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# Plasmid Profile of Environmental and Clinical Isolates of Multidrug Resistant *Salmonella Typhimurium* from Patients Attending General Hospital Asokoro, Abuja and Environ

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***Abstract***

*Plasmid is isolated from Salmonella typhimurium, an enteric bacterium responsible for gastro enteritis. Gastro enteritis remains a major clinical and public health problem all over the world, especially among children in the developing countries of the sub – Sahara Africa. The profiling of plasmid isolate of Salmonella typhimurium involved cultivation, isolation, identification, and characterization. The study aimed at investigating the multidrug resistance of salmonella typhimurium of patients attending General Hospital Asokoro with typhoid fever and the susceptibility of isolates to antibiotics. A total of thirty (30) samples, from blood, stool, and water were collected from patients attending General hospital, Asokoro and the environs. The specimens were analyzed using standard microbiological, biochemical, and serological techniques. Bacteriological analysis revealed that twenty-five (25) of the specimens were positive for Salmonella typhimurium. The other five (5) specimens were negative for salmonella typhymurium typhimurium. The biochemical test revealed that they were able to utilize sugar and are lactose fermenter. Anti-microbial susceptibility determination revealed that the most active drugs against salmonella include Ciprofloxacin, Ofloxacin, Amikacin, and Gentamicin. All the isolates were resistant to tetracycline and Ampicillin. There was marked resistance of all isolates to amoxicillin, doxycycline, and chloramphenicol. The result of findings revealed that the genes that encode resistance properties in Salmonella typhimurium are plasmid- borne in some of the bacteria. These plasmids can be cloned and used for genetic engineering and recombinant DNA technology. The antibiotic susceptibility determined the appropriate drugs efficient for therapy of Salmonella infections.*

***Keywords:*** *Antibiotic Susceptibility, Drug Resistance, Salmonella typhimurium, Plasmid.*

## Introduction

Plasmid is an extra chromosomal, small independent circle of DNA that replicates itself independently of chromosomal DNA especially in the cells of bacteria. Plasmids are autonomous extra chromosomal covalently closed circles of duplex DNA that range in size from about 1 to greater than 400 kilo bases (kb). They code for a wide variety of properties and most of these are extremely important to society and of great importance in medicine. These include resistance or sensitivity to antibiotics, production of enterotoxins and the ability to transmit themselves (and other genetic material) by various mechanisms that include conjugation with other bacteria.

In molecular biology, plasmids have been used extensively as model systems for the study

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of genetic material in particular, the structure, replication and some non – reciprocal forms of recombination. Furthermore, they are particularly useful in genetic engineering for instance, a variety of the commonly used cloning vectors are plasmids.

The profiling of plasmid isolate of *Salmonella typhimurium* normally involves the cultivation, isolation, identification, and characterization. Plasmid is isolated from *Salmonella typhimurium* which is an enteric bacterium that is responsible for gastro enteritis. Gastro enteritis remains a major clinical and public health problem all over the world, especially among children in the developing countries of the sub – Sahara Africa. More than 700 million cases of acute gastroenteritis are estimated to occur annually in children or children that are less than 5 years old. The mortality rate is estimated to be 3 – 5 million per year [1].

*Salmonella typhimurium* was isolated from water, stool and blood samples of patients having typhoid fever, gastroenteritis, and their environments such as their sources of drinking water using conventional methods for the purpose of this study. Many different pathogens have been found in the stool of children with gastroenteritis, such as bacteria in the form of *Salmonella* spp., *Shigella* spp., *Campylobacter* Spp., *Bergsma* spp., among others [2,3}. Viruses that can be found in the same stool include rotaviruses, adenoviruses and astro viruses all of which have been clearly established as causative agents of gastroenteritis in children.

A prerequisite for the identification of plasmid – coded pathogenicity factors is the determination of the plasmid distribution pattern in bacteria species. Therefore, several publications concerning the plasmid content of different *Bacteroides* spp. have appeared [4]. P W Stiffler *et al* first reported the occurrence of plasmids in *Bacteroides fragilies* in 1973.

More recently comprehensive surveys have been performed on the plasmid content of different species of *Bacteroides* [5, 6]. In their investigations, mostly small cryptic plasmids have been found in concentration of 40% of all strains either from intestinal or clinical origin.

*Salmonella spp*. has been extensively incriminated worldwide as a common cause of bacterial gastro enteritis in humans with food to animals serving as important reservoirs. However, the present study aimed at investigating the bacteria in multi drugs resistance gene of specimens such as human blood, human stool and water which were classified as clinical and environmental isolates obtained at Federal Capital Territory, Abuja (FCT). Basically, to isolate and determine their multi drug resistance genes as well as characterization [1, 7, 8]. This is very important in identifying factors involved in resistance, understanding the diversity of multidrug resistance strains, identifying genetic linkages among markers, understanding potential transfer mechanisms, and developing efficient detection methods.

## Methodology

Sterilization of Laboratory, Materials, glassware, and Media was done. The working laboratory was sterilized by fumigation. Reagents used in this study were prepared using either distilled or deionized water. All chemicals used were dissolved with distilled water and made up appropriately according to instructions. The pH was adjusted adequately as desired with the aid of pH meter with the use of sodium hydroxide (NaOH). Isolation and identification of *salmonella Salmonella typhimurium* from samples was using the various agars mentioned above and according to instructions (Refer to steps of plasmid isolation below/ Figure 1). Cultural and Morphological Characterization of *salmonella Salmonella typhimurium* isolates on agar plates were examined.

Gram staining was done. Gram reaction and cell morphology of the isolates were examined from appropriate stained, heat fixed smears

following Salle (1971). Tests on biochemical characterization of isolates include sugar fermentation, Kovac's indole test, citrate utilization and oxidase test. Antibiotics susceptibility testing on antibiotics chosen was based on the prescription practices for *Salmonella* in the selected study places and from the literature. Interpretation of the result was done according to Koneman *et al* (1997). Kirby Bauer sensitivity tests or disk diffusion antibiotic sensitivity testing, break point sensitivity tests and disk sensitivity tests were all done according to instructions. Isolated plasmid DNA was characterized by Agarose gel electrophoresis.

A total of thirty (30) specimens were processed for *Salmonella typhymurium typhimurium* isolation and subsequent profiling. Isolation and identification of *Salmonella typhimurium* was done by culturing specimens in Nutrient Agar broth, incubated at 30$^{o}$C for 48hrs. Colonies formed by bacteria were isolated and identified based on their cultural, mormorphological,d biochemical characteristics. The cultural and morphological characteristics of *Salmonella typhimurium* was conducted by appropriate staining and heat fixed smears. Biochemical characterization of isolates performed included sugar fermentation, kovac indole test, citrate utilization and oxidase test. Antibiotic susceptibility testing carried out was on Antibiotics chosen based on the prescription practices for *Salmonella* in the selected study places and from the literature. Interpretation of the result was done accordingly [6]. Plasmid isolation was performed on bacterial isolates that were found to be only Gram-negative. Isolates were first sub-cultured on Mueller Hinton Agar plates to get the pure strains of the *salmonella Salmonella typhimurium* for plasmid isolation. These plates were incubated at 37$^{o}$C for 24hrs. The cells were harvested on the second day for isolation of the plasmid DNA. Partial characterization of plasmid DNA was performed using 0.8% Agarose gel at pH 8.0. The buffer used for the electrophoresis was Tris borate EDTA (TBE).

## Steps in Plasmid Isolation

All steps below were applied to the different samples collected.

1. Cell pellets of bacterial strains grown on Mueller Hinton Agar were scrapped into eppendorf tubes containing 200 µl buffers **1**. Tubes were then kept on ice to maintain the cytoplasmic content or environment of the bacteria. The mixture of bacteria and buffer **1** turns milky.
2. Four hundred microliters (400µl) of lysing solution was added into the tubes containing the mixture (breaking of the bacterial cells to release the plasmid DNA) and the tubes were inverted 20 times at room temperature for 5minutes. The addition of the lysing solution produces a clear solution, ie the mixture now became clear.
3. Buffer **2** was placed on ice. 300µl of ice-cold buffer **2** was added and they were gently mixed by inversion 10 times. They were kept on ice for 30 minutes. The plasmid DNA flows to the top as a white liquid (whitish in color). The contents in the tubes (labeled) were centrifuged for 30 minutes at 12000rpm. The supernatants were decanted into other labeled tubes that correspond to the former tubes in use.
4. Seven hundred microliters (700µl) of chloroform was added to the supernatant and mixed by inversion 20 times. The solution was centrifuged for 10minutes at 3000rpm.There was foaming in the tubes which indicate the presence of Plasmid DNA. 500µl of the aqueous layer was removed from them and put in fresh tubes, and then 1ml of ice cold 95% ethanol was added. They were kept on the ice for 24hrs. The next day they were centrifuged at 12,000rpm for 30mins. Supernatant were discarded and the tubes were inverted on filter paper.

5. Hundred microliters (100µl) of buffer **3** was added to the residue in the tubes and were gently tapped for 5minutes. The tubes were stored in the refrigerator until needed for characterization by agarose gel electrophoresis.

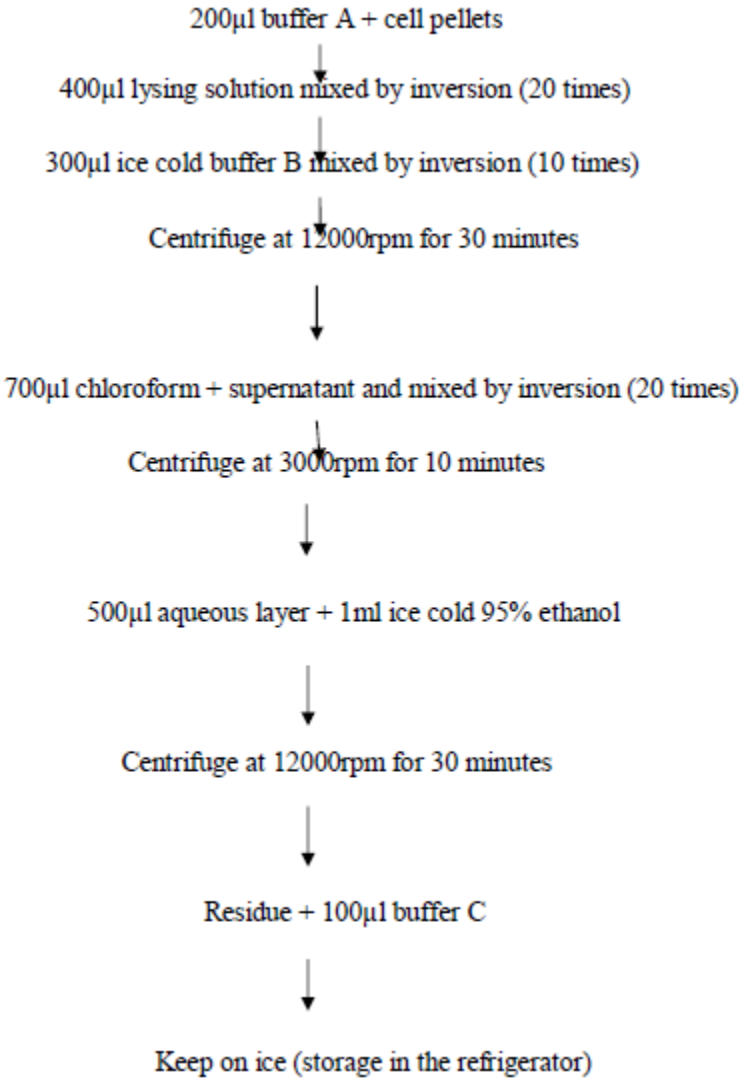



**Figure 1.** Flow Chart for Plasmid Isolation Steps

## Results

Tests were performed under aseptic techniques. The colony morphology was moist, gray and smooth. They were small gram-negative, non-spore forming enteric bacilli. 30 Strains were subjected to gram staining. 25 strains appeared like pink or red rods under the oil immersion objective lens of the light microscope. The other strains appeared as purple, assuming spherical shape. This shows that 25 strains were gram-negative while the five strains were gram positive.

30 strains were tested for sugar utilization. 25 strains were positive to lactose utilization and were lactose fermenters. Thus, they are known to be gram – negative bacteria. The rest were non- lactose fermenters.

Out of a total of 30 bacterial strains subjected to this test 12 strains tested positive and were able to utilize tryptophan as protein source. They therefore belong to *E. coli* family.

30 strains were subjected to this test and only 15 strains tested positive which were able to utilize citrate as carbon source. They therefore belong to the genus klebsiella Klebsiella.

Out of 30 strains subjected to this test, 22 strains tested positive and were able to utilize oxygen as energy source. They therefore belong to the genus Pseudomonas (Table 1).

**Table 1.** Results of Biochemical Characterization of *Salmonella Typhimurium* Isolates

| Bacterial | Lactose | Tryptophan | Citrate | Oxygen |
|---|---|---|---|---|
| ***S, typhimurium*** | --- | -- | -- | -- |
| ***E. Coli*** | -- | +++ | -- | -- |
| ***Klebsiella*** | -- | --- | +++ | --- |
| ***Pseudomonas*** | -- | -- | -- | +++ |

Number of colonies obtained on plates when compared with control plasmid as the determinant of antibiotic susceptibility. An absence of colonies on plates indicated that antibiotics were active. Presence of colonies on plates indicated that antibiotics were not active. Quinolones compound comprising of (ciprofloxacin 98.6%, Ofloxacin 93.3%) and the aminoglycosides (Amikacin 90.6%, Gentamycin 84%) and Neomycin 50.5% was the most active drugs against the isolates. There was no colonies formed i.e. complete absence of colonies on the plates. Tetracycline (100%) and Ampicillin (100%), Streptomycin (100%), and Chloramphenicol (100%), however, were the most resistant drugs. Colonies were formed. Amoxicillin (90.7%), Doxycycline (68%) and Cotrimozazole 61.4% displayed marked resistance. Colonies were formed but not up to Tetracycline and Ampicillin, the result is as shown in Table 2.

**Table 2.** Percentage and Interpretation of Result for Antibiotic Susceptibility and Resistance of Salmonella Typhimurium Isolates

| Names Of Antibiotics | Percentage Of Susceptibility | Percentage of Resistance | Interpretation Of Result |
|---|---|---|---|
| Ciprofloxacin | 98.6% | 1.4% | Sensitive |
| Ofloxacin | 93.3% | 6.7% | Sensitive |
| Amikacin | 90.6% | 9.4% | Sensitive |
| Gentamicin | 84% | 16% | Sensitive |
| Neomycin | 50.5% | 40.5% | Sensitive |
| Streptomycin | 0.0% | 100% | Resistant |
| Tetracycline | 0.0% | 100% | Resistant |
| Chloramphenicol | 0.0% | 100% | Resistant |
| Ampicillin | 0.0% | 100% | Resistant |
| Amoxicillin | 9.3% | 90.7% | Intermediate |
| Cotrimoxazole | 38.6% | 61.4% | Intermediate |
| Doxycycline | 32% | 68% | Intermediate |

Anti-microbial susceptibility determination (Table 2) revealed that the most active drugs against *salmonella* include Ciprofloxacin, Ofloxacin, Amikacin, and Gentamicin. All the isolates were resistant to Tetracycline and Ampicillin. There was marked resistance of all isolates to Amoxicillin, Doxycycline and Chloramphenicol as shown in Table 2 below.

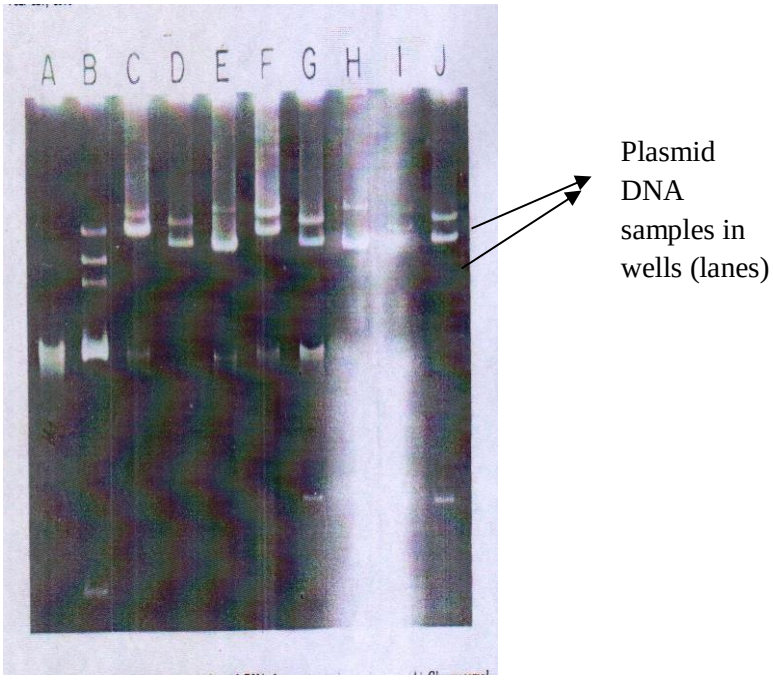
Bacteria that were resistant to different test antibiotics (at the breakpoint Concentration) grew to form colony, whilst those that were sensitive did not grow to form colony. A

control agar plate with no added antibiotic is used to check for viable bacterial growth.

Control: sensitivity agar plate without antibiotics incorporated but inoculated with ten different strains. Ampicillin- sensitivity agar plate incorporated with ten different strains inoculated on to the surface of the agar plate.10 strains grew on the control plate. Strains 1, 3, 6, 7, 9, &10 were sensitive to ampicillin while strains 2, 4, 5 & 8 were resistant.

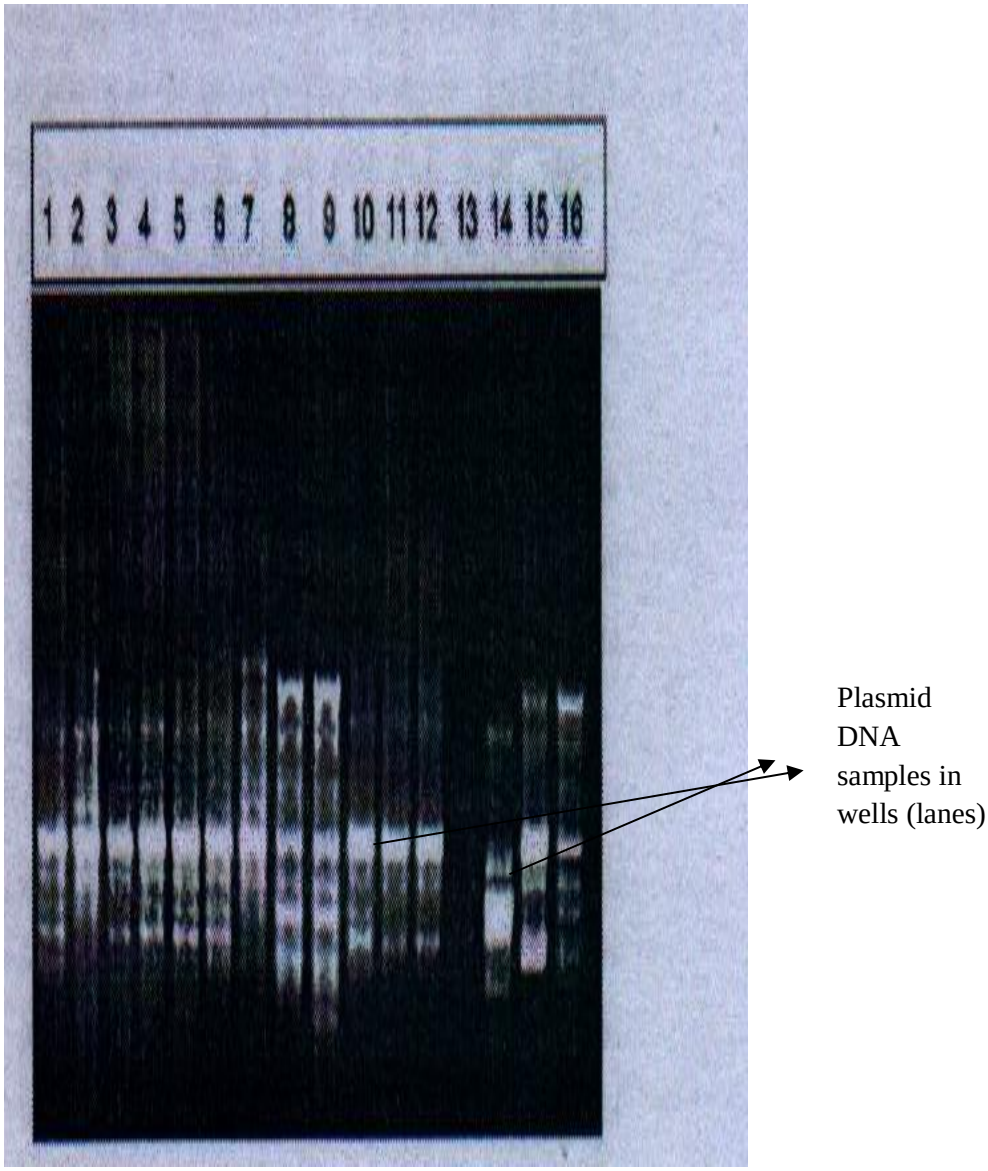
After allowing the bacteria to grow overnight, areas of clear media surrounding the disks indicate that the antibiotic inhibits bacterial growth. The concentration of antibiotic that diffuses into the media decreases with increasing distance from the source. Therefore, the more sensitive the bacteria are to a given antibiotic, the larger the clear bacteria-free zone that forms around the disk containing that antibiotic. The test results are reported as sensitive, intermediate, or resistant, based on the size of the zone of inhibition. Observed zone of inhibition greater than or equal to the size of the standard zone, meant the bacteria was sensitive to the antibiotic. Conversely, observed zone of inhibition smaller than the standard size, meant the bacteria was resistant. The size of a zone of inhibition in the test is inversely related to the minimum inhibitory concentration (MIC), which is the amount of antibiotic required to prevent bacterial growth in an overnight culture.

After the agarose gel electrophoresis of the sample's plasmid DNA along with the marker used as a standard (phage lambda DNA Hind III digest), it was discovered that out of 36 samples electrophoresed, 15 strains harbored plasmid DNA (see figure1 and 2). The relative molecular weights after interpolation from the standard curve have been listed in table 3. Resistance to different antimicrobial agents was mediated by a large plasmid. All plasmid DNA harbored large plasmid ≥ 15kbp in size which is responsible for multi- drug resistance. This result is in conformity with the findings of Liebana *et al*. 2002.




Legend: Lane A: Phage lambda (λ) DNA Hind III digest molecular weight marker (standard); Lanes B – J: Plasmid DNA isolates from *Salmonella typhimurium*

**Figure 2.** Results of Agarose Gel Electrophoresis of Plasmid DNA from the Samples




Legend; Lane 1: Phage lambda (λ) DNA Hind III digest molecular weight marker (standard/control); Lanes 2-11: Plasmid DNA isolates from Salmonella typhimurium

**Figure 3.** Results of Agarose Gel Electrophoresis of Plasmid DNA from Samples

**Table 3.** Plasmid DNA Sizes in Base Pairs

| Sample Plasmid DNA | Molecular weight (bp) |
|---|---|
| 1 | 1514, 1148 |
| 2 | 21514, 1148 |
| 3 | 31514, 1148 |
| 4 | 1000 |
| 5 | 1738 |
| 6 | 10058 |
| 7 | 1738,1318 |
| 8 | 1660,1202 |
| 9 | 1738,1318 |
| 10 | 1738,1380 |

| | |
|---|---|
| 11 | 1318 |
| 12 | 1148 |
| 13 | 1260 |
| 14 | 1514 |
| 15 | 1260, 1380 |

Table 3: 2: Results of the sizes (base pairs) of the sample plasmid DNA as determined from Agarose gel electrophoresis (estimation of the size of the DNA fragments was by charge). Plasmid sizes are all < 15kbp.

## Discussion

*Salmonella typhimurium* is a well – known zoonotic pathogen causing diarrhea, pyrexia, and septicemia in animals and humans [10.11.12]. Non – typhoid *Salmonella* serovars remain a potential threat to human health. Beef from cattle and broiler chickens are possible sources of these organisms in the environment [13, 14, 15]. Although non – typhoid *Salmonellosis* in human is usually a self-limiting disease confined to the intestinal tract, when infections spread beyond the intestine or when immune compromised persons are affected, it may have serious consequences requiring appropriate antimicrobial treatment [13, 16]. In animals such symptoms can be lethal, so prompt treatment with appropriate antimicrobial agents remain economically important. Hence, the surveillance of antimicrobial resistant strains is necessary for effective treatment prediction of occurrence of resistant populations of prevailing biotype [16]. Public health measures to reduce chances of infection, thus, take into consideration the presence of the organism in animals. The non-host specific *Salmonella* serovar such as *Salmonella enteritidisis* and *Salmonella typhimurium* are the agents of paratyphoid infections in domestic poultry and a major concern for food safety [14, 15,16].

Bacteriology analysis performed included colonial morphology. This is an important tool in the identification of bacterial culture. The colony morphology was moist, gray, and smooth. Microscopic examinations of the colonies confirmed that they were suspected *Salmonella typhimurium.* Colonies examined for motility also confirmed *Salmonella typhimurium* to be motile or peritrichous flagella. This also confirmed the conjugation and transmission activities [1, 17,18].

Thirty (30) strains were subjected to gram staining. Twenty-five (25) strains appeared like pink or red rods under the oil immersion objective lens of the light microscope. The other five (5) strains appeared as purple, assuming spherical shape. This shows that 25 strains were actually gram-negative while the five strains were gram positive.

Biochemical characterization performed included sugar utilization, indole test, citrate utilization and oxidase test according to techniques utilized by other researchers [5].

Sugar utilization result confirmed that twenty-five strains were positive to lactose utilization. Thus, they were known to be gram – negative bacteria. The rest five were non-lactose fermenters.

Out of a total of thirty (30) samples subjected to Kovac's Indole test twelve (12) strains tested positive and were able to utilize tryptophan as protein source. They therefore belong to *E.coli* family. Thirty (30) strains were subjected to Citrate utilization test fifteen (15) strains tested positive which were able to utilize citrate as carbon source. They therefore belong to the genus *Klebsiella* [18, 19].

Out of thirty (30) strains subjected to Oxidase test, twenty- two (22) strains tested positive and were able to utilize oxygen as energy source. They therefore belong to the genus *Pseudomonas*. Fifteen (15) of these strains belong to gram negative bacteria and others were gram positive. Of the fifteen (15) gram negative strains the bacterial harbored plasmid large enough to cause drug resistance

in infected patients [20]. These results were in conformity with the findings of other researchers [17, 18]. The taxonomy of the bacteria was over 30 genera and less than twenty-five (25) species in the taxonomy of enterobactericeae.

Plasmid isolation and characterization with Agarose gel electrophoresis was performed [21]. The isolated plasmid sizes from the host gram-negative bacilli ranges from 1.370kbp to 1.891kbp indicating that they were less than <15kbp. They are easily transferable into recipient strains and can be used in gene therapy. This is according to the findings of other researchers [18, 19].

Antimicrobial test of resistant *Salmonella typhimurium* has become necessary for effective treatment and prediction of occurrence of resistant populations. Antibiogram of the isolates revealed marked susceptibility of isolates to Quinolones compounds namely Ciprofloxacin and Ofloxacin and the aminoglycosides compounds namely Amikacin, Gentamycin and Neomycin which were the most active drugs against the isolates. The result of antimicrobial susceptibility in Table 1, Table 2 and figure 1 collaborates with the findings of other researchers [22, 23, 24].

Amoxicillin, Doxycycline and Cotrimozazole displayed marked resistance. This result was in conformity to the findings of other researchers [3, 25, 26].

There was complete resistance to tetracycline, Ampicillin, streptomycin, and Chloramphenicol. This result agrees with the findings of other researchers [2, 27, 28]. The analytics of susceptibility is shown in figure 1.

It is hoped that the biological characterization of *Salmonella* will become the necessary guide for diagnosis of *Salmonellosis* in hospital, environmental and community samples during Salmonellosis outbreak.

## Conclusion

This study basically provides d information on plasmid profile of multi drug resistant *Salmonella typhimurium.* It was discovered that in Nigeria particularly in Federal Capital Territory (FCT) Abuja and environ the most active drug against *Salmonella* infection were Ciprofloxacin, Ofloxacin, Amikacin and gentamicin. Though discrepancies may arise in other parts of the country due to differences related to prescription practices, Clinicians can use the test results to choose appropriate antibiotics to combat a particular infection in a patient. Administering antibiotics that specifically target the bacteria that are causing the infection can reduce the use of broad-spectrum antibiotics, which target many types of bacteria. Thus, clinical application of test results can decrease the frequency with which antibiotic-resistant bacteria evolve.

This study has provided baseline data for more extensive research to be conducted in the area of plasmid profile of multidrug resistance *Salmonella typhimurium* [27, 28, 29]. The isolation of plasmid DNA from bacteria is an important or crucial technique in molecular biology and it is an essential step in many procedures such as cloning, DNA sequencing, transfection, and gene therapy. Further research is recommended to determine the base sequence for genes that code for properties in *Salmonella typhimurium* to clone the genes into more viable, non- pathogenic and ubiquitous organism, in the use of recombinant DNA technology and the in genetic engineering as they can be transmitted between bacteria of the same species. Plasmids can potentially be used to increase the spread of antibiotic resistance (AR) genes as they are very important mediator factors of horizontal gene exchange.

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## Conflict of Interest

There is no conflict of interest declared.

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