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Detection of Antibiotics Resistance Genes and Molecular Characterization of *Salmonella* spp from Clinical and Poultry Samples in Yenagoa Metropolis of Bayelsa

Eboh D. D & Anyiam I. V
Federal University Otuoke, Bayelsa State, Nigeria
Eboh.dd@gmail.com

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Abstract

Salmonellae are facultative anaerobes, intracellular Gram-negative motile bacteria. They are one of the most important zoonotic pathogens transmitting among humans and animals and are responsible for high cases of morbidity and mortality in a wide range of hosts. Due to the similarity of antibiotic classes used to treat animals and humans, there is a high risk for the emergence of the multi-drug resistant (MDR) strains. Thus, this study was aimed at detection of antibiotics resistance genes and molecular characterization of *Salmonella* spp from clinical and livestock samples. Hundred (100) stool samples comprising of fifty (50) clinical and fifty (50) poultry were respectively collected from Federal Medical Centre (FMC) Yenagoa, Niger Delta University Teaching Hospital (NDUTH), Okolobiri, Diette Koki hospital, Opolo, and poultry farms at Swali, Kpansia and Azikoro. The samples were analyzed by culture using various enrichment, selective and differential media (selenite F broth, bismuth sulphite agar and MacConkey agar), biochemical tests, and molecular methods. Eleven (11) isolates were recovered from the samples comprising of two (2) clinical and nine (9) poultry, and resulting in a prevalence of 4% and 18% respectively. The result of the antibiotic profile showed that all isolates were multi drug resistant to ceftriaxone (63.6%), ofloxacin (0%), gentamicin (9.1%), co-trimoxazole (36.4%), levofloxacin (9.1%), clindamycin (90.9%), augmentin (72.7%), cefixime (100%),

nitrofurantoin (100%), tazobactam/piperacillin (72.7%) and ciprofloxacin (18.2%). Following the biofilm test analysis, none of the recovered isolates is a biofilm former hence, there is no relationship between biofilm formation and the high level of resistance recorded. Screening for the presence of resistance genes revealed that 83.3% of the isolates have *tet* A. 66.7% have *SHV* gene while 16.7% have *VEB* and *NDM* genes respectively. This depicts a strong correlation between the observed high level of antimicrobial resistance amongst the isolates and prevalence of resistance genes. The phylogenetic analysis result showed that the evolutionary distances as computed were in agreement with the phylogenetic placement of the 16S rRNA of the isolates within the *Salmonella, Shigella* and *Proteus* spp and revealed a closely relatedness to the *Salmonella enterica, Shigella flexneri, Proteus columbae* and *Proteus cibarius*. The study established that only the fluoroquinolones and aminoglycosides can be relied upon in the effective treatment of infections with MDR *Salmonella, Shigella and Proteus* spp and restriction of indiscriminate use of antibiotics in food animals will be an effective measure to stop/prevent uncontrollable outbreaks of infections due to these isolates even as antibiogram results are constantly monitored worldwide.

Keywords: Salmonella, Antibiogram, antibiotics resistance gene, Biofilm, 16SrRNA, Sequencing

INTRODUCTION

Salmonella infection remains a major public health concern worldwide, contributing to the economic burden of both industrialized and underdeveloped countries through the costs associated with surveillance, prevention, and treatment of disease. Gastroenteritis is the most common manifestation of *Salmonella* infection worldwide, followed by bacteremia and enteric fever (Majowicz et al., 2010).

Salmonella is a rod-shaped, Gram-negative facultative anaerobe that belongs to the family Enterobacteriaceae. Within the genus *Salmonella*, around 2600 serotypes have been identified with the use of the standard Kauffman–White scheme and most of these serotypes can adapt within a variety of animal hosts, including humans. *Salmonella* and Campylobacter are the most frequently isolated foodborne pathogens and are predominantly found in poultry, eggs, and dairy products (Silva et al., 2011). Other food sources that are involved in the transmission of *Salmonella* include fresh fruits and vegetables (Pui et al., 2011). In general, food animals such as swine, poultry, and cattle are the prime sources of *Salmonella* infections. The major dissemination routes of the pathogens involve trade in animal and uncooked animal food products. The slaughtering

process of food anim0als at abattoirs is considered one of the important sources of organ and carcass contamination with *Salmonella* (Gillespie et al., 2005). The emergence of antibiotic-resistant foodborne pathogens has raised the concern of the public as these pathogens are more virulent, causing an increase in the mortality rate of infected patients (Chiu et al., 2002).

Salmonella was first discovered and isolated from the intestines of pigs infected with classical swine fever, by Theobald Smith in 1855. The bacterial strain was named after Dr Daniel Elmer Salmon, an American pathologist who worked with Smith. The nomenclature of *Salmonella* is controversial and still evolving. Currently, the Centers for Disease Control and Prevention (CDC) uses the nomenclatural system of *Salmonella* recommended by the World Health Organization (WHO) Collaborating Centre (Popoff et al., 2003). According to this system, the genus *Salmonella* is classified into two species, *Salmonella enterica* (type species) and *Salmonella bongori*, based on differences in their 16S rRNA sequence analysis. The type species, *S. enterica*, can be further classified into six subspecies based on their genomic relatedness and biochemical properties (Reeves et al., 1989). The subspecies are denoted with roman numerals: I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salamae*; IIIa, *S. enterica* subsp. *arizonae*; IIIb, *S. enterica* subsp. *diarizonae*; IV, *S. enterica* subsp. *houtenae*; and VI, *S. enterica* subsp. *indica*. Among all the subspecies of *Salmonella*, *S. enterica* subsp. *Enterica* I is found predominantly in mammals and contributes approximately 99% of *Salmonella* infections in humans and warm-blooded animals. In contrast, the other five *Salmonella* subspecies and *S. bongori* are found mainly in the environment and also in cold-blooded animals, and hence are rare in humans (Brenner et al., 2000).

In addition to the classification of subspecies based on phylogeny, Kauffman and White developed a scheme to further classify *Salmonella* by serotype based on three major antigenic determinants: somatic (O), capsular (K), and flagellar (H) (Brenner et al., 2000). The heat-stable somatic O antigen is the oligosaccharide component of lipopolysaccharide located at the outer bacterial membrane. A specific serotype of *Salmonella* can express more than one O antigen on its surface (Hu & Kopecko, 2003). The heat-labile H antigens are found in the bacterial flagella and are involved in the activation of host immune responses. Most *Salmonella* spp. contain two distinct genes that encode the flagellar proteins; these bacteria have the special ability to express only one protein at a time and are, therefore, called diphasic (phase I and II). Each serotype expresses specific phase I H antigens which

are responsible for its immunological identity, whereas phase II antigens are non-specific antigens that can be shared by many serotypes (McQuiston et al., 2008). The surface K antigens are heat-sensitive polysaccharides located at the bacterial capsular surface and are the least common antigens found in the serotypes of *Salmonella*. Virulence (Vi) antigens, a special subtype of K antigen, are found only in three pathogenic serotypes: Paratyphi C, Dublin, and Typhi.

Salmonella pathogenicity is mediated by numerous genes such as *inv*A, *spv*, *fim*A, *stn, iro*B, *spi*C, and *pip*D, which code for effectors that induce successful host infection. Pathogenicity of *Salmonella* is expressed in three ways such as host cell invasion, intracellular survival, and colonization (Foley et al., 2013). Numerous virulence genes are essential for *Salmonella* pathogenesis and these genes are located on various elements of the genome including the chromosome, plasmids, integrated bacteriophage DNA, *Salmonella* pathogenicity islands (SPIs), and *Salmonella* genomic islands (SGIs) (Card et al., 2016; Bayoumi & Griffiths, 2010)

Based on the clinical patterns in human salmonellosis, *Salmonella* strains can be grouped into typhoid *Salmonella* and non-typhoid *Salmonella* (NTS). In human infections, the four different clinical manifestations are enteric fever, gastroenteritis, bacteremia and other extraintestinal complications, and chronic carrier state (Sheorey & Darby 2008).

The emergence of antimicrobial resistance in *Salmonella* strains is a serious health problem worldwide (Chiu et al., 2002). In the early 1960s, the first incidence of *Salmonella* resistance to a single antibiotic, namely chloramphenicol, was reported (Montville & Matthews, 2008). Since then, the frequency of isolation of *Salmonella* strains with resistance towards one or more antimicrobial agents has increased in many countries, including the USA, the UK and Saudi Arabia (Yoke-Kqueen et al., 2008). Antimicrobial agents such as ampicillin, chloramphenicol and trimethoprim–sulfamethoxazole is used as the traditional first line treatments for *Salmonella* infections. *Salmonella* spp. Resistant towards these agents is referred to as multi-drug resistant (MDR).

For many years, the phenotypic trait of MDR was widely distributed among *S*. Typhi and, at a lower rate, among *S*. Paratyphi (Rowe et al., 1997). Africa and Asia are two continents with a high isolation frequency of *S*. Typhi displaying MDR phenotype.

With the emergence of resistance towards traditional antibiotics, fluoroquinolones and extended-spectrum cephalosporins have been introduced as the antimicrobial agents of

choice in treating MDR *S.* Typhi (Sood et al., 1999). However, reports show an increase in the number of cases with typhoid *Salmonella* developing resistance towards fluoroquinolones. In countries with a higher incidence of MDR isolates, *S.* Paratyphi displays a higher level of resistance towards fluoroquinolones compared to *S.* Typhi (Hasan et al., 2008). Nalidixic acid resistance, which is used as an indicator of reduced susceptibility of ciprofloxacin and other fluoroquinolones, is displayed by isolates from Pakistan, India and Vietnam, with high incidence rates of 59%, 57% and 44%, respectively (Ochiai et al., 2008).

As for NTS, the number of strains developing MDR phenotype has increased in many countries since the first emergence of MDR *S.* Typhimurium DT104 strains in 1990 (Helms et al., 2005). Based on data from 2005 to 2006 presented by the National Antimicrobial Resistance Monitoring System (NARMS), 84% of clinical isolates of NTS displayed MDR phenotype and 4.1% of the isolates had reduced susceptibility to cephalosporins in the USA. NARMS presented data (from 1996–2007) which are more comprehensive, reporting the emergence of NTS isolates that are resistant to nalidixic acid and ceftriaxone. This phenomenon has raised concern among public health authorities regarding both clinical management and prevention of the infection (Crump et al., 2011). A surveillance study on 135,000 clinical isolates of NTS was conducted in Europe from 2000 to 2004, and the data showed that 15% of the isolates displayed MDR phenotype and 20% of the isolates were resistant to nalidixic acid (Meakins et al., 2008).

The emergence of *Salmonella* with antimicrobial resistance is mainly promoted by the use of antibiotics in animal feed to promote the growth of food animals, and in veterinary medicine to treat bacterial infections in those animals (Hyeon et al., 2011). This poses a high risk of zoonotic disease with the transmission of MDR *Salmonella* strains from animals to humans *via* the ingestion of food or water contaminated with the animals' feces, direct contact or the consumption of infected food animals (Holmberg et al., 1984). Moreover, MDR *Salmonella* strains were found in some exotic pet animals such as tortoises and turtles, as well as their water environment, and this could result in a higher risk of zoonotic infections in humans through direct contact with these animals (Shane et al., 1990).

The development of multi-drug resistance in the serotypes of *Salmonella* has a significant impact on the antibiotic treatment of *Salmonella* infections. Infections that involve the invasive serotypes are often life-threatening and require effective antibiotic treatment.

Quinolones and third-generation cephalosporins have been the antibiotics of choice in treating infections with MDR *Salmonella* (Karon et al., 2007). However, the emergence of *Salmonella* serotypes resistant to quinolones and cephalosporin poses a new challenge in treating infected patients, and the lack of an effective antibiotic therapy may lead to an increase in morbidity and mortality rates.

The emergence of MDR *Salmonella* has also resulted in the increased severity of bacterial infections in humans and animals. Epidemiological studies show that MDR *Salmonella* strains cause more severe or prolonged syndromes than susceptible strains, implying that the MDR strains are more virulent than the susceptible ones (Travers & Barza 2002). Thus, this study aims to detect antibiotics resistance genes and characterize Salmonella spp isolated from clinical and poultry sources in Yenagoa Metropolis of Bayelsa State.

MATERIALS AND METHODS

Study Area and Population: This research involves a cross-sectional study where 100 stool samples were collected. The samples comprised of fifty (50) clinical specimens from the Microbiology and Parasitology units of Federal Medical Centre (FMC) Yenagoa, Niger Delta University Teaching Hospital (NDUTH), Okolobiri and Diette Koki Hospital, Opolo; and fifty (50) livestock samples from Swali market, Kpansia market and Azikoro poultry farm.

Ethical Approval: This study was conducted following ethical standards and received approval from the Research Ethics Committee with application number FMC/REC/ECC/2024/JUNE/765 at Federal Medical Centre-Yenagoa. Informed consent was obtained from all participants involved in the study. Confidentiality was strictly maintained, with all data anonymized to protect participant privacy.

Sample Collection: A total of 100 stool samples were collected from clinical (50) and poultry (50) sources respectively into appropriate sterile containers. The samples were then transported to the laboratory for onward processing and microbiological analysis to recover *Salmonella* spp

Selective Isolation of *Salmonella* species: Aliquot portions of the respective samples were aseptically cultured into already prepared Selenite F broth (in test-tubes). The tubes were incubated at 37^0C for 18-24hrs. Thereafter, a loopful of the bacterial cells from the

selenite broth were respectively streaked on Bismuth Sulphite Agar (BSA) plates and incubated at 37°C for 24-48 hours. The resultant colonies were then examined for their morphological/cultural appearance on the BSA plates. Colonies suggestive of *Salmonella* spp will be picked and streaked out on nutrient agar for further confirmation/identification.

Identification and characterization of isolates*:* Each isolate was streaked with the aid of a flamed inoculating loop on MacConkey agar to check for lactose fermentation (*Salmonella* spp is a none lactose fermenter). The following tests were carried out to confirm no lactose fermenting isolates.

Gram Staining*:* A smear of each isolate was fixed to a clean grease-free microscope slide and flooded with 0.5% crystal violet stain. The stain was rinsed off with tap water after 30 seconds and thereafter flooded with Lugol's iodine for 30 seconds before being decolorized with acetone and then rinsed with water. The smear was then counterstained with aqueous safranin for 1 minute, rinsed with water allowed to dry. The stained smear was observed for shape, arrangement, and color by light microscope at x1000 magnification under oil immersion lens. Organisms the stained red were considered Gram-negative and those that stained purple as Gram-positive.

Oxidase test: Whatman number 1 filter paper was moistened with a few drops of freshly prepared oxidase reagent (1% aqueous tetramethyl-p-phenylenediamine dihydrochloride). 18-24 hours colonies of the test organism were then smeared on the paper. The development of a purple colour within 10 seconds was indicative of a positive oxidase test/reaction while the absence of coloration indicates a negative result.

Indole test: Sterilized peptone water (5ml) were inoculated with an aqueous suspension of each isolate and incubated at 37^0C for 24 hrs. About 0.5ml of Kovac's reagent was then added to each culture, shaken and allowed to stand. The development of a red colour in the organic layer is indicative of the test isolate being able to convert tryptophan to indole.

Citrate utilization test*:* The test detects the ability of an organism to utilize citrate as the sole carbon and energy source. Simmon citrate agar was prepared according to the manufacturer's directions, dispensed into Bijou bottles and sterilized at 121^0C for 15minutes. The bottles were then left to form slants which were later inoculated with a 18-24 hours culture and incubated at 37^0C for 24-48 hours. Citrate utilization was by an

increase in the pH of the medium which became alkaline; this was made evident by a change in the colour from green to blue.

Antibiotics Susceptibility Testing*:* Each of the biochemically confirmed isolates of *Salmonella* spp was standardized using the colony suspension method and the strain's suspension was matched with 0.5M McFarland standard to give a resultant concentration of 1.5 x 10^8cfu/ml. The antibiotic susceptibility testing was confirmed using the modified Kirby-Bauer diffusion technique (Cheesbrough, 2002) by swabbing the surface of fairly dried Mueller-Hinton agar (MHA) plates with the resultant saline suspension of each isolate and the multi-disc comprising the following antibiotics: ceftriaxone (30μg), ofloxacin (5 μg), gentamicin (10 μg), co-trimoxazole (25 μg), levofloxacin (5 μg), tetracycline (30 μg), clindamycin (2 μg), augmentin (10 μg), cefixime (5 μg), nitrofurantoin (300 μg), tazobactam/Piperacillin (110 μg) and ciprofloxacin (5 μg) was centrally placed onto the surface of the agar plates. The disc was firmly and gently pressed to the agar surface using sterile forceps. The plates containing the discs were allowed to stand for 30 minutes to ensure adequate diffusion of the antibiotics before incubation at 37^0C for 18-24 hours. The diameter of the zone of inhibition produced by each antibiotic was measured and interpreted using the Clinical and Laboratory Standard Institute interpretative chart (CLSI, 2023).

Biofilm test*:* Congo red agar was prepared following the method of Freeman et al ,(1989) and was aseptically poured into Petri plates. The plates were inoculated with the various *Salmonella* isolates and incubated at 37^0C for 24 hours. The appearance of black colonies with dry crystalline morphology was considered positive for biofilm formation while weak or non-biofilm-producing organisms appeared to be pink in colour.

Molecular characterization of *Salmonella* strains

16S rRNA Amplification

The 16s rRNA region of the rRNA gene of the isolates was amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microlitres for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by

Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.5uM, and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 40 seconds; annealing, 52ºC for 40 seconds; extension, 72ºC for 40 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 200V for 15 minutes and visualized on a blue light transilluminator for a 1500bp product.

Sequencing

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10ul, the components included 0.25 ul BigDye® terminator v1.1/v3.1, 2.25ul of 5 x BigDye sequencing buffer, 10uM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing conditions were as follows 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min.

Phylogenetic Analysis

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using ClustalX. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analysed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor 1969)

Detection of Resistance Genes

DNA Extraction*:* Extraction was done using a ZR fungal/bacterial DNA mini-prep extraction kit supplied by Inqaba South Africa. Heavy growth of the pure culture of the suspected isolates was suspended in 200 microliters of isotonic buffer in a ZR Bashing Bead Lysis tube, and 750 microliters of lysis solution were added to the tube. The tubes were secured in a bead beater fitted with a 2ml tube holder assembly and processed at maximum speed for 5 minutes. The ZR bashing bead lysis tube was centrifuged at 10,000x*g* for 1 minute.

Four hundred (400) microliters of supernatant were transferred to a Zymo-Spin IIIF spin Filter (orange top) in a collection tube and centrifuged at 10000*g* for 1 minute. One thousand two hundred (1200) microliters of fungal/bacterial DNA binding buffer were added to the filtrate in the collection tubes bringing the final volume to 1600 microliter, 800 microliter was then transferred to a Zymo-Spin IIC column in a collection tube and centrifuged at 10,000x*g* for 1 minute, the flow through was discarded from the collection tube. The remaining volume was transferred to the same Zymo-spin and spun. Two hundred (200) microliter of the DNA Pre-Was buffer was added to the Zymo-spin IIC in a new collection tube and spun at 10,000*g* for 1 minute followed by the addition of 500 microliter of fungal/bacterial DNA Wash Buffer and centrifuged at 10,000x*g* for 1 minute.

The Zymo-spin IIC column was transferred to a clean 1.5 microliter centrifuge tube, 100 microliter of DNA elution buffer was added to the column matrix and centrifuged at 10,000x*g* microliter for 30 seconds to elute the DNA. The ultra-pure DNA was then stored at -20 degree for other downstream reaction.

DNA quantification*:* The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was lunched by double clicking on the Nanodrop icon. The equipment was initialized with 2µL of sterile distilled water and blanked using normal saline. Two microliters of the extracted DNA were loaded onto the lower pedestal, the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the "measure" button.

Amplification of *SHV* genes: SHV genes from the isolates were amplified using the SHV F: 5-CGCCTGTGTATTATCTCCCT-3' and SHV R: 5'-CGAGTAGTCCACCAGATCCT-3' primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 microlitres for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and 50ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 40 seconds; annealing, 56ºC for 40 seconds; extension, 68ºC for 40 seconds for 35 cycles and final extension, 68ºC for 5 minutes. The product was resolved on a 1%

agarose gel at 200V for 15 minutes and visualized on blue light imaging system for a 293bp product size.

Amplification of *VEB* genes*:* VEB genes from the isolates were amplified using the VEB F: 5-CGACTTCCATTTCCCGATGC-3' and VEB R: 5'-GGACTCTGCAACAAATACGC-3' primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 microlitres for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and 50ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 40 seconds; annealing, 56C for 40 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 7 minutes. The product was resolved on a 1% agarose gel at 200V for 15 minutes and visualized on blue light imaging system for a 1050bp product size.

Amplification of *NDM* genes: NMD genes from the isolates were amplified using the NDM F: 5-GGTTTGGCGATCTGGTTTTC-3' and NDM R: 5'-CGGAATGGCTCATCACGATC-3' primers on ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 microlitres for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and 50ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 56ºC for 45 seconds; extension, 72ºC for 45 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 200V for 15 minutes and visualized on blue light imaging system for a 390bp product size.

Amplification of *tet* A genes: *Tet* A genes from the isolates were amplified using the TETA F: 5-CGCCTTTCCTTTGGGTTCTCTATATC-3' and TETA R: 5'-CAGCCCACCGAGCACAGG-3' primers on ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 microlitres for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and 50ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for

30 seconds; annealing, 55ºC for 40 seconds; extension, 72ºC for 40 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 200V for 15 minutes and visualized on a blue light imaging system for a 190bp product size.

Statistical Analysis

Data were subjected to statistical analysis using Graph Pad Prism version 10.3 with ANOVA and student's t-test and Pearson Correlation Coefficient (r) test. P -value ($p < 0.05$) was taken as a measure of statistical significance.

RESULTS

From the 100 samples collected from different poultry farms and clinical centre, 11 representing 11% were positive for *Salmonella* as evidenced by the isolation on selective media, followed by Gram staining and biochemical tests. The prevalence of Salmonella in each sample collected 4% for clinical and 18% for poultry. This is shown in table 1 below

Table 1: Identification and Biochemical Characterization of poultry and clinical isolates

Isolate	Lactose Ferm	Citrate rxn	Oxidase rxn	Indole rxn	Gram rxn	Shape
P3	-	+	-	-	-	Rod
P10	-	+	-	-	-	Rod
P20	-	+	-	-	-	Rod
P22	-	+	-	-	-	Rod
P23	-	+	-	-	-	Rod
P24	-	+	-	-	-	Rod
P27	-	+	-	-	-	Rod
P32	-	+	-	-	-	Rod
P49	-	+	-	-	-	Rod
H3	-	+	-	-	-	Rod
H37	-	+	-	-	-	Rod

P = Poultry sample, H = Clinical sample

As shown in table 2, the percentage occurrence of *Salmonella* isolates from poultry is 81.8% while its occurrence in clinical samples is 18.2%.

Table 2: Percentage occurrence of *Salmonella* isolates isolated from Poultry and Clinical samples in Yenagoa Metropolis

Sample source	Number of Isolates (%)
Poultry	9 (81.8)
Clinical (stool)	2 (18.2)
Total	11(100)

Table 3 below presents the antibiotic susceptibility pattern of both the clinical and poultry isolates. For the poultry isolates, the highest resistance was displayed against tetracycline, cefixime and nitrofurantoin with 100% resistance. Clindamycin, augmentin, piperacillin/tazobactam, ceftriaxone and co-trimoxazole had high resistance of 88.9%, 77.8%, 77.8%, 66.7% 44.4% resistance respectively while ciprofloxacin, levofloxacin and gentamicin had the lowest levels of resistance (22.2%, 11.1% and 11.1% respectively). 0% resistance was recorded against ofloxacin. For the clinical isolates, the 100% resistance was recorded against nitrofurantoin, clindamycin and cefixime, 50% resistance was recorded against ceftriaxone, tetracycline, augmentin and piperacillin/tazobactam while a 0% resistance (100% susceptibility) was obtained with ofloxacin, gentamicin, co-trimoxazole, levofloxacin and ofloxacin respectively. There was a significant difference ($P = 0.0192$, $P \leq 0.05$) in the resistance profile of the isolates from poultry and no significant difference ($P = 0.3505$, $P \leq 0.05$) in the resistance profile of the clinical isolates.

Table 3: Antibiotic Susceptibility of the *Salmonella* isolates

Antibiotics	Poultry (n=9)			Clinical (n=2)		
	Resistant (%)	Intermediate (%)	Susceptible (%)	Resistance (%)	Intermediate (%)	Susceptible (%)
Ceftriaxone	6 (66.7)	3 (33.3)	0 (0.0)	1 (50.0)	0 (0.0)	1 (50.0)
Ofloxacin	0 (0.0)	0 (0.0)	9 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)
Gentamicin	1 (11.1)	1 (11.1)	7 (77.8)	0 (0.0)	0 (0.0)	2 (100.0)
Cotrimoxazole	4 (44.4)	1 (11.1)	4 (44.4)	0 (0.0)	1 (50.0)	1 (50.0)
Levofloxacin	1 (11.1)	1 (11.1)	7 (77.8)	0 (0.0)	0 (0.0)	2 (100.0)
Tetracycline	9 (100.0)	0 (0.0)	0 (0.0)	1 (50.0)	1 (50.0)	0 (0.0)

Clindamycin	8 (88.9)	1 (11.1)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)
Augmentin	7 (77.8)	1 (11.1)	1 (11.1)	1 (50.0)	1 (50.0)	0 (0.0)
Cefixime	9 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)
Nitrofurantoin	9 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)
Tazo/Piperacillin	7 (77.8%)	1 (11.1)	1 (11.1)	1 (50.0)	1 (50.0)	0 (0.0)
Ciprofloxacin	2 (22.2)	1 (11.1)	6 (66.7)	0 (0.0)	0 (0.0)	2 (100.0)

Figure 1 presents a bar chart showing the percentage resistance of the isolates, from poultry and clinical sources against the various antibiotics. The chart indicates that 100% susceptibility was exhibited by all isolates to ofloxacin while 100% resistance was exhibited by all isolates against cefixime and nitrofurantoin respectively.

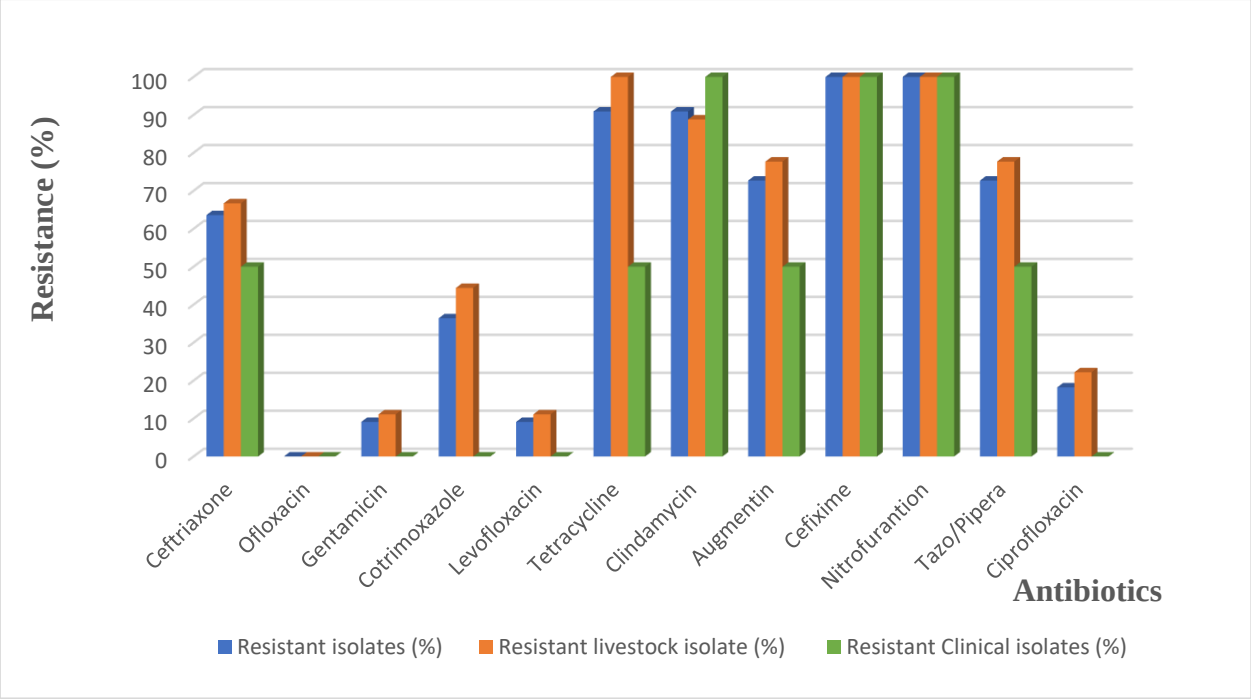

Figure 1: Percentage Antibiotic Resistance of the *Salmonella* Isolates, *Salmonella* Poultry Isolates and *Salmonella* Clinical Isolates

The biofilm formation analysis of the isolates as presented in table 4 shows that none of the isolates was a biofilm former as the congo red media remain unchanged in colour after the incubation period. Thus, there is no correlation between presence of antibiotics resistance genes and biofilm formation in this study.

Table 4: Biofilm formation analysis results

Isolates	Description	Result
P3	Colour of medium remain the same after 24hrs of incubation	Negative
P10	Colour of medium remain the same after 24hrs of incubation	Negative
P20	Colour of medium remain the same after 24hrs of incubation	Negative
P22	Colour of medium remain the same after 24hrs of incubation	Negative
P23	Colour of medium remain the same after 24hrs of incubation	Negative
P24	Colour of medium remain the same after 24hrs of incubation	Negative
P27	Colour of medium remain the same after 24hrs of incubation	Negative
P32	Colour of medium remain the same after 24hrs of incubation	Negative
P49	Colour of medium remain the same after 24hrs of incubation	Negative
H3	Colour of medium remain the same after 24hrs of incubation	Negative
H37	Colour of medium remain the same after 24hrs of incubation	Negative

Table 5 Presents the occurrence of the Antibiotic Resistance genes amongst the *Salmonella* isolates. There was no significant statistical difference ($P = 0.0756$, $P \leq 0.05$) in the occurrence of resistance genes amongst the isolates.

Table 5: Occurrence of Antibiotics Resistance genes in *Salmonella* isolates

S/N	Isolate code	Occurrence of Antibiotic Resistance gene			
		SHV	VEB	NDM	tet A
1	P20	Positive	Positive	Negative	Positive
2	P23	Positive	Negative	Negative	Positive
3	P27	Positive	Negative	Negative	Positive
4	P49	Negative	Negative	Negative	Negative
5	H3	Negative	Negative	Negative	Positive
6	H37	Positive	Negative	Positive	Positive

Plate 1 highlights the agarose gel electrophoresis showing SHV genes of some selected bacterial isolates. Lanes 2 and 4-6 represent positive SHV gene at 293bp. Lane D represents the 100bp Molecular ladder

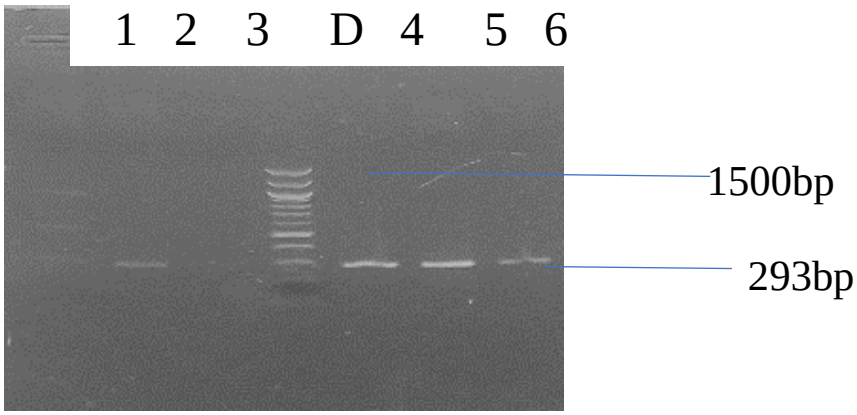

Plate 1: SHV gene in the bacteria isolates

Plate 2 shows the agarose gel electrophoresis of VEB gene of some selected bacterial isolates. Lane 6 represents the VEB gene bands (1050bp). Lane D represents the 100bp Molecular ladder.

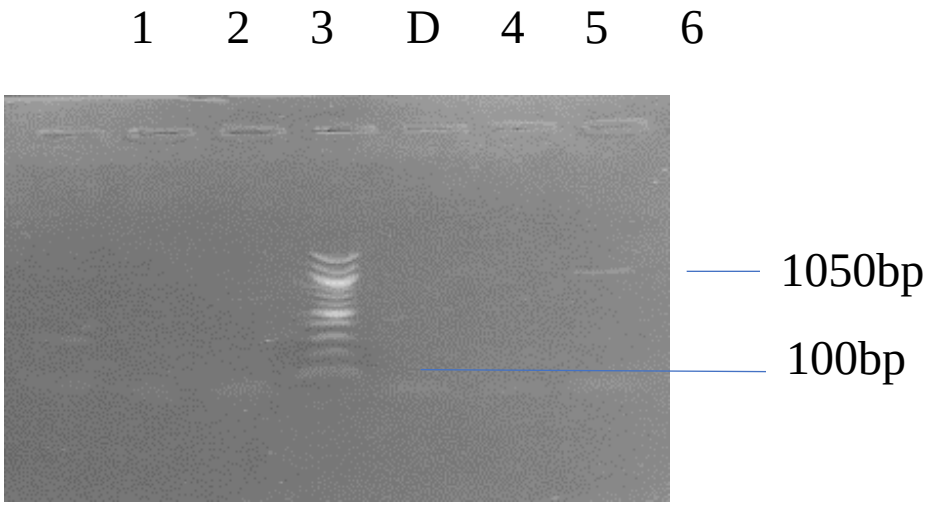

Plate 2: VEB gene in the bacteria isolates

Plate 3 captures the agarose gel electrophoresis of NDM gene of some selected bacterial isolates. Lane 2 represent the NDM gene band (390bp). Lane D represents the 100bp Molecular ladder of 1500bp.

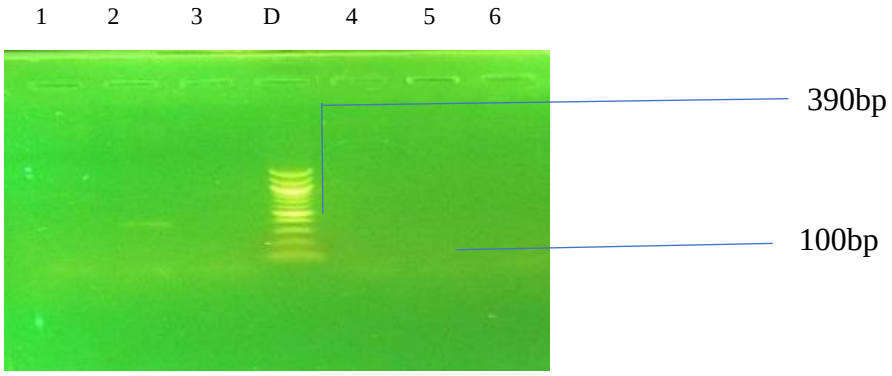

Plate 3: NDM gene in the bacteria isolates

Plate 4 presents the agarose gel electrophoresis of *tet* A gene of some selected bacterial isolates. Lanes 1, 2 and 4-6 represent the *tet* A gene band (190bp). Lane D represents the 100bp Molecular ladder of 1500bp.

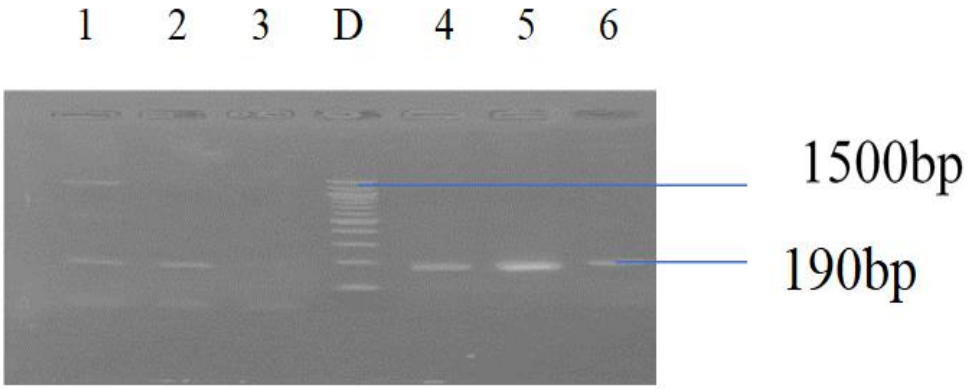

Plate 4: NDM gene in the bacteria isolates

Figure 2 below presents the isolates by percentage harboring resistance genes to be 66.7%, 16.7% 16.7% and 83.3% for *SHV, VEB, NDM* and *tet* A genes respectively. *tet* A gene is common to most of the isolates

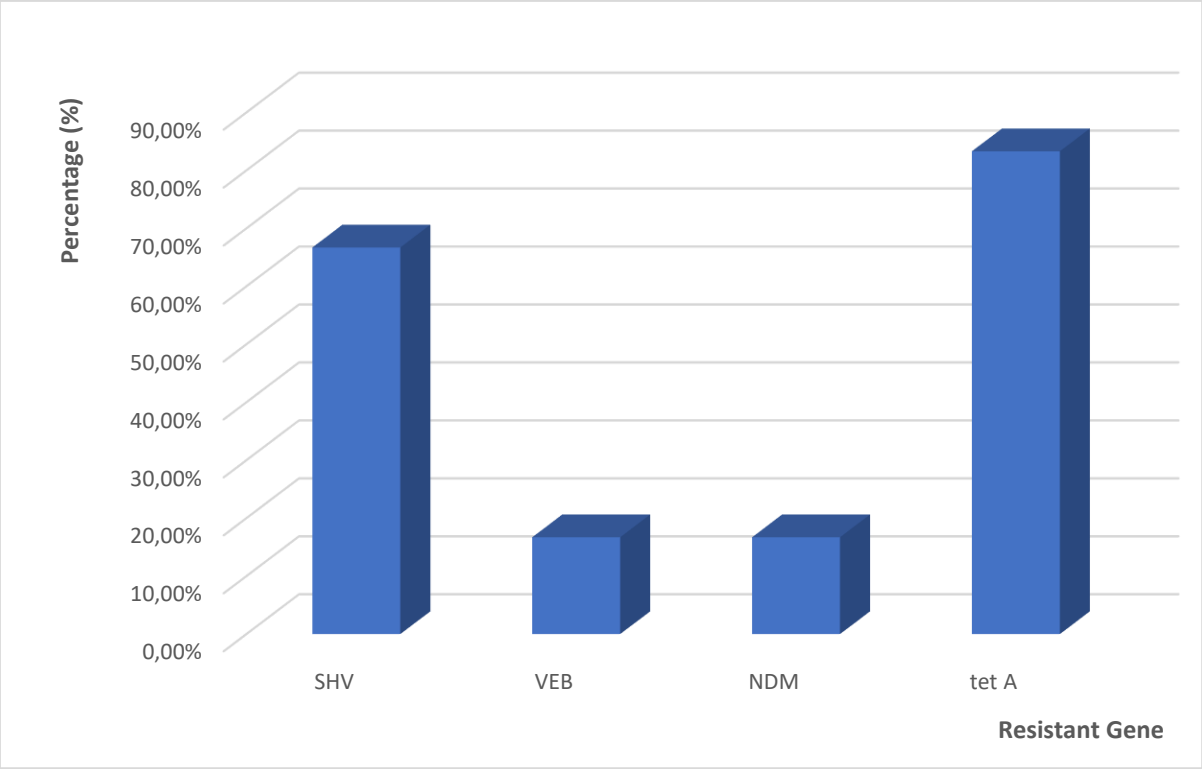

Figure 2: Percentage of isolates harboring Resistance genes

Table 6 Presents the Blastin analysis and identity of the sequenced isolates. From their respective sequence nucleotides obtained, D1, D3, D4, D5 and D6 which corresponds to isolates H1, P49, P27, P23 and P20, have been identified to be *Shigella flexneri, Salmonella enterica subsp enterica, Proteus columbae, Salmonella enterica subsp enterica and Proteus cibarius* respectively. Statistically, there is significant difference ($P = 0.0377$, $P \leq 0.05$) in obtaining *Salmonella* spp from the sequenced isolates.

Table 6: Characteristics for Blastin Analysis and identity of Isolates sequenced

S/N	Specimen Code/Type	Sequence Nucleotide obtained	Gene Bank Accession Number	Identity of Isolate obtained
1	D1 (H1)	AAGTAAGCTCGGGGGGCTTTCACCATCTGACTTAATTGACCCGCCTGCGGT GGCGCTTTACGCCCAGAATTCCGATTAACGCTTGCACCCTCCGATTACCGCG GCTGCTGGCACGGAGTAGCCGGTGCTTCTTCTGCGGGAACGCAATTGACA AGGGATTAACCTTATCACCTTCCTCCCCCTGAAAGACTTTACAACCCTAGGC TTTTCTACACCGGATGGCTGCACAGGCTGCCCCATGTGCATATTCCCACTGT GCCCCGAGGGCTGGGCGGCTCAGCCCAGGGGTGTCTCCCTAGACACTAGA GTCGCGCAGGGGCTTCCCACACACAACCATATGGTTATCGAAGGAAGGCCA AAGCCTCTTGGCCGAACTTTCGGATACCCGTCAGAGTACCCTCATCGGAAT CCAACTACCACGCCCCCCGCGAAAAGAAGTTCCTGTCGTCATGAGGTAGCC GCCACTCACTAGC	OP028031	*Shigella flexneri*
2	D3 (P49)	GGGTCGACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACC TCCAAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTT GCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTCGTCCGGGGGCCGCCTT CGCCACCGGTATTCCTCCCGATCTCTACGCATTTCACCGCTACCCTGGAATT CTACCCCCCTCTCGAGACTCAGCTTGCCAGTATCAGATGCAGTTCCCAGGT TGAGCCCGGGGATTTCACATCTGACTTAACAAACCGCCTGCGTGCGCTTTA CCCCAGAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGC ACGAGTAGCGGCGCTTCTTCTGCGGGTAACGTCAATGAGCAAAGGATTACT TTACTCCCTTCCTCCCCGCTGAAGTACTTTACAACCCAAAGCCTCTTATACA CG	PQ288806	*Salmonella enterica subsp enterica*
3	D4 (P27)	TTACAGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCG CACCTGAGCGTCAGTCTTTGTCCAGGGGGCCGCCTTCGCCACCGGTATTCC TCCACATCTCTACGCATTTCACCGCTACACATGGAATTCTACCCCCCTCTAC AAGACTCTAGCTAACCAGTCTTAGATGCCATTCCCAGGTTAAGCCCGGGGA TTTCACATCTAACTTAATTAACCGCCTGCGTGCGCTTTACGCCCAGTAATTC CGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAG CCGGTGCTTCTTCTGTCGGTAACGTCAATCGTTGATGGTATTAACATCAACG CCTTCCTCCCGACTGAAAGTACTTTACAACCCTAGGGCCTTCTTCATACACG CGGCATGGCTGCATCAGGCTTGCGCCCATTTGTGCAATATTCCCCACTGCTG CCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCC TCTCAGACCAGCTAGGGATCGTCGCCCTAGGTAAGCCATTACCTTACCTACT AGCTAATCCCATATGGGTTCATCCGACAGCGCAAGGCCCGAAAGGTCCCCT GCTTTGCTCCTAAGAGATTATGCGGTATTAGCCACCCGTT	PP556400	*Proteus columbae*
4	D5 (P23)	CTCTAAATCGACATCGTTTACAGCGTGGACTACCAGGGTATCTAATCCTGTT TGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTTGTCCAGGGGGCCGCC TTCGCCACCGGTATTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGA ATTCTACCCCCCTCTACAAGACTCTAGCCAACCAGTTTCAGATGCAATTCCC AAGTTAAGCTCGGGGCTTTCACATCTGACTTAATTGACCGCCTGCGTGCGC TTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCT GCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATTGCTAA GAGTATTAATCTTAACACCTTCCTCCCCGCTGAAAGTACTTTACAACCCTAA GGCCTTCTTCATACACGCGGCATGGCTGCATCAGGCTTGCGCCCATTGTGCA ATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCA GTGTGGCTGATCATCCTCTCAGACCAGCTAGAGATCGTCGCCTAGGTGAGC CTTTACCCCACCTACTAGCTAATC	MH356674	*Salmonella enterica subsp enterica*
5	D6 (P20)	GCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACAT CGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTT TCGCACCTGAGCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTAT TCCTCCAGATCTCTACGCATTTCACCGCTACACCTGGAATTCTACCCCCCTC TACGAGACTCAAGCTTGCCAGTATCAGATGCAGTTCCCAGGTTGAGCCCGG GGATTTCACATCTGACTTAACAAACCGCCTGCGTGCGCTTTACGCCCAGTA ATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGT TAGCCGGTGCTTCTTCTGCGGGTAACGTCAATGAGCAAAGGTATTAACTTTA CTCCCTTCCTCCCCGCTGAAAGTACTTTACAACCCGAAGGCCTTCTTCATAC ACGCGGCATGGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGC TGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGGCTGGTCAT CCTCTCAGACCAGCTAGGGATCGTCGCCTAGGTGAGCCGTTACCCCACCTA CTA	MK993517	*Proteus cibarius*

Figure 3 shows the phylogenetic tree of the evolutionary relationship between the isolates. The phylogenetic analysis, based on evolutionary distances calculated using the Jukes-Cantor method (Jukes & Cantor, 1969). The 16s rRNA sequence from the sequenced isolates produced an exact match to *Salmonella enterica subsp enterica*, *Shigella flexneri, Proteus*

columbae and *Proteus cibarius* respectively, during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide database.

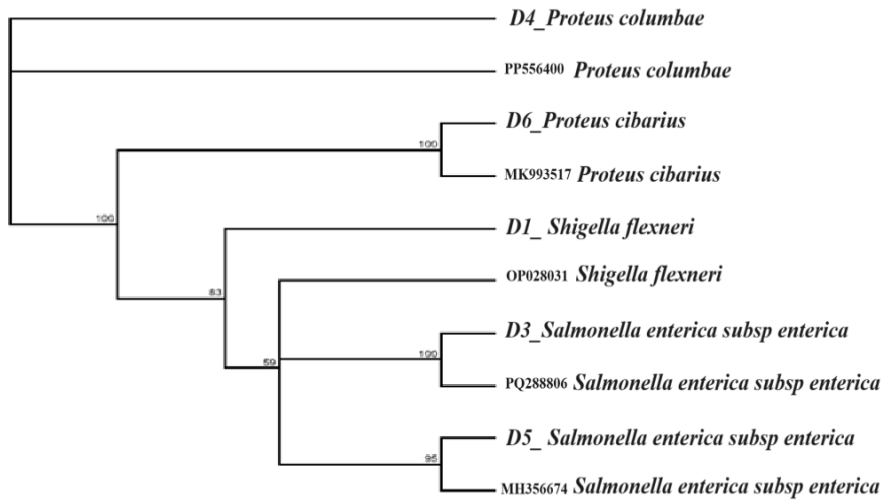

Figure 3: Phylogenetic tree of the evolutionary relationship between the bacterial isolates

DISCUSSION

Salmonella infections is a disease of distress and burden to human race, the coming onboard of antimicrobial resistance in strains of Salmonella has made it a more serious problem worldwide (Biswas et al., 2022). The first incidence of Salmonella resistance to a single antibiotic (chloramphenicol) was reported in the early 1960s (Lorenzo & Palmer, 2019). Ever since, the isolation of resistant strains of Salmonella have being on the increase according to findings of Yang et al., (2023).

The Fluoroquinolones and Extended-Spectrum Cephalosporins were introduced as antimicrobial agents of choice in treating multi-drug resistance (MDR) cases of salmonella infections following the emergence of resistance to towards the traditional antibiotics such as ampicillin, chloramphenicol and co-trimoxazole (Crump & Medalla, 2022).

The present study which seeks to determine and detect antibiotic resistance genes in Salmonella isolates from poultry and clinical sources in Bayelsa State, a South- South State in Nigeria, a prevalence of 11% Salmonella isolates recorded. A higher prevalence was obtained with the poultry samples than the clinical samples, 18% and 4% prevalence rates respectively. The marked difference in these two rates can be accounted for by factors such as environmental conditions, farm management and biosecurity practices. Studies by

Nworie et al., (2020) reported lower prevalence rates. The findings in this study are however consistent with 2.8%, 8.3% and 10.4% findings of Mathole et al., (2017); Jamshidi et al., (2010) and Manning et al., (2015) respectively. And contrary to our findings are the prevalence higher prevalence rates of 51% and 48% in the studies of Zishiri et al., (2016) and Abatcha et al., (2018).

It was observed that the poultry isolates have a greater incidence of resistance against the tested antibacterial agents when compared to the clinical isolates. This observed difference could be due to the indiscriminate usage of antibiotics in animal feed to promote the growth of food animals and veterinary medicine to treat bacterial infection in those animals (Graces et al., 2022). This creates selection pressure and poses a high risk of zoonotic disease with the transmission of MDR *Salmonella* strains from animals to humans via the ingestion of food or water contaminated with animal's faeces, direct contact or the consumption of infected food animals (Mughini-Gras et al., 2021).

The antibiotic susceptibility findings showed the isolates to be highly resistant to ceftriaxone (63.6%), tetracycline (90.9%), clindamycin (90.9%), augmentin (72%) and tazobactam/piperacillin (72%). A 100% resistance was recorded against cefixime and nitrofurantion. On the contrary, all the isolates were susceptible to ofloxacin (100%). Also, there were high susceptibility rates of 90.9%, 90.9%, 81.8%, and 63.6% to levofloxacin, and gentamicin. Ciprofloxacin and co-trimoxazole respectively.

With regards to the fluoroquinolones, Suez et al., (2013); Piekarska et al., (2023); Guerra et al., (2016) and Ali et al., (2023) reported diverse levels of resistance of *Salmonella* worldwide, ranging from 14.4% to 90%. They specifically reported 20.34% of the isolates to be resistant to ciprofloxacin and 38.98% of strains to be resistant to ofloxacin. These reports are inconsistent with the findings of this study as a relatively low percentage of resistance was recorded against the fluoroquinolones used in this study; levofloxacin and ciprofloxacin. Contradicting is the 100% susceptibility recorded for ofloxacin. The findings from this study are equally inconsistent with data from Taiwan, Trinidad, and Malaysia which reported high levels of resistant *Salmonella* isolates to aminoglycosides and quinolones (Chen et al., 2010; Abatcha et al., 2013; Hsu et al., 2023 and Kumar et al 2022).

On the β-lactam antibiotics, the susceptibility findings of this study are supported by findings of Arslan et al., (2010) with reports of high levels of resistance of *Salmonella*

isolates to the penicillins and cephalosporins. Contrary to this finding is the works of Reza et al., (2018) which recorded high susceptibility of salmonella isolates to β-lactam agents.

On biofilm formation, the report of this study is totally inconsistent with previous findings. None of the isolates in this study demonstrated biofilm formation capability using the congo red agar method. However, in separate studies conducted by Sereno et al., (2017) and Reza et al., (2018), a significant correlation between biofilm formation and the development of resistance to antimicrobial agents was established. In this study, such correlation was not established from the Pearson Correlation Coefficient test analysis done.

The findings of resistance gene in this study report five (5) of the isolates representing 83.3% to possess *tet* A gene, four (4) isolates representing 66.7% to possess SHV while VEB and NDM genes were harbored by one (1) poultry and one (1) clinical isolate respectively. Whereas, the *tet* A gene codes for resistance to the tetracycline family of antibiotics, the SHV, VEB, and NDM genes are β-lactamase genes that code for resistance to the β-lactam antibiotics (penicillins and cephalosporins).

The high resistance recorded by isolates against tetracycline and by extension other protein synthesis inhibitors viz a viz clindamycin and nitrofurantoin can be related to the presence of *tet* A gene in the majority of the isolates. Also, the remarkably high resistance recorded against the β-lactam antibiotics could be accounted for by the presence of the β-lactamase genes amongst the isolates. Indiscriminate usage of these agents in animal feed could account for cases of horizontal and vertical gene transfer thereby conferring notable resistance to human *Salmonella* isolates. The augmentation of human salmonellosis started with the emergence of foodborne MDR salmonella (Helmy et al., 2024; Khormi & Bhunia, 2023).

CONCLUSION

This study has demonstrated an alarming increase both in the prevalence of *Salmonella* in poultry samples in particular and the development of resistance by species of *Salmonella* to empirical antibiotics in general. Cases of MDR is on the rise and this has resulted to prolong hospital stay for patients with cases of salmonellosis. The economic burden consequences due to this cannot be over emphasized. The study established that the aminoglycosides represented by gentamicin and the fluoroquinolones represented by ofloxacin, levofloxacin and ciprofloxacin remain viable treatment options for infections of

Salmonella and there is a strong need to restrict and or halt every indiscriminate usage of these agents particularly in animal/poultry feeds even as proactive steps are taken in the search for newer and effective agents. There is equally the need to strengthen the antimicrobial stewardship program by all players in order to combat the menace of antibiotic abuse and misuse even as antibiogram results reported worldwide are constantly monitored.

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