5ug) and tetracycline (30ug). Isolates were categorized as either susceptible or resistant based upon interpretive criteria developed by the Clinical and Laboratory Standards Institute (CLSI) to antimicrobials according to guide lines of CLSI (2004). *Staphylococcus aureus* ATCC 25923 was used as quality control standard strain.

Chromogenic screening media

The oxacillin resistance screening agar base (ORSAB CM 1008; Oxoid**)** with the selective supplement SR195 (Oxoid) were prepared according to the manufacturer's instructions. Isolates were plated on the medium and incubated at 30^{o}C for 24h.

MIC of Oxacillin Resistant Strains of *S. aureus*

Five concentrations of 1µg oxacillin antibiotic, 0.625ug/ml, 1.25ug/ml, 2.5ug/ml, 5.0ug/ml and 10ug/ml were prepared. And 1ml of each concentration was dispensed in 5 test tubes respectively. Fresh 2-3 colonies of *S. aureus* were cultured in Brain Heart Infusion broth to 0.5 Mcfarland standard, and 1ml of the culture was dispensed to each test tube containing the oxacillin antibiotic, and incubated at 37^{o}C for 24 hours. *Staphylococcus aureus* ATCC 25923 was ran in parallel with the *Staphylococcus aureus* test samples as the

Table 1 Oligonucleotide primmer sequences and PCR condition used in the present study

Oligonucleotide Primers	Sequence	Program	Size of PCR product (bp)	Reference
mec A 1	5^{1-}AAAATCGATGGTAAAGGTTGGC -3^{1}	2	533	(Lee, 2003)
mec A 2	5^{l-} AGTTCTGCAGTACCGGATTTGC-3^{1}			
bla Z 1	5^{1}- ACTTCAACACCTGCTGCTTTC-3^{1}			Martineau *et al.*,(2000)
bla Z 2	5^{l}- TGACCACTTTTATCAGCAACC-3^{1}	2	173	
23SrRNA 1	5^{1}ACGGAGTTACAAAAGGACGAG-3^{1}			Straub *et al.*, (1999)
23SrRNA 2	5^{1}ACGTCAGCCTTAACGAGTAC-3^{1}	1	1250	

*PCR Programme
2.1x(940c,240s), 40x(940c, 30s; 550c, 30s; 720c, 60s), and 1 x (720c, 300s)
1.1x(940c,240s), 37x(940c, 40s; 640c,60s;720c,75s), and 1x(720c,300s)

positive control while sterile BHI served as negative control (Umoh *et al.,* 1990).

Preparation of whole-cell DNA for PCR of the 26 ORSA

A previously described method by Shuiep *et al*. (2009) was used. Briefly 3 – 4 colonies of freshly cultured strains were suspended in 180ul TE buffer (10mMTris-Hcl/l, 1mM ethylenediaminetetracetic acid (EDTA)/l, pH8 and 8ul lysostaphin (1.8U/ul; Sigma, Steinheim, Germany) was added to the suspension and incubated for 2h at 56°C. The DNA was subsequently isolated with DNeasy Tissue Kit (Qiagen) according to the manufactures instructions. A molecular identification was performed by PCR amplification of species- specific parts of the gene encoding the 23SrRNA. The single PCR reaction mixture (30ul) contained 1.0ul of each primer(10pmol/ul), 0.8 ul dNTP (10mM; MBI Fermentas, St-Leon, Germany), 3.0ul of 10x thermophilic buffer (Promega, Mannheim, Germany) with a final concentration of 1.8ul $MgCl_2$ (Promega), 0.1ul Taq polymerase (5U/ul; Promega/Boeringer) and 20ul of H_2O. Finally, 2.5ul of DNA preparation was added to each reaction tube. The tubes were then subjected to thermal cycling (Gene Amp PCR System 2400, Perkin-Elmer, Rodgau Jugesheim, Germany). The presence of 1250bp PCR product was determined by electrophoresis of 10ul of reaction product in an 1.5% agarose gel (Gibco BRL, Karlsruhe, Germany) with Tris acetate electrophoresis buffer (TAE, 4.0mmol/l Tris 1 mmol/l EDTA, pH 8.0) and gene ruler DNA Ladder Mix (Fermentas) as molecular size marker and visualized under UV (Image Master VDS, Pharmacia Biotech, Freiburg, Germany).

PCR detection of *mecA* and *blaZ* resistance genes

Details of the primer sequences and thermal cycler PCR programmes are summarized in (Table 1). The PCR reaction mixture 20ul contained 0.7ul of each primer (10pmol/ul), 0.8ul dNTP(10mmol; Genecraft, Munster, Germany), 2ul of 10x biotherm buffer with a final concentration of 1.5mM $Mgcl_2$ (Genecraft), 0.3ul biotherm polymerase (Genecraft) and 8.1ul H_2O . Finally 5ul DNA preparation was added to the PCR reaction mixture. The reaction mixture was subjected to thermal cycling (Gene Amp PCR system 2400, Perkin Elmer, Rodgau Jugeisheim, Germany). The Presence of PCR products were determined by electrophoresis of 10ul of reaction product in a 1.5% agarose gel (Gibco BRL, Karlsruhe, Germany) with Tris –acetate electrophoresis buffer (TAE, 4.0 mmol/l Tris, 1mmol/EDTA, pH 8.0) and visualized under uv light (Image Master VDS, Pharmacia Biotech, Freiburg, Germany).

RESULTS

Isolation and characterization

One hundred and three 103(30.9%) *S. aureus* were isolated from the 105 mastitic quarters investigated. Seventy three out of the 103 *S. aureus* positive isolates screened for oxacillin resistance, 26(35.6%) were positive. Two isolates were positive on ORSAB medium. Two mecA gene were detected by PCR.

Antibiotic susceptibility

The *mec* A-positive MRSA strains (isolates 1* and 2*) isolated in this study were susceptible to vancomycin and resistance to 11 antibiotics (Table 2). All the non mecA-positive isolates were resistance to penicillin and susceptible to vancomycin, they all possessed the *blaz* gene. Among the non- *mec*A isolates, 24, 23, 20, 21, 15, 16, 24, 24, 18, and 18 were resistance to methicillin, amoxicillin, cloxacillin, amikacin, erythromycin,

Table 2. Antibiotic susceptibility profile determined by agar diffusion method for ORSA isolates

isolates	Pen	Met	Amox	Clox	Ak	Eryt	Gen	Clin	Chl	Sxt	Van	Ka
1*	R	R	R	R	R	R	R	R	R	R	S	R
2*	R	R	R	R	R	R	R	R	R	R	S	R
3	R	S	R	R	S	R	S	R	R	S	S	R
4	R	R	R	S	R	R	S	R	R	R	S	S
5	R	R	R	S	S	R	S	R	R	R	S	S
6	R	R	R	S	R	S	S	R	R	R	S	S
7	R	R	R	S	R	S	S	R	R	R	S	R
8	R	R	R	R	R	S	S	R	R	R	S	R
9	R	R	R	R	R	S	S	R	R	R	S	R
10	R	R	S	R	R	S	S	R	R	R	S	R
11	R	R	S	S	R	S	R	R	R	R	S	R
12	R	R	S	R	R	R	R	R	S	R	S	S
13	R	R	R	R	R	R	R	R	S	S	S	R
14	R	R	R	R	S	R	R	S	R	S	S	R
15	R	R	R	R	R	R	R	S	R	S	S	S
16	R	R	R	R	R	S	R	R	R	R	S	R
17	R	R	R	R	R	S	R	R	R	R	S	R
18	R	R	R	R	R	S	R	R	R	R	S	R
19	R	R	R	R	R	S	S	R	R	S	S	S
20	R	R	R	R	R	S	S	R	R	S	S	S
21	R	R	R	R	S	R	R	R	R	S	S	R
22	R	R	R	R	R	R	R	R	R	S	S	R
23	R	R	R	R	R	R	R	R	R	R	S	R
24	R	R	R	R	R	R	R	R	R	R	S	R
25	R	R	R	S	R	R	R	R	R	R	S	R
26	R	R	R	R	S	R	R	R	R	R	S	S

Note: 1* and 2* *mec*A positive *Staphylococcus aureus* isolates

(Pen=Penicillin, Met= Methicillin, Amox= Amoxacillin, Clox=Cloxacillin, Ak= Amikacin, Eryt=Erythromycin, Gen=Gentamycin, Clin=Clindamycin, Chl=Chloramphenicol, Sxt= trimethoprim-sulfamethaxazole, Van=Vancomycin, Ka=Kanamycin)

gentamicin, clidamycin, chloramphenicol, trimethoprim-sulfamethaxazole and kanamycin respectively. All the non- *mec*A positive strains showed a multidrug resistance phenotype. The range of the oxacillin MICs for the *mec*A positive MRSA was between 2.5µg/ml and greater than 10µg/ml. While the non- *mec*A isolates were between 1.25µg/ml and 2.5µg/ml (Table 3).

PCR for 23SrRNA, *mec*A and *bla*Z gene detection

The PCR was used as a gold standard for all isolates. All the expected amplicons were as summarised on (Table 1). Gels were stained with ethidium bromide and photographed under UV light as shown in Figures I, 2 and 3.

DISCUSSION

This study of the prevalence of MRSA from sub-clinical bovine mastitic milk and the antibiotic susceptibility of these isolates to commonly used antibiotics, was done to evaluate the potential impact of transmission of resistant bacteria to humans via subclinical mastitic milk since milk is often consumed raw without any treatment. In this study, the predominant isolation of *S. aureus* from quarter milk samples,is in agreement with Cabral *et al*., (2004). The 26 Oxacillin resistance *S. aureus* investigated for *mecA* gene by PCR and on ORSAB medium, indicated an excellent relationship between PCR and ORSAB medium. In a similar study kwon *et al*. (2006) has shown that only 0.18% samples of unprocessed cow milk examined in South Korea contained MRSA. In Italy, out of the 160 *S. aureus* isolates from milk and dairy products analyzed 6(3.75%) were *mec*A positive (Normanno*et al*., 2007). All the 26 tested isolates in this study were positive for *blaZ* gene using PCR and also resistant to penicillin by in-vitro disk diffusion test showing a good relationship between the two techniques which also

Table 3. Minimum Inhibition Concentration (MIC) of Oxacillin Resistant *S. aureus* Isolates obtained from different LGA of Plateau State

Location of isolate	No. of isolates Tested	No. resistant at each indicated MIC concentration (µg ml^{-1})				
		0.625	1.25	2.5	5.0	10.0
Jos east	5	5	4	3	0	0
Jos North	7	7	7	3	1	1
Riyom	7	7	7	5	1	1
Jos South	1	1	0	0	0	0
Bassa	3	3	2	2	0	0
Barkin Ladi	3	3	2	2	0	0
Total	26	26	22	15	2	2
Percentage			(84.6)	(5.7)	(7.6)	(7.6)

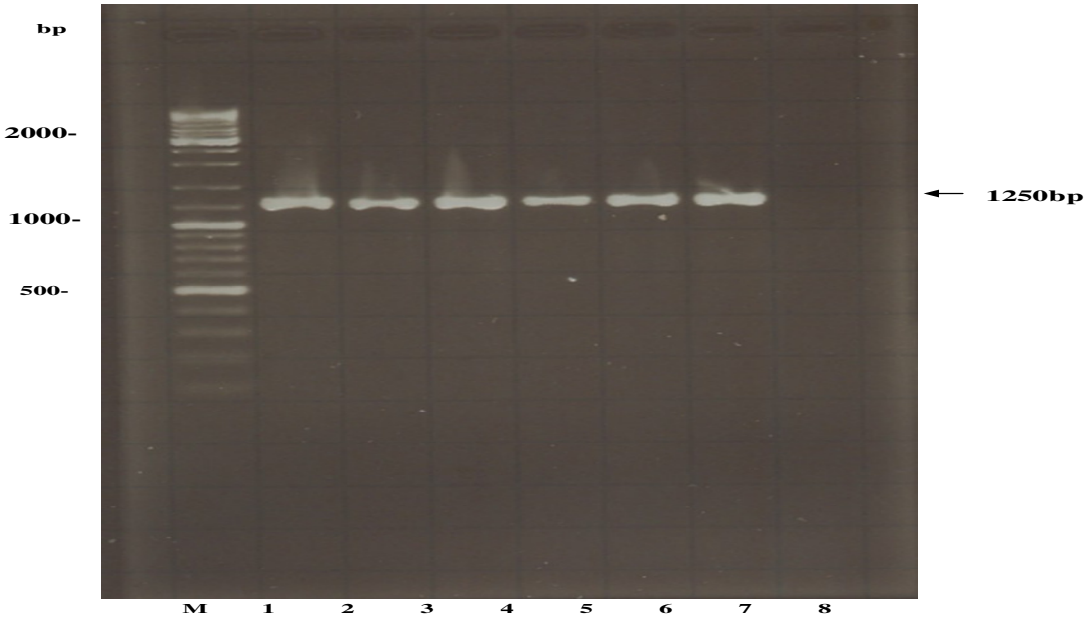

Figure 1. Amplicons of the *23Sr RNA* gene of S. *aureus* with a size of 1250bp. Lane 1 (FEF4), lane 2 (JES9A), lane 3 (BR24), lane 4 (RY4SM), lane 5 (JNT3A), lane 6 (BAKD17), lane 7 (JVM9), lane 8 (Water) control), M.= a 100bp ladder served as size marker

agrees with, a similar study (Lee, 2003) who reported a 96.1% resistance to penicillin. Antibiotics including oxacillin and methicillin were randomly used as a dry-cow treatment; this practice may contribute to the increasing incidence of MRSA strains in cows associated with mastitis, and also high resistance to these antibiotics due to selective pressure. The susceptibility of the entire *S. aureus* isolates to vancomycin could be due to the fact that the antibiotic was newly introduced to the area, while penicillin has been used overtime and is the antibiotic of choice for drying – off. This could be the reason for the 100% resistance by the *S. aureus* isolates against the antibiotic. The MIC result of this study showed a higher value for the *mec*A isolates as compared to non-*mec*A isolates. It can be speculated here, that the sub-optimal doses of antibiotic used against infection with MRSA could be the reason for that, because once discovered that the antibiotic treatment is not helpful the treatment is abandoned. Whereas in non-MRSA infection, treatment are always completed according to prescription.

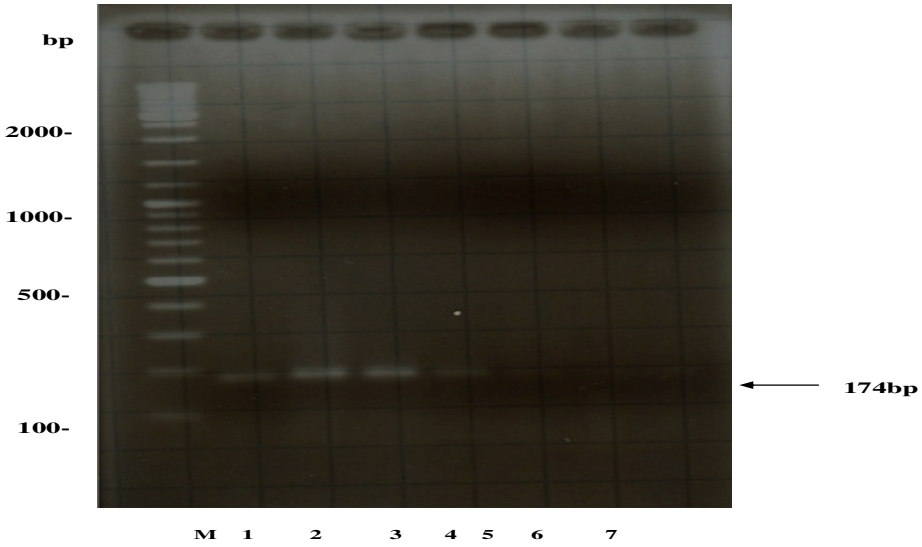

Figure 2. Amplicons of the *blaZ* gene of S. *aureus* with a size of 174bp Lane 1 (Positive control s. aureus strain), lane 2 (PSC10b), lane 3 (JES9A), lane 4 (BAKD17), lane 5 (BFR9A), lane 6 (JNT3A), lane 7 (RY4SM), M=100bp ladder served as a size marker.

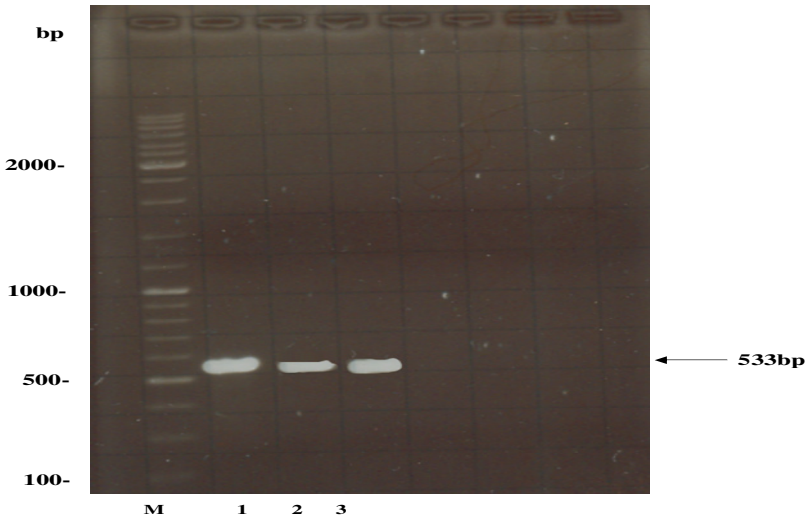

Figure 3. Amplicons of the mecA gene of S. aureus with a size of 533bp. Lane 1 (Positive control of s. aureus strain), lane 2 (RY4SM), lane 3 (BAKD17), lane 4-7 (Water Control), M.=100bp ladder as a size marker.

Generally, the MICs for MRSA in this study is lower than reported in a previous study (Lee, 2003).While the MICs result of non- *mec*A *S. aureus* in this study is higher than those of previous studies (de Oliviera *et al*., 2000; Gentilini *et al*., 2000; Yoshimura *et al*., 2002). The multi resistance, observed is necessary to initiate an active control measure(s) against antibiotic resistances in foods particularly milk and milk product, community and in nosocomial infections.

The rare isolation of MRSA from subclinical mastitic milk, at this stage seems to be of minor importance as a source of MRSA, but is plausible to say humans can be infected by animal MRSA through subclinical mastitic milk. The animal MRSA may have originally come from

humans, considering the prevalence of 52% (Fadeyi *et al*., 2010), when compared to the incidence of MRSA in bovine mastitis (7.6% in this study). Methicillin resistant *Staphylococcus aureus* (MRSA) in Nigeria has emerged and could threaten the successful treatment of staphylococcal diseases. Therefore, there is the need for continuous monitoring and surveillance of antibiotic resistance.

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