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(RESEARCH ARTICLE)

Sex distribution dynamics of plasmid mediated multi drug resistant *Salmonella serovars* isolates in Benue State, Nigeria

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Abstract

Multiplex Polymerase Chain Reaction for amplification of virulent genes was carried out on *Salmonella serovars* isolated. All the *Salmonella serovars* isolated and identified contained plasmids which were identified using replicon typing.The plasmids contained genes coding for virulence factors and multiple antibiotic resistance which include: ParcC (480bp), GyrA (251bp), CatA (198bp) were present the latter coded for chloramphenicol resistance. InvA plasmid genes (284bp and 389bp) typical of virulent *Salmonella serovars* were identified. Most of the serovars possessed plasmids that contained TetB gene in the 16sRNA region (571bp) coding for tetracycline resistance, SitC gene (578bp) and spvA gene (604bp) were also identified. Sex distribution dynamics of multidrug resistance of different*Salmonella serovars*compared the overall percentage susceptibility of the eight antibiotics used. GEN had the highest % susceptibility of 100% followed by NIT (72.2%) and COT (66.7%) before and after plasmid curing. Differences observed in curing status was insignificant (T = 0.33, P>0.05). The sex distribution of *Salmonella* infected Patients in relation to antibiotic resistance before plasmid curing was determined. The highest number of patients that resisted antibiotic was 18 as observed in AUG where 11 patients were male and 7 were female.More males compared to females showed significant antibiotic resistance before plasmid curing using the Mann whitney test (W = 84.0, P≤0.05 and after plasmid curing.There was no difference between both sexes after plasmid curing using the Mann Whitney test (W = 73.0, P>0.05).

Keywords: Salmonella; Serovars; Plasmid; Distribution and Dynamics

1. Introduction

Plasmids are extrachromosomal circular double stranded DNA found in most bacteria. Each bacterium may contain one or several plasmids. Plasmid profile analysis involves study of size, number and presence of certain genes of interests on plasmids. After the cells are lysed, the nucleic acids are subjected to electrophoresis. This gives the size and number of plasmids present in the cells. Plasmids are naturally present in some bacteria and they give special survival advantages to the organism. These include: resistance to antibiotics, pathogenicity, biodegradation, fermentation and physiological tolerance [1]. All these functions are coded for in the plasmid DNA. A plasmid must have restriction sites, resistance gene and origins of replication [2].

Plasmids can carry one or more antibiotic genes which confer resistance to a specific antibiotic to the bacteria carrying them. The presence of an antibiotic resistance gene on a plasmid allows researchers to easily isolate bacteria containing that plasmid by artificial selection when grown in a nutrient medium. Luria broth is a nutrient rich medium commonly used to culture bacteria in the lab. A liquid culture is capable of supporting a higher density of bacteria necessary to isolate enough plasmid DNA for research purposes [2]. Plasmid associated genes have been implicated in resistance to aminoglycosides, chloramphenicol, penicillins, cephalosporins, erythromycin, tetracycline, and sulphonamides [3]. They are clinically important if they contain virulence or resistance genes; the plasmid encoded fimbriae (*pefA*) gene contributes to the adhesion of *Salmonella* to epithelial cells [4]. Virulence plasmids carrying virulence genes such as

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the *spv* operon (*Salmonella* plasmid virulence) contribute to the colonization of deeper tissues among other functions [5]. Plasmids are very important in the epidemiology and spread of resistance to antibiotics. Plasmids can be horizontally transferred from one bacterial host to another even between bacterial species [6].

Curing of plasmids from bacteria strains also known as loss of plasmid is a way to eliminate the bacteria plasmid and determine the antibiotic resistance mediation. There are several methods involving chemical and physical agents that have been developed to eliminate plasmids [7]. Protocols for plasmid curing consist of chemical agents such as acridine orange, ethidium bromide and sodium dodecyl sulphate and physical agent. The mechanism of plasmid curing starts from the inhibition of plasmid replication resulted from a single nick, outside of the origin of replication of the superhelical structure. The process leads to further relaxation of plasmid DNA, an increase in melting point and circular dichroism. The intercalating agents would then break the superhelical form of plasmid DNA subsequently forming an open circular or linear form of plasmid DNA [7].

Intercalating agents such as acridine orange and ethidium bromide have been successfully used in curing bacterial plasmids. The modes of action of intercalating agents are through preferential inhibition of plasmid replication. Basically, overnight bacteria cultures are inoculated into enrichment broths, Tryptic Soy Broth (TSB), or Luria Bertani Broth (LB). Curing agents at a concentration ranging from 0.1 to 0.5mg/ml is added to the culture broth. The concentration depends on the organism and curing agent used. The cultures are then incubated overnight at 35 or 37^{o}C under constant agitation. After the treatment, antibiograms assay are again performed to find antibiotic resistance phenotypes [8, 9]. Apart from the two intercalating agents stated above, SDS (Sodium dodecyl Sulphate) is an anionic detergent that is used as a chemical curing agent. Plasmid containing cells are possibly more sensitive to SDS because of plasmid-specified pili on cell surface. The chemical acts in dislodging the indigenous plasmid from its site of attachment [10]. Physical agents such as elevated temperature have been used in curing plasmid partially or completely [11].

According to Ohad *et al*. [12], enteric fever should be treated immediately with antibiotics although widespread resistance to chloramphenicol, ampicillin and cotrimoxazole has been reported. Current treatment of salmonellosis using fluoroquinolones such as ciprofloxacin is under review as the typhoidal serotypes are gradually building strong resistance to the new therapy. New fluoroquinolones such as gatifloxacin and azithromycin hold promise. Multidrug resistance is an increasing problem in *S. enterica* serotypes. Three types of vaccines against *S*. Typhi are currently available but no licensed vaccine against *S*. Paratyphi A is available. There are vaccines against NTS serotypes Enteritidis and Typhimurium which are effective in poultry but no vaccine available for NTS in human yet. Effectiveness of treatment may depend on the presence of co-infections with other *Salmonella* species or other organisms such as worms. Precautionary approaches should be adopted. It is advisable that people should not eat foods containing raw eggs or milk such as undercooked fast food. Raw meat should not be cooked in the microwave as it may not reach a high temperature to kill *Salmonella* or it may be unevenly cooked. Hands must be thoroughly washed after handling reptiles or animal faeces [12].

Salmonella is sometimes antimicrobial resistant which can result in difficulty in treating infections [13, 14]. Because of the impact on human health, zoonotic transmission, and ability to acquire antibiotic resistance (AR), *Salmonella* has been chosen as the sentinel organism for foodborne disease and for AR monitoring [15, 16, 17]. In the U.S., *Salmonella* is estimated to cause over one million human infections each year [18]. Most of these infections result in gastroenteritis that resolves after a few days; however, some infections can be chronic or invasive, especially in the very young, the old, and groups of people with compromised immune systems [19]. In these cases *Salmonella* infections may require antimicrobial treatment to prevent further morbidity or mortality [19]. First line antibiotic treatment in the U.S. is typically a fluoroquinolone-like ciprofloxacin or a third generation cephalosporin β-lactam such as ceftriaxone, and folic acid pathway inhibitors are also available [20, 21, 22]. However, in children and pregnant women, treatment is usually limited to β-lactams due to fluoroquinolone's interference with cartilage formation; therefore resistance to β-lactams is a considerable concern in *Salmonella* [23]. In cases whereinfection is caused by *Salmonella* resistant to first line treatments, alternative second line antimicrobials may be used, such as aminoglycosides, or folic acid pathway inhibitors like sulfisoxazole or sulfamethoxazole with or without trimethoprim [24]. In MDR *Salmonella* infections, the last line treatments are usually the aminoglycoside, amikacin or the carbapenems, imipenem or meropenem which are administered intravenously. Due to observed increases in morbidity and mortality in antimicrobial resistant infections, it has been suggested that resistant *Salmonella* are more virulent than sensitive strains [25, 26, 27, 28]. However, research into this has been inconclusive, and some studies have demonstrated that resistance to some antimicrobials such as fluoroquinolones actually reduces virulence in *Salmonella* [27].

2. Material and methods

2.1. Plasmid profiling

Plasmid DNA of the strains were isolated using the alkaline lysis method as described by the protocol of Ponce *et al,* [29]. A 1.5 ml from overnight cultures of bacterial growth in Luria broth (LB, DIFCO) was centrifuged at 12,000g for 1 minute. The pellets were suspended in 1ml of SET buffer (20 % Sucrose, 50ml EDTA and 50 mM Tris-HCL, pH 7.6) and centriged for 1 min at 12, 000 x g and re-suspended in 150 µl of SET buffer. The cells were lysed by mixing with 350μl lysis buffer (1 % SOS and 0.2 M NaOH) and incubated for 30 min in ice. A 250μl of mutate buffer (3.0 M sodium mutate; pH 4.8) was added and the tubes mixed by inversion and incubated for 20 minutes in ice. After centrifugation at 12,000 x g at 4℃, 700μl of the upper aqueous phase was transferred to a clean tube and the DNA was precipitated by one volume of isopropyl alcohol. The Pellets were washed with 1ml ethanol and dissolved in 50μl of TE buffer (50 mM Tris, 1 mM EDTA, pH 8.0). The plasmids were separated on 1.0 % agarose gels in 1x Tris-autate-EDTA (TAE) buffer at 64 V for 2 hrs. The supercoiled DNA ladder (Invitrogen cooperation, Carlsbad, CA) was used as a molecular marker. The molecular sizes of the plasmids were determined using the S1 nuclease (Promega, Madison, WI) as described by Bosco *et al.* [30].

2.2. Plasmid curing

The curing curing of the plasmids of the organisms isolated was done using sub-inhibitory concentration of 0.1mg/ml of acridine orange as described by Bosco *et al.* (30). The isolates were grown for 24hrs at 37 ^{o}C in nutrient broth containing 0.10 mg/l acridine orange. After 24 hrs, the broth was agitated to homogenize the content and a loopful of the broth culture was was subcultured onto Mueller Hinton Agar (MHA) plates and plasmisds extracted as described by Ponce *et al.* (29).

2.3. PCR detection of virulence genes

All isolates of *Salmonella* were screened for virulence genes by a simplex PCR method as described by Skyberg *et al* [31]. Extraction of total genomic DNA from the isolates was done from overnight cultures using DN easy blood and tissue kit (Qiagen, Valancia, CA, USA). The composition of the PCR mixture was as reported by the methods of Skyberg *et al* [31]. Table 1 lists all the primers used in molecular characterization of *Salmonella*

2.4. Agarose Gel Electrophoresis

The extracted plasmids were separated on a 0.8 % Agarose gel and electrophoresis was carried out at 80V for 1hour 30 minutes. The DNA bands were visualized by Ethidium Bromide staining. Lamda DNA Hind III Marker (Jena Bioscience) was used as DNA molecular weight marker [31].

2.5. Sequencing

All PCR products were purified with Exo sap and sent to Epoch Life science (USA) for Sanger sequencing [3]. The corresponding sequences were identified using the online blast search at http://blast.ncbi.nlm.nih.gov/Blast.cgi

2.6. Antibiotic Susceptibility Test

Antibiotic susceptibility was tested using the Kirby-Bauer disk diffusion method according to the Clinical and Laboratory Standard Institute (CLSI) guidelines. Briefly, pure colonies of bacterial suspension were placed in test tubes and their turbidity adjusted to 0.5 McFarland turbidity standards [34]. The diluted bacterial suspensions were then transferred onto Mueller-Hinton agar plates using a sterile cotton swab and seeded uniformly. Antibiotic impregnated discs were placed on the plate surfaces using sterile forceps. *E. coli (*ATCC 25922) was used as control. Zones of inhibitions (ZIs) were measured to determine whether the bacteria are susceptible, intermediate or resistant in comparison to Clinical and Laboratory Science Institute critical points [35]. ZIs were classified as: Highly susceptible (≥20 mm); Intermediate (15-20 mm) and weakly susceptible (1-14 mm).

Table 1 Virulence Gene Primers and their Nucleotide Sequences

Primer	Target gene	Sequence (Forward/Reverse)	Amplified fragment size (bp)	Annealing Temp
16S rRNA	Genus specific	5'-TGT TGT GGT TAA TAA CCG CA -3' 5'-CAC AAA TCC ATC TCT GGA -3'	571	55
Salm3/salm4	invA	5'-GCTGCGCGCGAACGGCGAAG-3' 5'-TCCCGCCAGAGTTCCCATT-3	389	65
InvF/invar	invA	5-ACAGTGCTCGTTTACGACCTGAAT-3' 5'-AG CGACTGGTACTGATCGATAAT-3'	284	56
*Sit*C 3	*sit*C	5'-CAGTATATGCTCAACGCGATGTGG GTCTCC 5'- CGGGGCGAAAATAAAGGCTGTGATGAAC-3'	578	55
spvA	spvA	5'-GTC AGA CCC GTA AAC AGT-3' 5'-GCA CGC AGA GTA CCC GCA-3'	604	60
spvB	spvB	5'- ACG CCT CAG CGA TCC GCA -3' 5' – GTA CAA CAT CTC CGA GTA -3'	1063	60
Multiplex *inv*A/*spv*C	SpvA/ spvB	5'-ACAGTGCTCGTTTACGACCTGAAT-3' 5'-AGACGACTGGTACTGATCTAT-3' 5' GTCCTTGCTCGTTTACGACCTGAA T 3' 5' TCTCTTCTGCATTTCGTCA 3'	244 571	56
Bla SHV	ESBL	5'TGGTTATGCGTTATATTCGCC3' 5'GGTTAGCGTTGCCAGTGCT-3'	868	56
Bla TEM	ESBL	5'TCCGTCATGAGACAATAACC-3' 5'TTGGTCTGACAGTTACCAATGC-3'	972	56
Bla CTX-M1	ESBL	5'AAAAATCACTGCGCCAGTTC3' 5'AGCTTATTCATCGCCACGTT3'	415	56
Quinolone	ParC	5'ATGAGCGATATGGCAGAGCGCCTTGCGCTA3' ACGCGCCGGTAACATTTTCGGTTCCTGCAT3'	480	60
Quinolone	GyrA	5'CGTTGGTGACGTAATCGG3' 5'CCGTACCGTCATAGTTAT3'	251	60
Chloramphenicol	CAT A	5'ATGACCACCACAGCCCG3' 5'AGACGACTGGCGACTTCTTCG3'3'	198	60
Tetracycline	TET A-	5'TTGGCATTCTGCATTCACTC3' 5'GTATAGCTTGCCGGAAGTCG3'	495	55
Tetracycline	TET B	5'CAGTGCTGTTGTGTCATTAA3' 5'GCTTGGAATACTGAGTGTAA3'	571	55

3. Results

A study on the sex distribution dynamics of multidrug resistance of different *Salmonella serovars* in Benue State was undertaken. Figure 1 compares the overall percentage susceptibility of the eight antibiotics on *Salmonella* cases. GEN had the highest % susceptibility of 100% followed by NIT (72.2%) and COT (66.7%) before and after plasmid curing. %

susceptibility was lower before curing than after curing in CXC, CHL and TET. It was low (5.6%) in ERY while AUG recorded 0% susceptibility. Differences observed in curing status was insignificant (T = 0.33, P>0.05).

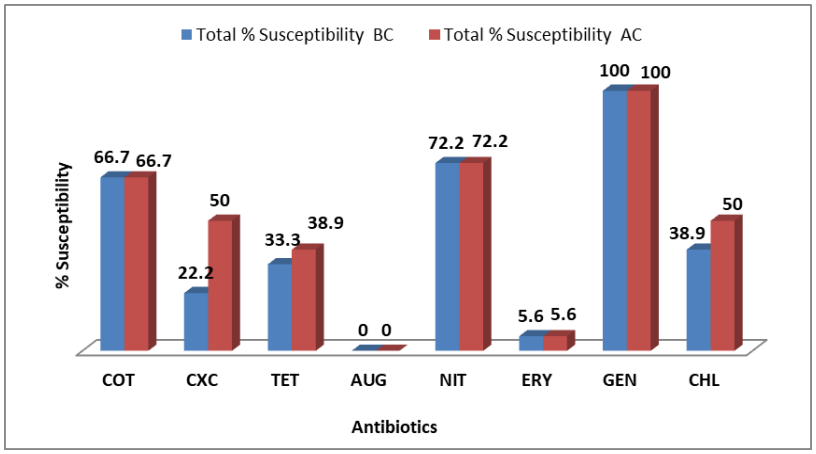

Figure 1 Total susceptibility of various antibiotics on Salmonella infection

BC = before plasmid curing; AC = after plasmid curing; COT = cotrimoxazole (25 µg); CXC = cloxacillin (5 µg); TET = tetracyclin (25 µg); AUG = augmentin (30 µg); NIT = nitrofurantoin (200 µg); ERY = erythromycin (5 µg); GEN = gentamicin (10 µg); CHL = chloraphenicol (30 µg)

T Statistics (BC and AC) = 0.33, P=0.749, P>0.05

Figure 2 shows the sex distribution of *Salmonella* infected Patients in relation to antibiotic resistance before plasmid curing. The highest number of patients that resisted antibiotic was 18 as observed in AUG where 11 patients were male and 7 were female. This was followed by ERY with a total of 17 patients comprising 10 male and 7 female. In CXC, resistance occurred in 8 male and 6 female. Similar trend was observed in TET (7 male, 4 female), CHL (9 male, 2 female) and COT (4 male, 2 female) As a result, more male than female showed significant antibiotic resistance before plasmid curing using the Mann whitney test (W = 84.0, P≤0.05).

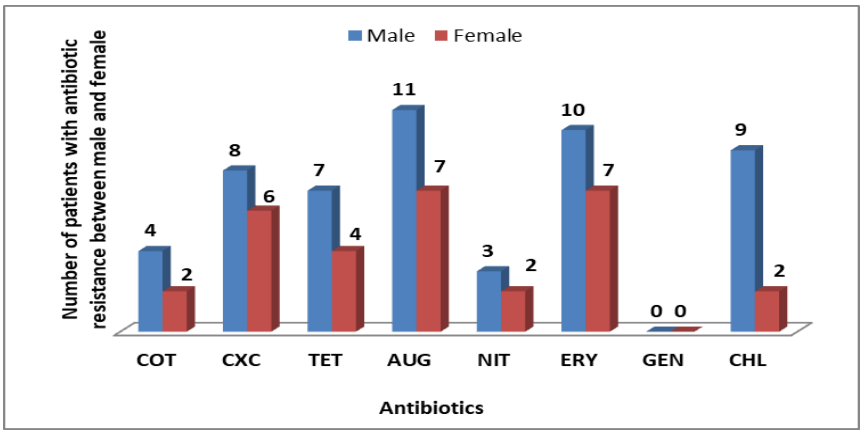

Figure 2 Sex Distribution of Antibiotic Resistance among *Salmonella* infected Patients(Before plasmid curing)

COT = cotrimoxazole (25 µg); CXC = cloxacillin (5 µg); TET = tetracyclin (25 µg); AUG = augmentin (30 µg); NIT = nitrofurantoin (200 µg); ERY = erythromycin (5 µg); GEN = gentamicin (10 µg); CHL = chloraphenicol (30 µg)

W (male and female)= 84.0, P=0.05 (P≤0.05)

Figure 3 shows the sex distribution of *Salmonella* infected Patients in relation to antibiotic resistance after plasmid curing. The highest number of patients that resisted antibiotic was 18 as observed in AUG where 11 patients were male and 7 were female. This was followed by ERY with a total of 17 patients comprising 11 male and 6 female. In CXC,

resistance occurred in 6 male and 3 female. More males showed antibiotic resistance to *Salmonella* infection in ERY, AUG, TET, CXC and COT. However, more female showed resistance to NIT (4 female, 1 male) and CHL (7 female, 2 male). As a result, there was no difference between both sexes after plasmid curing using the Mann Whitney test (W = 73.0, P>0.05).

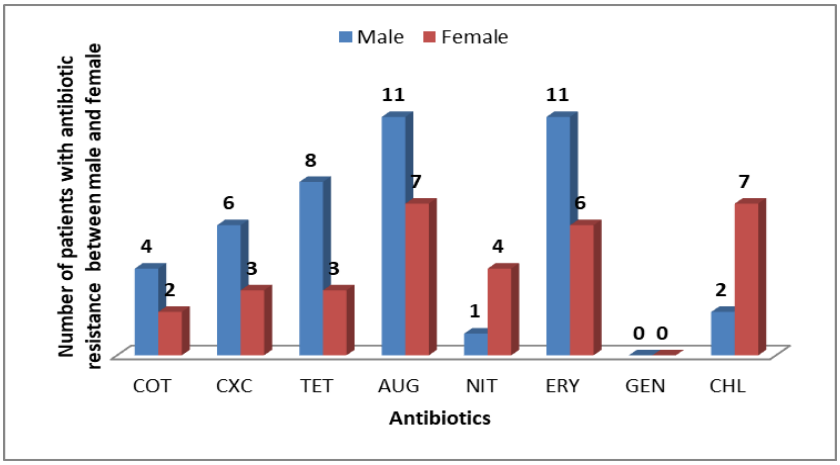

Figure 3 Sex Distribution of Antibiotic Resistance among *Salmonella* Infected Patients (After plasmid curing)

COT = cotrimoxazole (25 µg); CXC = cloxacillin (5 µg); TET = tetracyclin (25 µg); AUG = augmentin (30 µg); NIT = nitrofurantoin (200 µg); ERY = erythromycin (5 µg); GEN = gentamicin (10 µg); CHL = chloraphenicol (30 µg)

W (male and female) = 73.0, P=0.3174 (P>0.05)

Table 2 gives the results and statistics of plasmid gene sequencing of *Salmonella* species in Gboko tertiary health facility. One species of *S. enterica* of different serovars and strains were identified. Virulence gene was found either on the chromosome or on the 16S ribosomal RNA region of the plasmid. *S. enterica* subsp. *enterica* serovar Agona strain 392869-2 was identified and it possessed plasmid of 1498 bits capacity that was made up of 856 nucleotides with 98% sequence identity. *S. enterica* subsp. *enterica* serovar Paratyphi B strain JQ694526.1 was identified and it possessed plasmid of 1503 bits capacity that was made up of 843 nucleotides with 99% sequence identity. *S. enterica* subsp. *enterica* serovar Heidelberg strain MG663473.1 was identified and it possessed a smaller plasmid of 460 bits capacity that was made up of 620 nucleotides, 9 gaps and 73% sequence identity. *S. enterica* subsp. *enterica* serovar Heidelberg strain CP019176.1 was identified. It possessed a relatively larger plasmid of 1503 bits capacity that was made up of 872 nucleotides without gap but had 99% sequence identity.

Table 2 Plasmid Gene Sequencing Statistics of *Salmonella* species in Gboko General Hospital

Nomenclature	Identity	Gap	Size	Gene Location
Salmonella enterica subsp. enterica serovar Agona str. 392869-2	841/856 (98%)	1/856 (0%)	1498bits (1661)	Chromosome
Salmonella enterica subsp. enterica serovar Paratyphi B strain JQ694526.1	839/843 (99%)	1/843 (0%)	1503 bits(1666)	16S ribosomal RNA
Salmonella enterica subsp. enterica serovar Heidelberg strain MG663473.1	454/620 (73%)	9/620 (1%)	460 bits(509)	16S ribosomal RNA
Salmonella enterica subsp. enterica serovar Heidelberg strain CP019176.1	859/872 (99%)	3/872 (0%)	1522 bits(1687)	16S ribosomal RNA

Prevalence= 4/70= 5.71%

Table 3 gives the results and statistics of plasmid gene sequencing of *Salmonella* species in Makurdi tertiary health facility. One species of *S. enterica* of two serovars (Typhi and Typhimurium) and strains were identified Virulence gene was found on the 16S ribosomal RNA region of the plasmid. *S. enterica* subsp. *enterica* serovar typhi strain AK-1 was

identified. It possessed plasmid of 1102 bits capacity that was made up of 651 nucleotides with 92% sequence identity. *S. enterica* subsp. *enterica* serovar Typhimurium strain CP014981.1 was identified. It possessed plasmid of 860 bits capacity that was made up of 767 nucleotides with 81% sequence identity.

Table 3 Plasmid Gene Sequencing Statistics of *Salmonella* species in Makurdi General Hospital

Nomenclature	Identity	Gap	Size	Gene Location
Salmonella enterica subsp. enterica serovar typhi strain AK-1	600/651 (92%)	0/651 (0%)	1102 bits(543)	16S ribosomal RNA
Salmonella enterica subsp. enterica serovar Typhimurium strain CP014981.1	621/767 (81%)	2/767 (0%)	860 bits(405)	16S ribosomal RNA g

Prevalence= 2/70= 2.86%

Table 4 gives the results and statistics of plasmid gene sequencing of *Salmonella* species in Oju tertiary health facility. One species of *S. enterica* of three different serovars were identified. Virulence gene was found either on the chromosome or on the 16S ribosomal RNA region of the plasmid. *S. enterica* subsp. *enterica* serovar Enteritidis strain CP007325.2 possessed plasmid of 246 bits capacity that was made up of 346 nucleotides with 71% sequence identity. *S. enterica* subsp. *enterica* serovar Typhi strain AL513382.1 possessed plasmid of 80.6 bits capacity that was made up of 59 nucleotides with 90% sequence identity and 1% gap. *S. enterica* subsp. *enterica* serovar Typhimurium strain CP024619.1 had the largest plasmid size of 1210 bits capacity that was made up of 679 nucleotides with 99% sequence identity

Table 4 Plasmid Gene Sequencing Statistics of *Salmonella* species in Oju General Hospital

Nomenclature	Identity	Gap	Size	Gene Location
Salmonella enterica subsp. enterica serovar Enteritidis strain CP007325.2	246/346 (71%)	0/346 (0%)	246 bits(272)	16S ribosomal RNA
Salmonella enterica subsp. enterica serovar Typhi strain AL513382.1	53/59 (90%)	1/59 (1%)	80.6 bits(88)	16S ribosomal RNA
Salmonella enterica subsp. enterica serovar Typhimurium strain CP024619.1	674/679 (99%)	0/679 (0%)	1210 bits(1341)	16S ribosomal RNA

Prevalence= 3/70= 4.29%

Table 5 gives the results and statistics of plasmid gene sequencing of *Salmonella* species in Adikpo tertiary health facility. One species of *S. enterica* (two Typhimurium and one Enteritidis serovars) of different strains were identified. Virulence gene was found either on the chromosome or on the 16S ribosomal RNA region of the plasmid. *S. enterica* subsp. *enterica* serovar Enteritidis strain had the largest plasmid size of 1361 bits capacity made up of 346 nucleotides with 71% sequence identity. *S. enterica* subsp. *enterica* serovar Typhimurium strain CP023166.1 possessed plasmid of 1171 bits capacity that was made up of 346 nucleotides with 71% sequence identity.Table 6 gives the results and statistics of plasmid gene sequencing of *Salmonella* species in Katsina-Ala tertiary health facility. There were four *Salmonella* strains of two different species found. *S. enterica* had two serovars (Typhimurium and Huaian) and while *S. bongori* had a single serovar, *S.bongori* strain FR877557.1. It had the largest plasmid size of 1576 bits capacity made up of 881 nucleotides with 99% sequence identity. *Salmonella enterica* Serovar Huaian strain H52.1 also possessed a large plasmid sized 1216 bits but with the shortest nucleotides length of 647 and 98 % sequence identity. The two Typhimurium serovars (*S. enterica* subsp. *enterica* serovar Typhimurium strain LT795114.1 and *S. enterica* subsp. *enterica* serovar Typhimurium strain JQ228518.1) possessed plasmids of low capacities (714bits and 524 bits respectively.

Table 5 Plasmid Gene Sequencing Statistics of *Salmonella* species in Kwande General Hospital

Nomenclature	Identity	Gap	Size	Gene Location
Salmonella enterica subsp. enterica serovar Typhimurium strain MH196335.1	466/547 (85%)	1/547 (0%)	625 bits(316)	16S ribosomal RNA gene
Salmonella enterica subsp. enterica serovar Typhimurium strain CP023166.1	633/643 (98%)	2/643 (0%)	1171 bits(606)	16S ribosomal RNA gene
Salmonella enterica subsp. enterica serovar Enteritidis strain JF951181.1	709/714 (99%)	0/714 (0%)	1361 bits(704)	16S ribosomal RNA gene

Prevalence= 3/70= 4.29%

Table 6 Plasmid Gene Sequencing Statistics of *Salmonella* species in Katsina-Ala General Hospital

Nomenclature	Identity	Gap	Size	Gene Location
Salmonella enterica Serovar Huaian strain H52.1	637/647 (98%)	0/647 (0%)	1216 bits(626)	16S ribosomal RNA gene
Salmonella bongori strain FR877557.1	878/881 (99%)	1/881 (0%)	1576 bits(1747)	16S ribosomal RNA gene
Salmonella enterica subsp. *enterica* serovar Typhimurium strain LT795114.1	609/825 (74%)	0/825 (0%)	714 bits(319)	16S ribosomal RNA gene
Salmonella enterica subsp. *enterica* serovar Typhimurium strain JQ228518.1	636/876 (73%)	9/876 (1%)	524 bits(245)	16S ribosomal RNA gene

Prevalence= 4/70= 5.71%

Table 7 gives the results and statistics of plasmid gene sequencing of *Salmonella* species in Otukpo tertiary health facility. The two strains present were of *S.enterica*species, serovar Enteritidis. Virulence gene was on the 16S ribosomal RNA gene. *S. enterica* subsp. *enterica* serovar Enteritidis strain CP018642.1 had a large plasmid size of 1509 bits capacity made up of 817 nucleotides with 98% sequence identity. *S. enterica* subsp. *enterica* serovar Enteritidis strain TY1 had a small plasmid size of 182 bits capacity made up of 570 nucleotides with 67% sequence identity. No gap found between nucleotide sequences.

Table 7 Plasmid Gene Sequencing Statistics of *Salmonella* species in Otukpo General Hospital

Nomenclature	Identity	Gap	Size	Gene Location
Salmonella enterica subsp. enterica serovar Enteritidis strain TY1	384/570 (67%)	0/570 (0%)	182 bits(84)	16S ribosomal RNA gene
Salmonella enterica subsp. enterica serovar Enteritidis strain CP018642.1	799/817 (98%)	0/817 (0%)	1509 bits(775)	16S ribosomal RNA gene

Prevalence= 2/70= 2.86%

Amplified Plasmids extracted from 18 *Salmonella* strains are shown in the gel image (figure 4). The bands made up of 23,130 base pair. The plasmids contained genes coding for virulence factors and multiple antibiotic resistance. *Samonella* plasmids contained genes that conferred resistance to some antibiotics. Genes such as ParcC (480bp), GyrA (251bp), CatA (198bp) were present (figure 5). The latter coded for chloramphenicol resistance. InvA plasmid genes (284bp and 389bp) typical of virulent *Salmonella serovars* were identified (figure 6 and 7). Most of the serovars possessed plasmids that contained TetB gene in the 16sRNA region (571bp) coding for tetracycline resistance (figure 8 and 9). SitC gene (578bp) and spvA gene (604bp) were also identified (figure 10 and 11 respectively).

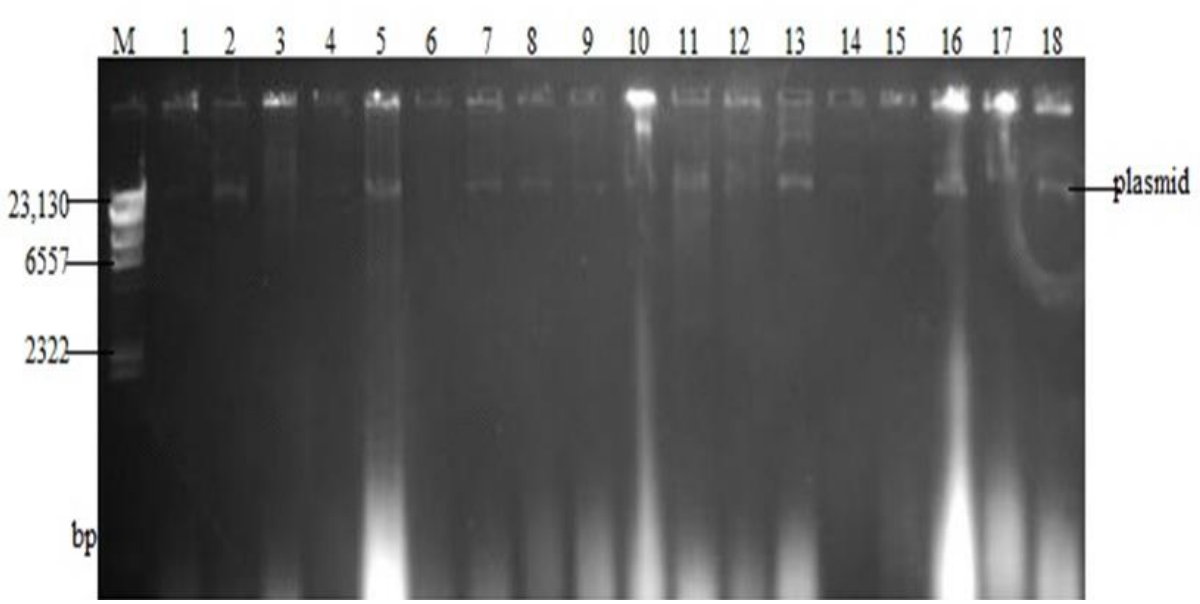

Figure 4 Amplified Plasmids of *Salmonella* strains (23,130bp)

M=DNA Ladder
1=*S.enterica*Agona-392869-2
2=*S.enterica*Paratyphi B-JQ694526.1
3=*S.enterica*Heidelberg-MG663473.1
4=*S.enterica*Heidelberg-CP019176.1
5=*S.enterica*Typhi-AK-1
6=*S.enterica*Typhimurium-CP014981.1
7=*S.enterica*Enteritidis-CP007325.2
8=*S.enterica*Typhi-AL513382.1
9=*S.enterica*Typhimurium-CP024619.1
10=*S.enterica*Typhimurium-MH196335.1
11=*S.enterica*Typhimurium-CP023166.1
12=*S.enterica*Enteritidis-JF951181.1
13=*S.enterica*Huaian-H52.1
14=*S.bongori*-FR877557.1
15=*S.enterica*Typhimurium-LT795114.1
16=*S.enterica*Typhimurium-JQ228518.1
17=*S.enterica*Enteritidis-TY1
18= *S.enterica*Enteritidis-CP018642.1

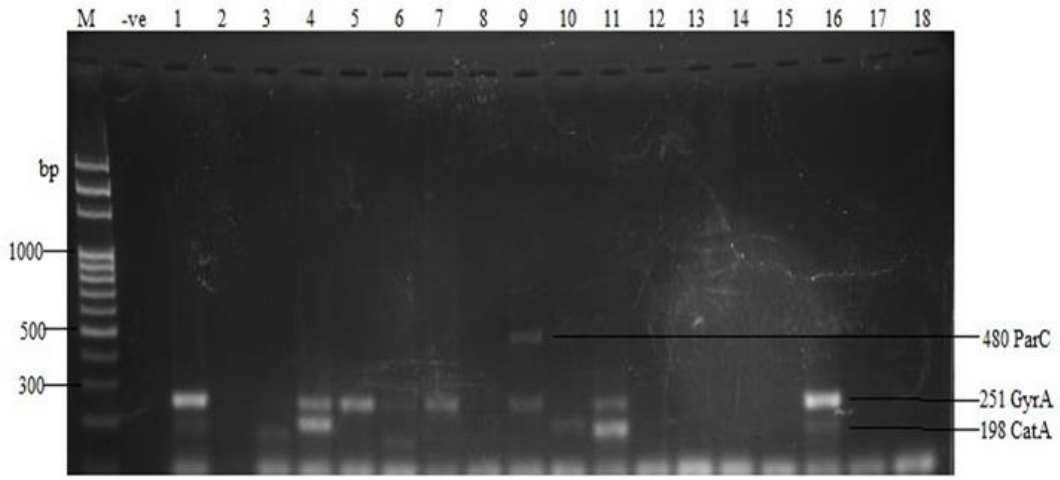

Figure 5 Quinolone Plasmid gene amplified by ParcC (480bp) and GyrA (251bp) primers and Chloramphenicol gene amplified by CatA (198bp) primer

M=DNA Ladder
1=*S.enterica*Agona-392869-2
2=*S.enterica*Paratyphi B-JQ694526.1
3=*S.enterica*Heidelberg-MG663473.1
4=*S.enterica*Heidelberg-CP019176.1
5=*S.enterica*Typhi-AK-1
6=*S.enterica*Typhimurium-CP014981.1
7=*S.enterica*Enteritidis-CP007325.2
8=*S.enterica*Typhi-AL513382.1
9=*S.enterica*Typhimurium-CP024619.1
10=*S.enterica*Typhimurium-MH196335.1
11=*S.enterica*Typhimurium-CP023166.1
12=*S.enterica*Enteritidis-JF951181.1
13=*S.enterica*Huaian-H52.1
14=*S.bongori*-FR877557.1
15=*S.enterica*Typhimurium-LT795114.1
16=*S.enterica*Typhimurium-JQ228518.1
17=*S.enterica*Enteritidis-TY1
18= *S.enterica*Enteritidis-CP018642.1
-ve= Blank

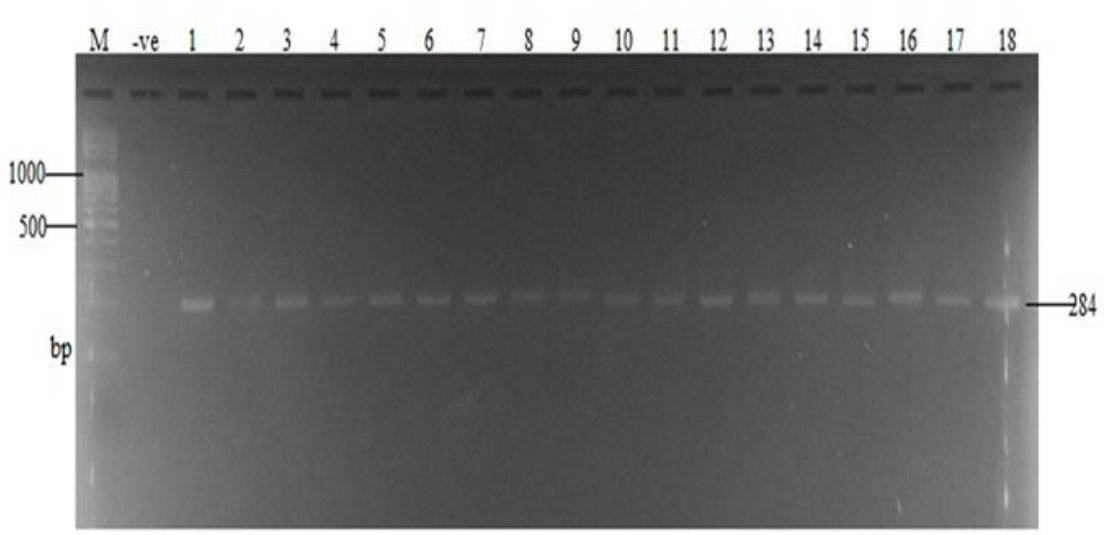

Figure 6 Amplified InvA Plasmid genes (284bp) by Inv A primer

M=DNA Ladder
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2=*S.enterica*Paratyphi B-JQ694526.1
3=*S.enterica*Heidelberg-MG663473.1
4=*S.enterica*Heidelberg-CP019176.1
5=*S.enterica*Typhi-AK-1
6=*S.enterica*Typhimurium-CP014981.1
7=*S.enterica*Enteritidis-CP007325.2
8=*S.enterica*Typhi-AL513382.1
9=*S.enterica*Typhimurium-CP024619.1
10=*S.enterica*Typhimurium-MH196335.1
11=*S.enterica*Typhimurium-CP023166.1
12=*S.enterica*Enteritidis-JF951181.1
13=*S.enterica*Huaian-H52.1
14=*S.bongori*-FR877557.1
15=*S.enterica*Typhimurium-LT795114.1
16=*S.enterica*Typhimurium-JQ228518.1
17=*S.enterica*Enteritidis-TY1
18= *S.enterica*Enteritidis-CP018642.1
-ve= Blank

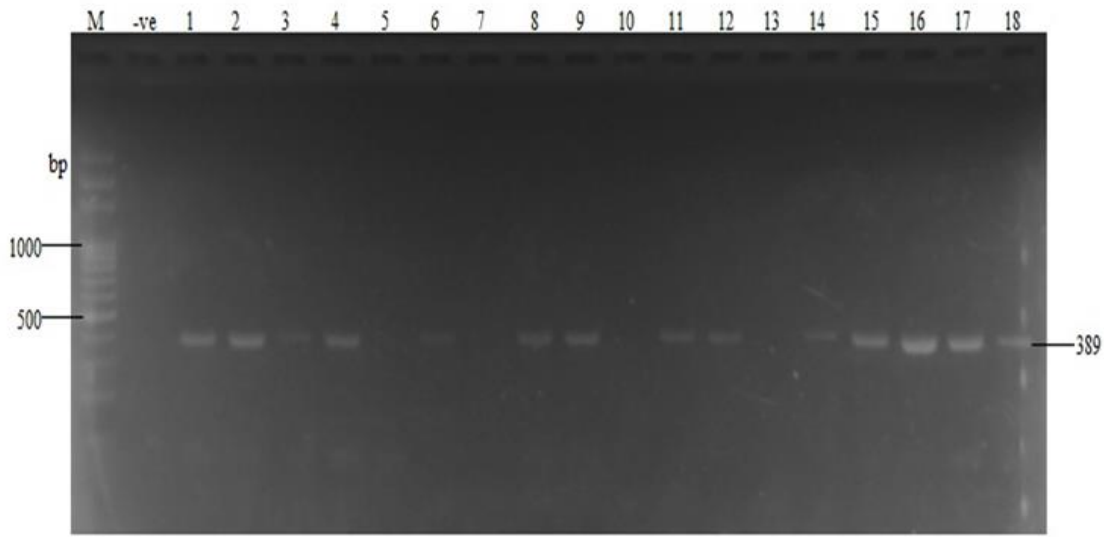

Figure 7 Plasmid Amplification of InvA gene (389bp) by Salm3/Salm4 primers

M=DNA Ladder
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2=*S.enterica*Paratyphi B-JQ694526.1
3=*S.enterica*Heidelberg-MG663473.1
4=*S.enterica*Heidelberg-CP019176.1
5=*S.enterica*Typhi-AK-1
6=*S.enterica*Typhimurium-CP014981.1
7=*S.enterica*Enteritidis-CP007325.2
8=*S.enterica*Typhi-AL513382.1
9=*S.enterica*Typhimurium-CP024619.1
10=*S.enterica*Typhimurium-MH196335.1
11=*S.enterica*Typhimurium-CP023166.1
12=*S.enterica*Enteritidis-JF951181.1
13=*S.enterica*Huaian-H52.1
14=*S.bongori*-FR877557.1
15=*S.enterica*Typhimurium-LT795114.1
16=*S.enterica*Typhimurium-JQ228518.1
17=*S.enterica*Enteritidis-TY1
18= *S.enterica*Enteritidis-CP018642.1
-ve= Blank

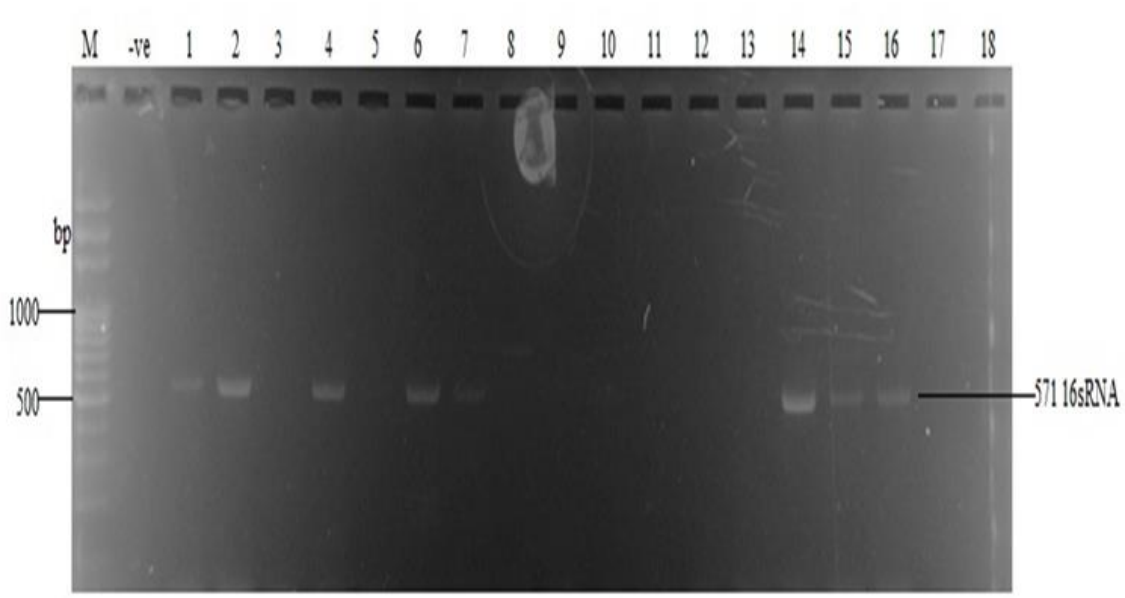

Figure 8 Plamsid Amplification of 16sRNA (1) region (571bp) coding for tetracycline resistance by TET B primer

M=DNA Ladder
1=*S.enterica*Agona-392869-2
2=*S.enterica*Paratyphi B-JQ694526.1
3=*S.enterica*Heidelberg-MG663473.1
4=*S.enterica*Heidelberg-CP019176.1
5=*S.enterica*Typhi-AK-1
6=*S.enterica*Typhimurium-CP014981.1
7=*S.enterica*Enteritidis-CP007325.2
8=*S.enterica*Typhi-AL513382.1
9=*S.enterica*Typhimurium-CP024619.1
10=*S.enterica*Typhimurium-MH196335.1
11=*S.enterica*Typhimurium-CP023166.1
12=*S.enterica*Enteritidis-JF951181.1
13=*S.enterica*Huaian-H52.1
14=*S.bongori*-FR877557.1
15=*S.enterica*Typhimurium-LT795114.1
16=*S.enterica*Typhimurium-JQ228518.1
17=*S.enterica*Enteritidis-TY1
18= *S.enterica*Enteritidis-CP018642.1
-ve= Blank

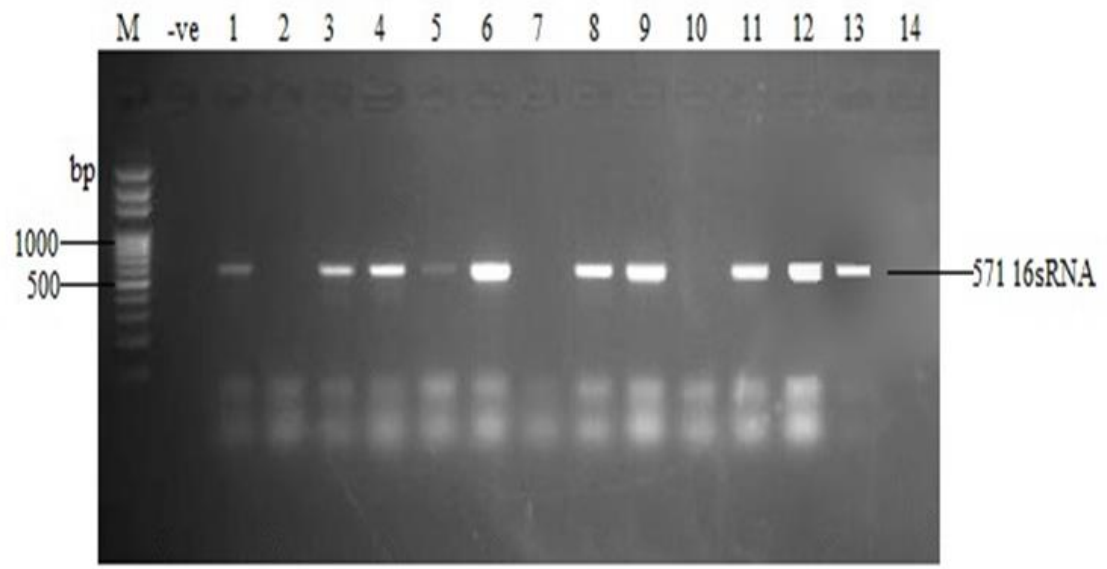

Figure 9 Plamsid Amplification of 16sRNA (2) region (571bp) coding for tetracycline resistance by TET B primer

M=DNA Ladder
1=*S.enterica*Agona-392869-2
2=*S.enterica*Paratyphi B-JQ694526.1
3=*S.enterica*Heidelberg-MG663473.1
4=*S.enterica*Heidelberg-CP019176.1
5=*S.enterica*Typhi-AK-1
6=*S.enterica*Typhimurium-CP014981.1
7=*S.enterica*Enteritidis-CP007325.2
8=*S.enterica*Typhi-AL513382.1
9=*S.enterica*Typhimurium-CP024619.1
10=*S.enterica*Typhimurium-MH196335.1
11=*S.enterica*Typhimurium-CP023166.1
12=*S.enterica*Enteritidis-JF951181.1
13=*S.enterica*Huaian-H52.1
14=*S.bongori*-FR877557.1
15=*S.enterica*Typhimurium-LT795114.1
16=*S.enterica*Typhimurium-JQ228518.1
17=*S.enterica*Enteritidis-TY1
18= *S.enterica*Enteritidis-CP018642.1
-ve= Blank

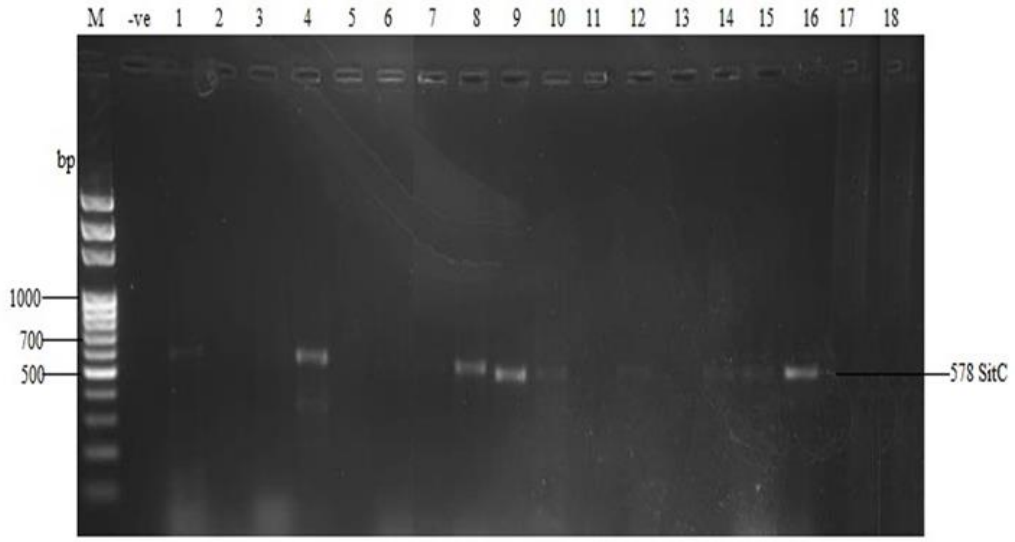

Figure 10 Plasmid Amplification of SitC gene (578bp) by SitC3 primer

M=DNA Ladder
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2=*S.enterica*Paratyphi B-JQ694526.1
3=*S.enterica*Heidelberg-MG663473.1
4=*S.enterica*Heidelberg-CP019176.1
5=*S.enterica*Typhi-AK-1
6=*S.enterica*Typhimurium-CP014981.1
7=*S.enterica*Enteritidis-CP007325.2
8=*S.enterica*Typhi-AL513382.1
9=*S.enterica*Typhimurium-CP024619.1
10=*S.enterica*Typhimurium-MH196335.1
11=*S.enterica*Typhimurium-CP023166.1
12=*S.enterica*Enteritidis-JF951181.1
13=*S.enterica*Huaian-H52.1
14=*S.bongori*-FR877557.1
15=*S.enterica*Typhimurium-LT795114.1
16=*S.enterica*Typhimurium-JQ228518.1
17=*S.enterica*Enteritidis-TY1
18= *S.enterica*Enteritidis-CP018642.1
-ve= Blank

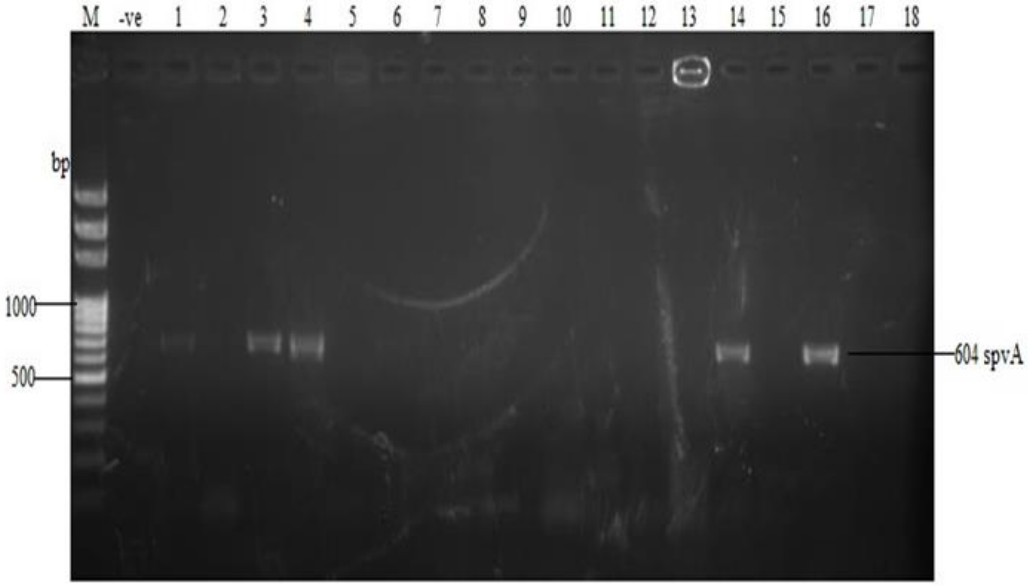

Figure 11 Plasmid Amplification of spvA gene (604bp) by spvA primer

M=DNA Ladder
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2=*S.enterica*Paratyphi B-JQ694526.1
3=*S.enterica*Heidelberg-MG663473.1
4=*S.enterica*Heidelberg-CP019176.1
5=*S.enterica*Typhi-AK-1
6=*S.enterica*Typhimurium-CP014981.1
7=*S.enterica*Enteritidis-CP007325.2
8=*S.enterica*Typhi-AL513382.1
9=*S.enterica*Typhimurium-CP024619.1
10=*S.enterica*Typhimurium-MH196335.1
11=*S.enterica*Typhimurium-CP023166.1
12=*S.enterica*Enteritidis-JF951181.1
13=*S.enterica*Huaian-H52.1
14=*S.bongori*-FR877557.1
15=*S.enterica*Typhimurium-LT795114.1
16=*S.enterica*Typhimurium-JQ228518.1
17=*S.enterica*Enteritidis-TY1
18= *S.enterica*Enteritidis-CP018642.1
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4. Discussion

The amplified plasmids contained genes coding for virulence factors and multiple antibiotic resistance. All strains except *S.enterica*Paratyphi B-JQ694526.1 possessed at least one of ParcC, GyrA, CatA genes in the plasmids. Most resistances to Quinolones and fluoroquinolones compounds are due to mutations within the genes that encode the enzymes such as gyrA, gyrB, parC, and parE. Most of these mutations occur in the quinolone resistance determining region (QRDR) which is a conserved site in these enzymes targeted by these antimicrobials (36, 37).

SitC3 was present in the plasmids of *S.* Heidelberg-CP019176.1, *S.enterica*Typhi-AL513382.1, *S.enterica* Typhimurium-CP024619.1 and *S.* Typhimurium strain JQ228518.1, although other faints bands were also present. Based on gel analysis, all strains possessed InvA gene in their plasmids. This finding is consistent with the fact that the most common virulence gene which in *Salmonella* is invA gene, and it is usually used as PCR target gene for detection of *Salmonella* (38, 39). Genes such as invA and hilA allow *Salmonella* to invade epithelial cells of the host (38, 38, 40). Strains that housed spvA gene in their plasmid include *S.* Heidelberg strain MG663473.1, *S.* Heidelberg strain CP019176.1, *S. bongori* strain FR877557.1 and *S.* Typhimurium strain JQ228518.1. The wide variety of outbreak sources and variability in genetics causing AR indicates resistance in *Salmonella* is multifaceted, and the trend of unusual food sources and antimicrobial resistant outbreaks may become more prevalent in the future (41). In order to predict the virulence potential of *Salmonella* isolates from different sources, the presence of virulence genes (spiA, pagC, msgA, sipB, spaN, and spvB) may be screened screened (41). In this work, antibiotic resistance gene was observed in the chromosome of *S.* Agona. Denyer *et al*. (42) reported that chromosome mediated-resistance has been attributed to a mutation in the gene that codes for either the target of the drug or the transport system in the membrane that controls the uptake of the drug. The chromosomal-mediated drug resistance phenomenon against fluoroquinolones has been reported recently as a result of selective pressure on the bacterial population due to their uncontrolled use.

The present report has agreed with the findings of Amenu (43) stating that antibiotic resistance is genetically encoded and can vary from mutations in endogenous genes, to horizontally acquired foreign resistance genes carried by mobile genetic elements (MGEs) like plasmids. Thus, plasmids are very important in the epidemiology and spread of resistance to antibiotics. Plasmids can be horizontally transferred from one bacterial host to another even between bacterial species (6). *Salmonella* resistance to aminoglycosides is usually an enzymatic modification of the compound; however, in other bacteria, active efflux of the compound or enzymatic modification of the 16s rRNA subunit to prevent the aminoglycoside from binding to its ribosomal target can lead to resistance (44).

5. Conclusion

The amplified plasmids contained genes coding for virulence factors and multiple antibiotic resistance genes. These include: ESBL (TEM and CTX-M genes), ParcC, GyrA, CatA, SitC3 and invA genes in the plasmids. Phylogenetics has revelaed the genetically divergent nature of some two strains namely: *S.* Typhimurium strain MH196335.1 found in Kwande and *S.*Agona strain 392869-2 found in Gboko. Other strains are grouped based on genetic similarity and differences, independent of location of isolation. This report has given sufficient information on the spectrum of *Salmonella serovars* in the six selected health facilities across the six LGAs. It has also given insight on the nature of antibiotic susceptibility or resistance profiling down to the strain level. The spectrum of genes that confer both virulence and antibiotic resistance on the *Salmonella* plasmids are well highlighted. The information provided is crucial in the control and management of *Salmonella* infections in Benue State, North Central Nigeria.

Further studies on multi drug resistance and susceptibility profiling should be undertaken on a routine basis since *Salmonella serovars* develop new strains incessantly.

Compliance with ethical standards

Acknowledgments

I acknowledge the contributions of my supervisors and Dr. Ichor Tersagh Smart for their roles in guiding this work to successful completion. The patience and endurance of my family for the busy period and my absence are well acknowledged and appreciated.

Disclosure of conflict of interest

The Authors declare no conflict of interest.

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