



Disinfectant Susceptibility of *Salmonella gallinarum* and *Pullorum* Isolates from Plateau State, Nigeria

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Abstract

Salmonella enterica serovar Gallinarum (SG) and Pullorum (SP) are non-motile poultry adapted Salmonellae. *Salmonella* Gallinarum causes fowl typhoid (FT), a severe systemic disease responsible for heavy economic losses to the poultry industry through morbidity, mortality and reduced egg production. Chickens of all ages are affected by SG and FT. Fowl typhoid is characterized by somnolence, droopy wings, ruffled feathers, anorexia, greenish yellow diarrhea, and mortality that can be as high as 100%. Pullorum disease (PD) caused by *Salmonella enterica* serovar Pullorum manifests as an acute disease mainly in young birds, presenting clinical signs resembling those of FT, but with watery whitish diarrhea. There are increasing reports of resistance regarding avian *Salmonella* species to available disinfectants. Vaccines against avian salmonellosis are available and in use in Nigeria; however varying results are being reported. Farmers are left with the option of prevention and control of FT and PD through bio-security, good hygiene and sanitation. However, there are increasing reports on the emergence and circulation of disinfectant-resistant salmonellae in the Nigerian poultry sector. There is paucity of information on disinfectant resistance of SG and SP in Plateau State. The aim of this study was to determine the antibiotypes and the pathotypes of *Salmonella enterica* var Gallinarum and Pullorum isolated from chickens in Plateau State, Nigeria. The specific objectives were to determine the occurrence of *Salmonella* Gallinarum and Pullorum in chickens in Plateau State, Nigeria and to document the susceptibility of the *Salmonellae* to available disinfectants. At recommended use concentrations, the 16(100%) most resistant isolates to antibiotics were susceptible to Purit[®], while susceptibility to Z-germicide[®], CID[®], Virkon[®] and Jik[®] were susceptible at 93.8, 87.5, 56.3 and 43.8% respectively. There was no association ($p > 0.05$) between *Salmonella* serotypes and resistance to antimicrobial agents. Descriptive analysis was used to analyze the data.

Keywords: *Salmonella*, Isolation, Disinfectants, Drug resistance, Fowl typhoid

Fowl typhoid (FT) has been eradicated in Australia, North America and most European countries, but the disease is still endemic in many African countries, the Middle East, Central and South America and Asia (Shah *et al.*, 2005). The disease causes substantial economic losses to the poultry industry by a reduction in egg production and also causing high mortality, which may reach 80% of affected flocks (Shivaprasad *et al.*, 2000; Celis-Estupinan *et al.* 2017). In Korea, the first outbreak was reported in 1992, since then FT has spread throughout the country, this could be possible because layer population of brown-shell egg layers are said to be more susceptible (Kwang *et al.*, 2019). In 2001, a live *Salmonella* Gallinarum (SG) 9R (SG9R) vaccine, was generated from a rough SG strain, and introduced for commercial layer chickens in Korea. There was no sufficient protection against SG infection (Kwang *et al.*, 2019). The red poultry mite has also emerged as a significant risk factor for promoting the transmission of FT and *S. Gallinarum* persistence in poultry houses (Kwang *et al.*, 2019). Antibiotic resistance and multiple resistance development among humans, animal and opportunistic pathogens is becoming a world public health problem (Jaja *et al.*, 2018). With an increase in Livestock farming the use of antimicrobials for prophylaxis, therapeutics, metaphylaxis and growth promoters have resulted in the emergence of drug resistance among commensals, opportunistic and foodborne flora of food animals (Jaja *et al.*, 2018). However, the recent emergence of multiple drug-resistant strains of SG has become a major hindrance in the effective treatment of fowl typhoid (FT) using antibiotics (Shah *et al.*, 2005). Antibiotic resistance is referred to as the genetic ability of bacteria to encode the

resistance genes that counterfeit the inhibitory effect of potential antibiotics for survival (Zeeshan *et al.*, 2019). This can be developed intrinsically or by natural recombination and by integration into the bacterial genome; it can also be acquired through horizontal gene mutation activities such as conjugation, transformation, and transduction (Zeeshan *et al.*, 2019). The most important activities in the generation of bacterial resistance include inactivation of the porin channel, modification of antibiotic targets, and neutralizing antibiotic efficacy through enzymatic action (Zeeshan *et al.* 2019). It was reported that SG between 2002 and 2007 were highly resistant to fluoroquinolones and aminoglycosides (Kwang *et al.*, 2019).

Many authors have studied *Salmonella* serovars of poultry worldwide (Agada *et al.*, 2014, Okwori *et al.*, 2014, Moses *et al.*, 2015, Parvej *et al.*, 2016, Nwiyi *et al.*, 2018). However, most of the research works on *Salmonella* in poultry have been limited to phenotypic characterization (Parvej *et al.*, 2016). Data on molecular typing, like polymerase chain reaction (PCR) and pulsed field gel electrophoresis (PFGE) are useful for epidemiological studies and also valuable in identifying the potential risk to public health associated with poultry meat, eggs and other poultry products (Parvej *et al.*, 2016).

The resistance of bacteria to disinfectants depends mainly on intrinsic factors and other environmental conditions. It is pertinent to conduct disinfectant susceptibility tests in order to know effective ones; to also note that in most cases, the more active a disinfectant is, the more toxic it would be. It is also good to know that resistance to disinfectants can occur and that this can be for a single or to several disinfectants

(Igizeneza *et al.*, 2021)

This study was carried out to detect the antimicrobial resistance genes of a number of antibiotics commonly used in the treatment of *S. Gallinarum* isolated from diseased bird carcasses in 2017.

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 30,913 km² (Ehizibolo *et al.*, 2013; Wikipedia, 2017).

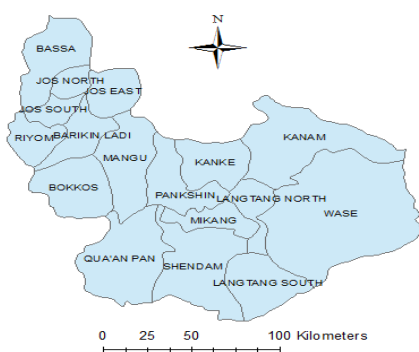


Fig. 1: Map of Plateau Showing the 17 Local Government Areas (LGAs)

2.2 Sample collection: A passive survey design was adopted for the study. All the suspected avian salmonellosis cases submitted to the Pathology Laboratory of National Veterinary Research Institute, Vom, were used for the study. A total of 494 avian cases were received 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 Rapoport Vassiliadis Soya (RVS); both were incubated at 37°C for 24 hours. 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 sub-cultured on MacConkey agar to have pure colonies.

2.4 Isolation and identification of *Salmonella*

2.4.1 Inoculation and discs application on test plates: 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 pure isolates were subsequently characterized biochemically (Chanhadradas *et al.*, 2017, Jajere, 2019). The serotyping was done where 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). 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 (2004). The standardization of the inoculum was done as described by CLSI (2016). Adjustment was made until 0.5 McFarland standards were obtained and applied unto the Mueller Hinton agar plates by uniform streaking unto the entire surface and incubated at 37°C for 24 hours. The following 14 antibiotic discs were used Ampicillin (Amp), Amoxicillin (Amc), Ceftoxitin (Fox), Tetracycline (Te), Ceftatoxone (CRO), Kanamycin (K), Chloramphenicol (C), Ciprofloxacin (CIP), Sulfamethoxazole/trimethoprim (SXT), Enrofloxacin (ENR), Meropenem (Mem), Norfloxacin (NOR), Streptomycin (S) and Ceftaxidime (CAZ).

Table 4: Showing Concentrations of Disinfectants per 20 ml

	Manufacturer's recommended concentration	(4 x) d i l u t e d conc/20ml	(2 x) d i l u t e d conc/20ml	(x) diluted conc/20ml	(0 . 5 x) d i l u t e d conc/20ml	(0.25x) diluted conc/20ml
A	10 ml/L	0.8 ml	0.4 ml	0.2 ml	0.1 ml	0.05 ml
B	100 ml/L	8 ml	4 ml	2 ml	1 ml	0.5 ml
C	12 ml/L	0.96 ml	0.48 ml	0.24 ml	0.12 ml	0.06 ml
D	100 g/L	8 g/ml	4 g/ml	2 g/ml	1 g/ml	0.5 g/ml
E	12.5 ml/L	1 ml	0.5 ml	0.25 ml	0.13 ml	0.07 ml

Key: A= CID^R; B= Purit^R; C= Z-germicide^R; D= Virkon^R; E= Jik^R

2.5 Preparation of Macconkey Agar for inoculation of isolates using the Macrodilution method

A suspension of 52 g powder was made in 1000 ml of distilled water. The suspension was boiled to completely dissolve and 20 ml was dispensed into universal bottles and sterilized by autoclaving at 121°C for 15 minutes. The five commonly used disinfectants in the study area selected were diluted using serial dilution method. The concentrations were made at x4, x2, x, 0.5x and 0.25x where x represented the manufacturers recommended concentration. The molten solution was allowed to cool to 45°C then the calculated concentration of each disinfectant was added and mixed thoroughly and then poured into sterile plastic petri plates and allowed to gel. The organism was inoculated onto the gelled agar immediately and incubator at 37°C for 24 hours for possible growth (Jorgensen & Ferraro, 2009).

2.6 Standardization of inoculum for inoculation

This was done as described by CLSI, (2016). A pure culture that was about 18 hours old of *Salmonella* Gallinarum/Pullorum isolates was used. Two to three colonies of the isolates were picked with a sterile wire loop and emulsified in 5 ml of sterile normal saline in a test tube. The emulsified bacteria in the tube were inserted into a sensititre nephelometer (TREK Diagnostic systems, UK) after calibration. Adjustments were made by adding or subtracting extra inoculum or diluents, where necessary, until 0.5 McFarland standards were obtained.

2.7 Inoculation of isolates

A loopful of the standardized inoculums of *Salmonella* Gallinarum/Pullorum were inoculated on to the solid agar and incubated at 37°C for 24 hours. This was checked for growth or no growth and recorded as described by Jorgensen & Ferraro, (2009). This was done in accordance with the calculated concentrations of the five disinfectants as shown below;

2.8 Data analysis: This was done using descriptive figures

3.0 RESULTS

3.1 Disinfectant Susceptibility tests results of *Salmonella* Gallinarum and Pullorum Isolates

At the manufacturers recommended concentration, 100% of the isolates were susceptible to Purit^R (Table 12), followed by Virkon^R (Table 14) which produced 93.7% susceptibility.

At double (2x) manufacturer's recommended concentration, 100% isolates were susceptible to Z-germicide^R (Table 13) and Virkon^R (Table 14) each, while CID^R (Table 11), Purit^R (Table 12) and Jik^R (Table 15) presented 93.7%, 88% and 88.0% susceptibility respectively.

At 4 times (4x) manufacturer's recommendation concentration Purit^R (Table 12) and Virkon^R (Table 14) performed at 100% susceptibility, while CID^R (Table 11), and Z-germicide^R (Table 13) performed at 75% each and Jik^R (Table 15) performed at 43% susceptibility.

At half (0.5x) manufacturer's recommended concentration only Jik^R (Table 15) performed at 43%. At one quarter (0.25x) or the least manufacturers recommended concentration none of the disinfectants yielded susceptibility.

There was no significant association ($p > 0.05$) between *Salmonella* serotypes and resistance to any of the disinfectants.

Table 1: Activity of CID^R on 16 *Salmonella* Isolates

Disinfectant Concentration	Growth	No Growth
4x	4	12 (85.7%)
2x	2	14 (87.5%)
X	14	2 (14.3%)
0.5x	16	0 (0.0%)
0.25x	16	0 (0.0%)

X= Recommended manufacturer's concentration

Table 2: Activity of Purit^R on 16 *Salmonella* Isolates

Disinfectant Concentration	Growth	No Growth
4x	0	16 (100.0%)
2x	1	15 (93.75%)
X	0	16 (100.0%)
0.5x	16	0 (0.0%)
0.25x	16	0 (0.0%)

X= Recommended manufacturer's concentration

Table 3: Activity of Z-germicide^R on 16 *Salmonella* Isolates

Disinfectant Concentration	Growth	No Growth
4x	4	12 (75.0%)
2x	0	16 (100.0%)
X	1	15 (93.75%)
0.5x	15	1 (6.25%)
0.25x	16	0 (0.0%)

X= Recommended manufacturer's concentration

Table 4: Activity of Virkon^R on 16 *Salmonella* Isolates

Disinfectant Concentration	Growth	No Growth
4x	0	16 (100%)
2x	0	16 (100%)
X	7	9 (56.25%)
0.5x	15	1 (6.25%)
0.25x	16	0 (0.0%)

X= Recommended manufacturer's concentration

Table 5: Activity of Jik^R on 16 *Salmonella* Isolates

Disinfectant Concentration	Growth	No Growth
4x	9	7 (43.75%)
2x	2	14 (87.5%)
X	9	7 (43.75%)
0.5x	10	6 (37.5%)
0.25x	16	0 (0.0%)

X= Recommended manufacturer's concentration

Discussion

In this study antimicrobial activity of five disinfectants was tested against *Salmonella* Gallinarum and *S. Pullorum* isolates. The five disinfectants were diluted to five graded concentrations each. The different concentrations produced various degrees of activity. Purit^R, Z-germicide and CID^R's activity at the manufacturer's concentration produced good activity. The high performance level of Purit^R could be due to low patronage by farmers so the development of minimal resistance and the low patronage of CID^R and Virkon^R is probably due to high cost even though they produced good activity agreeing with the report of Irene *et al.* (2013) who reported that CID^R and Virkon^R could demonstrate an effective performance on *Salmonella* sp. From this study only two disinfectants were active at the manufacturers recommended concentrations, hence, this result is in disagreement with Helder *et al.* (2019) whose work produced 100% activity from all disinfectants tested. And at

two double (2x) manufacturer's concentrations, all the 5 disinfectants (Z-germicide^R, Virkon^R, CID^R, Purit^R and Jik^R) were active against the *Salmonella* isolates signifying that a higher concentration of the disinfectants is required for a good activity; this is in agreement with Acsa *et al.* (2021) who reported that an increase in concentration increased the level of activity, but this was the opposite with the performance of Jik^R. It is possible that some of the resistance recorded in this study might have been due to the presence of resistance genes for the particular disinfectant (Russell, 2002). Other reasons could be that bacteria resistant to disinfectants may be genetically encoded by the organism or be carried by plasmids (Guimarães *et al.*, 2000; Acsa *et al.*, 2021) and also gram-negative bacteria could be less susceptible to biocides compared to their gram-positive counterparts.

The *Salmonella* isolates were resistant to Jik^R not only at manufacturer's recommended concentrations level and below the recommended user concentration, but also at four times (4x) the manufacturer's concentration and this is in agreement with Acsa *et al.* (2021). This finding could be attributed to the fact that chlorine disinfectant is easily affected by the concentrations of available chlorine and environmental factors such as pH, temperature, and organic matter (Jiang *et al.*, 2018). Jik being Sodium hypochlorite is a halogen that acts by denaturing bacterial proteins; for any disinfectant to properly work, it must cross the outer membrane of the bacteria to reach the target site (Russell, 2002; Acsa *et al.*, 2021).

No reasonable activity was recorded against any isolate among the five disinfectants at the concentrations 25% (0.5x) and 12.5% (0.25x) respectively. From the present study, concentrations of disinfectants should be adhered to or slightly increased to eliminate the confusion arising from the indiscriminate use of disinfectants that may arise from inappropriate reconstitution (Carrique-Mas *et al.*, 2009).

Disinfectants containing more active ingredients that target different sites of bacterial cells were found to be more effective (Jiang *et al.*, 2018). Concentrations and the methods used in the disinfection process should be explored based on the conditions of the farm to achieve disinfection and reduce resistance (Jiang *et al.*, 2018). In

this study there was no association ($p > 0.05$) between *Salmonella* serotypes and resistance to antimicrobial agents.

Conclusion

The choice of disinfectants too has to be carefully made in order to be sure the right disinfectants are selected for use. Farmers are supposed to be well informed or educated on the dilution of the various disinfectants. Farmers are to be educated on the typical clinical signs of salmonellosis so that they can report same to the veterinarians for early diagnosis and treatment. With all these in place farmers may have a sigh of relieve from the economic losses experienced from time to time.

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