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## Prevalence and molecular detection of *mecA* and *lukD* genes from livestock-associated methicillin-resistant *Staphylococcus aureus* isolated in raw chicken meats sold in Abakaliki, Nigeria

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### ABSTRACT

**Background:** Due to its deadly toxins and ability to produce enzymes, the commensal bacterium *Staphylococcus aureus* is found in animals, birds, and people. It may also be linked to certain medical disorders.

**Aim:** This study was aimed at the prevalence and molecular detection of *mecA* and *lukD* genes from livestock-associated methicillin-resistant *S. aureus* (LA-MRSA) in raw chicken meats sold in Abakaliki.

**Methods:** The butchers in the slaughterhouses provided 150 fresh raw chicken meat samples, which were then cultured and purified using mannitol salt agar. The LA-MRSA was determined using oxacillin and cefoxitin by a disk diffusion method. A polymerase chain reaction was used to investigate the presence of *mecA* and *lukD* genes in the isolates that were resistant to cefoxitin and oxacillin.

**Results:** Exactly, 61 (40.7%) out of the 150 samples analyzed were positive for *S. aureus*, and 39 (52.0%) and 22 (29.3%) were isolated from broiler and layer chicken meats, respectively. Out of the 61 (40.7%) samples colonized by *S. aureus*, 7 (11.5%) were identified to be LA-MRSA positive using the phenotypic method. Thus, 5 (12.8%) and 2 (9.1%) were recovered as LA-MRSA positive from broiler and layer chicken meats, respectively. All the LA-MRSA was reported to present three major multidrug resistance index patterns. The overall prevalence of *mecA* and *lukD* genes was reported as 2 (28.6%) and 3 (42.9%), respectively.

**Conclusion:** This study confirmed the presence of *mecA* and *lukD* in LA-MRSA isolated from chicken in Abakaliki, Nigeria, and is a serious public health threat.

**Keywords:** Chicken meats, *mecA*, LA-MRSA, *lukD*, public health.

### Introduction

The potential for antibiotic-resistant bacteria in animals to spread to humans as foodborne pathogens is a major cause for concern (Effendi *et al.*, 2018). Methicillin-resistant *S. aureus* (MRSA) is one of the main developing pathogenic strains causing significant issues in veterinary and human medicine, according to a growing number of reports. However, MRSA is drastically increasing among livestock, humans, and their environments (Wu *et al.*, 2019; Tyasningsih *et al.*, 2019). MRSA can spread through direct contact

between humans and animals as well as through the use of contaminated surfaces, food, water, and air (Suleyman *et al.*, 2018; Touimi *et al.*, 2020). MRSA has been linked to unsanitary conditions that occur when food is processed, such as when raw chicken meat is contaminated with bacterial toxins. In addition, poor cooking, inappropriate meat storage, incorrect food preparation using contaminated water, and careless handling by food handlers, including butchers and merchants of raw chicken meat, are additional sources of MRSA contamination in food (Abd El-Ghany, 2021).

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During the process of butchering birds or preparing meat, chicken flesh may become contaminated with MRSA (Abolghait *et al.*, 2020). The high incidence of MRSA in poultry meat, which is regarded as a major health risk for consumers, is strongly correlated with the rise of MRSA in human zoonotic illnesses (Benrabia *et al.*, 2020). Because of the potential for zoonotic transmission from contaminated retail meat to people or through the food chain, MRSA strains have previously attracted a lot of attention (Wu *et al.*, 2019). A particular kind of bacterium known as livestock-associated methicillin-resistant *S. aureus* (LA-MRSA) has become resistant to several antibiotics. It has been identified in various countries of the world. LA-MRSA has been recognized as a serious pathogen that presents a zoonotic risk to people, particularly those in intimate contact with the disease (Kinross *et al.*, 2017). LA-MRSA has always been associated with animals and animal products and is linked to transmission from animals to humans. LA-MRSA has been reported worldwide from farm animals such as poultry birds and pigs (Fox *et al.*, 2016).

Serious worries regarding potential food contamination have been raised by the discovery that LA-MRSA is present in food-producing animals. Research from Africa, Europe, Asia, and North America has demonstrated that MRSA can be retrieved from animals, retail meat, poultry, fowl, and their products (O'Brien *et al.*, 2012; Peter *et al.*, 2022; Tyasningsih *et al.*, 2022). Researchers have discovered that a number of genomic elements encode virulence and resistance genes carried by MRSA. The MRSA *mecA* gene, which contains insertion sites for genetic elements that promote the enhanced acquisition of resistance determinants to other antimicrobials, encodes PBP2a, which has decreased susceptibility to  $\beta$ -lactam antibiotics (Ito *et al.*, 2012). *Staphylococcus aureus* strains that carry the *mecA* gene become resistant to methicillin, which is penicillinase. Staphylococcal cassette chromosome *mec* (SCC*mec*) contains this gene, which confers resistance to many antibiotics such as penicillin and beta-lactam.

The *mecA* gene-related multidrug-resistant (MDR) *S. aureus* strains were found in ready-to-eat meat products, chicken giblets, and retail meat products (Darwish *et al.*, 2018; Morshdy *et al.*, 2018). MRSA from poultry chicken meat has been found to contain multiple toxin gene groups, including enterotoxins (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, and *sei*), exfoliative toxins (*eta* and *atb*), leukocidins (*lukE*, *lukD*, and *lukM*), Pantone-Valentine leukocidin (*lukS* and *lukF*), and hemolysins (*hla*, *hly*, and *hld*) (Abdallahman *et al.*, 2015). MRSA (*nuc* and *mecA* positive) has also been recovered from chicken, pork, and turkey (Fox *et al.*, 2016). There are limited studies about LA-MRSA strains that are capable of causing zoonotic infections, and other *S. aureus* infections in Nigeria, especially at Abakaliki in Ebonyi State. Thus, there is a need for this study, which aimed at the prevalence and molecular detection of *mecA* and

*lukD* genes from LA-MRSA isolated in raw chicken meats sold in Abakaliki.

## Materials and Methods

### Study design and sampling

A cross-sectional study was conducted; layer and broiler chicken fresh raw meat samples were collected from the chicken slaughterhouses located at the live chicken market in Ebonyi State International Market Abakaliki Nigeria. Abakaliki is the capital of Ebonyi State, located in the southeastern part of Nigeria. A total of 150 fresh raw chicken meat samples, which include 75 broilers and 75 layers, were collected randomly from the butchers in the slaughterhouses at the International Market in Abakaliki. Exactly, 10 g each of the fresh raw chicken meat samples were collected using a clean sharp cutting knife and stored in sterile screw-capped containers. The samples were properly labeled, packed in ice packs cool boxes and transported immediately to the Department of Applied Microbiology Laboratory at Ebonyi State University, Abakaliki, Nigeria, for bacteriological analysis.

### Isolation and identification of *S. aureus*

After being weighed, 5 g of the freshly obtained raw chicken meat samples were added to sterile flasks holding 10 ml of enhanced phosphate buffer saline (PBS). A meat grinder was used to thoroughly homogenize the samples in an aseptic setting. The homogenized raw chicken meat samples were diluted, inoculated into peptone water (TITAN Biotech, India) for enrichment, and incubated at 37 °C for 18–24 hours. The samples were subcultured onto a freshly prepared nutrient agar, further cultured on mannitol salt agar (Merck, Darmstadt, Germany), and incubated at 37 °C for 18–24 hours. The well-formed distinct colonies of *S. aureus* on mannitol salt agar (golden-yellow colonies) were identified phenotypically and biochemically verified using Gram staining, catalase, coagulase test, and characteristic pigment production (Sheet *et al.*, 2021; Tyasningsih *et al.*, 2022).

### Detection of LA-MRSA

Oxacillin and cefoxitin antibiotic discs were used to screen the *S. aureus* isolates from chicken meats for the presence of LA-MRSA. The cefoxitin and oxacillin-impregnated discs were inserted into the inoculated agar plates to identify MRSA. After that, the plates were incubated for 24 hours at 37 °C. The clinical laboratory standard institute was used to assess and interpret the zones of inhibition. The isolates with zone diameters of less or equal to 10 mm for oxacillin and less or equal to 21 mm for cefoxitin were considered resistant (Clinical and Laboratory Standards Institute, 2018; Ukpai *et al.*, 2021).

### Antibiotic susceptibility testing (AST) of LA-MRSA

The identified isolates of LA-MRSA were subjected to AST by testing six batteries of antibiotic, which were selected from five different antibiotic classes using the Kirby–Bauer disk diffusion technique according

to the guideline of Clinical and Laboratory Standards Institute (CLSI). The antimicrobials classes used in this study were beta-lactam [methicillin (oxacillin; OX 5 µg and cefoxitin; FOX 30 µg)], cephalosporins (cefotaxime; CTX 30 µg), macrolides (erythromycin; ERY 30 µg), tetracyclines (tetracyclines; TE 30 µg), and glycopeptides (vancomycin; VAN 30 µg) (Oxoid, UK). To summarize, using a sterile swab stick, each isolate was aseptically injected onto Muller-Hinton Agar (Oxoid, UK) plates after being standardized to 0.5 McFarland equivalents. The inoculated plates were allowed to stand for 15–20 min. Sterile forceps were used to insert the antibiotic discs onto the inoculation plates. Following an 18–24-hour incubation period at 37 °C, the zones of inhibition surrounding each disc were identified, measured to the closest millimeter using a meter rule, documented, and interpreted appropriately (Clinical and Laboratory Standards Institute, 2018).

#### **DNA extraction and screening for the presence of *lukD* and *mecA* gene**

In order to learn more about the LA-MRSA that was verified by oxacillin and cefoxitin, a polymerase chain reaction (PCR) was used to find the *lukD* and *mecA* genes (Sheet *et al.*, 2021). The QIAamp DNA Mini Kit (Promega, USA) protocol was followed for the DNA extraction procedure. The primers and PCR technique employed were previously reported by Sheet *et al.* (2021) and Couto *et al.* (2016), respectively. Briefly, the genomic DNA of LA-MRSA isolates was extracted from two to three colonies of 24-hour culture. PCR was used to validate the presence of the *mecA* and *lukD* genes in the LA-MRSA colonies.

Industrial ready-to-used prepared PCR master mix solution (GoTaq Green Master Mix), which contains Taq DNA polymerase, dNTPs, MgCl<sub>2</sub>, and a reaction buffer at a concentration of 12.5 µl, was used; 10 µl of Sigma water (nuclease-free water), 0.25 µl of forward primer, 0.25 µl of reverse primer, and 2 µl of the genomic DNA were mixed together in a PCR tube. The aliquot of DNA products was amplified using a C1000 Thermo Cycler machine (Bio-Red) for 35 cycles in 25 µl of the PCR reaction mixture. The PCR protocol condition comprised 35 cycles of initial denaturation step of 2 min at 95 °C, followed by denaturation at 94 °C for 30 s, annealing temperature of *mecA* gene at 54 °C, *lukD* gene at 55 °C for 30 s, an extension at 72 °C for 30 s, and a final extension at 72 °C for 4 min.

Electrophoresis was followed by the visualization of the PCR products. For the electrophoresis analysis, a 1% (w/v) agarose gel was prepared using 1.0 g of agarose (SeaKem, Lonza, USA) and 100 ml of 1X Tris-acetate-ethylenediaminetetraacetate (TAE; pH 8.0) buffer (Bio-Rad, USA). Precisely, one well was filled with 5 µl of Extend Quick-Load DNA Ladder (1 kb; New England, Bio Labs) to gauge band diameters, and another well was filled with 5 µl of negative control (water devoid of nuclease). Consequently, 5 µl of the PCR results from every isolate were meticulously added to the remaining wells. The gel was discolored after 60 minutes with an electric current of 80 V at 400 mA. A gel documentation system (Bio-Rad, USA) was used to visualize and take pictures of the gel.

#### **Ethical approval**

Not needed for this study.

#### **Results**

##### **Isolation of *S. aureus* bacteria**

In this study, 61 (40.7%) of the 150 chicken meat samples analyzed were positive for *S. aureus*, of which 39 (52.0%) and 22 (29.3%) *S. aureus* were isolated from broiler and layer chicken meats, respectively. Out of the 61 (40.7%) samples colonized by *S. aureus*, 7 (11.5%) were identified to be LA-MRSA positive using the phenotypic method. However, 5 (12.8%) and 2 (9.1%) were recovered as LA-MRSA positive from broiler and layer chicken meats, respectively (Table 1).

##### **Sensitivity test results**

The LA-MRSA isolates in this study were observed to show resistance to beta-lactams (oxacillin and cefoxitin), cephalosporins (cefotaxime), macrolides (erythromycin), tetracyclines (tetracyclines), and glycopeptides (vancomycin). All seven *S. aureus* isolates were observed to present three major patterns of MDR (Fig. 1). The isolates BM28 and LM17 had a similar pattern of MDR, which is OX-FOX-TE-ERY-CTX with a prevalence of 2 (28.6%) and a resistance index of 0.8. The isolates BM34, BM41, and BM70 presented an OX-FOX-TE-CTX resistance pattern and 3 (42.9%) prevalence with a resistance index of 0.7. In addition, isolates BM12 and LM55 showed the same MDR pattern as OX-FOX-TE-ERY-CTX-VAN and an average prevalence of 2 (28.6%), with a resistance index of 1.0 (Table 2).

##### **Detection of the MRSA encoding *mecA* and *lukD* gene**

The overall prevalence of *mecA* and *lukD* genes was discovered in LA-MRSA to be 2 (28.6%) and

**Table 1.** Prevalence of *S. aureus* and LA-MRSA from chicken meats.

Sources	Number of samples	Number positive for <i>S. aureus</i> (%)	LA-MRSA (%)
Broiler chicken meats	75	39 (52.0)	5 (12.8)
Layer chicken meats	75	22 (29.3)	2 (9.1)
Total	150	61 (40.7)	7 (11.5)

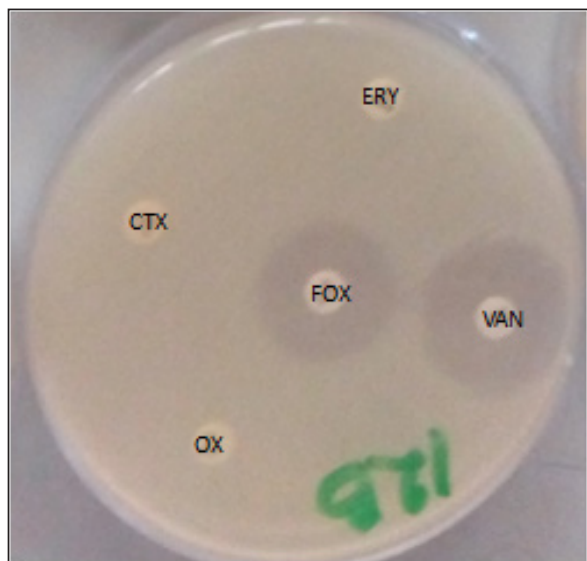


3 (42.9%), respectively (Fig. 2). Thus, out of the 5 LA-MRSA isolates recovered from broiler chicken meats studied, 1 (20.0%) and 2 (40.0%) isolates were identified to harbor *mecA* and *lukD* genes, respectively. The two LA-MRSA isolates isolated from layer chicken meats were observed to harbor 1 (50.0%) *mecA* and 1 (50.0%) *lukD* genes (Table 3).

## Discussion

### Isolation of *S. aureus* bacteria

LA-MRSA is a prominent drug-resistant bacterium that can spread from animals to humans and is responsible for a wide range of diseases around the world. The current study looked at meat samples from broilers and layers and found that one of the main pathogens causing zoonotic infection is *S. aureus*, which was shown to be highly prevalent. Exactly, 61 (40.7%) out of the 150 chicken meat samples studied were positive for *S. aureus*, 39 (52.0%) were from broilers, and 22 (29.3%) were from layer chicken meats. The prevalence of 40.7% reported agreed with the work of Waters *et al.* (2011) where the isolated 41.0% of *S. aureus* was found in poultry meat samples. According



**Fig. 1.** Multidrug resistance LA-MRSA showing resistant to antibiotics. Oxacillin (OX), cefoxitin (FOX), cefotaxime (CTX), erythromycin (ERY), and vancomycin (VAN).

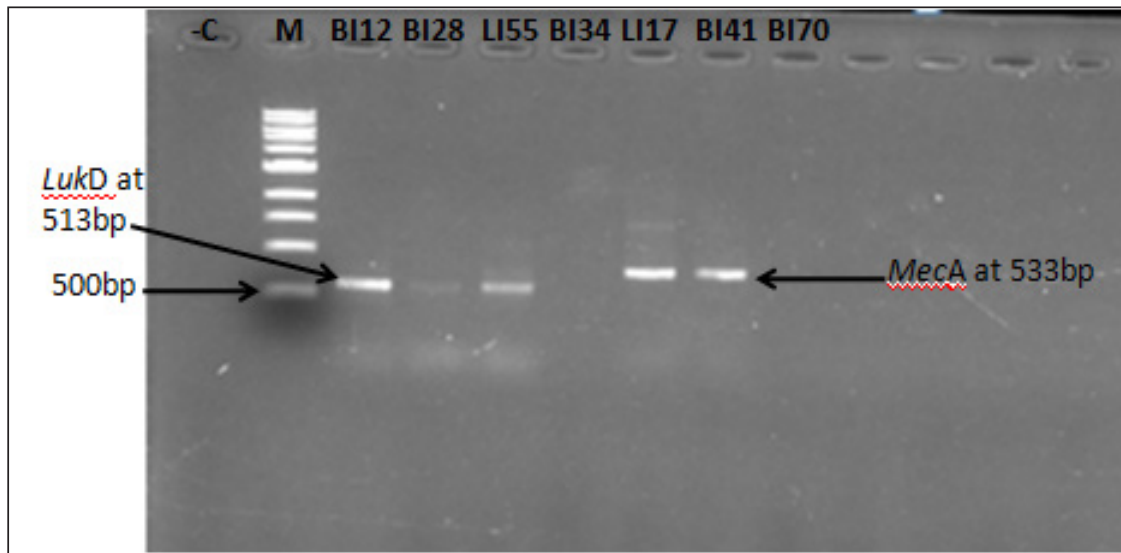
to Bounar-Kechih *et al.* (2018), laying chickens carried 42.0%, broiler chickens 12.0%, and bovines 55% of *S. aureus*. Variable numbers and percentages of *S. aureus* were also identified in these three species of chickens. The current observation of this study is also similar to several researchers in different parts of the world who reported different prevalences of *S. aureus* from poultry meats such as Mamza *et al.* (2010) who reported 52.5%, Bakheet *et al.* (2018) who reported 62.8%, and Nworie *et al.* (2016) who reported 76% prevalence of *S. aureus* strains. Another study on poultry meat revealed that 42 (72.41%) samples were found positive for *S. aureus*, which is higher than the report of this research (Sunitha *et al.*, 2017). In the food chain, the contamination of meats, such as chicken by *S. aureus*, is a serious global issue and the major sources of the contaminants are the food handlers such as the butchers, wholesalers, and retailers of the chicken meat who intend to carry *S. aureus* in their noses or hands (Tshipamba *et al.*, 2018). The length of time that animals are driven to markets in cages or other vehicles contributes to the spread of *S. aureus* among the animals, as does the food-producing animals themselves, which can carry the organisms on their skin, nose, and intestine (Ou *et al.*, 2017). *Staphylococcus aureus* is one of the most significant and widespread foodborne pathogens (Abd El-Ghany, 2021). *Staphylococcus aureus* produces enterotoxins, which are primarily responsible for food poisoning, and it can cause a wide variety of illnesses, such as septicemia and endocarditis. In 2005, it was determined that animals might also present a health danger to humans due to the discovery of MRSA strains known as LA-MRSA, which have a distinct reservoir in agricultural animals (Thwala *et al.*, 2021).

This study identified 7 (11.5%) isolates as LA-MRSA positive out of the 61 (40.7%) samples that were positive for *S. aureus*. However, 5 (12.8%) and 2 (9.1%) were reported as LA-MRSA positive from broiler and layer chicken meats, respectively. LA-MRSA was found in cattle and poultry at prevalence rates of 6.12% and 6.76%, respectively (Vanderhaeghen *et al.*, 2010). According to another study, there was a 6.76% prevalence of LA-MRSA in chicken flesh from African poultry (Ali *et al.*, 2017). Of the 250 pooled pharyngeal swabs of broiler chickens in The Netherlands, 11 (4.4%) included the LA-MRSA strains (Geenen *et al.*, 2013). LA-MRSA was recovered from nine (7.3%) samples comprising four chicken, three pork, and two

**Table 2.** Multidrug-resistant pattern of LA-MRSA isolated from chicken meats.

Isolates code	Multidrug-resistant pattern	Number of isolates (n = 7)	Resistance index
BI28 and LI17	OX-FOX-TE-ERY-CTX	2 (28.6)	0.8
BI34, BI41, and BI70	OX-FOX-TE-CTX	3 (42.9)	0.7
BI12 and LI55	OX-FOX-TE-ERY-CTX-VAN	2 (28.6)	1.0

BI = broiler meats isolate; LI = layer meats isolate.



**Fig. 2.** Gel image showing amplification of *mecA* and *lukD* genes among the LA-MRSA isolates. Lane M = 1 kb Marker, Lane LI17 and BI41 = *mecA* gene, Lane BI12, BI28, and LI55 = *lukD* gene, Lane BI34 and BI70 = *mecA* and *lukD* genes negative, and -C = negative control.

**Table 3.** Prevalence of *mecA* and *lukD* genes in LA-MRSA isolated from chicken meats.

Sources	<i>S. aureus</i> (%)	LA-MRSA (%)	<i>mecA</i> gene (%)	<i>lukD</i> gene (%)
Broiler chicken meats	39 (52.0)	5 (12.8)	1 (20.0)	2 (40.0)
Layer chicken meats	22 (29.3)	2 (9.1)	1 (50.0)	1 (50.0)
Total	61 (40.7)	7 (11.5)	2 (28.6)	3 (42.9)

turkey products obtained from different butchers and supermarkets (Fox *et al.*, 2016). Friese *et al.* (2013) revealed the presence of LA-MRSA in broiler samples at a rate of 50%–54% and in turkey samples at 62%–77%.

In western Nigeria, MRSA strains were found in broiler chickens with a high incidence rate of 90% (Adeyeye and Adewale, 2013). Comparable studies conducted in Northern Algeria showed that LA-MRSA was present in 23.9% and 6.4% of laying and broiler chicks retrieved from slaughterhouses, respectively (Bounar-Kechih *et al.*, 2018). Karmi (2013) reported a 44.0% prevalence of MRSA in poultry in Egypt; in Nigeria, Kenya, and Cameroon, the prevalence of MRSA range is between 30.0% and less than 10.0% in Tunisia (Bounar-Kechih *et al.*, 2018).

This current research indicated that the prevalence of LA-MRSA in chicken meat may be high, and indeed, meats can be contaminated by pathogens. The prevalence of LA-MRSA has been reported in pork meat (Verheghe *et al.*, 2016). Rather than coming from other sources, animal carcass contamination is the primary cause of LA-MRSA prevalence. Therefore, the frequency of LA-MRSA in the animal itself is closely correlated with its prevalence; however, this does not

rule out cross-contamination during processing and slaughter, which may result from the butchers' hands and utensils (Thwala *et al.*, 2021). The entry points are infected transport vehicles, direct contact with human colonists, and the transfer of MRSA-colonized animals from one farm to another (Grøntvedt *et al.*, 2016; Crespo-Piazuelo and Lawlor, 2021).

#### Sensitivity test results

The LA-MRSA presented different patterns of MDR, which include OX-FOX-TE-ERY-CTX, OX-FOX-TE-CTX, and OX-FOX-TE-ERY-CTX-VAN with an average index of 0.8, and is similar with the 0.6 MAR index reported in broiler chickens with resistance patterns as OXA-PEN-FOX-ERY and OXA-PEN-FOX-TCY-ENR-ERY (Bounar-Kechih *et al.*, 2018). Research done on LA-MRSA in Korea documented resistance to ampicillin, cefoxitin, erythromycin, and tetracyclines, the patterns of MDR, including AMP-CLI-ERY-FOX-TE (Seung *et al.*, 2020) and by the observation of this study.

Nworie *et al.* (2016), in their study on MDR *S. aureus* from layers and broilers chicken, reported MRSA to show resistance to cefotaxime, cefoxitin, erythromycin, tetracyclines, chloramphenicol, doxycycline, gentamycin, and levofloxacin, with a resistance

pattern as FOX-TE-ERY-CTX-CHL-DOX-GEN and an average index of 3.0. Another study on MRSA in village chickens from poultry markets demonstrated resistance patterns as ET-SXT-VAN-FOX-NA-ERY and VAN-FOX-NA-ERY (Kwoji et al., 2019). Gaddafi et al. (2022) reported MRSA to show resistance for erythromycin, oxytetracycline, neomycin, penicillin, sulfonamide, and gentamicin, with resistance patterns as ERY-OXY-NEO-PEN-SUL-GEN and OXY-NEO-PEN-SUL, and Fox et al. (2016) documented PEN-OXA-TET. The MDR found in LA-MRSA is linked to mobile genetic elements that carry the resistance marker. Due to antibiotic selection pressure in humans or livestock, these MDR determinants can spread throughout a community, region, or subregion.

#### **Detection of the MRSA encoding *mecA* and *lukD* gene**

The *mecA* gene was reported in MRSA, 40.0% from broiler chicken and 60.0% from the local chicken in Nigeria (Musawa et al., 2020). The presence of *mecA* gene has been reported from LA-MRSA to have a wide geographical distribution with a large diversity of hosts such as domestics/wildlife, companion animals, poultry birds, other food chain, and animal production wastewater samples, which is a threat to public health (Aires-de-Sousa, 2017; Decline et al., 2020; Yunita et al., 2020).

According to earlier studies, *S. aureus* developed resistance to beta-lactam and methicillin drugs by acquiring the *mec* gene, which is found in the SCC<sub>mec</sub> element (Li et al., 2017; Thwala et al., 2021). Multiple mutations can occur in the *mecA* gene promoter, which could be a factor in the decline in oxacillin activity (Chen et al., 2014; Khairullah et al., 2020). The *mecA* gene was detected in MDR-MRSA strains isolated from milk (Ramandinianto et al., 2020; Khairullah et al., 2022), chicken meat and giblets (Morshdy et al., 2018), ready-to-eat meat (Abd El-Ghany, 2021), and fresh retail meat (Darwish et al., 2018) and is by the observation of this study. The virulence toxin genes of the leukocidins (*lukE*, *lukD*, and *lukM*) and Panton-Valentine leukocidins (*lukS* and *lukF*) were found in MRSA from retail chicken and turkey meat in Oklahoma, and their results are consistent with the current data (Abdalrahman et al., 2015).

#### **Conclusion**

This research reported the prevalence of LA-MRSA at a reasonable height in raw broiler and layer chicken meat consumed by the general public in Abakaliki and this could lead to public health problems if not monitored. It further detected *mecA* and *lukD* genes in LA-MRSA from chicken, which may bring about a serious public health risk to the general population who depends on chicken meats as a source of protein and poultry farm workers or slaughters due to possible animal-to-human transmission. Therefore, there is a need for veterinarians and public health officers to

introduce policies and preventive measures to control the rate of LA-MRSA spread in poultry farms.

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#### **Conflict of interest**

The authors declare that there is no conflict of interest.

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#### **Authors' contributions**

ENU and AIU: conceived, designed, and coordinated the study. BNA and IBM: designed data collection tools, supervised the field sample and data collection, and laboratory work as well as data entry. MHE and WT: validation, supervision, and formal analysis. ACN: contributed reagents, materials, and analysis tools. SMY, RR, and ARK: carried out the statistical analysis and interpretation and participated in the preparation of the manuscript. All authors have read, reviewed, and approved the final manuscript.

#### **Data availability**

All data are available in the manuscript.

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