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MOLECULAR IDENTIFICATION AND DETECTION OF METHICILLIN RESISTANT *Staphylococcus aureus* CONTAINING PANTON VALENTINE LEUKOCIDIN (*LUKF-LUKS*) GENE IN POULTRY AND POULTRY FARM WORKERS IN KANO STATE, NIGERIA

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ABSTRACT
The pathogenesis of Staph. aureus is mediated by several cell surfaces and secreted virulence factors. The panton valentine leukocidin (PVL) encoded by LukF-LUkS genes associated with CA-MRSA are responsible for the wide spread of skin and soft tissue infection. This study aims to molecularly determine the PVL prevalence in methicillin S. aureus isolates. Isolates were obtained from poultry (Nasal and Cloacae swabs of chicks) and Nasal swab of workers from three senatorial zones in Kano. Phenotypic Identification of Staph.aureus was performed using coagulase both free and bound, catalase, DNase, and agglutination tests and using Microgen Staph ID kit. MRSA were phenotypically identified using cefoxitin. Genotypic characteristics were determined by spa typing, nuc gene and 16srRNA, detection of mecA gene and luks- lucf gene that encodes PVL. Out of 622 isolates, 98 (33.8 %) were S. aureus. Only 21 isolates showed the amplification of 16SrRNA and nuc gene. Seven isolates were Spa positive. Fifteen carried mecA gene. PVL gene was detected in 14 (14.3%) of S. aureus isolates. Majority of the PVL-positive isolates were obtained from nostril (8; 57.1%), four from cloacae (28.6%) and two from nostril of farm workers (14.3%). Most (10/14) of PVL positive isolates were methicillin resistant S. aureus. This study shows that 14.3% of S. aureus isolated in poultry and poultry farm workers carried PVL genes and 71.4% of the PVL positive were MRSA.
Keywords: PVL, S. aureus, Spa typing, MRSA and poultry

INTRODUCTION

Staphylococcus aureus, a Gram-positive coccus is a frequent cause of skin infections, such as boils and pimples. Since the 1970s the usual treatment for these infections has been penicillins and penicillinase resistant antibiotics such as methicillin. Methicillin resistant *S. aureus* is a major cause of hospital and community infections that are becoming increasingly difficult to combat because methicillin resistant *S. aureus* resist almost all currently available antibiotics (Shallcross *et al*., 2013). The pathogenesis of *S. aureus* is mediated by several cell surfaces and secreted virulence factors. Although produced by less than 5% of *S. aureus* strains, PVL is detected in large percentages of isolates that cause necrotic skin lesions and severe necrotizing pneumonia (Lina *et al*., 1999; Labandeira- Rey *et al*., 2007). The higher pathogenic potential and recurrence of CA-MRSA has been attributed to the ability of these organisms to express PVL (Breurec *et al*., 2011). The panton valentine leukocidin (PVL) encoded by *LukF-LUkS* genes associated with CA-MRSA are responsible for the wide spread of skin and soft tissue infection (David and Daum, 2010). PVL is a bi-component cytolysin (luks-lukf) with high affinity to leukocytes, while other bicomponents toxins, Y-hemolysin and leukocidin show cytotoxity to erythroyctes and leukocytes, respectively (Ferry *et al.,* 2005). Panton-Valentine leukocidin (PVL) and its genes (lukS - lukF) which elicit tissue necrosis are the most well-known community acquired methicillin resistant *S. aureus* virulence factor and though present in most cases of CA-MRSA infection worldwide (Lina *et al.,* 1999; Zetola *et al.,* 2005; Elston and Barlow, 2009). Molecular methods used to type PVL included multilocus sequence typing (MLST) as well as staphylococcus cassette chromosome mec (SCCmec) (Zhang *et al*., 2005). In addition, rapid and simple PVL and α -hemolysin genes detection procedures for *S. aureus* was developed to ease the previous methods that were cumbersome including oligonucleotide probes (Lina *et al*., 1999; Prevost *et al*., 2009). There have however, been little studies on PVL prevalence in methicillin resistance *S. aureus* isolates from poultry and poultry farm workers, especially in the northern part of the country particularly in Kano. This study aims to molecularly determine the PVL prevalence in methicillin resistant *S. aureus* isolates.

MATERIALS AND METHODS

The study was conducted at the Microbiology laboratories of the Department of Microbiology, Bayero University, Kano, Nigeria. DNA extraction and PCR-amplifications were done in molecular laboratories of International Institute for Tropical Agriculture (IITA, Ibadan).

Bacterial Isolates

Isolates were obtained from poultry (Nasal and Cloacae swabs of chicks) and Nasal swab of workers. A total of seven hundred and seventy-one Gram positive, catalase positive cocci occurring in pairs, short chains or clusters were selected and subjected to growth on Mannitol Salt Agar (MSA), coagulase and DNase tests.

Isolation and Phenotypic Identification of *S.aureus*

Each bacterial isolate was subjected to growth on MSA as described previously, incubated for 48 hours at 37^0C. All the isolates were tested for production of coagulase both free and bound (Chessbrough, 2000) and DNase tests (Baker *et al*., 1998). Agglutination test was carried out to further identify the isolates. Staphytect plus latex slide agglutination test kit (Oxoid Ltd England) was used for differentiation of *S. aureus* by detection of clumping factor, protein A. The test was carried out according to manufacturer's instructions. Microgen Staph ID Test kit (Oxoid Ltd England) was used and the test was carried out according to the manufacturer's instructions using 18-24 hrs culture to further screened the isolates.

Screening for methicillin resistant *S. aureus* (MRSA)

Using disk diffusion method, cefoxitin was used to determine methicillin resistance in *S. aureus* (CLSI, 2015). The test was carried out as described previously (Berger Bachi and Rohrer 2002). After incubation, strains that grew on the plates were selected for further studies. Diameter measured and compared with standard (CLSI, 2015). The cefoxitin disc was used as a surrogate for all penicillinase-stable penicillin and resistance was used to infer mecA - mediated methicillin resistance

Molecular Identification through PCR Amplification

Extraction of *S. aureus* DNA

Genomic DNA was extracted using DNA extraction Mini Kit ((Oxoid Ltd England)). The procedure was carried out according to the manufacturer`s instructions and the extracted DNA was stored at 4°C until used for PCR. The bacterial isolates which were found to be *S. aureus* by specific phenotypic identification were further tested by PCR for confirmation using specific primer pairs. The isolates were analyzed for 16SrRNA, the nuc gene and spa typing. These primers amplify 228 bp region of 16S rRNA gene fragment of *S. aureus*.

Preparation for Agarose Gel Electrophoresis

Gel electrophoresis was used to separate DNA on the basis of their sizes by applying an electric field to move the DNA through an agarose matrix. The volume of the TAE and the weight of the agarose used were dependent on the number of wells or gene product. Thirty wells were used and so 3g agarose was added to 200 ml× 1TAE and warmed to dissolve in a microwave for 2min, it was removed from the microwave oven and allowed to stand for a few minutes. Ethidium bromide 8µl (Et Br) was added to stain the DNA product. After the addition of the ethidium bromide to the viscous agarose and shaken to mix, it was poured into the gel mold with 30well comb and allowed to stand for 30minutes to harden. The comb was carefully pulled out of the gel. The clamp of the mold was removed and the gel was transferred into the electrophoretic tank. The tank was filled with 1xTAE buffer to the maximum limit and the PCR products were loaded into the wells. The gel was run at 120V for 1hour. Following electrophoresis, the PCR product was viewed and the picture of the bands was taken.

Spa typing

Amplification of the spa gene X region was performed as described previously (Shopsin *et al*., 1999). The primer used is shown in table 1.2. Spa products were purified before sequencing. Two vol. (20µl) of absolute ethanol was added to the PCR product and incubated at room temperature for 15minutes. This was spinned down at 1000rpm for another 15minutes and the supernatant decanted. Two vol (20µl) of 70% ethanol was added, and the supernatant decanted and air dried. About 10µl of ultrapure water was added and the amplicon was checked on 1.5% agarose. The purified PCR products were then sequenced by using 3130x1 genetic analyzer (applied biosystem, USA). Spa typing was determined with the ridom Spa server (Harmsen *et al*., 2003).

Detection of mecA Gene.

S.aureus isolates were subjected to the detection of *mec*A genes by PCR using specific primer pairs. The multiplex PCR carried out and the primers used were described and design by Lina *et al*., (1999); Shopsin *et al*., (1999).

Detection of lukS - lukF-PVL by PCR

The presence of lukS - lukF genes encoding Panton-Valentine leukocidin(PVL) was detected by PCR according to the method described by (Lina *et al*., 1999). PCR reaction mixture (25µ) consists of 1.5µl of genomic DNA, 12.5µl of Hot Star Red Taq Master Mix and 10µl PCR H2O, 0.5µl each of lukS-PV/ lukF-PV primers. PVL gene was amplified using the primers shown in Table 1. In each batch of the PCR reaction, PVL positive DNA sample and PCR water were added as PCR positive and negative control respectively. DNA amplification and the gel electrophoresis were carried out according to the protocol as described previously.

RESULTS

Prevalence of *S. aureus*

Out of six hundred and twenty-two (622) coagulase positive isolates obtained from the cloacae and nasal swab of chickens and nasal swab of workers only 290 (46.6%) were positive by agglutination test and were further screened and identified using microgen staph ID. Ninety-eight 98 of the isolates showed to be *S. aureus*, the rest were other species of Staphylococcus giving a prevalence of (33.8 %) (Table 1).

Table 1: Number of *S.aureus* identified by agglutination test and microgen Staph ID

Source	Coagulase positive	Agglutination positive	Microgen positive
Kano central	288	101	35
Kano North	155	88	39
Kano South	179	101	24
Total	622	290	98

Detection of 16SrRNA, and Nuc Gene

The 16SrRNA and the nuc gene were amplified and detected. Only 21 isolates showed the amplification of 800bp16SrRNA. This showed that all the isolates were *S. aureus* as 16SrRNA and nuc the thermostable nuclease gene were generally accepted as a genus species specific marker for detecting *S. aureus.* The result is shown in plate 1

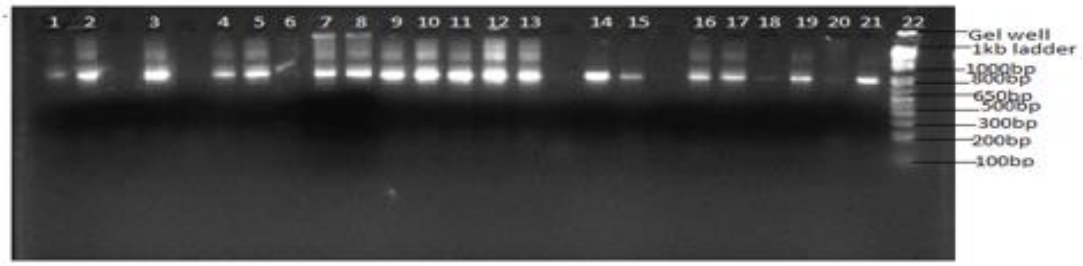

Plate 1: PCR product for 16SrRNA of *S. aureus* at 800bp

Lane 1-12: *S.aureus* isolates from cloacae. Lane 13-18: *S.aureus* isolates from nostril of the poultry. Lane 19-21: *S.aureus* isolates from poultry farm workers nostril. Lane 22: 1kb ladder.

Spa Typing of *S. aureus*

Sixteen of the isolates were sequenced for spa typing. Genes of seven (43.8 %) isolates were amplified and showed variation amongst the isolates and the amplicon size which ranges from 250 bp to 637 bp.

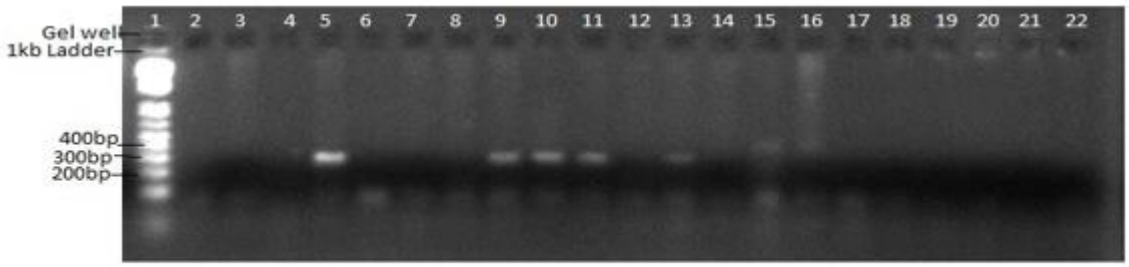

Plate 2: Spa typing of *S. aureus* on 1.5 % agarose gel electrophoresis

Lane 1: gel well ladder, Lane 5: Poultry MRSA from cloacae Spa positive. Lane 9-11: MRSA from cloacae, Spa positive. Lane 13: *S. aureus* from cloacae Spa positive Lane15 and16: MRSA from nostril Spa positive

Prevalence of mecA gene

mecA impart methicillin resistance and the presence of bands on the target area confirmed the organism to be MRSA. Out of the 98 *S. aureus,* 22 (22.4%) were found to be MRSA based on earlier MRSA detection method by cefoxitin-based susceptibility. Eight of the isolates possess mecA gene and the size of the amplicon correspond to 162 bp. Seven possess mecA gene and size of the amplicon correspond to 500 bp as represented by the mass ruler DNA ladder. The result is shown in plate 3.

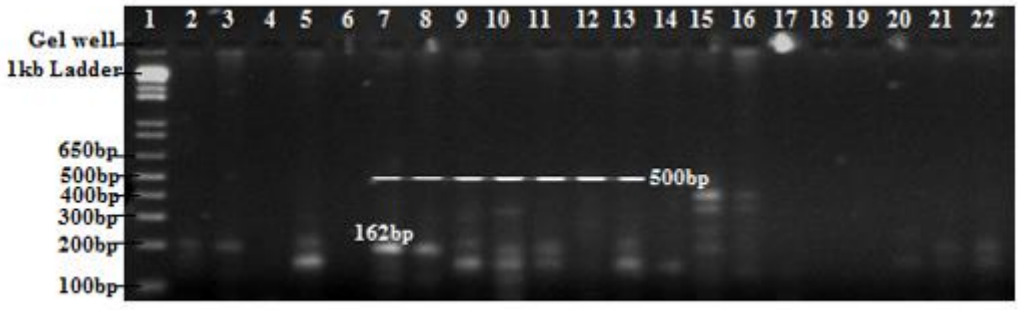

Plate 3: Multiplex PCR product for detection of mecA gene in *S. aureus*

Lane 1. 1kb ladder. Lane 2, 3 and 5: *S. aureus* from cloacae mecA positive. Lane 7- 13: *S. aureus* from cloacae mecA positive, Lane 20-22: *S.aureus* from nostril of farm worker mecA positive

PVL Detection

Staph. aureus isolates were tested for the presence of panton valentine leukocidin (PVL) luks-lukf genes. Genes from the fourteen isolates (14.3%) were amplified, (Plate 4).

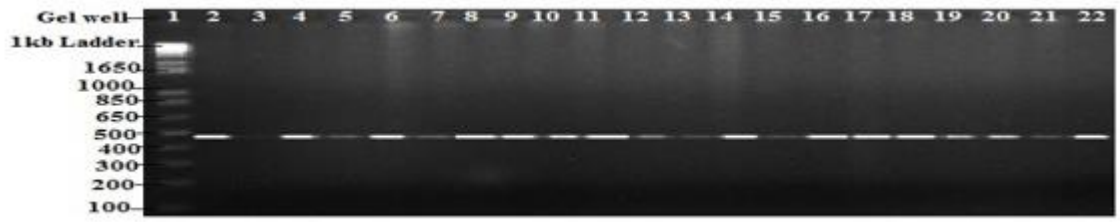

Plate 4: Amplification of PVL (lukS-luk F) gene in *S. aureus*

Lane 1: Ladder, Lane 2: MRSA PVL positive. Lane 4, 6*: S. aureus* from cloacae PVL- positive. Lane8-11 MRSA PVL positive. Lane14: *S. aureus* from nostril PVL- positive. Lane16-20 and 22: *S. aureus* from nostril PVL-positive.

DISCUSSION

S. aureus is a major cause of healthcare associated, community acquired and livestock associated infections throughout the world and the major problem associated with *S. aureus* infection is the acquisition of mecA gene by methicillin resistant *S. aureus (*MRSA). This study revealed a low (33.8 %) prevalence of *S. aureus* in poultry and poultry farm workers in Nigeria. Isolates were positive for the species-specific marker 16SrRNA.The *nuc* gene is used for identification for *S. aureus* strains (Braekstad *et al*., 1992). An intriguing finding in this study was the non-amplification of nuc the thermostable nuclease in most (63.6 %) of the isolates when screened, as compared to 16rRNA which showed more specificity and sensitivity. Thermostable nuclease gene nuc was reported to have 100% specificity and sensitivity for the identification of *S. aureus* isolates (Braekstad *et al*., 1992; Cai, *et al*., 2007). In India only, a few studies have reported the use of nuc along with mecA as molecular targets for identification of *S. aureus* and characterization of MRSA (Mathews *et al*., 2010; Chakraborty *et al*., 2010). *S. aureus* encodes many virulence factors including the surface Ig-binding protein A(Spa). Spa is an important virulence factor which enables *S. aureus* to evade host immune responses; it acts as an immunological disguise. Several studies have reported the use of mecA marker for detection of methicillin resistance and 16SrRNA and nuc for identification of *S. aureus* species. However, in Nigeria there are reported cases of detection of mecA gene in MRSA isolates from different parts of the country. In a research in Benin City, Nigeria, 4 (11%) isolates were confirmed to carry mecA gene (Obasuyi, 2013), while Clement, (2009) confirmed only one MRSA isolate from healthcare institutions from Ekiti and Ondo states. In another study carried out by Shittu *et al*., (2012), two MRSA isolates with mecA gene were detected in Ile-Ife, one from Lagos and two from Ibadan (South western Nigeria). In an international study of the prevalence of MRSA among Veterinarians, Wulf *et al*., (2008) reported that 12.5 % of participants from 9 countries carried MRSA and transmission of MRSA from pig to staff appears to be an international problem. The prevalence of MRSA in this study was 15.3 % out of the total *Staph. aureus* isolated (98) which is low compared to other studies in Morocco19.3 % (Elhamzavol *et al*., 2009) and Casablanca 35.4 % (Zriouil *et al*., 2012). Only 5 isolates out of 60 sampled from human source were found to be MRSA positive. Fourteen isolates were PVL positive (14.3 %). Several studies have reported an association between PVL genes and invasive diseases and are mostly associated with community acquired infections. PVL has been reported in China not only in CA-MRSA but HA-MRSA, which suggests that PVL is not an exclusive marker of CA-MRSA (Deurenberg *et al*., (2007); David and Daum, (2010)). Eleven of the PVL positive isolates in this study were from poultry. The finding of PVL genes amongst poultry isolates in this study portend great risks to those working in close contact with poultry birds due to its association with necrosis and skin infections. PVL positive isolates appeared to be more successful pathogens in skin and soft tissue infections (SSTIs) and because of its high transmissibility, these strains could cause serious clinical conditions if it makes its way into the community and hospital setting just like MRSA USA 300 (Omuse *et al,* 2013). This study confirms low (14.3 %) PVL prevalence in *S. aureus* compared to high prevalence of PVL in invasive disease that was found in Ghana (75%) (Dekker *et al*., 2016), 47% in Senegal, and across the globe (Breurec *et al*., 2011; Shallcross *et al*., 2013), prevalence of 40% was reported by Shittu *et al*. (2012) in Nigeria. The result of PVL prevalence observed in this study is comparatively higher than those reported in some other African countries and prevalence of 10.7% in Nigeria among isolates obtained from Maitama District Hospital (Orji *et al*., 2016). In contrast, our result was lower than the proportion of PVL-positive *S. aureus* reported in Maiduguri, Nigeria 52.1% by Okon *et al*., (2012). Our study, revealed a good relationship between MRSA and PVL with 10 out of the 14 (71.4%) PVL positive isolates also being MRSA. However, the prevalence of MRSA that harbors PVL in this study 71.4% is higher than that observed in many countries such as 9.4% in Zambia (Samutela *et al*.,2017), and 9% in South Africa (Abdulgader *et al*.,2015). It is also a suggestive of broader study to investigate the relationship between drug resistance and virulence in *Staph. aureus*.

CONCLUSION

In conclusion, it was observed that there was high PVL prevalence among *S. aureus* harboring mecA gene and there is no relationship between drug resistance and virulence

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