# METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* AMONG POULTRY IN UMUAHIA, NIGERIA

ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) among poultry was investigated.Methicillin-resistant *S. aureus* (MRSA) poses serious public health issues worldwide and infections resulting from these organisms present difficult therapeutic challenges. A total of 250 swab samples were collected from skin, cloaca, and egg shells of chickens as well as from feeds, droppings and environment of poultry farms located in rural communities of Umuahia, Nigeria.The respective specimens were inoculated onto mannitol salt agar using the streak plate technique. Inoculated plates were incubated at 35–37 °C for 18 to 24 h aerobically. Bacterial colonies showing typical characteristics of *S. aureus* (i.e., golden yellow pigmentation on mannitol salt agar resulting from fermentation of mannitol) were subcultured onto freshly prepared nutrient agar plates to obtain pure colonies. The pure isolates were characterized by Gram staining, biochemical testing and molecular identification. Antimicrobial assays of the isolates were performed using various antibiotics. Out of 250 samples, 66 isolates of *S. aureus* were obtained. The antibiotic resistant pattern of *S. aureus*varied. The isolates were highly resistant to tetracycline, ampicillin, erythromycin and cefuroxime. The multiple antibiotic resistant (MAR) profile of *S. aureus* showed that 20(30.3%) exhibited MARto nine antibiotics while 19(28.8%) isolates exhibited MAR to ten antibiotics. Out of the 66 isolates, 24(36.3%) isolates were Methicillin-Resistant *S. aureus* (MRSA). The highest prevalence of MRSA was obtained from skin and cloaca samples. This study has confirmed the existence of MRSA in poultry farm within the study area. The presence of *S. aureus* and MRSA in poultry poses a great challenge to food security, because most of the poultry where samples were collected in this study are smallholder farms that supply chickens to the public for human consumption as source of protein.

**Keyword**: *Staphylococcus aureus*, Poultry, Antibiotic susceptibility, Methicillin Resistant *S. aureus(Use different keywords and arrange them alphabetically)*

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**Introduction**

*Staphylococcus aureus* (*S. aureus*) is a Gram-positive coccus bacterium causing a wide variety of infections in both humans and animals.*Staphylococcus aureus* is present in the microbiota of both humans and some animal species, being recognized as one of the most important opportunistic human pathogens. It may reside asymptomatically on the skin and nose of animals (Basset *et. al*., 2011). However, under certain conditions, *S. aureus* causes skin infection, osteomyelitis and even death (Cheung *et. al*., 2021).*S. aureus* is recognized as the most important worldwide cause of foodborne poisoning (Fitzgerald, 2012).*S. aureus* is a potential pathogen that has been implicated as one of the major causes of foodborne diseases globally.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is recognized as one of the most common multi-drugresistant bacteria found in both human and veterinary medicine. MRSA pose a risk of zoonotic infections in humans, most often in people with direct contact with contaminated livestock and amongst poultry farmers.*Staphylococcus aureus* is an important pathogen of public health concern because of the emergence of methicillin resistant and multidrug resistant strains that can colonize and cause infections in humans and animals (Van Boeckel *et al.,* 2015).Therefore, identification of MRSA strains is important for both clinical and epidemiological implications.

Methicillin-resistant *S. aureus* (MRSA) is particularly important, as it is becoming increasingly prevalent in the population. Previously, *S. aureus* including MRSA was known to be a major cause of nosocomial infections. It is now prevalent in the community, and has recently emerged among livestock, pets and wildlife (Cuny*et al.,* 2015). These strains can be transmitted to people in contact with these animals. Notably, livestock- associated MRSA has been isolated from people without contact with animals, demonstrating the ability of these organisms to thrive in humans. This cross-transmission between animals and humans poses a considerable zoonotic threat and complicates treatment protocols.

The presence of *S. aureus* in poultry poses a great challenge to food security, because most of the farms where samples were collected in this study are smallholder farms that supply food of animal origin to the underprivileged rural communities.

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Poultry farming is common in rural communities, where they are practiced at subsistence level in addition to commercial poultry farms. It is possible that these animals carry methicillin-resistant *S. aureus* as reported by Olayinka *et al*. (2010). Persons that work in these poultry farms may acquire and transmit them across community settings where they may cause human staphylococcal infection.

The emergence of MRSA are thought to have arisen from increasing use of antibiotics in animal feeds and this gives good reason for this study since subsistence and commercial poultry farming is a common practice in several localities in Nigeria. This study will provide information on the prevalence of MRSA in poultry in Abia state.

The general aim of this study is to determine the prevalence of Methicillin Resistant *S. aureus* in poultry farms located in Umuahia

Specific Objectives are:

1. To isolate and identify *Staphylococcus aureus* fromdifferent samples of poultry.
2. To determine the drug susceptibility profile of the isolates
3. To determine the percentage occurrence of the MRSA.
4. Molecular characteristic of isolates

**Materials and methods**

Sample collection, inoculation and identification isolates

From June 2023 to December 2023, a total of 250 samples from skin, feed, cloaca, environment, droppings, and eggshells were collected hygienically from six (6) different poultry farms in Umuahia, Abia State. All samples were collected using sterile swab stick soaked in sterile saline, properly labeled, and then transferred to Microbiology Laboratories for analysis.

The different nutrient media (Nutrient agar, Mannitol Salt agar, Mueller Hinton agar and MacConkey agar) used for this study were prepared following the directives stipulated by the manufacturers of the media. They were then sterilized by autoclaving at 121$^{o}$C for 15minutes. In order to isolate *Staphylococcus*, the respective specimens were inoculated onto mannitol salt agar using the streak plate method of inoculation. Inoculated plates were incubated at 35–37 °C for 18 to 24 h aerobically. Bacterial colonies showing typical characteristics of *S. aureus* (i.e., golden yellow pigmentation on mannitol salt agar) resulting from fermentation of mannitol were sub

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cultured onto freshly prepared nutrient agar plates. The resulting pure colonies were stored in agar slants for characterization and antimicrobial assays.

The Gram status of each of the isolates were determined by Gram staining technique. Biochemical tests such as catalase and coagulase for each isolate were performed.

Using a small sterile applicator stick, 3-4 colonies of the test organism was immersed in 2ml of freshly prepared 3% $H_2O_2$ solution in a test tube. Immediate bubble production indicated a positive test and no bubbling indicated a negative test.

**Antimicrobial Susceptibility Testing**

The bacterial isolates were tested for susceptibility to 10 different antimicrobial agents by the disc diffusion method on Mueller Hinton agar (Bauer *et al.,* 1996). The antimicrobial agents tested were: Augmentin, 30μg (AUG), ampicillin, 10μg (AMP), erythromycin, 5μg (ERY), tetracycline, 30μg (TET), Meropenem, 10μg (MEM), gentamicin, 10μg (GEN), co-trimoxazole, 25μg (COT), Cefuroxime, 10μg (CRX), Ceftazidime, 10μg (CPZ), Cephalexin, 15μg (CP), vancomycin, 30μg (VAN) and Ciprofloxacin 5μg (CPR).~~A sterile cotton swab was dipped into the bacterial suspension and the swab pressed on the side of the tube to drain excess fluid. The entire surface of the agar plate was then inoculated with the same swab of inoculum, rotating the plate to ensure confluent growth of the bacteria. The antibiotics discs were placed on the agar plates already seeded with the isolates. The plates were incubated at 35°C for 24 hours and observed for zones of inhibition, measured using a ruler and recorded.~~The zones of inhibition produced by the antibiotics against the isolate was used to categorize them as either susceptible or resistant status after comparing the zone of inhibition produced by the antibiotics against the isolate with that of a reference guide provided by CLSI (2017). Multiple antibiotic resistance was defined as resistance to three or more classes of antimicrobial agents.No need to write whole procedure

**Detection of MRSA**

To determine the methicillin resistant status of the *S. aureus* isolates, the Kirby-Bauer disk diffusion test was employed. A suspension of each isolate was prepared from the colonies from an overnight growth on nutrient agar plate. A suspension of the overnight growth was prepared. A sterile swab was dipped into bacterial suspension and then inoculated on Mueller Hinton agar

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plate to create a lawn of the organisms. Cefoxitin disks, which are used for methicillin testing, were placed on the plates. The plates were incubated for 24hours at 37°C. The diameter of the zone around the disc was measured and the results were interpreted according to the CLSI guidelines. The isolates with a zone of inhibition less than 22mm were reported as MRSA strains. *Staphylococcus aureus* ATCC 25923 was used as the control strain.

**Molecular Detection of Methicillin-resistant *S. aureus* (MRSA)**

DNA extraction: Deoxyribonucleic Acid (DNA) was extracted from the samples (Six of them) using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo-Research Laboratory, California, USA) by following the manufacturer's protocol.

Agarose gel electrophoresis genomic DNA: The DNA samples were separated on 1% agarose at 100V for 1hr.The samples were loaded as follows: 10 µlof each sample was mixed with 2µl of DNA loading dye. The mixture was vortexed, while 10 µl of the mixture was loaded in the gel with micropipette. After separation, the gel was examined with a UV Trans-illuminator (TVD-1000R/FB) to view the DNA bands.

Polymerse Chain Reaction (PCR)(gene name should be italic)

*16S rRNA* Amplification and *MecA*gene screenning

Attempts were made to amplify the 16S rRNA and MecA genes in the six samples using 16S rRNA gene universal primers (27F/1492R and ATCC12600 F/ATCC12600R) and MecA gene primers respectively**.** A total of four primer pairs were used.However, samples 190, 191, 196, and 203 did not amplify with any of the primers, while samples 205 and 206 produced amplification with the **ATCC12600 primer pairs.** The primers and their sequences are shown in the chart below.

Chart 1: The primers and their sequences

| PRIMER | SEQUENCE (5'→3') | ANNEALING TEMPERATURE | TARGET GENE | SAMPLES AMLIFIED |
|---|---|---|---|---|
| **27F** | AGAGTTTGATCCTGGCTCAG | 50°C | | |

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| | | | | |
|---|---|---|---|---|
| **1429R** | TACGGCTACCTTGTTACGAC | | **16S rRNA** | NONE |
| ATCC12600F | CGCGGATCCATCTATAAGTGAC | 50°C | | |
| ATCC12600R | CCTGGCTCAGGATGAACG | | **16S rRNA** | 205, 206 |
| FORWARD | TTTGTGATAGCCACATCATTCG | | | |
| REVERSE | GCCTACACACAATCTGTATTCTCA | 50°C | **MecA** | NONE |
| FORWARD | AAAATCGATGGTAAAGGTTGGC | | | |
| REVERSE | AGTTCTGGAGTACCGGATTTGC | 50°C | **MecA** | NONE |

The fragments were sequenced using the Nimagen, Brilliant Dye™ Terminator Cycle Sequencing Kit V3.1, and BRD3-100/1000 according to manufacturer's instructions:

The labelled products were then cleaned with the ZR-96 DNA Sequencing Clean-up Kit (Catalogue No. D4053):

The cleaned products were injected on the Applied Biosystems ABI 3500XL Genetic Analyzer (Serial number 22309-040) with a 50cm array, using POP7: and sequence data collected.
The sequences were obtained in PDF and FASTA formats and then searched on the NCBI website.

## RESULTS

~~Poultry farms where samples were collected and the number are shown in Table 1. Out of 250 (100%) samples, 68 (27%) were collected from poultry farm located in Umudike, 45 (18%) from poultry farm in Amafor village, 40 (16%) each from poultry farms in Uzuakoli. From poultry farms located in Agbama and samples 32 (13%) and 25 (10%) samples were collected respectively.~~

~~In each poultry farm, samples for examination were collected from various areas such as skin, cloaca, egg shells of the birds as well as from the feeds, droppings and the environment. The distribution of types of samples collected among different poultry farms are shown in Table 2. A~~

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~~total of 53 skin swab samples, 34 samples from environment, 55 cloaca swab samples, 47 from birds' droppings, 26 eggshell swab samples, 35 samples from poultry feed were collected from various poultry farms. The actual number of each sample type collected in a particular poultry farm is also shown in the Table.~~(PUT this data in material and methods)

The antibiotic resistance pattern is shown in Table 3. The antibiotic resistance pattern varied based on the antibiotics tested. Some isolates were totally resistant (100%) to some antibiotics all fifteen (15) isolates from skin samples were completely resistant to CRX, AUG, AMP, MEM, COT, ERY but sensitive to VAN. All the 18 isolates from cloaca samples were resistant (100%) to TET, CRX, CPZ, AMP and COT. The isolates were highly resistant to TET, CRX, AMP and ERY.

Table 4 shows the multiple antibiotic resistance (MAR) profile of *S. aureus*. Interestingly, all isolates (n-66) exhibited different MAR patterns. Twenty isolates (30.3%) exhibited MAR to nine antibiotics while 19 (28.8%) isolated exhibited MAR to ten antibiotics. Only one isolate (1.5%) exhibited the MAR pattern to 5 TET-AMP-CRX-AUG-COT antibiotics.

As shown in Table 5, out of 66 isolates, 24 isolates were Methicillin-Resistant *Staphylococcusaureus* (MRSA) while 42 isolates were Methicillin-Sensitive *Staphylococcus aureus* (MSSA). The prevalence varied in sample sources. MRSA was detected in two or more samples sources from the poultry farms. The highest prevalence of MRSA was obtained from skin and cloaca samples. Fig. 1 is the result of molecular identification of isolates indicating *Staphylococcussciuri*

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Table 1. Number of samples collected from various poultry farm locations

| **S/N** | Location of poultry farm | No. of samples (%) |
|---|---|---|
| 1 | Umudike | 68(27) |
| 2 | Agbama | 32 (13) |
| 3 | Amafor | 45(18) |
| 4 | Orieugba | 25(10) |
| 5 | Umuana | 40(16) |
| 6 | Uzuakoli | 40(16) |
| | **TOTAL** | **250(100%)** |

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Table 2. Distribution of samples among different location of study

| S/N | Type of sample | Number collected from different location (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | **Umudike** | **Agbama** | **Amafor** | **Orieugba** | **Umuana** | **Uzuakoli** | |
| 1 | Skin | 18 | 1 | 10 | 5 | 6 | 8 | 48 (19.2%) |
| 2 | Environment | 8 | 2 | 5 | 4 | 8 | 7 | 34 (13.6%) |
| 3 | Cloaca | 10 | 20 | 8 | 4 | 8 | 10 | 60 (24.0%) |
| 4 | Droppings | 15 | 4 | 10 | 5 | 7 | 6 | 47 (18.8%) |
| 5 | Egg shell | 7 | 1 | 7 | 3 | 5 | 3 | 26 (10.4%) |
| 6 | Feed | 10 | 4 | 5 | 4 | 6 | 6 | 35 (14%) |
| | **TOTAL** | **68** | **32** | **45** | **25** | **40** | **40** | **250(100%)** |

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Table 3. Antibiotic resistance pattern of the isolates

| SOURCE OF ISOLATION | RESISTANCE TO (%) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **No. of isolates** | **TET** | **CRX** | **CPZ** | **AUG** | **AMP** | **MEM** | **VAN** | **CIP** | **CP** | **COT** | **ERY** | GEN |
| **SKIN** | 15 | 14(93.3%) | 15(100%) | 14(93.9%) | 15(100%) | 15(100%) | 15(100%) | 0 | 3 (53.3%) | 4(26.7%) | 15(100%) | 15(100%) | 10(66.7%) |
| **ENVIRONMENT** | 12 | 12(100%) | 12(100%) | 12(100%) | 12(100%) | 12(100%) | 12(100%) | 0 | 6 (50%) | 3(20%) | 12(100%) | 12(100%) | 8(66.7%) |
| **RECTUM** | 18 | 18(100%) | 18(100%) | 18 (100%) | 16(88.9%) | 18(100%) | 17 (94.4%) | 1(5.6%) | 10(55.7%) | 5(27.8%) | 18(100%) | 18(100%) | 12 (66.7%) |
| **DROPPINGS** | 10 | 10(100%) | 10(100%) | 8 (80%) | 8(80%) | 9(90%) | 10 (100%) | 2 (20%) | 5 (50%) | 2(20%) | 5 (50%) | 10(100%) | 8 (80%) |
| **EGG SHELL** | 6 | 5 (83.3%) | 6(100%)) | 5 (83.3%) | 6(100%) | 6(100%) | 5(83.3%) | 2 (33.3%) | 0 | 0 | 1(16.7%) | 6 (100%) | 4 (66.7%) |
| **FEED** | 5 | 4 (80%) | 4(80%) | 5 (100%) | 5(80%) | 5(100%) | 5(100%) | 1 (20%) | 4 (80%) | 0(0%) | 4 (80%) | 4 (80%) | **1 (20%)** |

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Table 4. Multiple antibiotic resistance (MAR) profile of *S. aureus*

| No. of Antibiotics | Antibiotics showing resistance | Antibiotics resistance (%) |
|---|---|---|
| **5** | TET, AMP, CRX, AUG, COT | 1(1.5%) |
| **7** | TET, AMP, CRX, MEM, AUG, CPZ, ERY | 2 (30%) |
| **8** | TET, COT, AMP, CRX, MEM, AUG, CPZ, ERZ | 10 (15.2%) |
| **9** | GEN, TET, COT, AMP, CRX, MEM, AUG, CPZ, MEM | 20 (30.3%) |
| **10** | CIP, TET, COT, AMP, CRX, MEM, AUG, CPZ, MEM, AUG | 19 (28.8%) |
| **11** | CIP, CP, GEN, CPZ, COT, TET, ERY, AMP, CRX, MEM, AUG | 8 (12.1%) |
| **12** | VAN, CP, CIP, GEN, CPZ, COT, TET, ERY, AMP, CRX, MEM, AUG | 6 (9.1%) |

Table 5. Prevalence of MSSA and MRSA in the poultry farms

| | Sample source | No. of Samples | No. of isolated | MSSA | MRSA |
|---|---|---|---|---|---|
| 1 | Skin | 48 | 15 | 10 | 6 |
| 2 | Environment | 34 | 12 | 7 | 4 |
| 3 | Cloaca | 60 | 18 | 10 | 6 |
| 4 | Droppings | 47 | 10 | 7 | 3 |
| 5 | Egg shell | 26 | 6 | 4 | 2 |
| 6 | Feed | 35 | 5 | 4 | 2 |
| | TOTAL | 250 | 66 | 42(63%) | 24(36%) |

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**PCR GEL IMAGE FOR THE AMPLICONS**
**(La=Ladder,1=206,2=205,3=203,4=196,5=191,6=190)**

**La 1 2 3 4 5 6**

Fig 1. PCR gel image for the amplicons

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**SEQUENCING RESULT**

**chart 2 : SUMMARY OF BLAST PREDICTION**

| *S/N* | *Seq ID* | *Matched organism* | *% Identity* | *Accession number* |
|---|---|---|---|---|
| *1* | 205 | *Staphylococcus sciuri* | 99.4% | MN71223.1 |
| 2 | 206 | *Staphylococcus sciuri* | 96.15% | MN960659.1 |

**DISCUSSION**

Methicillin-Resistant *Staphylococcus aureus* (MRSA) in poultry was investigated. *Staphylococcus aureus* is regarded as an opportunistic and commensal organism of birds, animals and humans. In poultry, *S. aureus* is found in feedstuff as well as on equipment and utensils present in farms. *S. aureus* is a gram-positive bacterium under certain circumstances, *S. aureus* can cause infections, and for instance staphylococcal food poisoning is associated with the secretion of enterotoxins by *S. aureus* which contaminate food causing foodborne poisoning.

Live birds are regarded as an important reservoir for pathogenic *S. aureus* strains. In this study, *S. aureus* were isolated from various samples in different poultry farms locations. Similarly, Mamza*et al.*, (2010) isolated *S. aureus* at a rate of 52.5% from apparently healthy chickens, whereas Suleiman *et al*, (2013) found coagulase – positive *S. aureus* in 54% of apparently healthy chicken samples. Nworie *et al.*, (2016) showed that 76% (247 of 325) of *S. aureus* isolated from nasal and cloacal swabs of chickens were from poultry. Two hundred and fifty broiler chicken samples including tracheal and cloacal swabs were collected from apparently healthy and diseased birds for isolation of *S. aureus*(Bakheet *et al.,* 2018) and their results showed the presence of 157 *Staphylococcus* isolates of which 81 (55%) were coagulase-positive and 76 (48.4%) were coagulase-negative.

The antibiotic resistance pattern of the isolates varied based on the antibiotics tested. Some isolates were completely resistant to antibiotics. Antibiotic-resistant bacteria in animals are growing concern because of their possible role in transmission to humans as foodborne pathogens. There is no doubt that the extensive usage of antimicrobials in the poultry farm enhanced the ability of *S. aureus* to acquire many resistance gene. (Al-Ashmawy, 2016).

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The results of this study indicated the presence of Methicillin-Resistant *Staphylococcusaureus* (MRSA) strains in poultry farms. The presence of MRSA in live poultry has been reported and has highlighted the increase in the antibiotic resistance rate Methicillin-Resistant *Staphylococcus aureus* (MRSA) strains have been increasingly reported as emerging pathogenic strains that cause great problems in veterinary medicine (Adeyeye and Adewale, 2013) demonstrated MRSA strains in 90 of 100 cloacal swabs of broiler chickens. Resistant MRSA strains represent a great threat to consumers health.

This study demonstrated the existence of multiple antibiotic resistant (MAR) *S. aureus* in samples collected from various poultry farms. *S. aureus* strains are resistant to three or more classes of antibiotics. Results showed 100% resistance to Ampicillin, Penicillin, and Tetracycline. Resistance of *S. aureus* isolates, especially those from broiler chicken origin to tetracycline has been reported (Nemati *et al.*, 2008; Delorme *et al.*, 2009). Coagulase-positive *S. aureus* strains from live chickens showed 100% resistance to tetracycline, penicillin, and erythromycin (Otalu*et al.,* 2011). The incidence of MAR. *S. aureus* strains has increased due to the hazardous use of antimicrobials in the prophylaxis and treatment of diseases in either animals or humans(Abubakar and Sulaiman,2018).

The ability of *S. aureus* to show multiple resistance to antibiotics might be due to the production of an exopolysaccharide barrier (Gündoğan *et al.,* 2006) and carriage of variable multi drug-resistant genes or plasmids that could be exchanged and spread to other *Staphylococcus* species (Neihart *et al.*, 1988). Apart from *S. aureus*, molecular characterization and identification confirmed coagulase-negative *Staphylococcus* as *Staphylococcussciuri*. The isolation of *S. sciuri* from poultry samples is surprising. *S. sciuri* is a bacterial pathogen associated with infections in animals and humans and represents a reservoir for the mecA gene encoding methicillin-resistance in staphylococci. It was speculated that the *S. sciuri* species group is a potential reservoir of virulence and antimicrobial resistance genes for other staphylococci.This study has confirmed the presence*S. aureus* as well as MRSA in the poultry farms where samples were collected and this may poses a great challenge to food security in the areas and health implications.

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