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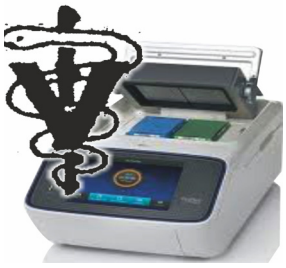

Isolation, Antibiotic susceptibilty testing and Detection of Antibiotic resistant Genes of Salmonella gallinarum and pullorum Isolated from Plateau State, Nigeria

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Abstract

The aim of this study was to determine the antibiotypes of S*almonella enterica var* Gallinarum and Pullorum isolated from chickens in Plateau State, Nigeria. The study adopted a passive survey design. Diseased chicken carcasses from Plateau State submitted to the Diagnostic Department of the National Veterinary Research Institute, Vom, Plateau State for post mortem examination and laboratory diagnosis were used for the study. The study was conducted between January and December, 2017. Based on case history and post mortem findings of suspected cases of salmonellosis, samples (heart blood, sections of the liver and gall bladder, spleen, lungs, ovary, kidneys and intestinal segments) from 494 chickens were collected and processed for *Salmonella* isolation, antibiotic sensitivity tests and antibiotic resistant genes type detection. The bacteriology samples were collected aseptically and inoculated into peptone water and incubated at 37^{o}C for 18 hours, then sub-cultured into Rappaport Vassiliadis broth and incubated at 42^{o}C for 18 hours, and finally sub-cultured on MacConkey agar (MCA) and incubated at 37^{o}C for 24 hours. Isolates were Gram stained and characterized phenotypically using standard biochemical tests such as citrate test, reaction on triple sugar iron agar, urease test, methyl red-Voges-Proskauer test, glucose and dulcitol fermentation and hanging drop motility test. Serotypes of the isolates were identified by slide agglutination test. Susceptibility of the isolates to 14 antimicrobial agents was carried out using the disc diffusion method. Antimicrobial resistance genes were assessed using polymerase chain reaction (PCR). Chi square was used to test association between resistance and the *Salmonella* serotypes. Significance was accepted at $p < 0.05$. *Salmonella* Gallinarum and Pullorum were isolated from 45(9.1%) and 5(1.0%) respectively of the chicken carcasses examined. Thirty-five (70%) of the 50 isolates exhibited resistance to at least one antimicrobial agent. Resistance to sulphamethoxazole/ trimethoprim was the highest (58%), followed by ampicllin (46%) and enrofloxacin (40%) while the least resistance was to meropenem 3 (6%). Of the 35 resistant isolates, 26 (74.3%) were multidrug-resistant. Genes encoding aminoglycosides (*aad*A), quinolones (*gyr*A) and sulphamethoxazole (*sul*1) were detected in 1(3.3%), 26 (86.7%) and 28 (93.3%) respectively of the isolates analyzed.

Keywords: Isolation, Salmonella Gallinarum and Pullorum, Antibiotic sensitivity tests (AST) and antibiotic resistant genes detection

Introduction

Fowl typhoid is a disease of poultry that is characterized by acute systemic infection, with mortality rates reaching 80% in affected flocks of any age (Rafael *et al*., 2016). The disease is caused by *Salmonella enterica* serovar Gallinarum biovar Gallinarum (*S*. Gallinarum) and S. enterica serovar Gallinarum biovar Pullorum (S. Pullorum) which causes pullorum disease. The disease is a severe septicemic disease that causes high morbidity and mortality in birds, specially newly hatched chicks, causing high losses to the poultry producers (Okwori *et al*., 2007, Salihu *et al*., 2014, Rafael *et al.*, 2016).

The transmission of this disease is both horizontal and vertical. This mode of transmission is important in the epidemiology of the disease worldwide. Vertical transmission of Salmonella to progenies through infected eggs and birds leads to chronic carriers in breeding flocks for both organisms (Agada *et al*., 2014, Moses *et al*., 2015). Ingestion of food or water already contaminated with faeces of clinically infected birds or carriers, presence of dead chickens, poultry farm attendants and contaminated feeds leads to horizontal transmission of salmonellosis (Agada *et al.*, 2014). In Nigeria, the expansion of the poultry industry has a direct proportion on fowl typhoid as the disease has gained ground as a major disease of poultry (Salihu *et al*., 2014). The prevalence of fowl typhoid has been put at 18.6% and 19.3% in local and exotic chickens respectively in Jos, Plateau State (Salihu *et al*., 2014), while that of Kaduna State is rated at 18.4% (Mbuko *et al.*, 2009).

The two biovars cause host-specific infections in birds (Nwiyi *et al.*, 2018). Some variants of *S. enterica* namely *Salmonella* Gallinarum (SG) and *Salmonella* Pullorum (SP) are non-flagellated, thus are non-motile, but majority of members in the genus Salmonella are motile by possessing a peritrichous flagella. The clinical disease in poultry caused by SG and SP result in considerable economic losses due to the replacement of infected flocks and associated treatment costs to poultry farmers, especially in developing countries of the world (Agada *et al.*, 2014; Jajere, 2019). Biosecurity, cleaning and disinfection of the facilities are the major ways of controlling the incidence of these bacteria in hatcheries and farms (Rafael *et al.,* 2016). The method of prevention of this disease is by vaccination (Lee *et al*., 2005, Rafael *et al.*, 2016). However, failures in biosafety measures and/or ineffective immunization, leading to outbreaks in commercial flocks have been reported worldwide (Shivaprasad, 2000).

Antibiotics have been successfully used in veterinary medicine as food animal growth promoting agents and as probiotics for prophylaxis and as therapeutics. However, they are being used indiscriminately thus causing enormous antimicrobial resistance among bacterial pathogens worldwide; this is often seen in Salmonella strains isolated from poultry and poultry environments (Agada *et al.*, 2014). Presently, there is increasing concern of the development of multidrug resistance in Salmonella strains causing fowl typhoid in poultry (Moses *et al.*, 2015). To achieve best results, testing is carried out in clinical microbiology laboratories to detect possible drug resistant pathogens and to assure susceptibility to first line drugs for particular bacterial isolates involved for all infections (Jorgensen and Ferraro., 2009). One of the most widely used testing methods today includes broth microdilution or rapid automated instrument methods. The manual disc diffusion and gradient diffusion methods are flexible and are possibly cost saving. Each method has its own strengths and weaknesses, including the organisms that may be accurately tested by the method. Quantitative results are provided by some methods (e.g., minimum inhibitory concentration), and all provide qualitative

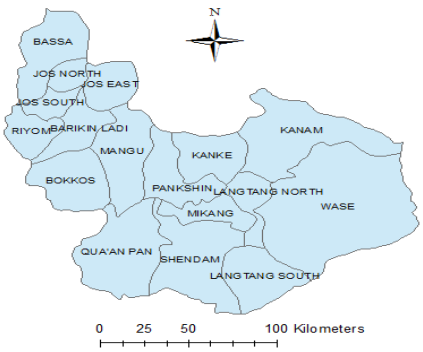
assessments using the categories susceptible, intermediate, or resistant. Generally, current testing methods do provide accurate detection of common antimicrobial resistance mechanisms. However, newer or emerging mechanisms of resistance require constant surveillance regarding the ability of each test method to accurately detect resistance (Jorgensen and Ferraro, 2009). Stringent control of antibiotic use coupled with effective surveillance of antibiotic resistance patterns, have successfully reduced the prevalence of antibiotic resistance to these agents in developed countries (Agada *et al*. ,2014). The situation in the developing countries like Nigeria is however different, where antimicrobial agents are readily available in local drug stores without prescription at most times (Agada *et al*., 2014).

The present study was aimed at detecting the antibiotypes of S*almonella enterica var* Gallinarum and Pullorum from diseased chickens in Plateau State, Nigeria

2.0 MATERIALS AND METHODS

2.1 Study area: The study area is Plateau State, Nigeria. The State (Plateau) is situated on coordinates 9° 10'N 9° 45'E and having a population of 3,178,712 at 2006 census and a total land area of 30,913Km^2 (Ehizibolo *et al*., 2013, Wikipedia, 2017).

Fig. 1: Map of Plateau Showing the 17 Local Government Areas (LGAs)



2.2 Sample collection: A passive survey design was adopted for the study. All the suspected avian salmonellosis cases submitted to the Diagnostic Department of National Veterinary Research Institute, Vom, were used for the study. A total of 494 avian cases were received from Plateau State within the period of January and December, 2017 and all samples were processed for salmonella isolates. At post mortem, the liver and gall bladder, spleen, heart, ovary or yolk sac and intestine were collected aseptically. Samples from each tissue were processed for *Salmonella* isolation and identification. Records of age, location, bird type, stock size, morbidity and mortality were obtained on each case presented.

2.3 Sample processing: Tissues collected from post mortem were directly streaked on MacConkey agar aseptically. The intestinal portions collected were pre-enriched in 225 ml of selective enrichment broth Rappoport Vassiliadis Soya (RVS); both were incubated at 37ºC for 24 hours. After 24hours of the pre-enriched Rappoport Vassiliadis (RVS), they were streaked on MacConkey agar. The growths were studied to differentiate the lactose and non lactose fermenters. The non lactose fermenters were Gram stained for the pinkish rods that are either single or in pairs. The non lactose fermenters were further subcultured on MacConkey agar to have pure colonies.

2.4 Isolation and identification of *Salmonella*

2.4.1 Presumptive isolation of *Salmonella*: The sub-cultured isolates on MacConkey agar plates were examined for the presence of typical colonies of Salmonella based on cultural

and morphological characteristics. The colonial morphology on MacConkey agar appeared colorless, translucent, smooth and raised. The pure isolates were subsequently characterized biochemically (Chanhadras *et al.,* 2017; Jajere, 2019).

2.4.2 Biochemical characterization of *Salmonella*

Isolation and identification of organisms were carried out as described by Jajere *et al*. (2019). A 24 hour pure culture of each isolate was used to determine their Gram stain reaction. The following biochemical tests were carried out: citrate test, Indole test, triple sugar iron test, methyl-red test, Voges-Proskauer test, lysine decarboxylase test, urease test, sugar (trehalose, sucrose, inositol, glucose, dulcitol, maltose, mannitol, melibiose, salicin, rhamnose and arabinose) fermentation test and motility test.

2.5 Serotyping of isolates: The somatic (O) and flagella (H) antigens were serotyped using the polyvalent Salmonella antisera (Oxoid, UK) according to Kauffmann White Scheme by slide agglutination test (Kauffman, 1974).

2.5.1 Antimicrobial susceptibility test: The *in-vitro* susceptibility testing of *Salmonella* isolates to fourteen commonly used antimicrobials was carried out by the standard disc diffusion technique using guidelines of NCCLS (2016).

2.5.2 Standardization of inoculums: The standardization of the inoculum was done as described by CLSI (2016). *Salmonella* isolates pure culture in a plate cultured for 18-hours was used. Two to three colonies of *Salmonella* were picked with a sterile wire loop and emulsified in 5 ml of sterile normal saline. The tube containing the bacterial suspension was inserted into a calibrated sensititre nephelometer (TREK Diagnostic systems, UK). Adjustment was made with addition of extra inoculum or diluents, until 0.5 McFarland standards were obtained. Fifty microliter of the broth was further transferred into 5 ml of Mueller-Hinton broth (Oxoid, UK) in a tube.

2.5.3 Inoculation and discs application on test plates: This was carried out as described by NCCLS (2016). When the turbidity of the inoculum suspension was adjusted and within 5-10 minutes, a sterile cotton swab was dipped into the standardized suspension in Mueller-Hinton broth, the dried Mueller- Hinton agar plate was inoculated by streaking with the cotton swab over the entire sterile agar surface. The inoculated plates were allowed to air dry before applying the antibiotic discs. The antibiotic discs (Oxoid, UK) were dispensed unto the surface of the inoculated agar plate using a disc dispenser and were gently pressed down to ensure complete contact with the agar surface. The plates were inverted and incubated at 37ºC for 18 hours. The following 14 antibiotic discs were used Ampicillin (Amp), Amoxicillin (Amc), Ceftoxitin (Fox), Tetracycline (Te), Ceftatoxone (CRO), Kanamycin (K), Chloramphenicol (C), Ciprofloxacin (CIP), Sulfamethoxasole/ trimethoprim (SXT), Enrofloxacin (ENR), Meropenem (Mem), Norfloxacin (NOR), Streptomycin (S) and Ceftaxidime (CAZ).

2.6 AMPLIFICATION OF TARGET DNA

The target DNA was amplified by polymerase chain reaction (PCR). The procedure according to Promega Corp., Madison USA was conducted in a volume of 25µl containing 20µl of the genomic DNA of all the isolates of *Salmonella.* The following two primers that are specific for *Salmonella* were used for the amplification (5-CCTACGGGAGGCAGCAG) and 3-CCGTCAATTCCTTTRAGTTT), Inqaba Biotechnical Industries, South Africa, was used. A typed *Salmonella* strain was used as positive control

2.6.1 Antimicrobial Resistance Gene Detection

Genes coding for resistance to antibiotics were investigated in the isolates using PCR in accordance with the procedures described below: the target genes, their corresponding primer sequences and amplicon sizes are presented in Table 2. The resistant genes targeted depended on the antibacterial resistance profile of each *Salmonella* isolate tested. The cycling conditions and primer sequences used were as described by Ishmael *et al*. (2019). The PCR was performed with Master buffer (Biolab-Rad, New England) 5 ul of DNA template, 10 µmol each of the forward and reverse primers and nuclease free water to make a final volume of 25 µl. PCR conditions for each antibiotic gene were as follows:

For tetA, there was 5 minutes of initial denaturation at 94°C followed by 35 cycles of denaturation at 94°C for 1 minute, then 55°C denaturation for 1 minute and 72°C for 1.5 minutes and a final incubation at 72°C for 5 minutes. For aminoglycosides, there was 94 °C of denaturation followed by 30 cycles of denaturation at 94 °C for 45s, annealing for 45s at 50 °C and an extension at 72 °C for 45s and a final extension for 7mins. For Beta-lactams (Blatem), there was an initial denaturation at 94 °C for 5 mins followed by 30 cycles of of denaturation of 94 °C for 30s and an annealing of 60 °C for 30s and an extension of 72 °C for 90s and a final incubation of at 72 °C for 5mins. For phenicols (cat1), there was an initial denaturation for 5 mins at 94 °C followed by 30 cycles of at 94 °C for 30s, then 50 °C for 30s and 72 °C for 90s and a final extension at 72 °C for 5 mins. And for sulphonamides (sul 1), there was an initial denaturation at 94 °C for 5 mins followed by 1 min of denaturation at 94 °C, 1 min of annealing at 55 °C, and 5 mins of extension at 72 °C for a total of 35 cycles and 5 mins of final extension at 72 °C. All PCR assays were performed using a thermocycler (Bio-Rad Mycycler, USA). And for the determination of gyrA the method was done according to the manufacturer's protocol as described by Mokhtari-Farsani *et al*. (2015). Polymerase chain reaction (PCR) was performed in final volume of 25 uL, PCR reactions containing 50–100 ng of genomic DNA template, 2.5 uL of 10X PCR buffer, 1.5 mM MgCl2, 200lM dNTPS, 40 ng of each primer and one unit of Taq DNA polymerase enzyme. Thermal PCR conditions consisted of initial 5 min at 95 °C and then 32 cycles of denaturing temperature at 94 °C for 1 min, annealing temperature at 56 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. Gene fragments were subjected to digestion by restriction enzymes in a total volume of 20 mL (10 mL reaction solution, 2 mL enzyme buffers, 0.2 mL enzymes and 7.8 mL distilled water) and placed in the incubator at 37°C for 5 hours and 100 bp ladder was used (Kumari *et al.,* 2013).

2.6.2 GEL ELECTROPHORESIS:

The amplified DNA products from *Salmonella* specific-PCR were analyzed by Electrophoresis on 1.5% agarose w/v gel stained with ethidium bromide and visualized by UV illumination. A current of 120V shall be applied to each gel. 10 µl of PCR product mixed with 2 µl of 6 x loading dye will be loaded on to the agarose gel. A 100 bp DNA will be used as a marker for PCR products. After electrophoresis, generated bands were screened and digitally photographed under UV light (Rahn *et al.,* 1992).

2.7 Data Analysis: This was done using Chi square

3.0 Results

Salmonella enterica serovar Gallinarum and Pullorum are poultry adapted bacteria that cause two diseases, fowl typhoid and pullorum disease respectively, in poultry (Shivaprasad, 2000). In this study, 50 out of 494 diseased carcasses (10.12%) submitted were diagnosed positive.

The isolation rate of SG was 45(9.1%) and SP was 5(1.0%). The highest percentage of isolation from the internal organs was, heart (32%), liver (24%), spleen (26%) and this was in agreement with Rafael et al. (2016), the ovarian follicles (10%) and (8%) intestines (Table1).

Table 1: Occurrence of *Salmonella* isolates by Organ Tissue

Organs	Occurrence	Percentage (%)
Liver	12	10(24.00%)
Heart	14	12(28.00%)
Spleen	13	10(26.00%)
Intestine	4	3(8.00%)
Bile	2	2(4.00%)
Ovarian follicles	5	5(10.00%)
Total	50	100.00%

The antibacterial resistance profile and the resistance pattern of *Salmonella* Gallinarum and Pullorum isolated were as presented below (Tables 2 and 3) respectively. Thirty five (35) isolates were resistant to at least an antibiotic. The highest resistance was that of sulphamethoxazole 29 (70.8%), ampicillin 23(56.1%), enrofloxacin 20(48.8%) and the least resistance was meropenem (7.3%). Twenty six (26) of the isolates recorded multidrug resistance (Table 2).

Table 2: Antibacterial Resistance Profile of *Salmonella* Isolates

Antibacterial agent	**No Resistant**	**% Resistant**
Ampicillin (Amc)	23	56.10
Amoxicillin (Amc)	6	14.63
Ceftoxitin (Fox)	7	17.10
Tetracycline (Te)	6	14.63
Ceftatoxone (CRO)	6	14.63
Kanamycin (K)	18	43.90
Chloramphenicol (C)	13	31.71
Ciprofloxacin (CIP)	11	26.83
Sulfamethoxasole/trimethoprim (SXT)	29	70.73
Enrofloxacin (ENR)	20	48.78
Meroperem (Mem)	3	07.32
Norfloxacin (NOR)	15	36.59
Streptomycin (S)	9	21.95
Ceftaxidime (CAZ)	4	09.80

Table 3: Showing Antibacterial Resistance Pattern of *Salmonella* Isolates

ISOLATE	Resistant Pattern	No. (%) of Isolate resistant
1	FOX, TE, AMP,CRO, S, AMC. K, C, CIP, SXT, ENR	11(78.57)
2	MEM, NOR, AMP, S, K, C, CIP, SXT	8(57.14)
3	AMP, S, K, CIP, SXT, ENR	6(42.86)
4	S	1(7.14)
5	S, K, SXT	3(21.43)
6	SXT	1(7.14)
7	NOR, AMP, CRO, K, C, CAZ, CIP, SXT, ENR	9(64.29)
8	FOX, TE, NOR, AMP, CRO, S, AMC, K, C, CIP SXT, ENR	12(85.71)
9	FOX, S, K, SXT, ENR	5(35.71)
10	TE, NOR, AMP, S, K, C, CIP, SXT, ENR	9(64.29)
11	CRO, SXT	2(14.29)
12	NOR, AMP, S, C, SXT, ENR	6(42.86)
13	TE, NOR, AMP, CRO, S, K, CAZ,	7(50.00)
14	FOX, TE, NOR, AMP, S, K, CIP, SXT	8(57.14)
15	NOR, AMP, AMC, CIP, ENR	5(35.71)
16	SXT	1(7.14)
17	AMP, S, SXT	3(21.43)
18	NOR, AMP, S, C, CIP, SXT, ENR	7(50.00)
19	NOR, AMP, S, K, C, SXT, ENR	7(50.00)
20	TE, AMP, AMC, K, C, SXT, ENR	7(50.00)
21	NOR, AMP, S, K, SXT, ENR	6(42.86)
22	FOX, TE, NOR, AMP, S, C, SXT, ENR	8(57.14)
23	TE, AMP, NOR, AMC, S, C, SXT, ENR	8(57.14)
24	AMP, S, SXT, ENR	4(28.57)
25	FOX, MEM, TE, NOR, CRO, S, AMC, C, CAZ SXT, ENR,	11(78.57)
26	NOR, AMP, K, C, SXT, ENR	6(42.86)
27	S	1(7.14)
28	AMP, S, SXT	3(21.43)
29	TE, NOR, AMP, AMC, K, CAZ, CIP, SXT, ENR	9(64.29)
30	NOR, AMP, K, CIP, SXT, ENR	6(42.86)
31	AMP, K, SXT	3(21.43)
32	NOR, AMP, S, CIP, SXT, ENR	6(42.86)
33	MEM, K, SXT	3(21.43)
34	FOX	1(7.14)
35	S, K	2(14.29)

Key: Amp = Ampicillin; Amc = Amoxicillin; Fox = Ceftoxitin; Te = Tetracycline; CRO = Ceftatoxone; K = Kanamycin; C = Chloramphenicol; CIP = Ciprofloxacin; SXT = Sulfamethoxasole/ trimethoprim; ENR = Enrofloxacin; Mem = Meropenem; NOR = Norfloxacin; S = Streptomycin and CAZ = Ceftaxidime.

3.1 Antibacterial Resistant Genes detected from *Salmonella* Gallinarum and Pullorum

Thirty (30) isolates were picked from the resistant isolates and analysed for antibiotic resistant genes. Among the 30 tested isolates, genes that encoded for aminoglycosides (*aad*A), quinolones (*gyr*A) and Sulphamethoxazole (*sul*1) were detected in 1 (3.3%), 26 (86.7%) and 28 (93.3%) strains, respectively. Table 4 presents the resistant gene primers used for the analysis and Table 5 and Plates 1, 2, 3 show the genes detected respectively.

Table 4: Antibiotic Resistant Gene Primers and their base pairs

Antibiotic class	Anti biotic type	Antibiotic Resistant Gene	Primer Sequence	bp
Aminoglycosides	*Kanamycin*	*aph(3)-Ia (aphA1)a*	F: ATGGGCTCGCGATAATGTC R: CTCACCGAGGCAGTTCCAT	600bp
	Streptomycin	*aadA*	F: GTGGATGGCGGCCTGAAGCC R: AATGCCCAGTCGGCAGCG	525bp
		Betalactams		
		blaTEM Amoxacillin	F: TTTCGTGTCGCCCTTATTCC R: CCGGCTCCAGATTTATCAGC	690bp
			F: TTCTATCAAMACTGGCARCC R: CCYTTTTATGTACCCAYGA	
		ampC Ampicillin		550bp
Sulphonamides	Sulfamethoxazole/ trimoprim	*sul1 Sulphamethoxazole*	F: TTCGGCATTCTGAATCTCAC R: ATGATCTAACCCTCGGTCTC	822bp
Phenicols				
	Chlorampheincol	*cat1 Chloramphenicol*	F: AGTTGCTCAATGTACCTATAACC R:TTGTAATTCATTAAGCATTCTGCC	320bp
Tetracyclines	Tetracyclines	*tetA Tetracycline*	F: GCTACATCCTGCTTGCCTTC R: CATAGATCGCCGTGAAGAGG	201bp
Quinolones				
	Norfloxacin, enrofloxacin and ciprofloxacin	*gyrA*	F-TACCGTCATAGTTATCCACGA R-GTACTTTACGCCATGAACGT	342bp

F=Forward, R=Reverse, Bp=Base pair (molecular weight)

1, 12, 28 &36 were positive for *aada*
Only 13, 30, 31, 33, 35 & 41 were negative for *sul*1
Only 11, 28, 31, 33, 35 & 36 were negative for *gyr*A (Table 5).

Table 5: Resistance genes detected in Salmonella Gallinrum and Pullorum isolated from diseased dead chickens

Salmonella strain	*aph*A$_1$	*Aada*	*bla*TEM	*ampC*	*sul*$_1$	*cat*$_1$	*tet*A	*gyr*A
1(SG)	-	+	-	-	+	-	-	+
2(SG)	-	-	-	-	+	-	-	+
3(SG)	-	-	-	-	+	-	-	+
7(SG)	-	-	-	-	+	-	-	+
8(SG)	-	-	-	-	+	-	-	+
9(SG)	-	-	-	-	+	-	-	+
10(SG)	-	-	-	-	+	-	-	+
11(SG)	-	-	-	-	+	-	-	-
12(SG)	-	+	-	-	+	-	-	+
13(SP)	-	-	-	-	-	-	-	+
14(SG)	-	-	-	-	+	-	-	+
18(SG)	-	-	-	-	+	-	-	+
19(SG)	-	-	-	-	+	-	-	+
20(SP)	-	-	-	-	+	-	-	+
21(SG)	-	-	-	-	+	-	-	+
22(SG)	-	-	-	-	+	-	-	+
23(SG)	-	-	-	-	+	-	-	+
24(SG)	-	-	-	-	+	-	-	+
25(SG)	-	-	-	-	+	-	-	+
26(SG)	-	-	-	-	+	-	-	+
28(SG)	-	+	-	-	+	-	-	-
29(SG)	-	-	-	-	+	-	-	+
30(SG)	-	-	-	-	-	-	-	+
31(SG)	-	-	-	-	+	-	-	-
32(SG)	-	-	-	-	+	-	-	+
33(SG)	-	-	-	-	-	-	-	-
35(SG)	-	-	-	-	-	-	-	-
36(SG)	-	+	-	-	+	-	-	-
41(SG)	-	-	-	-	-	-	-	+

+=Present; - = Absent; SG=*Salmonella* Gallinarum; SP=*Salmonella* Pullorum

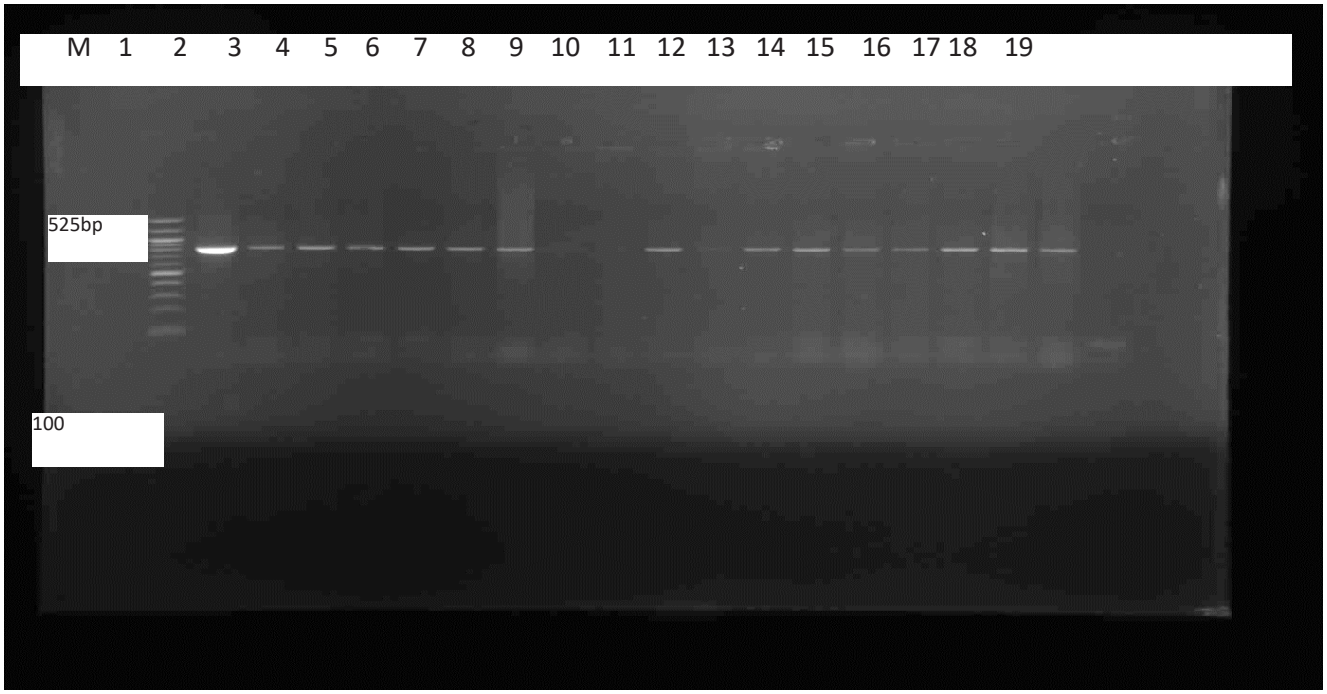


Plate 1: Showing agarose gel of amplified products using a primer set with M at 100bp-1kbDNA molecular marker. Lane 1 is positive for Aminoglycoside Streptomycin (*aad*A) resistant gene with band at 525bp positive for *Salmonella* Gallinarum.

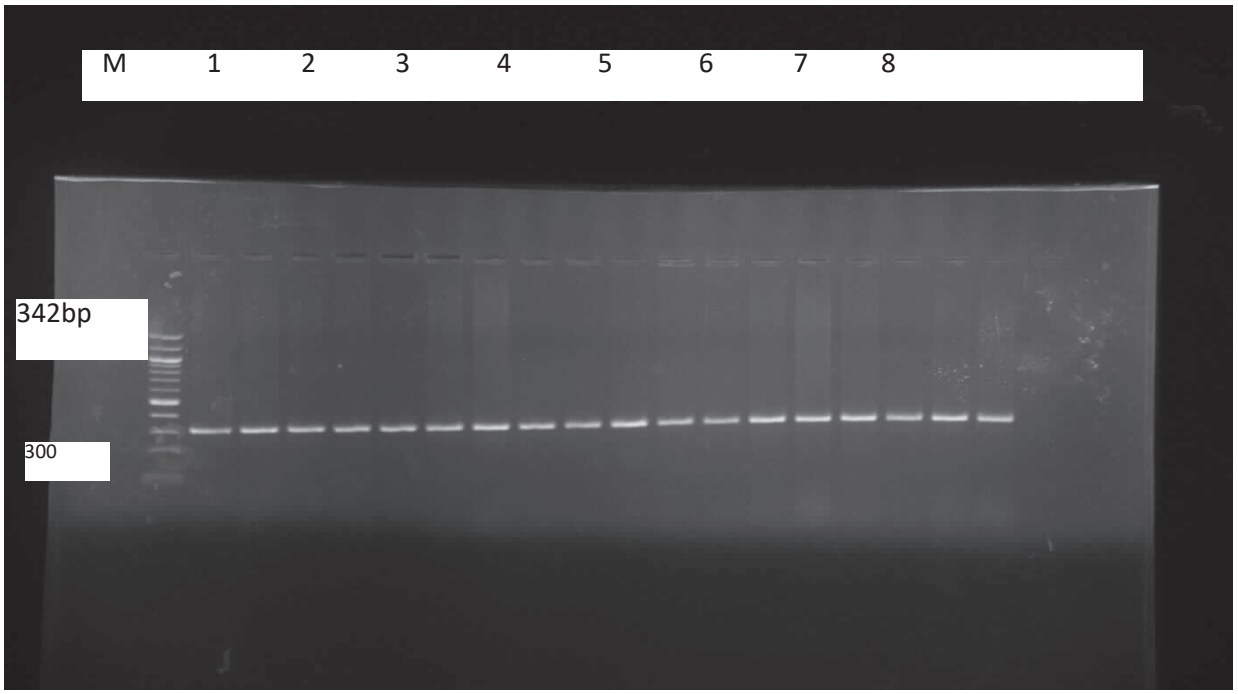


Plate 2: Showing agarose gel of amplified products using a primer set with M as molecular marker (100bp-1kbDNA). Lanes 1-8 were positive for Quinolones (*gyr*A) resistant gene with band at 342bp positive for *Salmonella* Galliarum and Pullorum.

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

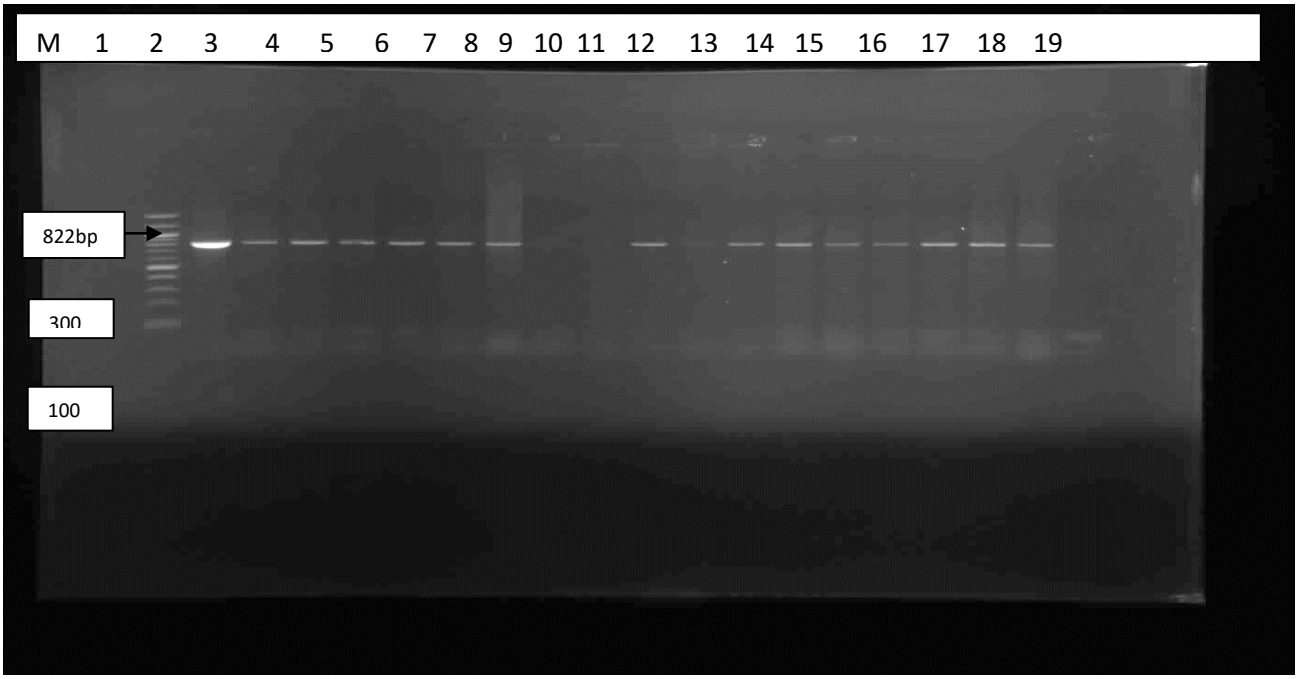


Plate 3: Showing agarose gel of amplified products using a primer set with M as molecular marker (100bp-1kbDNA). Lanes 1, 2, 3, 4, 5, 6, 7, 11, 13, 14, 15, 16, 17, 18, 19 were positive for Sulphonamides (*sul*1) resistant genes with band at 822bp positive for *Salmonella* Galliarum and Pullorum.

4.0 DISCUSSION

Salmonella Gallinarum and Pullorum belong to important bacterial pathogens that have resulted in enormous economic losses in poultry, particularly in the developing countries. The infection of *S.* Pullorum in young birds often leads to a high mortality. Infected adult birds show decreased egg production, weight loss, dysentery, and most importantly, persistent infection occurring in most recovered birds, which may lead to horizontal and vertical transmission. Chickens of all ages are affected by *Salmonella* Gallinarum and Pullorum (Shivaprasad, 2000; Chaunhan and Roy, 2003; Barrow and Freitas Neto, 2011).

In the study, there was agglutination of the *Salmonella* isolates in poly O, but there was no agglutination in poly H *Salmonella* antisera indicating that the isolates did not have flagella. The flagella are responsible for motility in *Salmonella* and if absent it suggests that the isolates are *Salmonella* Gallinarum and Pullorum, been non-motile which agree with Hossain *et al*. (2006). The *Salmonella* used for molecular studies and analyzed with 1.5% agarose gel electrophoresis proved that there was amplification with amplicon sizes at 284bp (Plate 1). This is in agreement with Kumari *et al.* (2013). The invA gene is considered a target for the molecular detection of the *Salmonella* genus (Astolfi-Ferreira *et al*., 2017).

The isolation rate of 10.1% of *S*. Gallinarum and Pullorum is higher than that of Moses *et al*. (2015) who recorded 7.4% from day old broiler chicks. Salihu *et al.* (2014) recorded a higher prevalence

of 18.30% and Okwori *et al*. (2007) recorded an isolation rate of 19.3%, all in Plateau State respectively. An isolation rate of 0.1-18% was obtained in chicken meat during pre-cleaning, post-cleaning and chilled carcasses in China (Jaja *et al*., 2019). The low isolation rate in the study area could be due to an improved hygiene level and or biosecurity. An improved hygiene and strict biosecurity will bring about a significant level of reduction in the infection rate of *Salmonella* Gallinarum and Pullorum in poultry. Other measures to reduce the rate of infection include the use of *Salmonella*-free feed and water, implementing effective cleaning and disinfection of poultry farms, appropriate good control measures against animate and inanimate vectors, and improving the sanitary status of the farms. The highest percentage of isolation from the internal organs was the heart (28%), the spleen (26%), and liver (24%). These findings agree with the report of Rafael *et al*. (2016).

In this study the isolates of *Salmonella* Gallinarum and Pullorum were evaluated for resistance against 14 antimicrobial agents. Some isolates showed resistance against some antibiotic agents. This is likely due to the indiscriminate use or underuse of these antimicrobial agents on the isolates leading to the development of resistance as reported by Moses *et al*. (2015). The highest number of isolates that developed resistance was recorded against sulfamethoxazole/trimethoprim which is in agreement with the findings of Agada *et al.* (2014) where all the isolates recorded 100% resistance. From field observation, the use of this drug is applied as a prophylactic drug against *Eimeria* parasites (coccidiosis). The high resistance to this antimicrobial agent was closely followed by ampicillin which is in agreement with Okwori *et al*. (2007) but is contrary to the work of Zishiri *et al*. (2016) who reported that ampicillin and amoxicillin are the best drugs of choice in the treatment of salmonellosis in poultry.

The isolates developed the least resistance to meropenem (7.9%), ceftaxidime (9.8%), amoxicillin and ceftriaoxone (14.6%). The low resistance of *Salmonella* isolates to meropenem and ceftriaoxone against the isolates is in agreement with Kakooza *et al.* (2021).

Low resistance of the isolates to these drugs may be due to low usage in the study area by many farmers in the management of salmonellosis. From this study these antibiotics can be recommended for use in the treatment of avian salmonellosis. The *Salmonella* isolates in the study exhibited 32 resistance patterns with S, SXT and AMP-SXT-S observed in 2 isolates each, respectively and recorded as the predominant patterns. Twenty six (26) isolates (74.3%) were resistant to 3 or more classes of antimicrobial agents, demonstrating multidrug resistance (MDR). One isolate showed resistance to 12 antimicrobial agents: AMC-AMP-FOX-TE-CRO-K-C-CIP-SXT-ENR-NOR-S. This multidrug resistance is likely due to an indiscriminate use of drugs, underuse of drugs and other environmental factors. Twenty nine of the isolates had a MAR index between 0.2-0.79. This multiple antibiotic resistance index (MAR I) exceeds the levels of > 0.2 (Jaja *et al.,* 2018). Any bacterium that exhibits a MAR index ≥ 0.2 is said to originate from a high-risk source of contamination where several antibiotics are used (Afunwa *et al*., 2020). For example, feed additives like fish meal can be one source of bacterial infection to poultry; therefore, the presence of antibiotic-resistant strains in the animal foods such as poultry can also raise concerns to both animals and man (Afunwa *et al*., 2020). It has resulted in disease burden, high cost of treatment and, a continuous spread of resistant microbial pathogens. Multiple Antibiotic Resistance (MAR) index is used in tracking bacterial infections and drug resistance (Afunwa *et al*., 2020).

Fowl typhoid and PD are currently controlled and prevented by strict biosecurity measures and vaccination. Despite the availability of vaccines in Nigeria, there still exist varying reports of efficacy (Moses *et al*., 2015). These bacteria are still present in the poultry environment with outbreaks often reported (Moses *et al.,* 2015; Rafael *et al*., 2016). In many countries, the control of salmonellosis in poultry is not done with antibiotics due to the poor history of these antimicrobials in eliminating *Salmonella* colonization leading to a great risk of promoting and selecting resistance in the fields (Rafael *et al*., 2016). Resistant genes develop due to antibiotic overuse, indiscriminate use and other environmental factors. Microbial agents easily develop resistance against antimicrobial agents that are abused either by overuse or under use. This seemed reflected in this study where the isolates tested had developed resistance to sulfur (sul1), quinolones (gyrA) and kanamycin (aadA). This could have been due to efflux pump mechanism (Rafael *et al*., 2016). For sulfur drugs as stated above, sulfur drugs are used indiscriminately in the study area by farmers in the prophylaxis against coccidiosis.

An aminoglycoside (kanamycin, *aadA*) was detected in this study. A class 1 integron that contains a variety of gene cassettes and conferring resistance to antimicrobials, such as aminoglycosides (*aad* A1, *aad* A2, *aac* A4, and *aad* B) have been reported in MDR of *S*. Enterica isolates (Kang *et al*., 2010). The isolates in the present study were averagely resistant (43.9%) to kanamycin corresponding to the percentage of resistant genes detected. The *aadA* genes, encoding resistance to aminoglycosides, have also been detected in *S.* Gallinarum since 1992 in Korea. The presence of *aad*A genes remains a serious problem in the selection of antimicrobials for the treatment of FT and PD. Antimicrobial resistance has been considered a serious problem because many drugs have become ineffective for the treatment of FT and PD. These results support the critical need for both passive and active surveillance of antimicrobial resistance in poultry as reported by Seo *et al.* (2019).

The gyrA result is in agreement with that of Lee *et al.* (2005) who reported mutations in the quinolone-resistance determining region (QRDR) of *gyr*A. This gene as reported confers resistance to quinolone drugs in a variety of microorganisms including *Salmonella* species. In this study the isolates were resistant to ciprofloxacin 26.8%, norfloxacin 36.5% and to enrofloxacin 48.8%, which is low compared to the percentage detection of the resistant genes of 86.7%. Currently WHO guidelines suggest that the quinolones are the optimal group of antimicrobials for uncomplicated typhoid treatment in adults (Koirala *et al*., 2012). The widespread quinolone usage may lead to emergence of isolates with elevated MICs that may be characterized by formation of point mutations within the gyrA (DNA gyrase) gene and sometimes an additional nucleotide substitution in the parCgene (Koirala *et al*., 2012). The formation of these organisms in particular is a concern when there is lack of feasible alternatives and an explicit correlation between increasing MICs to quinolones leading to treatment failure due to resistance (Koirala *et al*., 2012). This is likely in the study area because the antibiotic is abused so much by farmers, and because many of them use the antibiotic for brooding young chicks instead of using it to treat adult chickens.

Genes conferring resistance to sulfamethoxazole/ trimethoprim (sul_1) were also detected in 93% *Salmonella* isolates that exhibited resistance to trimethoprim-sulfamethoxazole. This result showed that most of the isolates were even harbouring the resistant genes. This result agrees with the report of Zishiri *et al.* (2016) who

detected both sul_1 and sul_2 in most of the isolates screened. Sulfonamide resistance arises from the acquisition of sul_1 and *sul2* genes, which encode dihydropteroate synthase.

Detection of 6 virulence genes (*Inv*A, *sef*C, *stn*, *Inv*H, *sop*B and *sop*) in the present study is similar to the works of Alstofi-Ferari *et al*. (2017) and Kim *et al*. (2020) who detected 3 same genes in all isolates analyzed. These genes have roles in facilitating the virulence of the disease in invasion, pathogenicity, host recognition and acquisition (Kim *et al*., 2020). Their presence in the study area could promote the virulence of the disease. The *pef*A gene was absent because it is related to fimbriae (Kim *et al*., 2020). However, this work agrees with those of Bäumler *et al.* (1997) and Dodson *et al*. (1999) who did not detect this gene in *S*. Gallinarum or *S*. Pullorum.

InvA gene was detected in this study proving that the isolates sequences were unique to this genus (Choudhury *et al*., 2016), and this served as target for the molecular detection of the *Salmonella* genus (Rahn *et al.,* 1992). This gene serves as agent for recognition and invasion of the host; it was detected in all the strains tested and this agrees with Astolfi-Ferreira *et al*. (2017). The invH gene that serves to stabilize and regulate protein in the host was also detected in all the *Salmonella* isolates agreeing with the report of Choudhury *et al*., (2016).

The *stn,* inv and sef genes that function in the production of enterotoxins, host recognition and invasion were also detected meaning that the *Salmonella* organisms in the study area will easily adhere and also invade the host cells thus, causing disease. Also detected were the *Sop* genes that are known as the effector molecules of type-III secretion system (TTSS) which are known to facilitate disease in early stage of *Salmonella* infection, thus facilitating disease spread as it is reported by Prager *et al*. (2000) and Choudhury *et al*. (2016).

The protein sop (*sopA–sopE*) that was detected is known to be associated with *Salmonella*-induced diarrhoea and gastroenteritis and many other polymorphisms will also increase fatalities in the study area due to dehydration and electrolyte loss. Their detection agrees with the findings of Choudhury *et al*. (2016) and Singh *et al.* (2018).

The detection of virulence genes from these *Salmonella* isolates should serve as an early warning to farmers to stop visiting other farms to avoid the possibility of transfer of pathogenic *Salmonella* strains from one farm to another. Although virulence genes can also be present in a location, but with an introduction of new strains, the prevalence of genes encoding for pathogenicity and increasing genetic diversity of *Salmonella* strains could increase if importation of chicken is not controlled (Zishiri *et al*., 2016).

5.0 Conclusion

This study has revealed the isolation rate of *Salmonella* Gallinarum and Pullorum (10.12%) to be high with a corresponding antibiotic resistance that could have resulted due to indiscriminate use of antibiotic. Manufacturers have also been incriminated in the production of sub-standard products. These could result in proliferation antibiotic resistance genes from these salmonellae. It is advisable for farmers to use antibiotics only after testing in the laboratory and to follow strictly the recommended dosages. Continuous surveillance should be encouraged at all times and to also strictly observe bio-security and good sanitary measures.

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