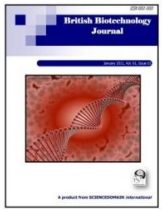

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# Genetic and Phenotypic Identification of Vancomycin-Resistant *Staphylococcus aureus* Isolates from Retail Poultry Carcasses in Omu-Aran, North-Central Nigeria

**C. E. Okolie[1*], U. C. Essien[2] and J. Idoko[3]**

*[1]Department of Biological Sciences, Landmark University, Omu-Aran, Kwara State, Nigeria.*
*[2]Department of Medical Laboratory Sciences, University of Jos, Plateau State, Nigeria.*
*[3]Department of Histopathology and Cytology, Ahmadu Bello University, Teaching Hospital, Zaria, Kaduna State, Nigeria.*

***Authors' contributions***

*This work was carried out in collaboration between all authors. Author CEO designed the study, wrote the protocol, and wrote the first draft of the manuscript. Authors CEO, UCE and JI managed the analyses of the study. All authors performed the wet laboratory experiments and statistical analysis, read and approved the final manuscript.*

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## ABSTRACT

*Staphylococcus aureus* is a well-known agent of zoonotic infections. Livestock-associated methicillin-resistant *S. aureus* (LA-MRSA) had been receiving public health attention for over a decade. Recently, the genomes of some MRSA strains evolved further by enabling acquisition of *vanA* gene from enterococcus which drives the emergence of vancomycin-resistant *S. aureus* (VRSA), thus signaling a higher threat to antimicrobial chemotherapy and diagnostic microbiology. This study was designed to examine slaughtered chicken carcasses in Omu-Aran, North-Central Nigeria for the presence of VRSA using vancomycin agar screen (VAS) as recommended by the Clinical and Laboratories Standards Institute (CLSI). To provide independent witness to further support the evidences from VAS, a 235 bp marker for *vanA* gene was simultaneously detected by

**Corresponding author: Email: charlesokolie@hotmail.com;*

PCR. From April 2013 through May 2014, chicken carcasses (n=784) were collected and studied. Among 155 (19.8%) samples which yielded *S. aureus*, VAS and *vanA* PCR methods unequivocally identified VRSA in 22 (14.2%). Compared with 46.2% VRSA report from Zaria, North-Western Nigeria, the incidence of VRSA is much less in Omu-Aran chicken carcasses than those of Zaria. Further investigation in other parts of Nigeria is recommended in order to generate nation-wide data on VRSA in this country.

*Keywords: Staphylococcus aureus; poultry; VRSA; PCR; vancomycin agar screen.*

## 1. INTRODUCTION

The evolution of antimicrobial resistant clones in bacteria including the staphylococci is impacting adversely on humans as well as on domesticated animals. Especially interesting is the widespread methicillin resistant *Staphylococcus aureus* (MRSA) strains some of which recently evolved further into vancomycin resistant *S. aureus* (VRSA). Several reports have provided evidences for the mechainism of acquisition of *mecA* gene which drives MRSA as prerequisite to the acquisition via horizontal gene transfer (HGT) of *vanA* gene which encodes staphylococcal vancomycin resistance [1-3]. While the world battles the menace visited upon public health by HGT-associated resistance to antibiotics, the recent report of MRSA which transformed into VRSA within the same patient [4] makes the story of VRSA more complex than MRSA.

Expert reviews have highlighted the need for correct identification of the genetic capacity for and expression of vancomycin resistance in staphylococcal isolates [5,6]. Also, given the high chances of inter-species transmission, livestock-associated *S. aureus* should not be seen as very distant from humans. Furthermore, the frequency of VRSA in domesticated animals should be seen as an emerging threat to food security. The Food and Agricultural Organization (FAO) estimated that domesticated birds in Nigeria with a population of 140 million supplies about one billion eggs and 500,000 metric tons of poultry meat annually [7]. The poultry industry obviously makes meaningful input to Nigeria's economy. Recently we isolated VRSA during routine testing of poultry and human specimens in Omu-Aran. In addition to this new finding, the possibility of emission via the airborne route [8] and there cent report of high prevalence of VRSA among poultry *S. aureus* in Zaria, North-Western Nigeria [9] all informed our interest in investigating chickens in Omu-Aran, North-Central Nigeria for VRSA by random sampling of retail carcasses.

## 2. MATERIALS AND METHODS

### 2.1 Control Bacterial Strains Used in this Study

Bacterial strains used as controls for this study are listed (Table 1). They include recently isolated strains of VRSA (n=2) harbouring the *vanA* gene and used as PCR positive controls for the gene detection assay as well as the vancomycin agar screen (VAS) method. Coagulase-negative staphylococcus (CoNS) strains (n=24) and vancomycin-susceptible *S. aureus* (VSSA) strains (n=22) were used as negative controls.

### 2.2 Sample Population Size

Chicken carcasses (n=784) were examined over a period of fourteen months (April 2013 through May 2014). They were obtained at slaughter from the poultry section of Omu-Aran abattoir in New Market, Sabo, Omu-Aran.

### 2.3 Sample Collection and Transportation

Following the method of Persoons et al. [10], chicken parts were placed in a disposable sterile bag containing 400 mL of brain-heart infusion (BHI) broth supplemented with nalidixic acid and colistin each at a concentration of 10 µg/mL. At the end of each day of sample collection, all collected samples were transported to the laboratory which is less than 30 minutes away.

### 2.4 Isolation and Identification of *Staphylococcus aureus* in Samples

Following overnight incubation of the broth at room temperature (30°C), an aliquot (10.0 µL) was streak-inoculated unto a BHI plate containing 4% NaCl and incubated overnight at 35°C. Based on colonial appearance, discrete colonies suspected to be staphylococci were subjected to conventional identification procedures including Gram staining, catalase, and coagulase tests.

**Table 1. Characteristics of control bacterial strains (n=48) used for this study[a]**

| Strain identity | Type of staphylococcus (CoNS, S. *aureus* or VRSA) | *vanA* gene[b] |
|---|---|---|
| Omu-SA1 | *S. aureus* | - |
| Omu-SA2 | *S. aureus* | - |
| Omu-SA3 | *S. aureus* | - |
| Omu-SA4 | *S. aureus* | - |
| Omu-SA5 | *S. aureus* | - |
| Omu-SA6 | *S. aureus* | - |
| Omu-SA7 | *S. aureus* | - |
| Omu-SA8 | *S. aureus* | - |
| Omu-SA9 | *S. aureus* | - |
| Omu-SA10 | *S. aureus* | - |
| Omu-SA11 | *S. aureus* | - |
| Omu-SA12 | *S. aureus* | - |
| Omu-SA13 | *S. aureus* | - |
| Omu-SA14 | *S. aureus* | - |
| Omu-SA15 | *S. aureus* | - |
| Omu-SA16 | *S. aureus* | - |
| Omu-SA17 | *S. aureus* | - |
| Omu-SA18 | *S. aureus* | - |
| Omu-SA19 | *S. aureus* | - |
| Omu-SA20 | *S. aureus* | - |
| Omu-SA21 | *S. aureus* | - |
| Omu-SA22 | *S. aureus* | - |
| Omu-VRSA1 | VRSA | + |
| Omu-VRSA2 | VRSA | + |
| Omu-CoNS1 | CoNS | - |
| Omu-CoNS2 | CoNS | - |
| Omu-CoNS3 | CoNS | - |
| Omu-CoNS4 | CoNS | - |
| Omu-CoNS5 | CoNS | - |
| Omu-CoNS6 | CoNS | - |
| Omu-CoNS7 | CoNS | - |
| Omu-CoNS8 | CoNS | - |
| Omu-CoNS9 | CoNS | - |
| Omu-CoNS10 | CoNS | - |
| Omu-CoNS11 | CoNS | - |
| Omu-CoNS12 | CoNS | - |
| Omu-CoNS13 | CoNS | - |
| Omu-CoNS14 | CoNS | - |
| Omu-CoNS15 | CoNS | - |
| Omu-CoNS16 | CoNS | - |
| Omu-CoNS17 | CoNS | - |
| Omu-CoNS18 | CoNS | - |
| Omu-CoNS19 | CoNS | - |
| Omu-CoNS20 | CoNS | - |
| Omu-CoNS21 | CoNS | - |
| Omu-CoNS22 | CoNS | - |
| Omu-CoNS23 | CoNS | - |
| Omu-CoNS24 | CoNS | - |

*aControl strains were isolated and characterized recently in Omu-Aran hospitals and poultry farms.*
*b + means positive, - means negative*

## 2.5 Phenotypic Detection of VRSA by Vancomycin Agar Screen (VAS) Method

The Clinical and Laboratory Standards Institute (CLSI) recommended vancomycin agar screen (VAS) for identification of VRSA [11]. Briefly, vancomycin supplement (Oxoid, Basingstoke, UK) was incorporated into BHIagar to a concentration of 6.0 mg/L. Four VAS plates were inoculated per test sample to provide duplicate plates for the two temperature zones used for isolation. To obtain an inoculum with $10^4$ CFU following isolation, standardized (0.5 McFarland) inoculum was diluted by transferring 10 µL of the standardized inoculum into 900 µL of sterile normal saline. VAS test plates were then inoculated with a 10 µL drop of the diluted inoculum by using a micropipette to deliver onto the agar surface, spotting an area of 10mm to 15 mm at the centre of the VAS plate. As soon as they were inoculated, one set of duplicate plates was incubated at 30°C. The second set of duplicate plates was incubated at 35°C. All VAS plates were incubated for 24 hours and examined carefully for evidence of small colonies (>1 colony) or a film of growth, suggesting reduced susceptibility to vancomycin.

## 2.6 Gene Detection by PCR

PCR was performed following a recent PCR assay targeting 235 bp marker within the *vanA* gene encoding staphylococcal vancomycin resistance [12]. Briefly, an aliquot (0.5 mL) of 0.5 McFarland standardized bacterial suspension was heated for 10 min at 95°C. Following centrifugation (13000 RPM, 20 s), the supernatant was used as template DNA for PCR amplification on an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). An initial single cycle for 4 min at 94°C was followed by 40 polymerization cycles consisting of 15 s at 94°C and 10 s at 60°C with a final polymerization at 72°C for 1 min and cooling at 8°C before gel electrophoresis. PCR products were resolved by horizontal submarine electrophoresis (Bio-Rad, USA) at 200 V for 30 min in Agarose gel (2%) containing ethidium bromide (0.5 µg/mL). Following visualization (UV-transilluminator: UVP, Cambridge, UK), gel images were captured with the associated Vision Works™ software. To obtain clean DNA for sequencing, PCR products were purified from Agarose gels using Gen Elute™ according to the manufacturer (Sigma, UK). Sequencing reactions were prepared following the Applied Biosystems Big Dye™

protocol. Sequencing reaction was analyzed in a Prism 310 Genetic analyzer (Abbott Laboratories, USA) following which the obtained sequence data was followed by BLAST.

To allow for direct blind comparison of independent tests, PCR detection of *vanA* marker from the isolates was performed simultaneously without waiting for VAS to complete. In the end, both VAS and PCR results were examined together to confirm corroboration and/or discrepancies.

## 3. RESULTS AND DISCUSSION

### 3.1 Concordance between *vanA* PCR and Identification of VRSA by VAS

All (100%) of the 48 control staphylococcal strains listed in Table 1 showed absolute concordance between *vanA* PCR and VAS methods. Following on this concordance which is important to ascertain assay accuracy, the collected samples revealed 22 (14.2%) strains of the 155 *S. aureus* isolates were VRSA while the remaining 131 strains were VSSA (Fig. 1).

All the strains identified as VRSA by VAS tested positive for *vanA* gene. *vanA*-negative strains did not grow on the vancomycin (6 mg/L) agar used for VAS. These findings are nosurprises as the 235 bp *vanA* PCR yielded 100% positive predictive values (100% PPV) and 100% negative predictive value (100% NPV) when it was developed [12]. Several reports show varied levels of correlation between gene detection and agar-based identification of staphylococcal vancomycin-resistant phenotype. In India, report of staphylococcal isolates which expressed vancomycin resistance phenotypically for which detection of *vanA* and *vanB* genes by PCR was not possible had been published [13]. Failure of PCR to detect the genes encoding vancomycin resistance could be multifactorial ranging from poor priming to absence of the target gene (or sequence) within the genome. However, in another report from India, Saha et al. [14] documented what was described as the first successful recovery of VRSA by PCR as well as agar-based phenotypic methods.

### 3.2 Monthly Frequencies of VRSA

Between April 2013 and May 2014, 784 samples were collected for this study and *S. aureus* was isolated from 155 (19.8%) samples (Table 2).

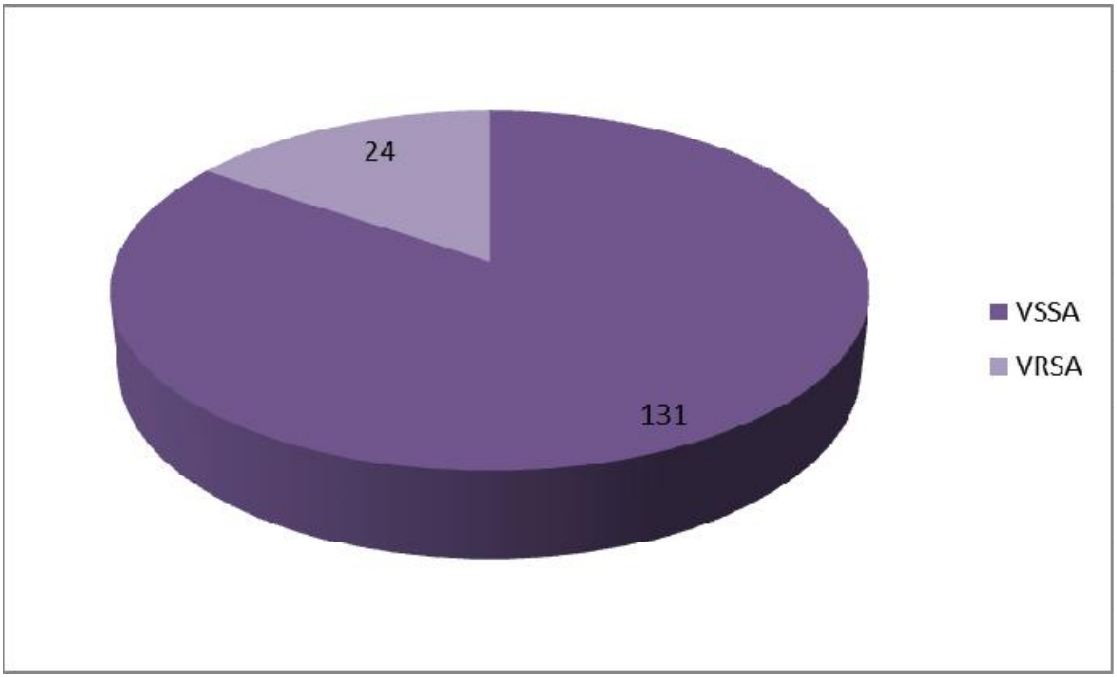


**Fig. 1. Abundance of *Staphylococcus aureus* and VRSA recovered in the study**

**Table 2. Monthly prevalence of *S. aureus* and VRSA isolates from poultry carcasses**

| Month of experimentation | Number of samples collected | Number (%) positive for | |
|---|---|---|---|
| | | For *S. aureus* | For VRSA |
| 2013 April | 48 | 4 (14.6) | 1 (2.1) |
| 2013 May | 55 | 10 (18.2) | 2 (3.6) |
| 2013 June | 62 | 7 (11.3) | 2 (3.2) |
| 2013 July | 65 | 10 (15.4) | 3 (4.6) |
| 2013 August | 58 | 10 (17.2) | 2 (3.4) |
| 2013 September | 54 | 11 (20.4) | 2 (3.7) |
| 2013 October | 58 | 11 (19.0) | 0 (0.0) |
| 2013 November | 57 | 14 (24.6) | 3 (5.3) |
| 2013 December | 52 | 15 (28.8) | 1 (1.9) |
| 2014 January | 55 | 9 (16.4) | 1 (1.8) |
| 2014 February | 54 | 11 (20.4) | 1 (1.9) |
| 2014 March | 54 | 15 (27.8) | 2 (3.7) |
| 2014 April | 58 | 11 (19.0) | 2 (3.4) |
| 2014 May | 54 | 17 (31.5) | 2 (3.7) |

Although our findings show low prevalence of VRSA compared with the Zaria study which reported 46.2% VRSA [9], it still calls for attention in the use of antibiotics in animal production. Despite the efforts of the National Agency for Food and Drug Administration and Control (NAFDAC) especially in human drug (mis) use, it seems that more effort needs to be applied to the regulation of drug use in food animal production. However, given the low level of hygiene in poor settings (http://www.who.int/water_sanitation_health/hygiene/en/), of which this part of Nigeria is not excluded, the VRSA could also be of origins other than food animal production. It could even be one of the repercussions of poor hygiene.

## 4. CONCLUSION

Studies of *Staphylococcus aureus* isolates from retail chicken carcasses have been reported elsewhere, most of which showed varying levels of resistance to antibiotics including methicillin [15-17]. The report of 46.2% VRSA in Zaria chickens by Otaluetal (2011) is probably the first in Nigeria. Whether they originate from poor hygiene or from uncontrolled and indiscriminate sale and use of antibiotics as the authors mentioned, larger scale testing for the presence of antibiotic resistance especially VRSA in animals is recommended. As VRSA in humans is not widespread at the moment, the possibility of inter-species transmission of *S. aureus*, makes the recommendation for further investigations imperative.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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