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ANTIBIOTICS RESISTANCE PROFILE AND EXTENDED SPECTRUM BETA-LACTAMASES (ESBLS) PRODUCTION BY *SALMONELLA* SPECIES ISOLATED FROM HIV/AIDS SUBJECTS IN AKWA IBOM STATE, NIGERIA

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ABSTRACTS
The present study investigated the antibiotics resistance profile and ESBLs producing strains of *Salmonella* isolated from HIV/AIDS seropositive subjects in Akwa Ibom State. Stool samples from 300 HIV/AIDS seropositve persons and 300 HIV/AIDS seronegative subjects (control) were cultured for isolation of *Salmonella* organisms using standard microbiological techniques. Isolates were characterized and identified using conventional tests and Microgen- Gram Negative Identification System (Microgen Bioproducts, USA). Sensitivity testing on the isolates was performed by Kirby-Bauer method while ESLB - production was detected using double disc synergy antibiotics (Hardy diagnostic, USA). Plasmid profiling and plasmid curing were also carried out. A total of 79 and 13 *Salmonella* isolates were obtained from HIV/AIDS persons and control group. The resistance profile of *Salmonella* isolates from HIV/AIDS subjects showed 44.3% of the isolates expressed resistance to Ampicillin- Sulbactam, 60.8% to Tetracycline, 46.8% to Ceftaxidime, 53.2% to Ciprofloxacin and 79.8% to Sulfamethoxazole- Trimethoprim. .Isolates from control subjects expressed resistance of 61.5% to Ampicillin-Sulbactam, and Amoxicillin respectively. Isolates from HIV seropositive subjects produced ESBLs activities of 35.4%, against Amoxicillin + Clavulanic acid, Cefotaxime + Clavulanic acid (29.1%) and Cefotaxidime + Clavulanic acid (32.9%).The organisms produced TEM and CTX- types of ESBLS. Plasmids extracted exhibited heavy molecular weight ranging 23.13kbp and above. Some isolates retained plasmids after curing. Multi-drug resistance by salmonellae in the study area was a repertoire of high molecular weight materials that are plasmid and chromosomal mediated. This calls for enforcement of laboratory examination before antibiotics prescription and administration especially in immunocompromised conditions.

KEYWORDS: *Salmonella*, HIV/AIDS, Antibiotics, Resistance, Plasmids.

INTRODUCTION
Antibiotics have been used to fight infections caused by bacteria. However, heavy use of these drugs measured in hundreds of tons per year, progressively eliminated sensitive strains so that greater percentages of bacteria are now developing resistance to these modes of action.[1, 2] Antibiotic resistance by some strains of bacteria such as *Salmonella* is a very serious and global problem of deep scientific concern both in hospital and community settings. *Salmonella* organisms are gram-negative bacilli of the family Enterobacteriaceae. They are important food and water - borne pathogens. They are known to cause typhoidal and non-typhoidal illnesses.[3] Human immunodeficiency virus and Acquired immunodeficiency syndrome (HIV/AIDS) seropositive patients are prone to opportunistic infections from bacteria including *Salmonella* which tends to be more severe in them due to their immunocompromised condition.[4,5]

There are various ways in which bacteria acquire resistance. These may be generated by one or more mechanisms which include the production of some enzymes such as the β-lactamases. Beta-lactamases are enzymes produced by some bacteria which are responsible for the resistance in Penicillin, Cephalosporins and other related antibiotics that contain a common element in their molecular structure - a four carbon-ring known as beta-lactam. [6] Worst still is the production of extended-spectrum β-lactamases (ESBLs) which is a significant resistance-mechanism that impedes the antibiotics treatment of infections caused by

Enterobacteriaceae and is a serious challenge to the currently available antibiotics.[7] According to[8], ESBLs are enzymes produced because of continuous and persistent exposure of bacterial strains to a variety of β-lactam drugs, and these induced dynamic and continuous production leading to mutation of β-lactamases, thus conferring these bacteria the additional ability to hydrolyze the β-lactam rings. ESBLs are classified into several groups according to their amino acid sequence homology. These may include the SHV type and according to[9], this type of β-lactamases appears to be derived from *Klebsiella* species confers resistance to broad-spectrum Penicillin such as Ampicillin, Tigecycline and Piperacillin but not to the Oxyimino substituted Cephalosporins. Another is TEM type which has many derivatives. TEM-1 is capable of hydrolyzing Penicillin and first generation Cephalosporins but is unable to attack the Oxyimino Cephalosporin. The first TEM variant with increased activity against extended spectrum Cephalosporins was TEM-3.[10,11] Yet another one is CTX type and according to[12, 13], they reported that CTX- type is discovered as a new family of β-lactamases and that it has been found in isolates of *Salmonella* serovar, *E. coli* mainly and some other species of *Enterobacteriaceae.*

There are quite a number of wide spread reports of the existence of antibiotics resistant and ESBLs-producing among salmonellae in many countries like Europe, America and Asia which create great problems and challenges for clinicians in the treatments of patients especially in immunosuppressed conditions.[14, 15, 16] Similarly, in some developing countries such as Africa, particularly in South Africa, the occurrence of resistant *Salmonella* spp and extended-spectrum beta-lactamases (ESBLs) producer have been reported.[17, 18] but there is limited report of its existence in sub-Saharan Africa like Nigeria. Rapid detection of their presence, resistance profile and mechanisms in clinical laboratories is essential for proper treatments of infections caused by these bacteria since these modes of actions are becoming a major threat for patients in the hospitals, long-term care facilities and community and most especially in HIV/AIDS condition.[19] Hence, the present study investigated the presence, resistance profile and ESBLs production of some strains of *Salmonella* isolated from HIV/AIDS seropositive patients in Akwa Ibom State.

MATERIALS AND METHODS

Study Design: This research investigating antibiotics resistance, ESBLs producers and plasmid profiles of Salmonella isolates from HIV/AIDS patients in Akwa Ibom State, Nigeria, was a cross - sectional descriptive study.

Ethical approval and Collection of Samples: Approval from Ethical Committee of the University of Uyo Teaching Hospital, Uyo, Akwa Ibom State, Nigeria was obtained and consents were also sought from the subjects who were willing to volunteer. Stool samples were collected from 300 HIV- seropositive subjects and 300 HIV- seronegative subjects.

Isolation, Characterization and identification of *Salmonella* isolates: Stool samples collected were cultured for isolation of *Salmonella* organisms using standard Microbiological technique. Isolates were characterized and identified with conventional biochemical methods as described by[20] and Microgen™ - GN ID System (Microgen Bioproducts, USA). Pure *Salmonella* isolates were maintained in agar slant at temperature of 4°C for further analysis.

Antibiotic Sensitivity Test: Antibiotic susceptibility test was performed on the isolates by means of Kirby-Bauer disc diffusion method using the guidelines provided by Clinical Laboratory Standard Institute.[21] Isolates were tested for their susceptibility to 10 routine antibiotics (Hardy diagnostic, USA). The antimicrobial agents and their concentrations were: Ampicillin-Sulbactam (20µg) Gentamicin (10 µg) Amoxicillin,(10 µg) Tetracycline,(10 µg) Chloramphenicol, (10 µg) Ceftaxinole, (30 µg) Ceftrioxone,(30 µg) Ciprofloxacin (5 µg) Sulfamethoxazole – Trimethoprim (25 µg) and Ceftazidime (30 µg). The plates were incubated for 24 hours at 37°C after which they were read. The diameters of the zone of inhibition of the growth were measured by the use of scale ruler in milliliter (mm). Clear zones of inhibition indicated the susceptibility of the organism to the antibiotics while absence of such zones showed resistance of the test organism to the antibiotics. The values less than 12mm were recorded as resistance while values greater than 12mm were recorded as sensitive.

Detection of Extended-Spectrum Beta-Lactamases (ESBLs) and types: Extended spectrum beta-lactamases (ESBLs) producing *Salmonella* isolates and types was detected using a double disc synergy test (DDST) method as described by[22] Akujobi and Ewuru, (2010) with slight modifications. Antibiotics used were Amoxicillin (20 µg) + Clavulanic acid (10 µg), Cefotaxime (20 µg) + Clavulanic acid (10 µg), Cefotaxidime (20 µg) + Clavulanic acid (10 µg) (Hardy diagnostic, USA). The plates were incubated for 24 hours at 37°C after which they were read. Isolates that exhibited a distinct shape potentiating towards double disc synergy were recorded as ESBLs producers. Results of ESBLs were interpreted based on the enhanced zone of inhibition between any one of the beta-lactam disc and other double synergy discs as the presumptive evidence for the presence of ESBL.[21] (CLSI, 2006). Some Salmonellae that produced ESBLs were selected for plasmid analysis.

Plasmid Profiling Using Agar Gel Electrophoresis Analysis

Profiling of plasmids using agar gel electrophoresis analysis on the resistant *Salmonella i*solates was carried out using the methods described by[23, 24] Ehrenfeld and

Clewell,(1987), Akinjogunla and Enabulele, (2010) with slight modification.

Plasmid Curing: The curing or elimination of the resistant plasmids of the *Salmonella* was done using sub- inhibitory concentration of 0.10mg/ml of acridine orange as described by[25] Akortha and Filgona, (2009) with slight modification. The plates incubated at 37°C for 24 hours. After incubation the test organisms were re-subjected for ESBL production using DDST.

RESULTS AND DISCUSSIONS

A total of 79 and 13 *Salmonella* isolates were obtained from HIV/AIDS persons and control group respectively. The resistance profile of *Salmonella* isolates from HIV/AIDS subjects showed a total of 44.3% of the isolates expressed resistance to Ampicillin- Sulbactam, 55.7% to Gentamicin, 38.0% to Amoxicillin, 60.8% to Tetracycline, 39.7% to Chloramphenicol, 35.4% to Ceftaxinole, 30.4% against Ceftrioxone 46.8% to Ceftaxidime, 53.2% to Ciprofloxacin and 79.8% to Sulfamethoxazole- Trimethoprim. Some percentages of resistance were also recorded for isolates from control subjects as 61.5% to Ampicillin- Sulbactam, 30.8% to Gentamicin, and Amoxicillin respectively, 46.2% to Tetracycline, 38.5% to Chloramphenicol, 53.8% to Sulfamethoxazole- Trimethoprim, 15.4% to Ceftaxinole and Ceftaxidime 23.1% against Ciprofloxacin and Ceftrioxone (**Figure 1).**

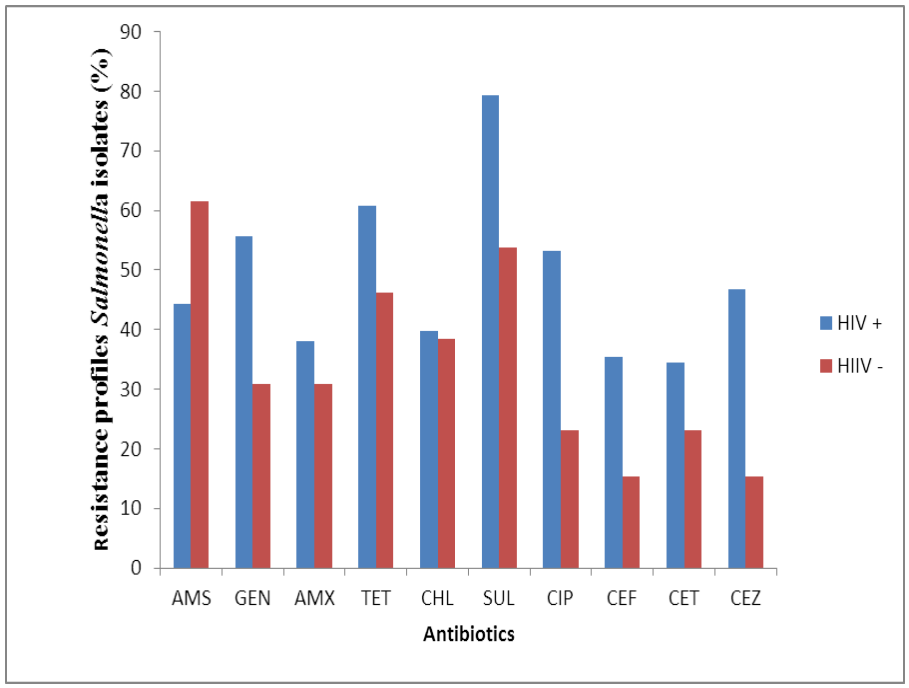

Figure 1: **Comparative resistance profile on *Salmonella* isolates from HIV/AIDS patients and HIV negative (control) subjects.**

Keys: **AMS**- Ampicillin-Sulbactam, **GEN**- Gentamicin AMX- Amoxicillin**TET**- Tetracycline, **CHL**- Chloramphenicol, **SUL**-Sulfamethoxazole – Trimethoprim, **CIP**- Ciprofloxacin, **CEF**- Ceftaxinole, **CET**- Ceftrioxone, CEZ- Ceftazidime

Some *Salmonella* isolates produced ESBLs activity against double disc test (DDST) drugs used in the study. A total of 79 *Salmonella* isolates from HIV- seropositive subjects tested for ESBLs production, out of which a total of 35.4% of the isolates produced ESBLs against Amoxicillin + Clavulanic acid, 29.1% against Cefotaxime + Clavulanic acid and 32.9%against Cefotaxidime + Clavulanic acid. Similarly from HIV/AIDS negative (control) subjects, 23.1% produced ESBL against Amoxicillin + Clavulanic acid, 15.4% against Cefotaxime + Clavulanic acid, 15.4% from control had against Cefotaxidime + Clavulanic acid respectively. The organisms produced TEM- type of ESBLS which is capable to hydrolyze Penicillin and Cephalosporin related antibiotics with Beta –lactam ring used in the study. Also, the *Salmonella* strains isolated were observed to produce CTX- types of ESBLS as some were able to hydrolyze double synergy drug such as Cefotaxime to the extent that their resistance was not inhibited by Cluvalanic acid **(Table 1**).

Table 1: *Salmonella* isolates producing Extended Spectrum Betalactamases (ESBLs) activity against double disc drugs used in the study.		
Antibiotics Used	**Total no. of *Salmonella* isolates from HIV/AIDS that produced** TEM **and** CTX **ESBls (%) n=79**	**Total no. of *Salmonella* isolates that produced** TEM **and** CTX **ESBls from control subjects (%) n =13**
Amoxicillin + Clavulanic acid	28 (35.4)	3 (23.07)
Cefotaxime + Clavulanic acid	23(29.1)	2 (15.4)
Cefotaxidime + Clavulanic acid.	26(32.9.)	2 (15.4)

Extraction of plasmids carried out on 13 randomly selected *Salmonella* isolates from HIV/AIDS seropositive patients and the control subjects that produced high resistant pattern against double synergy antibiotics used for identification of beta-lactamases producers, of which 10 came from HIV seropositive patients while 3 came from HIV seronegative (control) subjects and observation under UV transilluminator showed various plasmid bands from 6 isolates from HIV subjects and 1 from the control subjects. They exhibited heavy molecular weight plasmids ranging 23.13kbp and above **(Figures 2).**

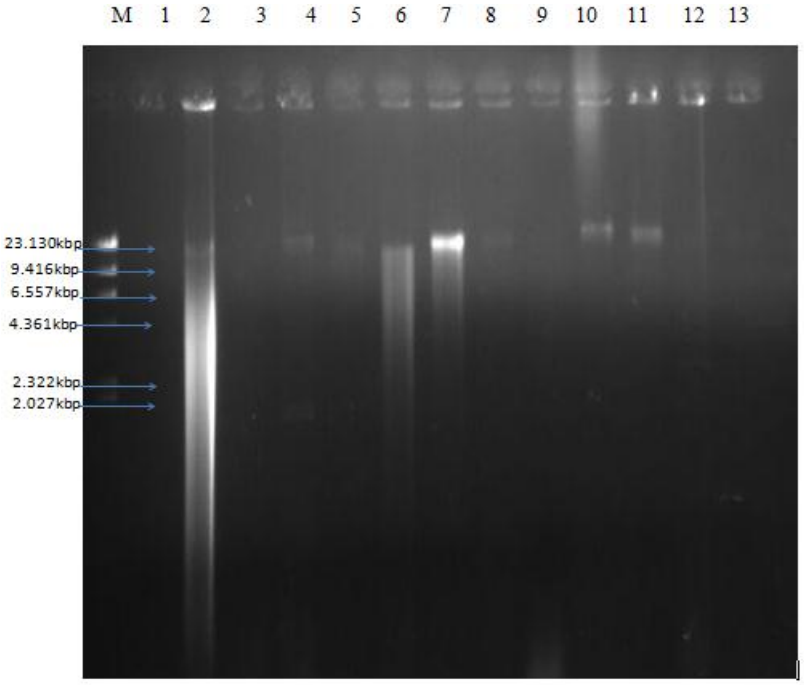

Figure 2: Plasmid profile of *Salmonella* isolates from HIV/AIDS Seropositive and HIV Seronegative (control) subjects.
MK: Molecular weight marker (Hind 111 digest), lanes 1-10 (HIV positive patients) lanes 11-13 (HIV negative samples-control). Plasmids found on Lanes 2, 4, 5, 6, 7, 10 and 11. Molecular weight of plasmids ≥23.1kbp

However, plasmid curing on the 7 isolates showed that some *Salmonella* isolates still retained plasmids as they were resistance when further subjected to DDST drugs. A total of 50%, 16.7% and 33.3% of the isolates from HIV subjects retained resistance against Amoxicillin + Clavulanic acid, Cefotaxime + Clavulanic acid, Cefotaxidime + Clavulanic acid. Similarly from the control subjects, the isolate retained resistance with 100% against Amoxicillin + Clavulanic acid **(Table 2).**

Table 2: Susceptibility of *Salmonella* isolates to ESBLs after curing of plasmids.		
Antibiotics Used	**No. of *Salmonella* isolates from HIV/AIDS subjects that produced ESBls against double-disc drugs after curing of plasmids (%) n=6**	**No. of *Salmonella* isolates from control subjects that produced ESBls against double-disc drugs after curing of plasmids (%) n =1**
Amoxicillin + Clavulanic acid	3 (50)	1 (100)
Cefotaxime + Clavulanic acid	1(16.7)	0 (0.00)
Cefotaxidime + Clavulanic acid.	2(33.3)	0 (0.00)

Salmonella isolates in this study exhibited several percentages of resistance against common antibiotics used in the study. Similar reports were documented by[26, 27] Study conducted by[28] reported many strains of *Salmonella* to be resistant to Ceftazidime, Gentamicin, Chloramphenicol and Ciprofloxacin. Some *Salmonella* isolates from both the HIV/AIDS subjects and the HIV/AIDS negative (control) subjects in the study produced ESBLs which covered them greater resistance to multiple drugs. In the study by[29], they reported the emergence of resistant bacteria because of the widespread use of extended-spectrum Cephalosporins such as Ceftazidime, Cefotaxime or Ceftriaxone as the mainstay of treatment of serious infections due to *Salmonella* species especially in children immediately after usage of Fluoroquinolone was discouraged. According to[22] many of the resistant cases have been attributed to the production of these extended spectrum beta-lactamases (ESBLs) of which these group of enzymes that enable the bacteria possessing them to hydrolyze antibiotics and thus confer resistance to expanded spectrum Oxyimino-cephalosporins, Penicillin and Aztreonam among enterobacteriaceae and other gram-negative bacteria. Moreover[30] reported the ESBL isolates that showed high resistance to tetracycline, gentamicin, pefloxacin, ceftriaxone, cefuroxime, ciprofloxacin and Augmentin at a tertiary hospital in Nigeria. The observation of TEM and CTX type of ESBLs in this study agrees with[11] Soughakoff *et al*.., (1988) who reported that TEM- type of ESBLs has increased activity against extended spectrum cephalosporins and Bush and Fisher,(2011) reported that CTX- type found often found in isolates of *Salmonella* serovar and some other species of *Enterobacteriaceae. Moreover*, according to[31] Tzouvelekis *et al.*, (2000), CTX types are observed to carry out hydrolysis of Cefotaxime and other related drugs. However, Clavulanic acid, Sulbactan and Tazobacter are β-lactamase inhibitors that have a high affinity for and irreversibly bind some β-lactamase but in this study it was observed that some *Salmonella* isolates were seen hydrolyzing Clavulanic acid in combination thus preventing the inhibition and binding of these enzymes leading the some *Salmonella* to become resistance to the double synergy drugs. Studies by[32,33] reported that another unique feature of CTX types is that they are better inhibited by the β-lactamase inhibitor Tazobactam than by Sulbactam and Clavulanate Moreover, reports from[34 ,35] showed that ESBLs enzymes are indeed the largest source of resistance presently and are most commonly produced by *Klebsiella* species, and *Escherichia coli* but may also occurs in other Gram-negative bacteria including *Salmonella, Proteus, Pseudomonas, Citrobacter, Morganella, Serratia* and *Shigella* species.

The *Salmonella* strains in this study have been detected to produce high molecular plasmid-mediated beta-lactamases (PMBLS). Difference in the molecular weight of the plasmids from the *Salmonella* isolates may probably be due to different serovars. According to[36] the plasmids code for enzymes that acetylate, adenylate or phosphorylate various amino-glycosides that determine the active transportation of beta-lactam antibiotics across the cell membrane of the organism. Additionally, the detection of resistant *Salmonella* strains which produce plasmid-mediated beta-lactamases agrees with[37, 38] who reported plasmid mediated type beta-lactamases. Moreover, chromosomal resistance of some bacteria to a class of antimicrobial agents may arise as a result of mutation in the chromosome of the organisms. Thus, the antibacterial resistance caused by acquisition of plasmids observed from the isolated salmonellae is wholly attributed to routine indiscriminate use of those antibacterial agents in Akwa Ibom State, leading to rapid chromosomal mutation and acquisition of some of specific plasmid DNA. These findings are in support of other studies like[39, 40] who reported that the indiscriminate and unwise use of antibiotics normally leads to susceptible strains been resistant due to these organisms acquiring these extra–chromosomal materials. Moreover, in the study, it was observed that some bacteria were resistant to the drugs but with no plasmids. This agrees with[41] that some bacteria used non-genetic mechanism to become resistant to antimicrobials which include active replication of bacteria, microorganisms that are metabolically inactive and non-multiplying may be phenotypically resistant to drugs

Some *Salmonella* strains in the study lost of their plasmids after curing. The removal of conjugative plasmids using acridine orange agrees with[42, 25] who stated that acriflavine has ability to inhibit plasmid replication and remove the plasmids that are found between the DNA bases, without interfering in the replication of chromosomal DNA. However, the non-susceptibility by some *Salmonella* isolates to double-disc drugs after plasmids curing was in suggestion that the multidrug resistance pattern in the *Salmonella* isolates in this study was plasmid and chromosomal mediated. The plasmids were eliminated while those resistant genes in the chromosomes were unable to be removed by the acridine substance. Correspondingly, similar report was made by[30] that the plasmid curing in their studies revealed that the acridine orange could not affect the plasmids on the isolates as they still retained high resistance to the antibiotics after the treatment.

CONCLUSION

High resistance of *Salmonella* isolates to conventional antibiotics, production of TEM and CXT-type of ESBLs, plasmid profile and curing results analysis of some of these isolates from both HIV/AIDS positive and negative subjects showed that the resistance was a repertoire of high molecular weight particles of both plasmids and chromosomes. Thus this work provides data based information that there are multi-drug resistant salmonellae that exhibited ESBLs from both the immunocompetent and immuncompromised individuals in of Akwa Ibom State, Nigeria. Therefore, as these

bacteria developed different strategies to counter the effects of antimicrobial drugs, the identification of the resistance mechanism will help in the discovery and design of new antimicrobial agents. This calls for enforcement and caution of laboratory examination in the study area before antibiotics prescription and /or administration as this is vital in the management of this infection and continuous surveillance to combat infections from *Salmonella* isolates.

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