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MOLECULAR CHARACTERIZATION OF METHICILLIN RESISTANT *Staph. aureus* FROM POULTRY FARMS IN KANO STATE, NIGERIA

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ABSTRACT

The increasing rate of drug resistance associated with Methicillin resistant Staph. aureus is not only a problem in clinical sector but also in livestock disease treatment and management. This study was set out to evaluate the incidence of Methicillin resistant Staph. Aureus from poultry farm birds and the farm workers, and also to evaluate the likelyhood of cross-infection between the birds and the farm workers in Kano State, Nigeria, using standard microbiological and molecular methods. The results showed that out of the 1260 samples collected, 98(33.8%) isolates were confirmed to be Staph. aureus. Using disc diffusion method, the antibiotics susceptibility test results showed that 68 (69.4%) of the isolates were susceptible to cefoxitin while 30 (30.6%) were resistant. While 100% of the isolates were resistant to Ampicillin and Amoxicillin, 93.3% to Oxytetracycline, 90% to Chloramphenicol, 80% to Erythromycin, 76.7% to Oxacillin, 63.3% to Trimethroprim/Sulphamethozaxole, 30% to Ciprofloxacin and 26.7% to Gentamicin. The result also showed that 83.3% of the isolates had multiple antibiotic resistance index (MARI) of > 0.3 and were also multidrug resistant (MDR) while 16.7% had MARI ≤ 0.3. The molecular analysis showed that all the isolates were Staph. aureus of 800bp, 66.7% of the MDR isolates possess MecA gene (162bp), while 33.3% had MecA of500bp. Further analysis showed that 3 of the seven housekeeping genes (pta, gmk and yqil) were also present in the MDR isolates at 43.3%, 20% and 16.7% respectively while 10% express spa typing. The results also showed that there is a correlation between phenotypic cefoxitin resistance and carriage of MecA gene. The implication of these results in cross infection between the farm workers and the poultry birds as well as the danger of transfer of these resistant isolates to the community is discussed in this report.

Key words: Staph. aureus, cefoxitin resistance, poultry farms, housekeeping genes, spa typing, MDR.

INTRODUCTION

In poultry management, antibiotics such as β-lactams are often used in animal food production for growth promotion and routine disease prevention without prescription or control measures. This has necessitated the development of drug resistance superbug such as MRSA, which is now a major emerging public health problem. The incidence of both communities associated and hospital associated MRSA has been observed to increase due to drug abuse and zoonotic transfer of resistance gene through horizontal gene transfer (Adelisa *et al.,* 1992; Igwe *et al.,* 2013). The emergence of resistance against antibiotics of choice for the treatment of MRSA (e.g. Glycopeptides) has been highlighted as a significant cause of treatment failure to MRSA bacteremia due to increased Vancomycin MICs range (George *et al.,* 2004). Surveys conducted by the National Antimicrobial Resistance Monitoring System (NARMS) indicated that retail meat and poultry products are frequently contaminated with multidrug-resistant *Campylobacter* species, *Staph.* spp., *Salmonella* species, *Enterococcus* species, and *Escherichia coli* (Food and Drug Administration (FDA, 2007); but little information is known about the occurrence of MRSA in poultry in Kano State, Nigeria. Hence the need for re-evaluation of periodic antibiotics resistance surveillance and control strategies are advocated in all sectors of human endeavor including in poultry farm management.

MATERIALS AND METHODS

Sample collection, *Staph.* spp. Isolation, Microscopy and Biochemical Identification

One thousand two hundred and sixty (1260) poultry samples consisting of 600 cloacae samples, 600 nostril samples and 60 from poultry farm workers were collected aseptically in a clean sterile universal bottle from 12 poultry farms in Kano State and transported in an ice park to the laboratory for bacteriological examination. *Staph.aureus* isolation and microscopy was carried out using the method described by Cheesbrough (2000). Preliminary identification of *Staph.aureus* was carried out using catalase, coagulase and deoxyribonuclease (DNase) tests as described by Cheesbrough (2000) while further confirmatory test was carried out using STAPH Agglutination kit and Microgen STAPH kit (Oxoid Ltd. England).

Antibiotic Susceptibility Test, Multiple Antibiotic Resistance Index (MARI) Evaluation and Classification of Drug Resistance

The susceptibility profiles of the identified *Staph. aureus* was tested against Cefoxitin. Isolates that showed resistance to Cefoxitin were also evaluated for resistance against other antibiotics such as Oxytetracycline, Ampicillin, Ciprofloxacillin, Vancomycin, Gentamicin, Chloramphenicol, Erythromycin, Augmentin and Oxacillin using disc diffusion method as described by Cheesbrough (2000) and the corresponding results interpreted using Clinical Laboratory Standard Institute criteria CLSI (2014). The multiple antibiotic resistant (MAR) index was determined (as the number of antibiotic resistant to/ total number of antibiotics tested) for each isolate as described by Paul *et al.,* (1997). Classification of drug resistance (MDR, XDR) was also carried out according to the method described by Magiorakos *et al.,* (2012).For CLSI break point see appendix

Molecular Analysis

DNA Extraction

DNA extraction was carried out using Zymo research fungal/bacteria DNA miniprep™ kit with catalog number D6005 using manufacturer procedure, while the purity of the extracted DNA was quantified using Nano drop Thermo machine.

Polymerase Chain Reaction

PCR was carried out using a cocktail mix of 3.5µl of 2X master mix from Promega, 0.5µl of 5pMol forward primer, 5pMol reverse primer, 3µl of 25ng/µl of the extracted DNA and 2.5µl H_2O to form a 10µl PCR cocktail. The following pathogenic genes were evaluated in this study, *MecA*, the staphylococcal protein A (*spa*) which code for the polymorphic region of protein A (repeat polymorphism of the X-region of the *spa* gene) and the seven housekeeping genes (*arcC, aroE, glpF, gmk, pta, tpi, and yqiL*) using the under listed primers (Table 1) and PCR condition (Table 2):

Table 1: Primers for Molecular Characterization of MRSA in Poultry Farm

S/N	Primer name	Forward	Metabolic Function	Base pair
1	*mecA*$_{1F}$	TCCAGATTACAACTTCACCAGG		162
	mecA$_{1R}$	CCACTTCATATCTTGTAACG		
	mecA$_{2F}$	AAA ATC GAT GGT AAA GGT TGG C		
	mecA$_{2R}$	AGT TCT GCA GTA CCG GAT TTG C		500
2	*StaphF*	AAT CTT TGT CGG TAC ACG ATA TTC ACG		800
	StaphR	CGTAATGAGATTTCAGTA GAT AAT ACA AC		
3	*arcCF*	TTG ATT CAC CAG CGC GTA TTG TC	Carbamate kinase	456
	arcCR	AGG TAT CTG CTT CAA TCA GCG		
4	*AroEF*	ATC GGA AAT CCT ATT TCA CAT TC	Shikimate dehydrogenase	456
	AroER	GGT GTT GTA TTA ATA ACG ATA TC		
5	*glpF*	CTA GGA ACT GCA ATC TTA ATC	Glycerol kinase	465
	glpR	TGG TAA AAT CGC ATG TCC AAT TC		
6	*GmkF*	ATC GTT TTA TCG GGA CCA TC	Guanylate kinase	429
	GmkR	TCA TTA ACT ACA ACG TAA TCG TA		
7	*PtaF*	GTT AAA ATC GTA TTA CCT GAA GG	Phosphate acetyltransferase	474
	PtaR	GAC CCT TTT GTT GAA AAG CTT AA		
8	*TpiF*	TCG TTC ATT CTG AAC GTC GTG AA	Triosephosphateisomerase	402
	TpiR	TTT GCA CCT TCT AAC AAT TGT AC		
9	*yqiLF*	CAG CAT ACA GGA CAC CTA TTG GC	Acetyl-CoA acetyltransferase	516
	yqiLR	CGT TGA GGA ATC GAT ACT GGA AC		
10	*spa* 2F	GAACAACGTAACGGCTTCATCC		250-637
11	*spa* 1514R	CAGCAGTAGTGCCGTTTGCCT		~425

PCR Conditions

Table 2: Conditions for Molecular Characterization of MRSA in Poultry Farm.

Initial Den.	Denatu.	Ann. Temp	Extension	No. of circles	Final Ext.	Hold Temp.
94°C	94°C	56°C	72°C	72°C	72°C	10°C
5mins	30 Sec	30 Sec	45 Sec.	36	7mins	∞

Enright *et al.,* (2000)

Gel Electrophoresis

The amplified PCR products for each of the genes were run on a large gel in which 3grams of agarose were gradually heated to dissolve in 200ml of TAE buffer and 8µl of ethidium bromide was added to stain the amplicons. The PCR products were loaded on the gel and run on a Bio-radelectrophorectic machine at 120V for 1.5hrs. At the end, the gel bands were viewed using the Bio-rad Gel Doc XR transilluminator Machine.

Statistical Analysis

Data was entered in micro soft excel 2007 and then transferred to SPSS for analysis. Comparison between proportions was made using one way ANOVA for three means. Differences showing a critical value less than F value confidence level 0.05 or 0.01 were considered significant.

RESULTS AND DISCUSSION

Sample collection, Isolation and Biochemical Tests

Out of 1260 isolates evaluated 98 (33.8%) of the presumptive isolates were confirmed as *Staph. aureus*.

Antibiotics Susceptibility

Figure 1 showed the resistance profile of *Staph. aureus* resistant to cefoxitin in poultry farms in Kano State.

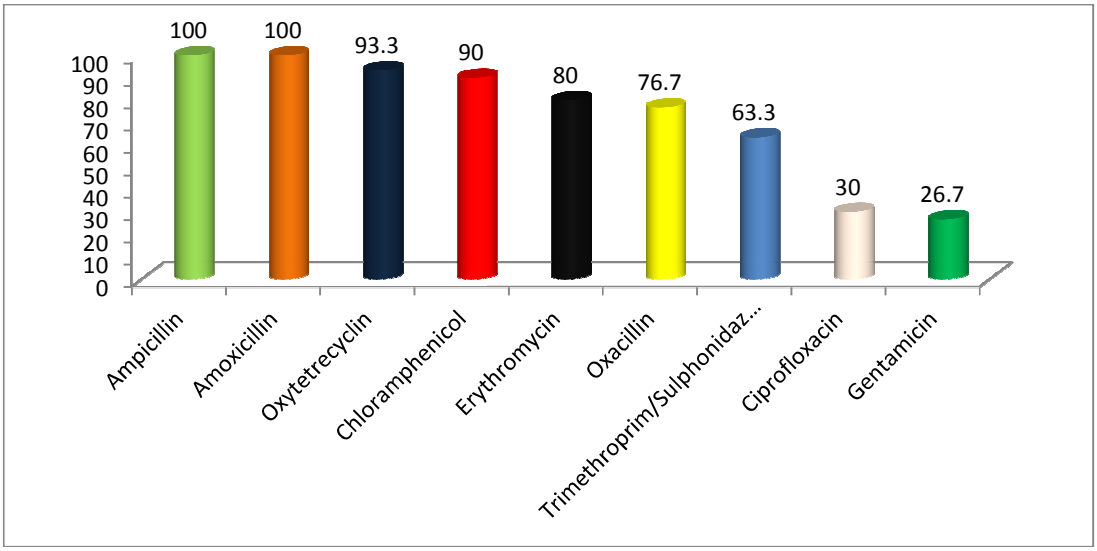

Figure 1: Antibiotics Resistance Profile of Cefoxitin Resistant *Staph. aureus* from Poultry Farms in Kano State, Nigeria.

The result below showed that 83.3% of the isolates had MARI of > 0.3 while 16.7% had MARI of ≤ 0.3.

Table 3: Antibiotic Resistance Pattern, Classification and MARI of *Staph. aureus* from Poultry Farms in Kano State, Nigeria.

S/N	Isolate Code	Resistance Pattern	NART	ARC	MARI
1	12C	AMP,AMX,OT,	3	XDR	0.3
2	7N	AMP,AMX,OT,C,E,OX,SXT	7	MDR	0.7
3	83C	AMP,AMX,OT,C,E,OX,CN, SXT	8	MDR	0.7
4	88N	AMP,AMX,OT,C,E,OX,SXT	7	MDR	0.7
5	3C	AMP,AMX,OT,C,E,OX,SXT	7	MDR	0.7
6	90C	AMP,AMX,OT,C,E,OX,SXT	7	MDR	0.7
7	93C	AMP,AMX,OT,C,E,OX,CN	7	MDR	0.7
8	151C	AMP,AMX,OT,C,OX,SXT,E	7	MDR	0.7
9	67N	AMP,AMX,OT,C,OX,E,SXT,CIP,CN	9	MDR	0.9
10	73C	AMP,AMX,OT,C,E,OX,SXT	7	MDR	0.7
11	91N	AMP,AMX,OT,C,E,OX,SXT	7	MDR	0.7
12	86C	AMP,AMX,OT,C,E,SXT,CIP,CN	8	MDR	0.8
13	68C	AMP,AMX,OT,C,OX,SXT	6	MDR	0.6
14	14C	AMP,AMX,OT,C,E,OX,SXT,CIP	8	MDR	0.8
15	12N	AMP,AMX,C,	3	XDR	0.3
16	39C	AMP,AMX,OT, E,OX, SXT, C,	7	MDR	0.7
17	90C	AMP,AMX,OT,C,E,OX,CIP,CN	8	MDR	0.8
18	76C	AMP,AMX,OT,C,CIP,E,	6	MDR	0.6
19	31N	AMP,AMX,OT,C,E,OX,CN	7	MDR	0.7
20	145C	AMP,AMX,OT,C,E,OX,CN	7	MDR	0.7
21	69C	AMP,AMX,C,	3	XDR	0.3
22	64C	AMP,AMX,OT,C,E,OX,SXT,CIP	8	MDR	0.8
23	182C	AMP,AMX,OT,C,OX,SXT,E	7	MDR	0.7
24	184C	AMP,AMX,OT,C,E,SXT,CN,OX	8	MDR	0.8
25	95C	AMP,AMX,OT,C,E,OX,SXT,CIP	8	MDR	0.8
26	41C	AMP,AMX,OT,C,E,OX,SXT	7	MDR	0.7
27	35C	AMP,AMX,OT	3	XDR	0.3
28	39N	AMP,AMX,OT,	3	XDR	0.3
29	190N	AMP,AMX,OT,C,E,OX,CIP, SXT	8	MDR	0.8
30	199C	AMP,AMX,OT,C,E,CIP,OX,	7	MDR	0.7

Keys: NART = number of antibiotics resistance to, ARC = antibiotics resistance classification, MARI = multiple antibiotics resistance index, OT= Oxytetracycline, AMP = Ampicillin, CIP= Ciprofloxacillin, VAN=Vancomycin, CN=Gentamicin, C=Chloramphenicol, E=Erythromycin, and OX= Oxacillin, SXT= Trimethomprim/sulfamethaxazole

MOLECULAR ANALYSIS

Twenty three (76.7%) of the cefoxitin resistance *Staph. aureus* isolates that showed MDR to at least 7 antibiotics were subjected to molecular analysis for the presence of *MecA* genes.

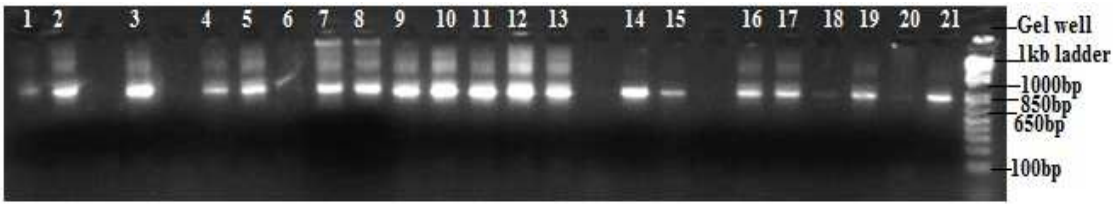

Figure 2: PCR product for 16SrRNA of *Staph. aureus* at 800bp

The molecular analysis showed that all the isolates 23 (76.7%) were *Staph. aureus* of 800bp, 66.7% of the MDR isolates posses 9 *MecA* gene (162bp), while 33.3% had *MecA* of 500bp.

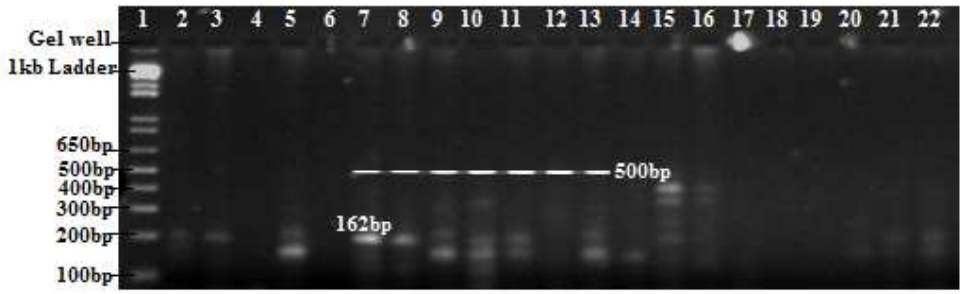

Figure 3: Molecular Characterization of *MecA* (162bp and 500bp respectively)

Further analysis showed that 3 of the seven housekeeping genes (*pta, gmk*and*yqil*) were also present in the MDR isolates at 43.3%, 20% and 16.7% respectively (Figure 5) while 10% express spa typing gene (Figure 6).

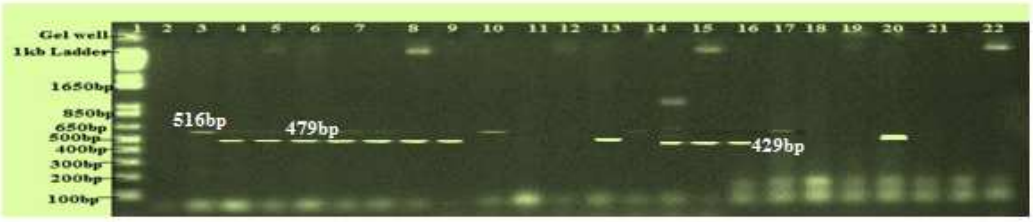

Figure 5: Molecular Characterization of the Seven Housekeeping genes (*pta*(479bp)*, gmk*(429bp)and*yqil*(516bp).

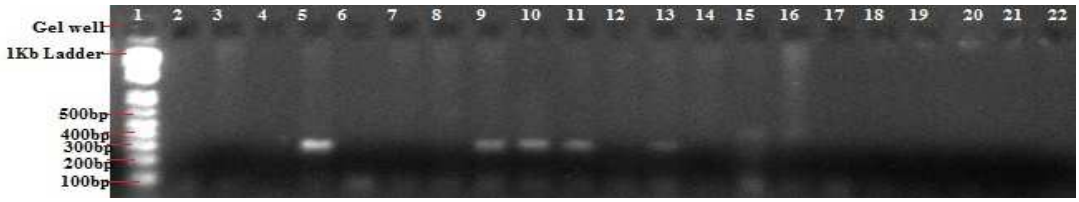

Figure 6: Molecular Characterization of Spa gene (250-637bp)

Staph. aureus remains one of the predominant health care emerging problems as a result of increase in pathogenicity and Methicillin-resistant *Staphylococcus aureus* (MRSA) in hospitals, community and in poultry, with multidrug resistance characteristics. The occurrence rate of *Staph. aureus* from poultry farm in Kano State (33.8%) observed in this study is lower than that reported by of Suleiman *et al.,* (2013), who reported the occurrence of 83% *Staph. aureus* out of 100 tracheal swabs from 100 apparently healthy chickens among small holder flocks in Maiduguri, Nigeria and Mohamed (2013) in Egypt who reported 40.8% (51/125) occurrence of *Staph. aureus*. But higher than the report of Neela *et al.,* (2013) who reported 1.4% (7/503) in Malaysia. However our result substantiates other reports that *Staph. aureus* is a normal flora of human and animal skin and nasal trachea. Significant numbers of the isolates (30.6%) were observed to show resistance to cefoxitin; a presumptive identification for MRSA in *Staph. aureus* and subsequently showed varying antibiotics resistance patterns and percentage resistances. This finding also concur with the report of Suleiman *et al.,*(2013) who reported that coagulase positive *S. aureus* isolates were susceptible to ciprofloxacin and gentamicin but showed varying degrees of resistance to other antibiotics with most resistance to betalactam (Ampicillin and Amoxicillin). Thirty three percent (83.3%) of the isolates are MDR and have MARI > 0.3 showing that the isolates have been pre-exposed to antibiotics tested in this study with high multidrug resistance potential. This might be as a result of uncontrolled usage of antibiotics in poultry feeds for growth and diseases. Molecular characterization of *mecA* gene among these isolates showed that all the MDR isolates were *Staph. aureus* of 800bp , 66.7% of the MDR isolates also possess *MecA* gene (162bp), while 33.3% had *MecA* of 500bp.These finds concur with the report of Adeyeye and Adewale (2013) in South West Nigeria, who recorded 83.3%MRSA incidence in poultry attendants, and 95% in chickens from a poultry farm.

MRSA of human-origin has been isolated from raw chicken meat or carcasses in Korea (Lee, 2006) and Japan (Kitai *et al.,* 2005). Though the pathogenicity of MRSA from different sources varies, those associated with poultry (livestock associated MRSA (LA-MRSA)) rarely cause disease (s). Evolutionary analyses on the seven multilocus sequence typing (MLST) genes that encode for proteins of central metabolic functions and causes phenotypic differences (Enright *et al.,* 2000; Lamers *et al.,* 2011) was also carried out on the MDR MRSA to analyze the genetic diversity present in the isolates. The result showed that 3 of the seven housekeeping genes (*pta, gmk* and *yqil*) were also present in the MDR isolates at 43.3%, 20% and 16.7% respectively while 10% express Spa typing. The results showed that there is a relationship between phenotypic cefoxitin resistance and carriage of *MecA* gene.

CONCLUSION

The high prevalence of *Staph. aureus* means an extensive distribution of the organism amongst poultry farms. It is concluded that; the use of conventional biochemical method characterizing *Staph. aureus* is no longer reliable, isolates are generally resistant to β- lactam antibiotic and oxytetracycline. All the MRSA in this study were multi drug resistant.

RECOMMENDATION

This study therefore recommends proper food hygiene and cooking in all abattoir and eateries, which is believed to reduce the risk of infection and possible colonization. Also strong awareness is advocated in otder for consumers to understand the importance of proper handling of poultry products to avoid/reduce cross contamination of MRSA.

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APPENDIX ONE

INTERPRETATIVE CHART FOR ANTIMICROBIAL SUSCEPTIBILITY TESTING

Antibiotics	Disc Potency	Diameter of zone of growth inhibition (mm)		
		Susceptibility	Intermediate	Resistance
Vancomycin	30µg	≥15	_	_
Cefoxitin	30µg	≥22	_	≤21
Amoxicillin clavulanic acid	30µg	≥20	_	≤19
Ampicillin	10µg	≥29	_	≤28
Gentamicin	10µg	≥15	13-14	≤12
Oxacillin	1µg	≥13	11-12	≤10
Erythromycim	15µg	≥23	14-22	≤13
Neomycin		≥17	_	≤16
Ciprofloxacin	5µg	≥21	15-12	≤15
Sxt	25µg	≥16	11-15	≤10
OXT	30µg	≥15	12-14	≤11

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