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ANTIBIOTIC SUSCEPTIBILITY PROFILE AND PLASMID CURING IN STAPHYLOCOCCUS AUREUS FROM CHICKEN DROPLETS IN ZARIA, NORTHWEST NIGERIA

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ABSTRACT

Staphylococcus aureus is one of the major bacterial pathogens associated with hospital, community acquired infection and recently livestock associated methicillin resistant *Staph. aureus* (MRSA). The aim of this study was to isolate and determine the antibiotic susceptibility profile and plasmid curing in *Staph*. aureus from chicken droplets in Zaria. A total of 250 samples were collected from 5 poultry farms. Sample were evaluated for the presence of staphylococci using Gram staining reaction, growth on mannitol salt agar, catalase, coagulase and DNAse tests. Antibiotic susceptibility was carried out using disc agar diffusion method. Plasmid curing was demonstrated using 0.5mg/ml acridine orange on antibiotic resistant isolates and beta lactamase production using nitrocefin test. The results revealed 98 *Staph.aureus,*88 was β- lactamase producers. Most isolates showed high resistance pattern to tetracycline (74.7%) and ampicillin (50.4%). A high percentage of the isolates were sensitive to ciprofloxacin 90.9%, vancomycin 76.1%, pefloxacin 72.3%, and gentamycin 65.9%. The percentage of phenotypic MRSA was 39.8%. Most (97.7%) of the β- lactamase producers had multiple antibiotic resistant index ≥0.3 indicating they originated from the environment where antibiotics are frequently used. The result of plasmid curing revealed that ampicillin (22%), oxacillin, (19%) and gentamycin (17%) showed the highest percentage reduction in 40% of the isolates after plasmid curing, indicating that the genes were not largely resided within the plasmid which may suggest partial involvement of chromosome in resistance. This study shows that, there is high incidence of *Staph.aureus* and high level of resistance to antibiotics which is mediated by β-lactamase and partial involvement of R-plasmid in mediating resistance

Keywords: Antibiotics, beta-lactamase,MRSA, poultry, resistance

INTRODUCTION

The rising incidence of methicillin resistance *Staphylococcus aureus* (MRSA) infection is concern for public health. Originally MRSA was a nosocomial pathogen causing infections in various group of individuals with defined risk factors. There are now numerous reports of community-acquired MRSA infections, worldwide, and MRSA strains have been isolated from various animals and animal derived products (Wall *et al.,* 2016). Carriage of *S. aureus* Sequence Type (ST) 398 has primarily been reported as occurring among persons in contact with livestock, including swine and cattle This association has given rise to the characterization of this strain as livestock associated (Jensen *et al*.,2010)). Antimicrobial such as penicillin and other beta-lactam are commonly used in livestock production for the treatment of disease associated with the *Staph aureus* and to improve production (Kadariya *et al*.,

2014). The development of resistance through the production of β-lactamase enzymes against β-lactam antibiotics by *S. aureus* creates a huge sum of economic burden (Wall *et al.,* 2016). This limitation had promoted the development and used of methicillin antibiotic, a new β-lactam drug that resisted β-lactamase producing *S. aureus*. The used of methicillin was stopped due to its toxicity, development of mecA resistant gene and widespread of resistance after a few years of production (Middleton, 2013).

Antimicrobial resistance plasmids of staphylococci are circular, double stranded DNA molecules that range in size from about 2kbp to larger than 100kbp. R plasmids share a number of basic properties with other plasmids such as replicating independently from the chromosomal DNA by using specific replication genes, distribute to daughter cells during bacterial cell division and can be exchanged between bacteria by horizontal gene transfer (Haaber *et al*., 2017). Numerous plasmids which differ considerably in size and structure have been described to mediate resistance in staphylococci of human and animal origin (Jensen *et al*.,2010). The range of staphylococcal resistance plasmids varies from small plasmids that carry only a single antimicrobial resistance gene to large plasmids that carry a number of different antimicrobial resistance genes occasionally coupled with genes that confer resistance to heavy metal ion, disinfectants and biocides (Lozano *et al*.,2012). Although, it is known that antibiotics are administered to control infection, but most of the antibiotics are administered at low level in feed for long durations to increase the rate of weight gain and improve the efficiency of converting animal feed to unit of animal production (Hao *et al*.,2014). These antibiotics belongs to the same chemical class to those used to treat human infections. This raises concern on the possibility of human cross infecting with chicken infecting bacteria (Garcia *et al*., 2014). It is known that overuse and misuse of antibiotics undoubtedly have favored the emergence and survival of resistant strains of microorganism. The aim of this study was therefore to isolate and to determine the antibiotic susceptibility profile and plasmid curing in *S. aureus* in poultry farms in Zaria.

METHODS

Study Design and Sample Collection

A cross-sectional study design was implemented to determine the incidence of *S. aureus* in chicken droplets in Zaria, Kaduna State, Nigeria. Five poultry farms were selected randomly and fifty samples were collected from each farm. Two hundred and fifty chicken droplet samples were aseptically collected using sterile spatula and deposited into a clean sterile universal bottle from the selected poultry farms in Zaria and transported to the Microbiology Laboratory, Ahmadu Bello University, Zaria for analysis.

Sample Size Determination

The sample size for the study was determined by the formula as described by Niang, (2006):

$N = [Z^2 (pq)]/d^2$

Where: N= the desired sample size

Z= Normal standard distribution that corresponds to confidence interval as 1.96

p= Prevalence

q = 1-p

d= degree of accuracy / precision expected at 0.05

Isolation and Purification of *Staphylococcus aureus*

Each sample was suspended in 5ml sterile normal saline and allowed to sediment. A Loop of the supernatant was spread plated on sterile Nutrient Agar (NA) plates and incubated at 37^0C for 24 hours (Kennedy and Wilbard,2015). After incubation period microscopic Gram staining was carried out. Gram positive isolates were plated on sterile Mannitol Salt Agar (MSA) plates using the streak plate method and incubated at 37^0C for 24 hours Cheesbrough (2006). The distinct tentative Staphylococcus colonies on the MSA plates were further purified on freshly prepared Nutrient Agar (NA) plates by repeated subculturing until pure colonies were obtained as described by Sampson, *et al.,* (2022). Obtained pure colonies were inoculated aseptically into nutrient agar slants in Bijou bottles and incubated for 24 hours at 37^0C. After incubation period, slants were refrigerated at 4^0C until needed.

Biochemical and Confirmatory Test

Catalase test

The ability of the isolates to produce an enzyme catalase was demonstrated by the addition of 1ml of 3 % w/v hydrogen peroxide solution to 24 hrs. culture of the isolate. Rapid evolutions of gas bubbles indicated the breakdown of hydrogen peroxide into oxygen and water by catalase peroxidase enzyme Cheesbrough (2006).

$$2H_2O_2 \rightarrow H_2O + O_2$$

Coagulase test

The tube coagulase method was carried out as described by Cheesbrough (2006). Pooled, EDTA-anticoagulated human plasma was diluted 1 in 10 with sterile normal saline. Aliquots of 0.5 ml were pipetted into sterile test tubes into which 0.1ml of 24 hrs nutrient broth (NB) culture of the isolate was added, mixed and incubated in water bath thermostat at 37^0 C for 4 hrs. At intervals of 30 minutes, the test tubes were observed for clotting of tube contents or fibrin clot in tubes. Two different test tubes were set up for positive control test (containing 24 hrs culture of a known *Staph. aureus*) and negative control (containing only sterile NB).

Deoxyribonuclease (DNAse) test

Deoxyribonucleic acid agar media (containing 0.2 % W/V of deoxyribonucleic acid) was prepared. An overnight broth culture of the test organism was spot inoculated onto the surface of the plate and incubated at 37^0 C for 24 hrs. The surface of the overnight plate was covered with 1 mol/1 hydrochloric acid solution and excess decanted. Clear zone around the colonies within 5 minutes of adding the acid indicated the production of the enzymes deoxyribonuclease by the organism (Cheesbrough, 2006).

Antibiotics susceptibility test

Antibiotics susceptibility testing of the *Staph aureus* isolate was carried out by the modified Kirby Bauer disc agar diffusion method as described by Cheesbrough, (2006). The results were interpreted using CLSI, (2017) guidelines. The susceptibility of the identified *Staph aureus* was tested against the following antibiotics: Ampicillin, oxacillin, methicillin, tetracycline, vancomycin, gentamicin, pefloxacin, and ciprofloxacin. Antibiotics selected were commonly used in our environment. A sterile swab stick was dipped into the tube containing the bacterial suspension and its turbidity was equivalent to 0.5m McFarland turbidity. The swab stick was pressed against the tube above the fluid level to remove excess broth. The swab was used to streak over the entire Mueller Hinton agar plate surface. The agar plate was allowed to dry for 5 minutes then the antibiotic disk was impregnated to the agar using a sterile

forcep on the surface of the inoculated plate 15mm away from the edge of the plate. Using the head of the sterile forcep the disk is slightly pressed down to ensure good contact with the agar. After applying the disk, the plates were incubated in an inverted position at 35^0C for 16 to 18 hours. The diameter of each zone of inhibition was measured and recorded accordingly (CLSI 2017).

β-lactamase production test

The ability of the identified *Staph aureus* to produce β-lactamase enzymes was determined using plate acidimetric method as described by Cheesbrough, (2006). Nitrocefin test was carried out to determineβ-lactamase hyper producers. Oxoid identification stick was used to detect beta lactamase producers. Nitrocefin is a cephalosporin developed by Glaxo Research Ltd. This compound exhibits a rapid distinctive color change from yellow to red as the amide bond in the beta lactam ring is hydrolyzed by a beta lactamase. The test was carried out according to manufacturer's instructions.

Multiple antibiotic resistance index (MARI)

Multiple antibiotic resistance is the resistance of isolates to three or more antibiotics. MARI was determined for all the isolates by using the formula:

MAR = A/B

where A denoted the number of antibiotics to which the test isolates depicted resistance

B = Total number of antibiotics to which the test isolate has been evaluated for susceptibility (Sampson *et al.,*2022).

Phenotypic Determination of Methicillin Resistance

Methicillin resistance was determined for all resistant isolates. CLSI recommends using cefoxitin when using disk diffusion method to determine methicillin resistance in *Staph. aureus* (CLSI, 2017). Cefoxitin results are easier to interpret and are thus more sensitive and enhance induction of PBP2a for the detection of mecA mediated resistance. A direct colony suspension of each *Staph. aureus* isolate was prepared equivalent to 0.5 McFarland standards and plated on Mueller Hinton agar surfaces. Cefoxitin 30µg disc was placed on the plate and was incubated at 36^0C for 24h. The zones of inhibition were measured and compared to that of CLSI interpretative chart (CLSI, 2017).

Plasmid curing

Plasmid present in the staphylococcal isolates which could be responsible for antibiotic resistance was eliminated by growth in the presence of acridine orange. Nutrient Broth (NB) broth was used as medium and acridine orange (0.5 mg/ml) was the curing agent. Standard inoculum of each isolate was prepared to obtain about 10^6cfu/ml. A loopful of each isolate was aseptically inoculated into NB tubes containing the acridine orange and incubated at 37^0C for 24hrs. The tubes were agitated at intervals to maintain an even distribution of the curing agent. After incubation, cured isolates were further subjected to antibiotic resistance screening to detect for plasmid-borne antibiotic-resistant species (Sampson *et al*., 2020)

RESULTS

Isolation and Purification of Isolates

The number of isolates identified on Gram reaction, mannitol fermentation, catalase test, coagulase test, DNAse test was shown in figure 1.

Phenotypic Determination of MRSA

A total of 35 of the 88 *S. aureus* isolates were resistant to cefoxitin 30µg (zone ≤

21mm). This shows that 39.8 % of the isolates were methicillin resistant *S. aureus* (MRSA) phenotypically

Antibiotic Susceptibility Profile

The number of isolates resistant/sensitive to each of the antibiotics is shown in table II. Out of the 88 *Staph. aureus* isolates recovered from the five farms, high percentage of resistance was observed against tetracycline and ampicillin

Beta – Lactamase Production Test

The result of β- lactamase production test showed that 88(89.8 %) produced β - lactamase. Ten were non β lactamase producers. The result is presented in figure II.

MAR Index

MARI showed that 77(87.5 %) isolates were resistant to three or more antibiotics. Eight isolates showed 100 % resistance to all the antibiotics tested. All the isolates were consistently resistant to tetracycline, ampicillin and oxacillin

Plasmid Curing

The overall percentage reduction in antibiotic resistance of *Staph. aureus* before and after plasmid curing is as shown in Figure III. The result revealed that ampicillin, oxacillin and gentamycin showed the highest percentage reduction 22%, 19% and 17% respectively. Following the treatment with acridine orange, no reduction was observed for tetracycline, pefloxacin and ciprofloxacin.

Table I: Prevalence of *Staphylococcus. aureus* and MRSA in Poultry Farms

Farm	No. of *Staph aureus* (%)	No. of MRSA (%)
1	11(12.5)	6(17.1)
2	20(22.7)	9(25.7)
3	20(22.7)	7(20.0)
4	24(27.3)	8(22.9)
5	13(14.8)	5(14.3)
Total	88(89.8)	35(39.8)

Table II: Antibiotics Susceptibility Pattern of the *Staph Aureus* and MRSA Isolates

Antibiotics	Staph aureus (n=88)		MRSA (n=35)	
	Sensitivity level (%)	Resistance level (%)	Sensitivity level (%)	Resistance level (%)
Ciprofloxacin	80(90.9)	8.0(9.1)	29(80.9)	6.0(19.1)
Vancomycin	67(76.1)	27(23.9)	27(77.1)	8.0(22.9)
Pefloxacin	64(72.3)	24(27.7)	19(54.3)	16(45.7)
Gentamicin	58(65.9)	30(34.1)	19(54.3)	16(45.7)
Methicillin	52(59.1)	36(40.9)	20(57.1)	15(42.9)
Oxacillin	51(57.9)	37(42.1)	21(60.0)	14(40.0)
Ampicillin	43(48.9)	45951.1)	14(40.2)	21(60.0)
Tetracycline	23(26.1)	65(73.9)	9.0(25.7)	26(74.3)

Table III: Antibiotic Resistant Indices of *Staphylococcus aureus* Isolates

No. of antibiotic to which resistant	Resistant isolates	MAR index	% of *Staphylococcus aureus* to MARI
1	2	0.1	2.3
2	9	0.3	10.2
3	13	0.4	14.8
4	10	0.5	11.4
5	14	0.6	15.9
6	15	0.8	17.0
7	17	0.9	19.3
8	8	1.0	9.0

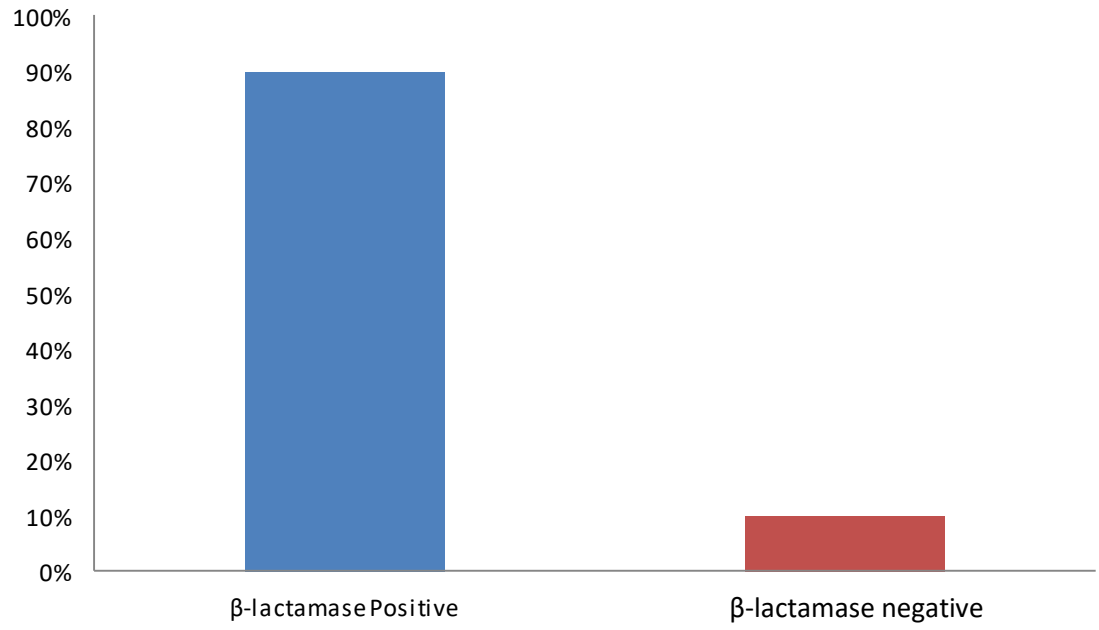

Figure 2: Percentage of β-lactamase Production

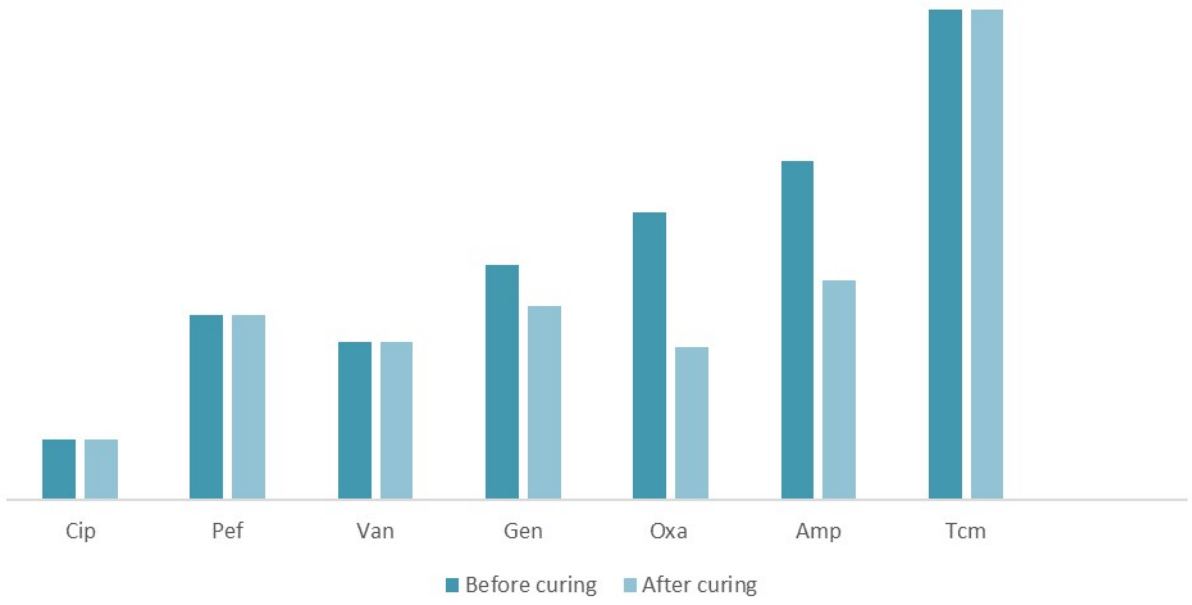

Figure 3: Percentage Reduction in Antibiotic Resistance of *Staphylococcus aureus* Before and after Plasmid Curing

Cip: ciprofloxacin Pef: perfloxacin Van: vancomycin Gent: gentamicin Oxa: oxacilin Amp: ampicillin Tcn: tetracycline

DISCUSSION

S. aureus have been previously recognized on poultry farms as an important pathogenic organism. Based on geographical locations and site of sample collection, different percentages of *Staph. aureus* from poultry farms have been reported (Hao, 2014). The percentage of *Staph. aureus* recovered from chicken droppings in this study was 39.2% as shown in table I. This might be associated with the feed which has been reported to carry contaminants including *Staph aureus* (Crump *et al*., 2017).

The bacteria isolated from chicken droppings in this study exhibited multiple antibiotic resistance that is clear indication of misused and overused of antibiotic which might have an important role in the development of antibiotic resistance in both humans and animals. The susceptibility profile observed in this study indicated that the isolates showed different pattern of susceptibility to conventional antibiotics. While majority of the isolates were resistant to tetracycline, ampicillin and oxacillin, the isolates were resistant to the other antibiotics in different proportion. Several factors have been known to account for bacterial resistance to antibiotics, including beta – lactamase production, plasmid mediated factors as well as some chromosomal factors. A good number of traits are encoded by plasmids (extra chromosomal pieces within the bacterial cytoplasm, which are expressed by the bacterial species. Genes for virulence factors, antibiotic resistance, detoxifying agents and enzymes for secondary metabolism have been found to be associated with plasmids (Mah,2012). The pattern of antibiotics susceptibility of the isolates reported in this study is similar to previous studies by other researchers

(Foster, (2017); Simpson, (2007); Kennedy and Wilbard,2015). *Staph aureus* isolates are still sensitive to ciprofloxacin, vancomycin, gentamycin, and pefloxacin with high level of resistance to tetracycline (73.9%) and ampicillin (51.1%) (table III). Some of the bacterial infections caused by *Staph. aureus* is contracted from under cooked meat or from drinking contaminated water or from surfaces contamination of raw produce such as vegetables. Given the fact that chicken droppings are applied on farm crops as manure. The antibiotic-resistant bacteria that persist in chicken manure can thus be transmitted to humans through consumption of vegetables and undercooked meat stuff, However, chicken manure is not treated before it is applied to farms (Thong and Modarressi,2013). The detection of β-lactamase enzymes in 89.8% of the *Staph. aureus* isolates points to the possible role of enzymes in mediating resistance especially to β-lactam antibiotics used in this study.

From this study, the overall percentage reduction in antibiotic resistance of *Staph. aureus* after plasmid curing was determined. 40% showed zones of inhibition (plasmid cured) while 60% showed lower zones of inhibition. The data indicated that oxacillin, ampicillin and gentamycin showed the highest percentage reduction in antibiotics resistance following the treatment with acridine orange. This result concurs with the work of earlier researchers (Adegoke and Okoh,2011; Onyeadi and Agbagwa,2019). The notable reduction in antibiotic resistance of the species to these antibiotics could therefore be attributed to the presence of resistance plasmid genes, which were eliminated after plasmid curing. No reduction was observed for tetracycline, pefloxacin and ciproploxacin. This is an indication that genes responsible for the resistance in those isolates were not largely resided within the extrachromosomal element (plasmid) which may suggest partial involvement of chromosome in the resistance (Onyeadi and Agbagwa,2019).

MAR index is an indication of the level of exposure of a given organism to different antibiotics, as it is an index to measure antibiotic resistance level. The MAR indices determined in this study is a good indication that a very large proportion of the isolated bacteria had been exposed to several antibiotics. An index of ≥ 0.2 is an indication of resistance to more than one drug, and increasing values relates with the number of drugs the isolate is resistant to (Garcia-Migura *et al*., 2014). As presented in results before and after curing, average MAR index from the different farms ranged from 0.1 to 1.0. This is a clear indication that different chickens from different farms were consistently exposed to different classes of antibiotics during the course of treatment or feeding thereby bringing about the high indices in MAR. High MAR indices have been reported to contribute to the development of superbugs (Hao *et al*., 2014)

CONCLUSIONS

There is high incidence of *Staph aureus* and MRSA in poultry farms in Zaria, Nigeria. The production of inactivating enzymes is the major mechanism that conferred resistance to most of the antibiotics used in this study rather than, possession of R-plasmid. It can be inferred that the *Staph. aureus* isolated from chicken are associated with multiple antibiotics resistance. The high average MAR index value indicated that the organism may have been previously exposed to different classes of antibiotics.

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