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Research Article

Molecular Identification of Penicillin Resistant *Staphylococcus aureus* Isolated from Bovine Mastitic Udder in Sokoto Metropolis

[1]Kasarawa A. B. [*2]Salau I. A. and [3]Lawal S. M.

[1]Department of Science Laboratory Technology, Umaru Ali Shinkafi Polytechnic, Sokoto
[*2]Department of Biological Sciences, Federal University Gusau, Zamfara, Nigeria
[3]Department of Veterinary Medicine, Usmanu Danfodiyo University, Sokoto, Nigeria
*Corresponding address: Department of Biological Sciences, Federal University Gusau, Zamfara, Nigeria
Tel: +234(0) 8053569551 Email: ibrahimasalau@gmail.com

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This study was carried out to identify Penicillin Resistance *Staphylococcus aureus* isolates isolated from milk of mastitis cattle in Sokoto metropolis using Antibiogram and PCR assay. Twenty (20) milk samples of mastitis cattle were collected from cattle herds in Sokoto metropolis and were analyzed using Biochemical tests, antibiogram and PCR assay. Samples were inoculated using streaking methods and cultures were incubated at 37^{o}C for 24 hours. The results of the study revealed eighteen (90%) were positive for bacterial growth and there were no growth from two (10%) samples. The bacterial pathogens recovered from mastitis milk samples were *Staphylococcus aureus* 15 (75%) and 3(15%) were isolates of *Streptococcus agalactiae*. Antibiogram of the most prevalent Staphylococcus aureus showed that the isolates were sensitive to Gentamycin (GEN), Sorofloxacin (SP), Ciprofloxacin (CPX) and Pefloxacin (PEP)but resistant to Cefradine (CH).The PCR assay confirmed the isolates to be Penicillin resistant *Staphylococcus aureus* and the genomic DNA was confirmed to harbor the SPA genes. It is recommended that a control strategy should be developed to prevent and control the deleterious consequences of the pathogen on human health since the milk is consumed by the general populace in the Study area.

Key words: - Antibiogram, Staphylococcus, Molecular, Bovine and Mastitic Udder

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INTRODUCTION

Milk is a very nutritional food that is rich in carbohydrates, proteins, fats, vitamins and mineral salt. However, health risk to consumers can be associated with milk, due to the presence of zoonotic pathogens and antimicrobial drug residues (Bradely, 2012). The quality of milk may be lowered by a numbers of factors such as adulteration, contamination during and after milking and the presence of udder infections (Esron *et al.,* 2015). Pathogenic organisms in milk can be derived from the cow itself, the human hand or the environment (Bradely, 2012). Mastitis, inflammation of the mammary gland, is a highly prevalent problem in dairy cattle and is one of the most important threats affecting the world's dairy industry (Wallenberg *et al.,* 2010). Staphylococcal mastitis is the commonest and economically the greatest concern wherever dairy farming is practiced. The chief reservoir of this bacterium is an infected udder. The organism is well adapted to survive in the udder and usually establishes mild sub clinical infection of long duration. Bacteria are shed into milk from infected quarters (Tsegaye, 2010).

The genus Staphylococci were first described by the Scottish surgeon, Sir Alexander Ogston as the cause of a

number of pyogenic (pus forming) infections in humans. In 1882, he gave them the name Staphylococcus (Greek: staphyle, bunch of grapes; coccus, a grain or berry), after their appearance under the microscope (Cheng *et al*., 2010). The pathogens are Gram-positive spherical bacteria that occurred in clusters resembling grapes. The configurations of the cocci help to distinguish Staphylococci from Streptococci, which are slightly oblong cells that usually grow in chains (Afroz, 2008). They are non spore-forming and non-motile organisms which grow readily on most bacteriologic media under aerobic or micro aerobic conditions with their optimum growth temperature being 37^{o}C. The young culture of *Staphylococcus spp.* stain strong Gram-positive but on aging many cells become Gram-negative. This is considered as „Gram variation" (Cheng*et al*., 2010). Bacteriological culture of the nose and skin of normal humans invariably yields Staphylococci. In 1884, Rosenbach described the two pigmented colony types of *Staphylococcus* and proposed the appropriate nomenclature: *Staphylococcus aureus* (Yellow) and *Staphylococcusepidermidis* (White) (Afroz, 2008). The *S. aureus* and *S. epidermidis* are significant in their interactions with humans, *S. aureus* colonizes mainly the nasal passages but it may be found regularly in most other anatomical locales. *S epidermidis* is an inhabitant of the skin. It lives completely harmless on the skin and in the nose of about one third of the normal healthy people. This is referred to as colonization or carriage. *S. aureus* can cause actual infection and disease, particularly if there is an opportunity for the bacteria to enter the body e.g. via a cut or an abrasion (Afroz, 2008).

Penicillin Resistant *Staphylococcus aureus* (PRSA) refers to a group of Gram-positive bacteria that are genetically distinct from other strains of *Staphylococcus aureus*. PRSA is responsible for several difficult-to-treat infections in humans. PRSA is any strain of *S. aureus* that has developed, through horizontal gene transfer and natural selection, multiple drug resistance to beta-lactam antibiotics. β-lactam antibiotics are a broad-spectrum group that include some penams (penicillin derivatives such as methicillin and oxacillin) and cephems such as the cephalosporins. Strains unable to resist these antibiotics are classified as penicillin-susceptible *S. aureus*, or PSSA (Cheng *et al*., 2010).

In present day of Nigeria, there is a national drive to alleviate the existing food deficit by devising different agricultural strategies including improvements of the productivity of livestock sector by controlling some of the major infectious disease through regular monitoring. Mastitis, as a disease, has received little attention in Ethiopia, especially the sub clinical form which is mainly caused by *S. aureus* (Mekonnen *et al.,* 2005).

However, the information generated from this study will provide a baseline data for pastoral, herders and milk production industries on the importance of hygienic practice in camel milk processing and pasteurization. The results obtained from this study will help the Agricultural Development Project to formulate guidelines for proper hygienic management in processing cattle milk and also provide opportunity for the chain actors and other stakeholders to determine appropriate mitigation measures at the various points.

The study is aimed at isolating and identifying Penicillin Resistance *Staphylococcus aureus* phenotypically and confirmed the isolates recovered from milk of mastitis cattle using Polymerase Chain Reaction (PCR) assay

Materials and Methods

Study area

The study was conducted in Sokoto the capital city of Sokoto State, Sokoto metropolis comprises of Sokoto North, Sokoto South, part of Wamakko, Dange Shuni and Kware Local Government Area. Sokoto is located at latitude 13.05N 05 15E and longitude 13,083N 5.250E of the equator. Sokoto is located in the extreme North west of Nigeria, near the confluence of the Sokoto River and Rima River, and falls within the Sudan Savannah ecological zone. It is populated with various categories of people some of which are peasant farmers, traders and artisans. The dominant ethnic groups are Hausa/Fulani. As of 2006 National census, it has a population of 4,277,60 people. The central parts are occupied mostly by the middle class whose Hausas have water closets with soak away pits. The drainage systems consist of open shallow gutters often littered with refuse and eroded soil.

Microbiological Analysis

Sample Analysis

The pure isolate from the culture was inoculated into the sterile selective medium of Manitol Salt Agar (MSA) and were incubated aerobically at 37^{o}C for between 18-24hours.

Identification of Bacteria

Biochemical Tests

Biochemical test refers to the chemical identification of unknown substances within a living thing. The test quantitatively and qualitatively determines a particular substance lie bacteria, fungi and enzyme with the blood. A biochemical test can be used to diagnose a given disease. It can also be used to find the metabolic properties of bacteria. The biochemical tests carried out for identification of the bacterial isolates were as

Catalase Test

Three (3) millitres of hydrogen peroxide (3%v /v) solution was disposed into a series of test tubes labeled with the isolate numbers. Using sterile wooden spatula, good growths of the test organisms was removed from the plates and immersed into the respective test tubes appropriately. Budding was observed in organisms that catalase was produced. The absence of bubbles showed catalase negative result (Abraham 2010).

Coagulase Test

This test was used to distinguish *Staphylococcus aureus* (coagulase positive) from *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* (coagulase negative). The test was carried out using the tube coagulase technique (DIFCO) capable of demonstrating both free and bound coagulase. Tube coagulase: In the tube coagulate method, human plasma was diluted 1 in 10 using normal saline as diluent. One milliliter of the diluted plasma was added to 0.1ml of a 24hour nutrient broth culture of the organism. The mixture was indicated by the formation of a solid clott within the tube while there was no clot formation in the coagulase negative isolates.

Triple Sugar iron Agar

The test organism was picked with inoculating needle, the media was firstly spread with the isolated organism and stabbed with the inoculating needle and incubated for 24hours at 37^{o}C in incubator (Birru*,* 1989).

Haemolysis Tests

Blood agar was prepared by adding blood into the nutrient agar. Then media was transferred into a Petri dish and allow to gel for some minutes. The inoculums was inoculated into the culture media and incubated for 18-24 hours. The culture media was observed for beta haemolysis

Sensitivity Testing

Drug susceptibility

All isolates were tested for susceptibility to the following antibiotic Streptomycin (STR), Gentamycin (GEN), Ofloxacine (OFX), Augmentin (AU), Trimothoprim sulfamethoxazole (TSX), Cefradine (CH), Sorofloxacin (SP), Amoxicillin (AM), Ciprofloxacin (CPX) and Pefloxacin (PEP)

. ***In vitro*** **sensitivity tests**

All isolates were tested using sensitivity disc impregnated with different antibiotics was tested against the tests organisms to determine their susceptibility.

Molecular Identification of *Staphylococcus aureus*

DNA Extraction

DNA extraction was done using the Qiagen® protocol described in DNeasy Blood and Tissue Handbook (2006). However, the suspension was centrifuged at 20,000 x g for 2 min instead of 300 x g for 5 min as recommended. A total of 3 isolates were processed.

DNA Principle

The isolation of DNA usually begins with the lysis of cells or tissues inorder to destroy the protein structures and allows the release of nucleic acids from the nucleus. Lysis is carried out in a lysis solution containing important ingredients: sodium chloride which provides an osmotic shock to the cells; Tris HCl, which is a buffer to retain constant pH; EDTA, which sequesters the divalent metal ions that is required for nuclease activity and thereby inhibiting its action; a detergent, usually SDS, which disrupts the cell membrane and nuclear envelope, causing the cells to burst open and release their DNA. The DNA is still rapped very tightly around histone proteins. Proteinase K (a serine protease) is the common enzyme used in DNA extraction that cuts apart the histones to free the DNA and finally results in the breakdown of cells and dissolving of membranes (Chambers, 1997).

The nucleic acids are then purified from the protein-nucleic acid complex by phase extraction with a mixture of organic solvents namely Phenol, Chloroform and Isoamyl alcohol in a ratio of 25:24:1. Phenol dissociates proteins from DNA. Chloroform denatures the proteins and lipids and helps to maintain the separation of the organic and aqueous phase. It also makes the DNA less soluble in the phenol, thus reducing losses to the organic phase. Isoamyl alcohol is often added to prevent foaming. At pH 7-8, the DNA partitions to the aqueous phase while the protein is denatured and extracted into water- immiscible organic phase, which is separated from the nucleic acid containing aqueous phase by centrifugation. When large amount of protein is present, it forms a white precipitate between the organic and aqueous phase. The DNA is then precipitated with cold ethanol or isopropanol after adjustment with 3M sodium acetate and then centrifuging. The DNA is insoluble in the alcohol and will come out of solution, and the alcohol serves as a wash to remove the residual salts. The alcohol is then removed, and DNA is stored in a biological buffer, like TE (Tris-EDTA) buffer. Contaminating RNA in the DNA sample can eliminated by digestion with an RNase (Chambers, 1997).

Polymerase Chain Reaction Methodology

PCR reaction mix of *mec*A gene from the extracted DNA

Analyzer 25 µl reaction mix containing, 12.5 µl of qiagen Toptaq PCR premix, 0.5 µL of each primer and 2.5 µL of coral load . The remaining volume was completed with nuclease free water to make 17 µL, and then vortexed for 15 seconds and finally 8 µL of template DNA added to make final 25 µL. *mecA* gene was amplified with the following primers: *mecA*-f: (5'-AAAATCGATGGTAAAGGTTGGC-3'); *mecA*-r: (5'-AGTTCTGCAGTACCGGATTTGC-3') with 533bp. Negative control contains all material except template DNA, so instead of that distilled water was added. DNA amplification was carried out for 35 cycles according to the following protocol: denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min with a final extension at 72 °C for 5 min. The PCR products were then analyzed in 2% (w/v) agarose gels stained with ethidium bromide and visualized under UV light.

PCR reaction mix for SPA gene from the extracted DNA

The primers SPA-Forward 5' TCAACAAGAACAACAAAATGG 3' and SPA- Reverse 5' GCTTTGGTGCTTGAGATTC 3' with 180-bp (Chambers, 1997) were selected for this study. PCR mixture was set up in a total volume of 25 µL including 12.5 µl of qiagen Toptaq PCR premix, 0.5 µL of each primer and 2.5 µL of coral load . The remaining volume was completed with nuclease free water to make 17 µL, and then vortexed for 15 seconds and finally 8 µL of template DNA added to make final 25 µL. Negative control contains all material except template DNA, so instead of that distilled water was added. PCR reaction tubes were centrifuged briefly to mix and bring the contents to the bottom of the tubes, and placed into thermocycler PCR instrument where DNA was amplified. DNA amplification was carried out for 35 cycles according to the following protocol: initial denaturation at 95 °C for 3 minutes denaturation at 94 °C for 30 Seconds, annealing at 56 °C for 30 seconds, and extension at 72 °C for 1 min with a final extension at 72 °C for 10 min. The PCR products were then analyzed in 1.5 % (w/v) agarose gels stained with ethidium bromide and visualized under UV light (Chambers, 1997).

Agarose Gel Electrophoresis

Gel electrophoresis is used to separate DNA on the basis of their sizes by applying an electric field to move the DNA through an agarose matrix.

Agarose Gel Preparation

Agarose powder (Amresco lifescience, Solon) was used in preparing the 1.5% agarose gels used in this study. The agarose was dissolved in 1x TAE: Tris-Acetate-EDTA and microwaved to dissolve the agarose. The molten agarose was then placed on a stirrer and allowed to cool down and 7 µL of ethidium bromide added into the gel to allow visualization of the fragments during imaging. Casting of the gel was done by placing a comb into the cast and then pouring the agarose gently into the cast. The gel was then allowed to solidify after which the comb was removed.

Gel Electrophoresis

The electrophoresis chamber was filled with the running buffer (1x TBE) and casting tray was then placed in the chamber. The amplicon (10µL) was loaded into each well. A 100-bp plus molecular weight marker was then loaded into the first and the last well as a standard for estimating the size of the resulting DNA fragment. The electrophoresis chamber was then connected to the power source and the DNA run at 70V for 1 hour (Chambers, 1997). The DNA bands (180-bp for SPA, 533-bp for *mec*A) were then viewed by illumination with UV light and images recorded by photography (Biorad imager).

RESULTS

Of the twenty (20) mastitis milk samples collected and analyzed in this study, there were growth in eighteen 18 (90%) samples while two 2 (10%) samples revealed no growth. The bacterial pathogens isolated from the mastitis milk samples were *Staphylococcus aureus* 15 (75%) and 3 (15%) were isolates of *Streptococcus agalactiae.* The Frequency of occurrence of bacteria isolated from the mastitis milk samples is presented in Table 1.

Table 1: Frequency of occurrence of bacteria isolated from the mastitis milk samples

Organisms isolated	Mastitis milk samples (%)
Staphylococcus aureus	15 (75%)
Streptococcus agalactiae	3 (15%)
Negative sample	2 (10%)
Total	**20(100%)**

The result obtained from this study revealed that species of *Staphylococcus aureus occurred most and were* highly prevalent.

Antibiotic Sensitivity Pattern of *Staphylococcus aureus* isolates

The antibiotic sensitivity pattern of exhibited by *Staphylococcus aureus* isolates on antibiogram showed that all the isolates were sensitive to Gentamycin (GEN), Sorofloxacin (SP), Ciprofloxacin (CPX) and Pefloxacin (PEP) but resisted the effect of Cefradine (CH). This pattern exhibited by the isolates presumptively signifies penicillin resistant *Staphylococcus aureus* strains. Detailed of the sensitivity pattern is demonstrated in table 2 below

Table 2: Antibiotic sensitivity pattern of *Staphylococcus aureus isolates*

Antibacterial Agent	No of isolate		Percentage (%)	
	Sensitivity	Resistivity	Sensitivity	Resistant
Streptomycin (STR)	13	5	72%	28%
Gentamycin (GEN)	15	3	83%	17%
Ofloxacine (OFX)	14	4	78%	22%
Augmentin (AU)	8	10	44%	56%
Trimothoprim sulfamethoxazole (TSX)	9	9	50%	50%
Cefradine (CH)	7	11	39%	61%
Sorofloxacin (SP)	15	3	83%	17%
Amoxicillin (AM)	14	4	78%	22%
Ciprofloxacin (CPX)	15	3	83%	17%
Pefloxacin (PEP)	15	3	83%	17%

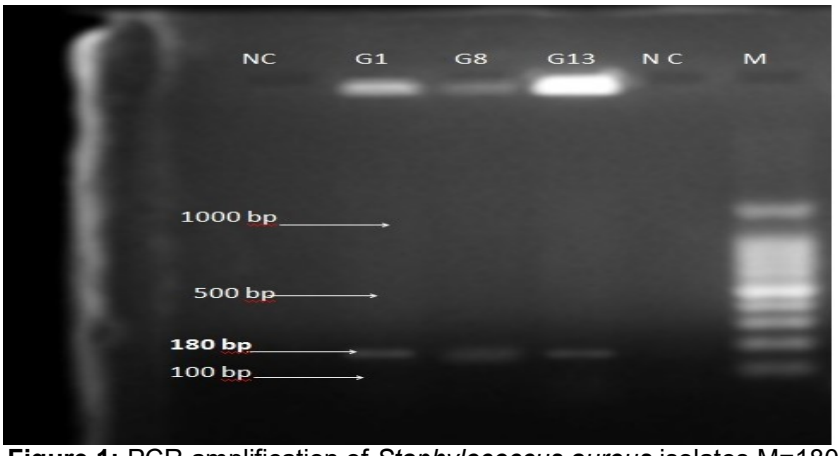

Figure 1: PCR amplification of *Staphylococcus aureus* isolates M=180 bp size.

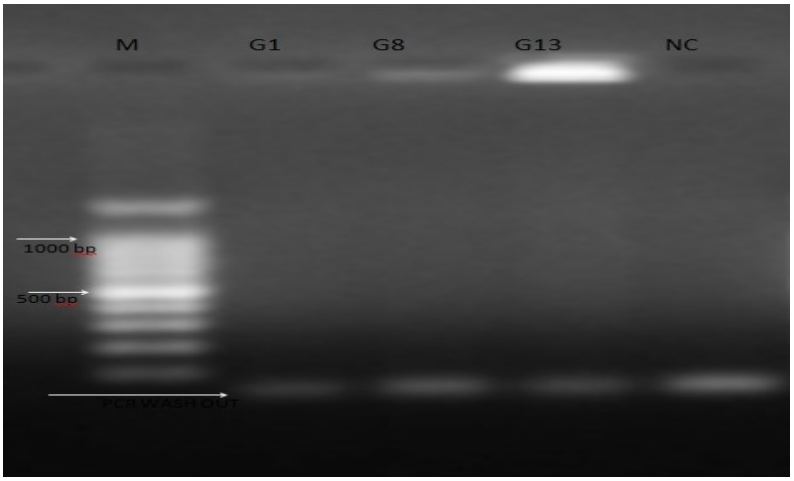

Figure 2: PCR amplification of SPA gene of *Staphylococcus aureus* M=533 bp size.

The results of the molecular identification by PCR assay among the 3 suspected isolates of penicillin resistant *Staphylococcus aureus* confirmed the presence of the strains (Penicillin resistant *Staphylococcus aureus* strains) and were all positive for SPA gene (Figure 2)

DISCUSSION

The results of this study showed that of the twenty (20) mastitis milk samples collected and analyzed in this study, there were growth in eighteen 18 (90%) samples while two 2 (10%) samples revealed no growth. The bacterial pathogens isolated from the mastitis milk samples were *Staphylococcus* aureus 15 (75%) and *Streptococcus agalactiae* were 3(15%). However, the overall prevalence of mastitis observed in the present study 18 (90%) was caused by *Staphylococcus aureus.* This was in contrast to result of findings reported by Abdulqader *et al.* (1990), who found a prevalence of 45.8% in Sudan, Talossa (1987) in Debere, Tsegaye (2010) in Holeta, Tolossa (1987) in Kallu province and Esron (2005) in Morgora region reported 52.8%, 53.35%, 53.5%, 61.11%, 63%, and 68.1% respectively. However, the report of this study is in agreement with the report recorded by Aiken *et al.* (2014) reported 71.1% prevalence of bovine mastitis from Holeta.

In this study, the dominant bacterial pathogens isolated from milk samples were S*taphylococcus aureus* 15 (75%) however; this was lower than that of the 42.1% reported by Cefai *et al.* (2004). Similarly, *S. aureus* was isolated as main etiological agent of mastitis in cattle in many African and Asian countries. *S. aureus* is considered as typical contagious pathogen causing bovine mastitis. Accordingly, the wide spread *S. aureus* mastitis might be cows positive in herd which act as primary reservoir and infected others especially during milking. EFSA. (2007) asserted that *S. aureus* is well adapted to survive in the udder and usually establishes a mild sub clinical infection of long duration from which it shed in milk facilitating transmission to healthy animals mainly during milking. Generally, *S. aureus* has been designated as a causative agent of both clinical and subclinical mastitis.

Bovine mastitis remains a serious problem to the dairy producers in the study area which costs the farmers from losses associated with reduced production, increased replacement cows, drug costs, veterinary fees and labour costs. It might be due to lack of coordinated actions on prevention and control of bovine mastitis. The numbers of dairy farms has increased in the study areas as compared to previous years. However, most of these farms have poor housing facilities and this might contribute to the contamination and exposure of teats to environmental pathogens and could be reason for increased prevalence of bovine mastitis.

The antibiogram in this study revealed that all the isolates were sensitive to antibiotics tested. However, Gentamycin (CN), Sorofloxacin (SP), Ciprofloxacin (CPX) and Pefloxacin (PEP) has the highest sensitivity with of 15(83%) while Cefradine (CH) showed the least sensitivity of 7(39%). while Augmentin (AU) showed the least sensitivity of 2(10%). Similarly, the resistant pattern of the isolate showed that Cefradine (CH) has the highest resistivity of 11(61%) while Gentamycin (CN), Sorofloxacin (SP), Ciprofloxacin (CPX) and Pefloxacin (PEP) showed the least resistivity of 3(17%). The result of this study is comparable with the report of high resistance of 75 to ampicillin, cefradine and streptomycin by Mekonnen *et al*. (2005). The resistance of *S. aureus* to the aforementioned antibiotics may be attributed to the production of betalactamase, an enzyme that inactivates penicillin and closely related antibiotics. It is believed that around 50% of mastitis causing *S. aureus* strains produce betalactamase (Bradely, 2012).

The results of the molecular characterization by PCR among the 3 isolates of Penicillin resistant *Staphylococcus aureus* were all positive for SPA gene (Figure 2). However, this was in consonance with the

report by Donnio *et al.* (2004).

Similar findings were previously illustrated by Donnio *et al.* (2004) who identified enterotoxigenic *Staph*. *aureus* with the percentages of 40% (2 out of 5) in raw milk using SPA gene, however, (Broens *et al.* 2011) were determined the enterotoxigenic *Staph. aureus* from milk with the percentages of (7.1 (1out of 48), 21.37 (28 out of 131) and 11.8 (2 out of 17), respectively. In the current study, 20% from apparently healthy human stool samples were found to be contaminated with the target sea gene. Nearly similar finding was previously reported by Broens *et al.* (2011) who detected enterotoxigenic pathogens.

CONCLUSION

Evidently, it is concluded that bovine mastitis is a major challenge to the dairy producers in and around Sokoto metropolis. This study showed an overall prevalence of mastitis to be 18 (90%). However, the bacterial pathogens isolated in the mastitis milk samples were *Staphylococcus aureus* 15 (75%) and *Streptococcus agalactiae* was 3(15%). By the antibiogram test, it may be concluded Gentamycin (GEN), Sorofloxacin (SP), Ciprofloxacin (CPX) and Pefloxacin (PEP) were more sensitive drug for *Staphylococcus aureus* and Cefradine (CH) was highly resistant to *Staphylococcus aureus.* Out of the randomly selected three *Staph aureus* culture positive, all isolates were identified as *Staph aureus* using SPA gene. However, all the recovered *Staph aureus* isolates were free from mecA gene. This finding therefore revealed that the milk and dairy product sold in Sokoto metropolis are contaminated with *S. aureus* which are very alarming for both human and animal health

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