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Research article

Detection of *inv*A and *bla*$_{CTM}$-genes in *Salmonella* spp. isolated from febrile patients in Lagos hospitals, Nigeria

Kabiru O. Akinyemi*, Christopher O. Fakorede, Rebecca O. Abegunrin, Samuel O. Ajoseh, Abdul-azeez A. Anjorin, Kehinde O. Amisu, Bolanle O. Opere and Dauphin D. Moro

Department of Microbiology, Faculty of Science, Lagos State University, Ojo, Lagos, Nigeria

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***Corresponding author:**
Kabiru O. Akinyemi
akinyemiko@yahoo.com

Abstract

Salmonella infections remain a global challenge. The culture method is the gold standard for the detection of genus *Salmonella*. The application of Polymerase Chain Reaction (PCR) has become an effective tool for the detection of virulence and antimicrobial resistance genes. This study investigated the prevalence of *Salmonella* by culture and detection of *inv*A gene, *bla*$_{CTX\text{-}M}$, and *bla*$_{CTX\text{-}M\text{-}3}$ gene markers by PCR. A total of 612 blood samples were collected from hospitalized febrile patients between March 2020 and April 2021. The samples were cultured and the isolates were identified by standard method with Analytical Profile Index (API 20-E) kits, and were subjected to *in-vitro* antimicrobial susceptibility test (AST) using disk diffusion method. The Extended-spectrum beta-lactamase (ESBL) detection was carried out by double-disc synergy test. Detection of *inv*A gene and antibiotic-resistant genes makers was done by qPCR. A total of 24 *Salmonella* isolates were identified given a prevalence of 3.9% *Salmonella*-associated bacteraemia. Children within 1-10 years with persistent pyrexia of unknown origin (PUO) accounted for 50% of the *Salmonella* isolated with a mean age of 5.299 years. Specifically, 75% (18/24) *Salmonella* isolates and their corresponding samples of positive *Salmonella* culture were positive for the *inv*A gene. The AST results indicated 100% *Salmonella* isolates developed resistance to ceftazidime, cefotaxime, augmentin, ampicillin, ertapenem, and doripenem. None of drug resistant-*Salmonella* isolates expressed ESBL enzyme phenotypically. Seven resistance patterns were observed, and the pattern CAZ-CTX-OFL-AUG-NIT-AMP-ETR-DOR was the most encountered pattern. Twelve (50%) *Salmonella* isolates harbored the *bla*$_{CTX\text{-}M}$ and *bla*$_{CTX\text{-}M\text{-}3}$ genes and were mostly from children. The study has added to the growing knowledge on the suitability of the *inv*A gene primer set as a PCR target for the detection of *Salmonella*. It also revealed a paradigm shift in the occurrence of invasive *Salmonella* harboring *bla*$_{CTX\text{-}M}$ and *bla*$_{CTX\text{-}M\text{-}3}$ genes in PUO cases. There is a need for judicious use of cephalosporin and carbapenem antibiotics to preserve their efficacies.

Keywords: *Salmonella*, qPCR, *inv*A, *bla*$_{CTX\text{-}M}$, Resistance, Febrile patients

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Introduction

Salmonella infections remain a global challenge. In sub-Saharan Africa countries and other low- and middle-income countries, the disease is one of the leading causes of community-acquired bloodstream infections (Reddy et al., 2010; Anejo-Okopi et al., 2016). Typhoid fever is an acute, life-threatening, febrile illness and the case fatality rate of typhoid fever without treatment reached 10–30%, dropping to 1–4% with appropriate therapy (WHO, 2018). Transmission of typhoidal *Salmonella* (*S.* Typhi and *S.* Paratyphi) is via the fecal-oral route through ingestion of water or food contaminated with typhoidal *Salmonella* bacteria and characterized by fever, which is usually accompanied by chills, nausea, diffuse abdominal pain, rash, anorexia, and diarrhoea or constipation (Radhakrishnan et al., 2018).

Non-typhoidal *Salmonellae* (NTS) are the most frequently isolated foodborne pathogens and are predominantly found in poultry meat, eggs, and dairy products. Other food sources that are involved in the transmission of NTS include fruits and vegetables. Food animals such as swine, poultry, and cattle are the primary sources of salmonellosis (Pui et al., 2011). Invasive non-typhoidal *Salmonella* disease (iNTS) has been associated mainly with *Salmonella enterica* serovars *S.* Typhimurium and *S.* Enteritidis, but other serovars have been isolated from febrile patients and diagnosed cases of pyrexia of unknown origin (PUO) (Akinyemi et al., 2007; MacLennan and Levine, 2013). An es-

timated 2.1–6.5 million cases of iNTS disease occur annually, however, iNTS is endemic in sub-Saharan Africa and other underdeveloped countries, accounting for 25% of the mortality rate, occurring more in children under the age of 3 years including severe anaemia, malaria, malnutrition, and human immunodeficiency virus (HIV) infected patients (Gordon et al., 2008; Ao et al., 2015).

The severity of *Salmonella* infections in humans varies depending on the serotype involved and the health status of the human host. Children below the age of 5 years, elderly people, and patients with immunosuppression are more susceptible to *Salmonella* infection than healthy individuals (Crump et al., 2004). Blood culture is considered the gold standard for detection of *Salmonella* serovars after subjecting the culture to different biochemical assays, it is, however, time-consuming which involves multiple processes (Kadry et al., 2019). A rapid molecular detection method to identify *Salmonella* spp. in critically ill patients is paramount for quick intervention and administration of appropriate therapy. The use of different genes and primer sets have been employed to detect *Salmonella* isolates through Polymerase Chain Reaction (PCR) techniques. PCR has been successfully used in the detection of *Salmonella* (Kim et al., 2006; Trafny et al., 2006; Silva et al., 2011).

Almost all *Salmonella* strains can cause disease because of their ability to invade, replicate and survive in human host cells, resulting in disease conditions that may eventually lead to death (Quinn et al., 2005). The ability of *Salmonella* to invade the host cells is encoded in genes located in *Salmonella* Pathogenicity Island 1 (SPI-1) and one of such genes is the invasion gene (*inv*A) (Vieira, 2009). However, *inv*A gene contains sequences unique to *Salmonella* isolates and has been established as a suitable PCR target with an invaluable diagnostic application (Shanmugasamy et al., 2011). However, these techniques are yet to be fully exploited in most low- and middle-income countries due to the major limitations of the requirements for a thermal cycler; relatively expensive reagents and skilled personnel (William, 2021).

Salmonella infection requires the use of antibiotics and administering of the inappropriate antibiotics may result in increased mortality and morbidity (Butler-Laporte et al., 2018). Antibiotics used in the treatment of bacteraemia include ampicillin, tetracycline, and cotrimoxazole. A previous study has revealed that infections caused by multi-drug resistant (MDR) strains are more severe than those caused by susceptible strains (Djeghout et al., 2017). Fluoroquinolone and extended-spectrum cephalosporin became the first line of antibiotics in the early 80s for the treatment of *Salmonella* infections because of the emergence of MDR strains (Kanungo et al., 2008). Resistance to cefotaxime and ceftriaxone and reduced susceptibility to ciprofloxacin had been documented (Harish and Menezes, 2011; Akinyemi et al., 2021).

Resistance to extended-spectrum cephalosporin most times is due to the production of the enzyme extended-spectrum β-lactamases (ESBLs) with CTX-M-group as the most widespread enzymes globally in clinical settings (Zhan et al., 2019). Other ESBL genes reported in *Salmonellae* order than CTX-M genes include TEM, SHV, and PER (Jin and Ling, 2006). In 2015, the first case of CTX-M1 group producing strains of *Salmonella* was reported in Lagos, Nigeria (Akinyemi et al., 2015) and since then, there has been increasing awareness of emergence and circulation of *Salmonella* isolates carrying CTX-M genes and other antibiotic resistance gene markers. Despite this, there is a paucity of information regarding the spate of emergence and circulation of invasive *Salmonella*-associated bloodstream infections harboring $bla_{CTX\text{-}M}$ genes. Besides, the issue of prolonged illness and hospitalization as well as frequent treatment failure with empirical therapy observed in patients has been a great concern and thus requires rapid and effective diagnosis methods.

Accordingly, this study investigated the prevalence of *Salmonella* by culture and detection of *inv*A gene using qPCR from positive culture samples and carriage of $bla_{CTX\text{-}M}$ and $bla_{CTX\text{-}M\text{-}3}$ gene markers in the isolated *Salmonella* spp.

Material and Methods

Study population, case definition and sample collection

A total of 612 patients who sought treatment at public and private medical centers including Alimosho General Hospital (AGH), Randle General Hospital (RGH), and Life-font Medical Centre (LMC) Lagos; from March 2020 to April 2021 were recruited for the study. The clinical history of the patients was obtained. Ethics approval from the ethics committee of each institution was obtained before patients' enrollment. Moreover, the consent of the patients was sought. Data of the patients, including age and sex, were noted. In total, 612 blood samples were aseptically collected from patients diagnosed with PUO, general fever, with the manifestation of headache and diarrhoea. AGH, RGH, and LMC contributed 240, 150, and 222 blood samples, respectively.

Case definition

The PUO in this study was defined as a patient who had been diagnosed by a clinician of persistent febrile illness ($\geq$37.5°C) in the last 72 hours with or without one or more of the following symptoms: diarrhea, headache, loss of appetite, abdominal colic, vomiting, and/or nausea for5 consecutive days.

Sample size

The sample size was estimated using the formula for estimating the minimum sample for descriptive studies

$$N = \frac{Z2p(1-p)}{d2}$$

where, N= sample size, Z= 1.96 (standard error) at 95% confidence interval, p= prevalence of the disease as reported by (Smith et al., 2012)= 74%, d= level of precision at 5% (0.05). Therefore; N= 295649536 per

100,000 population. To make the inference valid, a total of 612 samples were collected in order to increase the accuracy of the estimate.

Bacterial isolation

Three ml of venous blood aseptically obtained from adults were inoculated into bottles containing 27 ml of brain heart infusion (BHI) broth (HIMEDIA, Mumbai, India), while 2 ml of blood from children were inoculated into bottles containing 18 ml of BHI broth and was gently mixed. The bottles were incubated at 37^{o}C aerobically for 7 days. Turbid bottles were sub-cultured onto blood agar, xylose lysine agar (XLD) (HIMEDIA, Mumbai, India), Bismuth sulfite agar (BSA) (Lab M, Lancashire, UK), *Salmonella-Shigella* agar (SSA) (Lab M, Lancashire, UK), and MacConkey agar (MCA) (Lab M, Lancashire, UK) and were further incubated for 18- 24 hours at 37°C. In negative blood samples, sub-cultures were repeated daily from the BHI broth cultures for 7 consecutive days, after which the samples were finally discarded. All isolates were picked, and Gram-stained as described by Barrow and Feltham (1993). All isolates were further identified using an API 20E identification system (Institut Mérieux, Marcy l'Etoile, France) that uses 20 biochemical parameters for microbial identification and confirmation. The profile code generated was used for the identification of all the isolates in the APIWEB database.

Antimicrobial susceptibility testing

All *Salmonella* isolates were investigated for their *in-vitro* susceptibilities to 10 commonly prescribed antibiotics in Nigeria by disk diffusion method, as described by the guideline established by the Clinical and Laboratory Standard Institute guidelines (CLSI, 2019). Disks with the following preparations were used for susceptibility testing: ceftazidime (CAZ) 30 μg, cefotaxime (CTX) 30 μg, gentamicin (GEN) 10μg, ciprofloxacin (CPR) 5μg, ofloxacin (OFL) 5μg, augmentin (AUG) 30μg, nitrofurantoin (NIT) 300μg, ampicillin (AMP) 10μg, doripenem (DOR) 10μg, and ertapenem (ETR) 10μg (Oxoid, Hampshire, UK). The plates were incubated aerobically at 37°C for 18–24 hours. The diameters of the zones of inhibition were measured with a ruler in millimeter and compared with a zone interpretation chart of CLSI (CLSI, 2019). The *E. coli* American Type Culture Collection (ATCC) 25922 was used as a control. MDR phenotype was defined as resistance to three or more classes of antibiotics.

Extended spectrum β-Lactamase (ESBL) assay

All the *Salmonella* isolates that exhibited reduced susceptibility and/or resistance to 3rd generation cephalosporin (3GCs) were screened for ESBL production, using the double disk synergy test method with little modification as described by Akinyemi et al. (2015). The test was conducted by placing the indicator cephalosporin (3GC antibiotics), CAZ (30 μg), CPD (30 μg) and CTX (30 μg at a 15 mm distance from β-lactam inhibitor AUG (30 μg) (20 μg amoxicillin combined with 10 μg clavulanic acid). The *Escherichia coli* ATCC 25992 was used as negative control while *Klebsiella pneumoniae* ATCC 700603 was used as positive control.

DNA extraction

DNA extraction from bacterial isolate

Stock culture of *Salmonella* isolates previously store at 4°C were sub-cultured on BHI. The inoculated plates were incubated at 37°C for 18-24 hours. The DNA extraction was carried out using QIAamp DNA mini kit (Qiagen, Stockach, Germany) according to the manufacturer's instruction. Briefly, fresh bacteria isolate (18-24 hours old) was removed from the culture plate and suspended in 180 μl of buffer ATL. 20 μl proteinase K was added, mixed, and incubated at 56°C for 2 hours. After incubation, 200 μl buffer AL was added and was mixed by pulse-vortexing for 15 seconds. The mixture was further incubated at 70°C for 10 min, followed by addition of 200 μl of ethanol (96%) and was mixed by pulse-vortexing for 15 seconds.

The mixture was carefully dispensed into labelled QIAamp Mini spin columns. The column was placed in a 2 ml collection tube. The cap was closed and centrifuged at 6000 xg for 1 min. The QIAamp Mini spin column was removed and was placed in another clean 2 ml collection tube and the tube containing the filtrate was discarded. 500 μl of buffer AW1 was added to the QIAamp, the cap was closed, and centrifuged at 6000 xg for 1 min. The QIAamp Mini spin column was removed and placed in another clean 2 ml collection tube, and the tube containing the filtrate was discarded. 500 μl of buffer AW2 was added to the QIAamp Mini spin column and centrifuged at full speed (20,000 xg) for 3 min. The QIAamp Mini spin column was removed and was placed in a clean 1.5 ml microcentrifuge tube, and the filtrate in the collection tube was discarded. The QIAamp Mini spin column was opened and 200 μl buffer AE was added and the mixture was incubated at room temperature for 1 min. Thereafter centrifuged at 6000 x g for 1minute. The QIAamp Mini spin column was finally discarded, and the filtrate was collected as DNA extract.

DNA extraction from buffy coat

The buffy coat was prepared by centrifuging 3 ml of whole blood at 2500 xg for 10 minutes at room temperature (15–25°C). After centrifugation, 3 different fractions were distinguishable: the upper clear layer (which is plasma) was carefully aspirated and discarded. The intermediate layer (buffy coat) containing concentrated leukocytes was carefully aspirated into a clean sterile eppendorf tubes ready for use. 20 μl of QIAGEN protease (or proteinase K) was dispensed into a 1.5 ml microcentrifuge tube, followed by addition of 200 μl of the prepared buffy coat. 200 μl of buffer AL was added and was mixed by pulse-vortexing for 15 s. The suspension was incubated at 56°C for 10 min. The mixture was centrifuged briefly to remove drops from the inside of the lid and 200 μl of ethanol (96%) was added and was mixed by pulse-vortexing for 15 s. QIAamp

Mini spin columns were labelled with the appropriate sample number and was placed into a 2 ml collection tube.

The mixture was carefully dispensed into the QIAamp Mini spin columns. The cap was closed and centrifuged at 6000 xg for 1 min. The QIAamp Mini spin columns were removed and placed in another clean 2 ml collection tube and the tube containing the filtrate was discarded. 500 μl of buffer AW1 was added to the QIAamp Mini spin column. The cap was closed, and centrifuged at 6000 xg for 1 min. The QIAamp Mini spin column was removed and placed in another clean 2 ml collection tube, and the tube containing the filtrate was discarded. 500 μl of buffer AW2 was added to the QIAamp Mini spin column and was centrifuged at full speed (20,000 xg) for 3 min. The QIAamp Mini spin column was removed and placed in another clean 1.5 ml microcentrifuge tube, and the filtrate in the collection tube was discarded. The QIAamp Mini spin column was opened and 200 μl buffer AE was added. The mixture was incubated at room temperature for 1 min, and was centrifuged at 6000 xg for 1 min. The QIAamp Mini spin column was finally discarded, and the filtrate was collected as DNA extract.

Real-time PCR (qPCR)

The real-time PCR Rotor gene Q 2plex (Qiagen, Germany) was used for the amplification and detection of *Salmonella* virulence genes *inv*A, and antibiotics resistance genes, $bla_{CTX\text{-}M}$ and $bla_{CTX\text{-}M\text{-}3}$ amplicon by the use of the TaqMan technology. The used oligonucleotides sequence specific for detection of *inv*A, $bla_{CTX\text{-}M}$ and $bla_{CTX\text{-}M\text{-}3}$ in *Salmonella* are shown in Table 1. The *E. coli* NCTC 13461 strain was used as positive control for $bla_{CTX\text{-}M}$ gene.

All qPCR assays were performed on a Qiagen Q 2 plex Real-Time PCR System (Qiagen, Germany) with 72 well reaction tubes, closed with 4-Cap Strip. The reaction was performed using appropriately, 12.5 μl of 2x SYBR®Green Taq PCR master mix (Qiagen, Germany) was dispensed into a 0.2ml PCR tubes. 1.5 μl of forward and reverse primers was added. 10.5 μl of nucleic acid free water was added. Finally, 1.5 μl of bacterial DNA (104 copies of gDNA) was added to the mixture and amplified using the following protocol: Hold temperature of 95°C for 3 min, and subsequently 40 cycles of denaturation at 94°C for 60 s, annealing 64°C for 30 s and extension at 72°C for 30 s.

The fluorescent reporter signal was normalized against the internal reference dye (ROX) signal and the threshold limit setting was performed in automatic mode, No template controls using nucleic acid free water (Biosystem, USA)) were included in each reaction. Only signal generated in SYBR®Green qPCR analysis that displayed amplification above the threshold level were considered positive. While those signals with no Cq value obtained were considered as negative. The qPCR reactions were performed with approximately 104 copies of genomic DNA calculated according to the genome size of each targeted *Salmonella* isolate using the following formula:

$$CN = \frac{M \times AC}{M_{\mathrm{W}} \times G_6}$$

Statistical analysis

The data generated from the independent predictor to determine the correlation between the demography, clinical prognosis and the isolates were entered into Microsoft Excel spread sheet, edited, coded, and analyzed by statistical methods using statistical software program (SPSS version 15.0). The prevalence of invasive salmonellosis was calculated as number of positive cultures for *Salmonella* divided by the total number of samples collected. The data were summarized using Chi-square test of independence. The statistical test was considered significant if the p-value is $\leqq$0.05.

Results

A total of 243 bacterial isolates were recovered from blood samples of 612 hospitalized febrile patients. Twenty-four *Salmonella* isolates were identified with 3.9% prevalence of *Salmonella*-associated bacteremia recorded. The proportion of other bacterial pathogens isolated from the positive samples included *Proteus* spp. 49.8% (120/243), *Klebsiella pneumoniae*19.8% (48/243), *K. oxytoca* 18.5% (45/243) and *Citrobacter* spp. 2.5% (6/243). From 24 (9.9%) positive *Salmonella* isolates, the independent predictor for sex distribution revealed a prevalence of 5.7% and 2.6% among male and female subjects respectively.

Of the 24 *Salmonella* isolates, 75% (18/24) were from male and 25% from female subjects. Children with persistent PUO within the age bracket 1-10 years accounted for 3.7% (12/321) prevalence and 50% (12/24) of the total *Salmonella* recovered with a mean age of 5.299 years, standard deviation (SD) of $\pm$3.01 years and a margin of error (ME) of $\pm$0.2 years at 95% confidence level (Figure 1). Furthermore, teenagers (11-17 years) with 4.8% (9/189) prevalence accounted for 37.5% (9/24) of the invasive *Salmonella* infection among the patients. A mean average age of children within the age bracket (11-17) was $\pm$15.14 years, with SD of $\pm$2.5 years and a ME of $\pm$0.31. Meanwhile subjects above 18 years were grouped as adults and accounted for prevalence of 2.9% (3/102) and 12.5% of the total *Salmonella* detected in the study.

Detection of *Salmonella* spp. within the different age groups showed a statistically significant association with persistent pyrexia with a p-value of 0.0341 (Figure 1). The result of 24 *Salmonella* isolates and the corresponding positive blood samples screened for *inv*A gene primer sets using qPCR revealed the presence of *inv*A in 75% (18/24) *Salmonella* isolates and the respective blood samples of positive *Salmonella* culture. Interestingly, six of the *Salmonella* isolates with absence *inv*A gene were identified by API kit with two of the profile indices 6504552 and 6104512 (Table 2).

The result of the *in-vitro* AST conducted revealed that 100% of *Salmonella* isolates were resistant to ceftazidime, cefotaxime, augmentin, ampicillin, ertapenem, and doropenem. Also, 50% (12/24) of the

Table 1: Primers used in this study.

Primers	Oligonucleotide sequence	Accession number	References
*inv*A-F	5'- ACCACGCTCTTTCGTCTGG-3'	AE006468.1	(Choudhury et al., 2016)
1-3 *inv*A-R	5'- GAACTGACTACGTAGACGCTC-3'	AE006468.1	
CTX-M-F 60C	5'-CGCTGTTGTTAGGAAGTGTG-3'	-	(Mohammed et al., 2016)
1-3 CTX-M-R 62C	5'-GGCTGGGTGAAGTAAGTGAC-3'	-	
CTX-M-3-F	5'-GGTTAAAAAATCACTGCGTC-3'	AY044436	(Weill et al., 2004)
1-3 CTX-M-3-R	5'-TTACAAACCGTCGGTGACGA-3'	AY044436	

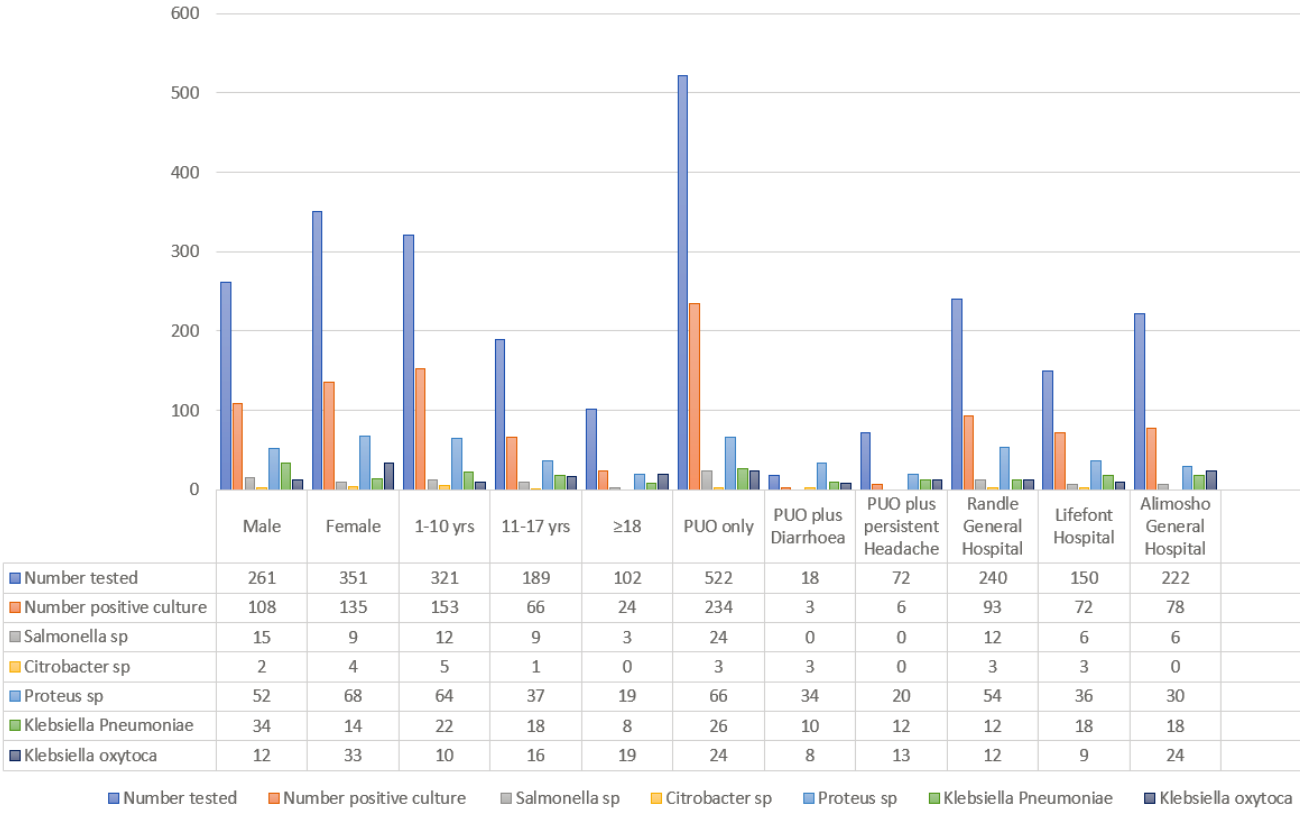

Figure 1: Prevalence of *Salmonella* and other pathogens with independent predictors in hospitalized patients in Lagos, Nigeria

isolates developed resistance to ciprofloxacin, ofloxacin and nitrofurantoin. While a low level of resistant-*Salmonella* (25%) was recorded for Gentamycin.

All the *Salmonella* isolates exhibited resistance to between 7 and 10 different antibiotics and are MDR. Seven resistance profiles were observed with resistance pattern CAZ-CTX-OFL-AUG-NIT-AMP-ETR-DOR being the most frequently encountered pattern. However, none of the MDR *Salmonella* isolates in this study expressed ESBL enzyme phenotypically (Table 3). In this study, it is interesting to note that three *Salmonella* isolates with resistant pattern CAZ-CTX-GEN-CPR-OFL-AUG-NIT-AMP-ETR-DOR harbored $bla_{CTX\text{-}M}$ gene, while resistant pattern CAZ-CTX-CPR-AUG-AMP-ETR-DOR was restricted to two *Salmonella* $bla_{CTX\text{-}M\text{-}3}$ producing strains. Twelve isolates of *Salmonella* (50%) harbored antibiotics resistant gene coding for $bla_{CTX\text{-}M}$ and $bla_{CTX\text{-}M\text{-}3}$.

Discussion

Salmonella enterica-associated community-acquired bloodstream infection remains a serious health challenge in Africa. In this study, twenty-four *Salmonella* isolates were recovered from 612 hospitalized patients with febrile illness indicating 3.9% prevalence rate of *Salmonella* bacteraemia-associated illnesses. This result contrasts with a similar study conducted in Lagos 15 years ago, in which a higher prevalence rate of 16.2% *Salmonella*-bacteraemia was reported (Akinyemi et al., 2007). Similarly, less than a decade ago, about 30% of the *Salmonella* isolates in Lagos were reported from patients with persistent pyrexia (Akinyemi et al., 2015). It has been documented that *S.* Typhi percent positivity ranged between 7% and 18.6% in Lagos. and in Abuja, the percent positivity of *S.* Typhi ranged from 0.8% to 2.4% across the period of 2008–2017 (Akinyemi

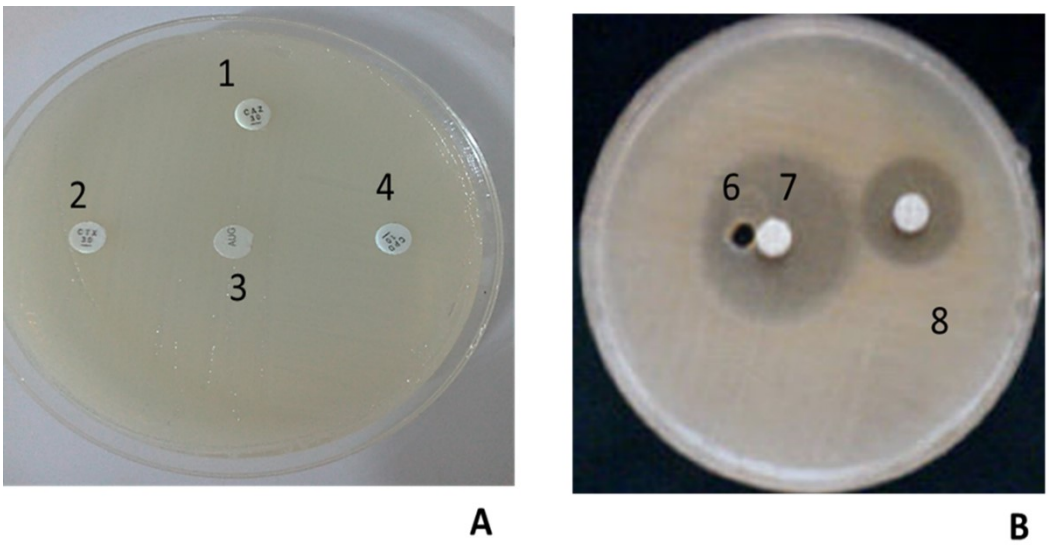

Figure 2: A) *Salmonella* spp. showing negative ESBL result with double disc synergy test (DDST), and complete resistance to all 3GCs including β-lactam inhibitor, B) Phenotypic expression of positive ESBL result with double disc synergy test (DDST) by *Klebsiella pneumoniae* ATCC 700603. An increase in zone of inhibition for ceftriaxone-impregnated-clavulanic acid compared with ceftriaxone alone. 1= ceftazidime (CAZ), 2= cefotaxime (CTX), 3= amoxicillin/clavulanic acid (AUG), 4= cefpodoxime (CPD), 5= clavulanic acid, 7= ceftriaxone, 8=ceftriaxone (CTR)

et al., 2021) and in Warri, Delta State, Nigeria, 14% prevalence was recorded (Ehwarieme, 2011). The low prevalence rate recorded when compared with our previous studies may be attributed to the improved sanitation and constant awareness campaign instituted by the Lagos State Government in the last few years.

Recently, the prevalence of *Salmonella*-bacteraemia was reported to be 1.9% in a systemic review of human *Salmonella enterica* serovars in Nigeria (Akinyemi et al., 2021). In Kenya, 26.1% prevalence of *Salmonella* blood stream infection was found (Kariuki et al., 2020) and 32% in Bangladesh (Ghurnee et al., 2021). Generally, it has been reported that contribution of typhoidal and nontyphoidal *Salmonella* serovars to invasive disease varies considerably from one geopolitical region to another, and even from one state to another (Crump and Heyderman, 2015; Akinyemi et al., 2021). Unfortunately, *Salmonella* isolates identified in this study were not serotyped and the 3.9% prevalence of *Salmonella* bacteraemia obtained still needs serious attention of health policy makers. These results indicated circulation of *Salmonella* bacteraemia and a paradigm shift in the occurrence of invasive *Salmonella* in pyrexia cases in Lagos, Nigeria.

Other bacterial pathogens isolated in this study were: *Proteus* spp. 49.8% (120/243), *Klebsiella pneumoniae* 19.8% (48/243), *K. oxytoca* 18.5% (45/243) and *Citrobacter* spp. 2.5% (6/243). The isolation of bacterial pathogens in the family *Enterobacteriaceae* from cases of PUO is not uncommon. These observations support the growing evidence that invasive *Salmonella* infections do not always present with a distinct clinical picture, and that other bacterial infections as revealed in this study, as well as viral and protozoan infections may mimic its presentation (Grange, 1994; Crump and Heyderman, 2015).

In this study, 75% (18/24) detection rate of *inv*A gene was found in *Salmonella* isolates and their respective blood (buffy coat) samples of positive *Salmonella* culture, an indication of the suitability of *inv*A gene primer set as PCR target with potential diagnostic applications. This result is in line with the study of Heymans et al. (2018) and D'Souza et al. (2009), who reported the presence of *inv*A genes in *Salmonella* as a diagnostic tool for the detection of *Salmonella* isolates. In a similar study, 50% detection rate of *inv*A gene was obtained among *Salmonella* isolates (Kadry et al., 2019), while 55% detection rate was reported by Sharma and Das (2016). However, Anejo-Okopi et al. (2016) reported 90.1% detection rate of *Salmonella* isolates by *inv*A gene specific primers and 100% detection rate was recorded by Ammar et al. (2016).

There are various reasons for the variation in these detection rates, including but not limited to, the differences in the study methods, the specific nucleotide sequences, sample types, sample size, geographical locations as well as the serovars involved. It is worthy of note that six of the 24 *Salmonella* isolates with absence of *inv*A gene were identified with two of the profile indices 6504552 and 6104512 of API kit and were different from other profile indices used to identify other 18 *Salmonella* isolates (Table 1). The different profile indexes as revealed by API 20E kit might indicate possibilities of different *Salmonella* strains belonging to different clades and may have been responsible for the absence of *inv*A gene.

Although, studies had shown 99.6% sensitivity and 100% specificity PCR target of *inv*A gene in the detection of genus *Salmonella* (Malorny et al., 2003; Shanmugasamy et al., 2011), and that accuracy can be at-

Table 2: Comparison of cultural, biochemical and PCR identification of *Salmonella* from hospitalized febrile patients

S.No	Isolate code	Isolate	Blood culture	Analytical profile index	*inv*A gene detection by qPCR			
					Culture positive isolate	Cycle threshold	Buffy coat	Cycle threshold
1	S1	*S. Typhimurium* (control)	+	6706752	+	31.09	+	31.09
2	S2	*Salmonella* spp.	+	6704752	+	31.60	+	31.60
3	S3	*Salmonella* spp.	+	6504552	-	-	-	-
4	S4	*Salmonella* spp.	+	6104512	-	-	-	-
5	S5	*Salmonella* spp.	+	6504752	+	28.06	+	28.06
6	S6	*Salmonella* spp.	+	6504752	+	23.30	+	23.30
7	S7	*Salmonella* spp.	+	6504752	+	23.59	+	23.59
8	S8	*Salmonella* spp.	+	6704752	+	39.53	+	39.53
9	S9	*Salmonella* spp.	+	6504552	+	27.15	+	27.15
10	S10	*Salmonella* spp.	+	6704752	+	27.60	+	31.60
11	S13	*Salmonella* spp.	+	6504752	+	28.06	+	28.06
12	S14	*Salmonella* spp.	+	6504752	+	23.30	+	23.30
13	S15	*Salmonella* spp.	+	6504752	+	22.21	+	23.59
14	S16	*Salmonella* spp.	+	6704752	+	39.53	+	39.53
15	S17	*Salmonella* spp.	+	6504552	+	27.15	+	27.15
16	S11	*Salmonella* spp.	+	6504552	-	-	-	-
17	S12	*Salmonella* spp.	+	6504512	-	-	-	-
18	S18	*Salmonella* spp.	+	6704752	+	31.60	+	31.60
19	S19	*Salmonella* spp.	+	6504552	-	-	-	-
20	S20	*Salmonella* spp.	+	6104552	-	-	-	-
21	S21	*Salmonella* spp.	+	6504752	+	28.09	+	28.06
22	S22	*Salmonella* spp.	+	6504752	+	23.30	+	23.30
23	S23	*Salmonella* spp.	+	6504752	+	25.70	+	23.59
24	S24	*Salmonella* spp.	+	6704752	+	32.53	+	39.53
25	S25	*Salmonella* spp.	+	6704752	+	37.53	+	39.53

Table 3: Antibiotic resistance patterns of *Salmonella* sp isolated from hospitalized febrile patients habouring antibiotics resistance gene.

Pattern of resistance[a]	No. of Antibiotics resisted	No of isolate with profile (N=24)	Phenotypic expression of ESBL[b] (DDST)[c]		$bla_{CTX\text{-}M}$ gene		$bla_{CTX\text{-}M\text{-}3}$ gene	
			Positive	Negative	Positive	Negative	Positive	Negative
CAZ-CTX-GEN-CPR-OFL-AUG-NIT-AMP-ETR-DOR	10	3	3	0	0	3	3	0
CAZ-CTX-GEN-CPR-OFL-AUG-AMP-ETR-DO	9	2	2	0	2	0	2	0
CAZ-CTX-CPR-AUG-NIT-AMP-ETR-DOR	8	4	4	0	0	4	0	4
CAZ-CTX-OFL-OAU-GNI-TAM-PET-RDOR	8	6	6	0	3	3	3	3
CAZ-CTX-AUG-NIT-AMP-ETR-DOR	7	4	4	0	0	4	0	4
CAZ-CTX-OFL-AUG-AMP-ETR-DOR	7	3	3	0	0	3	0	3
CAZ-CTX-CPR-AUG-AMP-ETR-DOR	7	2	2	0	2	0	0	2

[a]CAZ=Ceftazidime, CTX = Cefotaxime, GEN=Gentamycin, CPR=Ciprofloxacin, OFL= Ofloxacin, AUG = Augmentin, NIT= Nitrofurantoin, AMP = Ampicillin, DOR = Doripenem, ETR = Ertapenem.
[b]ESBL =Extended spectrum beta lactamase.
[c]DDST = Double disc synergy test.

tributed to *inv*A primer sequences used in PCR assay (Kadry et al., 2019). The present study revealed that amplification of *inv*A gene as a PCR target is a good tool for the detection of *Salmonella*, but it is not without shortcomings of possible deletion or insertion due to mutation. Natural deletion of the *inv*A gene in the

centisome 63 Pathogenicity Island of some *Salmonella* sp isolated from the environment had been reported (Ginocchio et al., 1997), and this region has been reported to constitute an unstable pathogenicity island in certain *Salmonella* serotypes. The implication of negative result using only *inv*A primer set as PCR target for the detection of *Salmonella* will preclude the detection of those *Salmonella* whose *inv*A virulence genes have been lost due to possible mutation.

The result of the *in-vitro* antimicrobial susceptibility conducted on all the *Salmonella* isolates revealed high level of resistance (100%) to ceftazidime, cefotaxime, augmentin, ampicillin, ertapenem and doripenem. Similarly, 50% (12/24) *Salmonella* isolates exhibited resistance to ciprofloxacin, ofloxacin and nitrofurantoin, while 25% resistance to gentamycin was recorded. The resistance to many classes of antibiotic such as β-lactam inhibitor containing antibiotics, 3GCs, carbapenems and fluoroquinolone evaluated in this study is worrisome and call for serious concerns. This is because, in Nigeria, empirical antibiotics such as ampicillin, tetracycline, co-trimoxazole, and chloramphenicol are cheap and affordable for treating of *Salmonella* bacteraemia and their efficacies had long been documented to be questionable and are such no longer effective (Obaro et al., 2015; Akinyemi et al., 2018), due to drug abuse, fake and adulterated drugs, indiscriminate and widespread uses of antimicrobials both in clinical and veterinary medicine, as well as the easy access to over-the-counter antimicrobials without prescription.

The observed fluoroquinolone resistance may likely be the consequence of widespread replacement of these traditional first-line antibiotics (ampicillin, tetracycline, co-trimoxazole, chloramphenicol) with fluoroquinolones. MDR and/or reduced fluoroquinolone susceptibility had been an issue in Nigeria (Akinyemi et al., 2021) and other West African countries such as Malawi and Tanzania (MacLennan and Levine, 2013) and are becoming a public health challenge.

Since the introduction of antibiotics over a century ago, there has been an increasing report of antibiotic resistance (Harish and Menezes, 2011; Nordmann and Poirel, 2014). The resistance of invasive *Salmonella enterica* serovars to 3GCs, fluoroquinolone, and macrolides has been documented elsewhere. For example, in Lebanon, 74% *Salmonella* resistance to cefotaxime was observed (Harakeh et al., 2005) and in China, between 60% and 75% resistance to 3GC and fluoroquinolones antibiotics were recorded from different studies (Jin and Ling, 2006; Mshana et al., 2011; Fischer et al., 2014). Furthermore, in China as low as 8.4% *Salmonella*-bacteraemia resistance to cefotaxime was reported (Zhan et al., 2019). The 100% resistance to carbapenems (doripenem and ertapenem) observed in this study is quite worrisome with a serious health implication. This is because these antibiotics are expensive and less abused, and the cost for five days treatment in our environment is about 25 US dollars.

Carbapenem antibiotics are the last resort in combating severe infections including invasive *Salmonella* infections (Guerra et al., 2014; Nordmann and Poirel, 2014). Nevertheless, the observed resistance could be attributed to number of factors which may include carbapenemase production, and AmpC-lactamases with porin deficiency, a resistance mechanism which had been documented in invasive and non-invasive *Salmonellae* (Nordmann et al., 2012; Su et al., 2012; Huang et al., 2013; Fernández et al., 2018). Our results indicated that all *Salmonella* isolates exhibited heterogeneous resistance profiles, between 7 and 10 different antibiotics with CAZ-CTX-OFL-AUG-NIT-AMP-ETR-DOR being the most frequently encountered pattern produced by six *Salmonella* isolates.

None of the *Salmonella* isolates that developed resistance to 3GCs expressed ESBL enzyme phenotypically. However, 50% (12/24) of *Salmonella* revealed the carriage of antibiotic resistance, $bla_{CTX\text{-}M}$ and $bla_{CTX\text{-}M\text{-}3}$ genes, an indication of the continuous circulation and burden of antibiotic resistant-*Salmonella* strains in our environment. Three *Salmonella* isolates with resistant pattern CAZ-CTX-GEN-CPR-OFL-AUG-NIT-AMP-ETR-DOR harbored $bla_{CTX\text{-}M}$ gene, while resistant pattern CAZ-CTX-CPR-AUG-AMP-ETR-DOR was restricted to another two *Salmonella* $bla_{CTX\text{-}M\text{-}3}$ producing strains.

It is worthy to note that, $bla_{CTX\text{-}M}$ and $bla_{CTX\text{-}M\text{-}3}$ producing *Salmonella* strains in this study were isolated from patients with the clinical history of persistent PUO and mostly of children under 10 years of age with cases of treatment failure. In India, invasive *Salmonella* strains carrying resistant gene $bla_{CTX\text{-}M}$ to cefotaxime and ceftriaxone had been reported (Ramachandran et al., 2017). Also, the occurrence of $bla_{CTX\text{-}M}$ genes had been documented in Egypt (Adel et al., 2021) and in Nigeria (Akinyemi et al., 2015; Ugwu et al., 2017). The implication of this finding is that there is a reservoir of ESBLs and CTX-M genes among *Salmonella* isolates which may likely constitute a huge risk factor for the spread of resistance to other pathogenic *Enterobacteriaceae*.

Conclusions

This study recorded 3.9% prevalence of *Salmonella*-bacteraemia in PUO cases. The study revealed 75% of *Salmonella* isolates contained the *inv*A gene, which adds to the growing knowledge of *inv*A gene primers as PCR targets and with blood cultures as the gold standard for diagnosis. *Salmonella* isolates were 100% resistant to ceftazidime, cefotaxime, augmentin, ampicillin, ertapenem and doripenem an indication that efficacies of 3GC and carbapenem antibiotics are becoming doubtful in our environment. It also revealed a paradigm shift in the occurrence of invasive *Salmonella* harboring, $bla_{CTX\text{-}M}$ and $bla_{CTX\text{-}M\text{-}3}$ genes in PUO cases. There is a need for judicious use of cephalosporin and carbapenem antibiotics to preserve their efficacies and to curtal likely risk factors arising from the spread of resistance to other pathogenic *Enterobacteriaceae* in our environment.

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