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Molecular characterization and antibiotic resistance of *Salmonella* in children with acute gastroenteritis in Abuja, Nigeria

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Abstract

Introduction: In Nigeria, acute gastroenteritis in children under five years of age is a major cause of mortality and morbidity; identification and characterization of microbial agents of acute gastroenteritis, including *Salmonella*, remains a powerful tool for effective management, surveillance, and control.

Methodology: Diarrheal stool samples were directly plated onto differential and selective media to isolate *Salmonella*. Extended-spectrum beta-lactamases were screened using the double disk diffusion technique and by PCR targeting the *bla*_{TEM} and *bla*_{CTX-M} genes. Pulsed-field gel electrophoresis (PFGE) was performed using the PulseNet Canada Laboratory protocol for molecular subtyping using the restriction enzymes *Xba*I and *Bln*I.

Results: The serotypes identified were *Salmonella enterica* serovar Zanzibar (n = 5), *Salmonella* Brancaster (n = 3), and one isolate of *Salmonella* Enteritidis (phage type 1). The following levels of resistance were found among the *Salmonella* strains: amoxicillin, five strains (55.6%); amoxicillin-clavulanic acid, two strains (22.2%); cephalexin, five strains (55.6%); and cefuroxime, five strains (55.6%). Intermediate resistance was found in five strains (55.6%) only to amoxicillin-clavulanic acid. All isolates were susceptible to nalidixic acid, ciprofloxacin, and ceftriaxone, and no ESBL-producing *Salmonella* were detected.

Conclusions: Our findings demonstrated the involvement of three *Salmonella* serovars in acute gastroenteritis; resistance to penicillins and cephalosporins was common.

Key words: acute gastroenteritis in children; pulse-field gel electrophoresis; *Salmonella*; antibiotic resistance; Nigeria

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Introduction

Salmonella infections are associated with acute gastroenteritis, one of the most common alimentary diseases; they are caused by the consumption of contaminated water and food, especially meat [1]. The prevalence rate of acute diarrhea in Nigeria is 18.8%, one of the worst in sub-Saharan Africa. It accounts for over 16% of child deaths in Nigeria and an estimated 150,000 deaths – chiefly amongst children under five years of age – occur annually due to this disease, mainly caused by poor sanitation and hygiene practices. These diseases are especially common in developing countries with poor hygiene and sanitation and with limited access to safe drinking water [2]. The identification of the pathogenic organisms responsible for these diseases is highly crucial for surveillance, prevention, and control activities. TEM β-lactamases

are encoded by plasmid-mediated *bla*_{TEM} gene clusters, which have various coding region frameworks and types of promoters [3]. The *bla*_{TEM} gene cluster encodes by plasmid mediation resistance mechanisms such as penicillinase hyper production, extended-spectrum beta-lactamases (ESBL), or inhibitor-resistant TEM beta-lactamase [4]. Variations in sugar content of the O-polysaccharide contribute to the wide variety of antigenic types between species and even strains of Gram-negative bacteria [5]. The variability defined by immunologic characterization of two surface structures, O-polysaccharide (O antigen) and flagellin protein (H antigen), provides the basis for serotyping *Salmonella* spp. into serogroups [6]. Polymorphism in the *bla*_{TEM} genes has already been noted for clinical isolates with > 150 variants [7]. The highly polymorphic *bla*_{TEM} gene has been targeted as a

molecular marker to detect antimicrobial resistance associated with *Salmonella* serovars in acute gastroenteritis in Abuja.

Antimicrobial resistance in enteric pathogens is of great importance in developing countries, where the rate of diarrheal disease is high. The progressive increase in antimicrobial resistance among enteric pathogens in developing countries is becoming a critical area of concern. Among the bacterial causes of diarrheal illnesses, *Salmonella* spp. continues to be a major public health problem [8]. Although most *Salmonella* infections are self-limiting, serious sequelae, including systemic infection and death, can occur [9]. In potentially life-threatening cases, the antibiotics of choice are fluoroquinolones and extended-spectrum cephalosporins [10]. These cephalosporins are commonly used in the treatment of *Salmonella* infections in children because of their pharmacodynamic properties and the very low prevalence of resistance to these agents. Very few studies have reported on the problem of ESBL in Africa in general and in Nigeria in particular. There have been reports of SHV-12 in *Salmonella enterica* serotype in Mali [10] and TEM-3 in *S. Typhimurium* in Morocco [11].

The aim of this study was to determine serotype, antibiotic resistance, and extended-spectrum beta-lactamase (ESBL) gene production of *Salmonella* species associated with childhood acute gastroenteritis in Federal Capital Territory Abuja, Nigeria. In addition, the study evaluated the molecular characteristics and clonal relatedness among the strains.

Methodology

Microbiological diagnosis

Stool samples were collected in sterile plastic cups, with prior instruction given for correct sampling. The samples were obtained by spontaneous emission, and given to the investigator. They were inoculated within two hours of collection onto selective and differential media: MacConkey (MAC) agar, *Salmonella Shigella* (SS) agar, and xylose lysine deoxycholate (XLD) agar, using a calibrated inoculating loop in the spread plate method. The media were then incubated aerobically at 35°C for 18 to 24 hours. Samples were also inoculated into selenite cystine enrichment broth – which favors the development of potentially pathogenic microorganisms and contains substances that inhibit the native flora [12] – and incubated at 35°C for 8 to

12 hours before re-inoculation onto MCK and SS agars.

Colonies with a presumptive *Salmonella* morphology as per the phenotypic characteristics in the different culture media were identified biochemically according to the following procedure [13]: five probable *Salmonella* colonies were selected from the SS or XLD agars, inoculated into 3.5 mL brain-heart infusion broth (BHI), incubated at 35°C for 10 minutes, and then inoculated onto the different media. Biochemical tests were then carried out for: the determination of glucose and lactose; gas formation and the production of ferrous sulfite from *sodium thiosulfate* in Kligler agar; the presence of cytochrome oxidase; citrate utilization; urea hydrolysis in Christensen's agar; the presence of phenylalanine deaminase; the liberation of indole; acid production from sugar fermentation; the decarboxylation of lysine, ornithine, and arginine; and the utilization of malonate as the sole carbon source. In addition, a nutrient agar plate was inoculated from each BHI broth and incubated at 35°C for 24 hours; the colonies were used for the oxidase test.

Serotyping and phage typing

Serotyping and phage typing of isolates were performed at the National Microbiology Laboratory (NML), Public Health Agency (PHC) of Canada according to the Kauffmann–White scheme and the recommendations of Edwards and Ewing [14].

Molecular characterization

The serovars that were resistant to either amoxicillin-clavulanic acid, cephalexin, or cefuroxime alone or in combinations were screened by PCR using two primer sets sequence specific for *bla*_{TEM} and *bla*_{CTX-M} genes to amplify the DNA template for genes coding for ESBL production [3].

DNA was extracted from *Salmonella* isolates using Wizard Genomic DNA purification Kit (Promega, Fitchburg, USA) according to manufacturer's specifications. Oligonucleotide pairs that amplified 708 bp and 593 bp products of *bla*_{TEM} and *bla*_{CTX-M} genes, respectively, were used (Table 1). PCR amplification employed a final volume of 25 µL containing 1.5 mM MgCl₂, 100µM deoxyribonucleoside triphosphate, 0.2µM of each oligonucleotide, and 1U of Taq DNA polymerase. The PCR amplification reaction for the detection of *bla*_{TEM} consisted of 36 cycles of 30 seconds of denaturation at 94°C, 30 seconds of hybridization at 42°C, and 60 seconds of extension at 72°C, with a final extension

Table 1. Primers used in identifying ESBL gene production in *Salmonella* serogroups

Primer	Primer sequence	Primer target	Amplicon size (bp)	Reference
TEM - G	5'-TTGCTCACCCAGAAACGCTGGTG-3'	<i>bla</i> _{TEM}	708	Boyd <i>et al.</i> , 2004
TEM -R	5'-TACGATACGGGAGGGCTTACC-3'			
CTX-M-F	5'-TTTGGCGATGTGCAGTACCAGTAA-3'	<i>bla</i> _{CTX-M}	593	Boyd <i>et al.</i> , 2004
CTX-M-R	5'-CGATATCGTTGGTGGTGCCATA-3'			

step at 72°C for 10 minutes [3]. The *bla*_{CTX-M} gene was identified with PCR conditions similar to those described for *bla*_{TEM}, except for the alteration of the annealing temperature to 58°C [3]. The amplified products were visualized electrophoretically on 2% agarose gel [15]. For quality control, *S. branderup* H9812 ATCC BAA-664 was used.

Pulsed-field gel electrophoresis analysis

Pulsed-field gel electrophoresis (PFGE) was performed using the standardized protocol of PulseNet Canada Laboratory for molecular subtyping by using the restriction enzymes *Xba*I and *Bln*I according to the standardized protocol for *E. coli* (O157:H7). *Salmonella* switch times of 2.2-63.8 seconds was used for the molecular typing of *Salmonella* isolates [15]. *S. branderup* strain H9812 (ATCC BAA 664) was used as a reference strain. DNA macrorestriction profiles were analyzed with BioNumerics software version 2.5 (Applied Maths, Saint Martens-Latem, Belgium) using a band tolerance of 1.5% and optimized at 1.0%. Dendrograms were constructed on the basis of the unweighted pair group method of averages, position tolerance of 1%. Clusters were defined as DNA patterns sharing ≥ 85% similarity [16]. PFGE pattern designations were assigned according the PulseNet Canada database.

Antimicrobial susceptibility testing

Characterized strains were tested for susceptibility to the following antibiotics: nalidixic acid (30 µg), ciprofloxacin (5 µg), cephalexin (30 µg), cefuroxime (30 µg), amoxicillin (25 µg), ceftriaxone (30 µg), and amoxicillin-clavulanic acid (30 µg), using the standard Kirby Bauer disk diffusion susceptibility testing method. *Escherichia coli* ATCC 25922 was used as a control. Multidrug resistance (MDR) for this study was defined as resistance to two or more classes of antimicrobials agents. Minimum inhibitory concentrations (MICs) of isolates were performed by the broth microdilution method (Sensititre Automated Microbiology System; Trek Diagnostic Systems Ltd., Westlake, OH, USA). Using Clinical and Laboratory

Standards Institute (CLSI) guidelines, each organism was classified either resistant or susceptible [17].

Detection of ESBL production was done by a double disk diffusion technique with the following disks: three containing ceftazidime 30 µg (CAZ30 C), cefotaxime 30 µg (CTX30C), and cefpodoxime 30µg (CPD30C), and three with a combination of the same antibiotics with the addition of clavulanate: ceftazidime 30 µg/clavulanic acid 10 µg (CAZ30/CLAV10); cefotaxime 30µg/clavulanic acid 10 µg (CTX30/CLAV10); cefpodoxime 30µg/clavulanic acid 10 µg (CPD30C/CLAV10) (CA; Oxoid, Basingstoke, UK). The zones of inhibition of each isolate were tested on Mueller-Hinton agar plates (Oxoid, Basingstoke, UK). Interpretive criteria established by the CLSI were used to categorize the results of antimicrobial susceptibility testing and ESBL production [17].

Ethical considerations

Ethical approvals were obtained from the University of Abuja Teaching Hospital Gwagwalada, Abuja, National Hospital Abuja, and Health and Human Services Secretariat of the Federal Capital Administration. Informed consent was obtained from the parents of the patients who were to participate in the study. A questionnaire was used to collect epidemiological data following the principles proposed by the model ethical protocol in accordance with the Helsinki declaration [18].

Results

The nine strains of *Salmonella* isolates were typed into three serovars with specific antigenic formulae. The serotypes identified were *Salmonella* Zanzibar (n = 5), *Salmonella* Brancaster (n = 3), and one strain of *Salmonella* Enteritidis. The *S. Enteritidis* strain was phage type 1; the phage types of *S. Zanzibar* and *S. Brancaster* were not determined (Table 2).

There was no genetic diversity in the PFGE patterns of *Xba*I digested chromosomal DNA of the five isolates of *S. Zanzibar*, as all had the same restriction pattern.

Table 2. Demographic and phenotypic characteristics of *Salmonella* serotypes isolated from diarrheic children younger than five years of age in FCT, Abuja, Nigeria

Patient identity	Locality	Age group (months)	Type of diarrhea	Organism identity/serotype	Antigenic formulae	Phage type
087	Municipal	4	Watery	<i>Salmonella</i> Zanzibar	3,10:k:1,5	ND
099	Peri-urban	12	Watery	<i>Salmonella</i> Brancaster	4,12,27:z29:-	ND
155	Municipal	12	Mucoid	<i>Salmonella</i> Zanzibar	3,10:k:1,5	ND
159	Municipal	24	Mucoid	<i>Salmonella</i> Zanzibar	3,10:k:1,5	ND
167	Municipal	18	Watery	<i>Salmonella</i> Brancaster	4,12,27:z29:-	ND
280	Municipal	4	Watery	<i>Salmonella</i> Brancaster	4,12,27:z29:-	ND
298	Peri-urban	12	Mucoid	<i>Salmonella</i> Zanzibar	3,10:k:1,5	ND
302	Municipal	24	Watery	<i>Salmonella</i> Enteritidis	9,12:g,m:-	1
326	Municipal	18	Mucoid	<i>Salmonella</i> Zanzibar	3,10:k:1,5	ND

ND: not determined

Table 3. The PFGE patterns of *Xba*I and *Bln*I digested chromosomal DNA of *Salmonella* serovar isolates from diarrheagenic children younger than five years of age in FCT Abuja, Nigeria

<i>Salmonella</i> serovar	PFGE pattern		Inference
	<i>Xba</i> I	<i>Bln</i> I	
<i>S. Zanzibar</i> (5)	ZanXAI.0001	ZanBNI.0001	New <i>Xba</i> I, new <i>Bln</i> I
	ZanXAI.0001	ZanBNI.0002	New <i>Bln</i> I
	ZanXAI.0001	ZanBNI.0001	nil
	ZanXAI.0001	ZanBNI.0001	nil
	ZanXAI.0001	ZanBNI.0001	nil
<i>S. Brancaster</i> (3)	BranXAI.0001	BranBNI.0001	nil
	BranXAI.0001	BranBNI.0001	New <i>Xba</i> I, new <i>Bln</i> I
	BranXAI.0001	BranBNI.0001	nil
<i>S. Enteritidis</i> (1)	SENXAI.0001	SENBNI.0017	Common patterns

Table 4a. Antimicrobial resistance pattern of *Salmonella* isolates (n = 9)

Antibiotic used	Number of resistant strains (%)
Amoxicillin	5 (55.5)
Cephalexin	5 (55.5)
Cefuroxime	5 (55.5)
Ciprofloxacin	0 (0.0)
Ceftriaxone	0 (0.0)
Amoxicillin-clavulanic acid	2 (22.2)
Nalidixic acid	0 (0.0)

Table 4b. Antibiotic resistance pattern of nine strains of *Salmonella* serovars isolated from children younger than five years of age in FCT, Abuja Nigeria.

<i>Salmonella</i> serovars	Antibiotic resistance profile	No of Strain	Total No
<i>Salmonella</i> Zanzibar (5)	AMX,CEPH	1	4
	CEPH ,CEFU	1	
	AMX,CEPH,CEFU	1	
	AMX,AUG,CEPH,CEFU	1	
<i>Salmonella</i> Brancaster(3)	AMX,CEPH,CEFU	1	2
	AMX,AUG,CEPH,CEFU	1	
<i>Salmonella</i> Enteritidis(1)	AMX,CEPH,CEFU	1	1

AMX: amoxicillin; CEPH: cephalixin; CEFU: cefuroxime; CEFTR: ceftriaxone; AUG: Augmentin (amoxicillin-clavulanic acid)

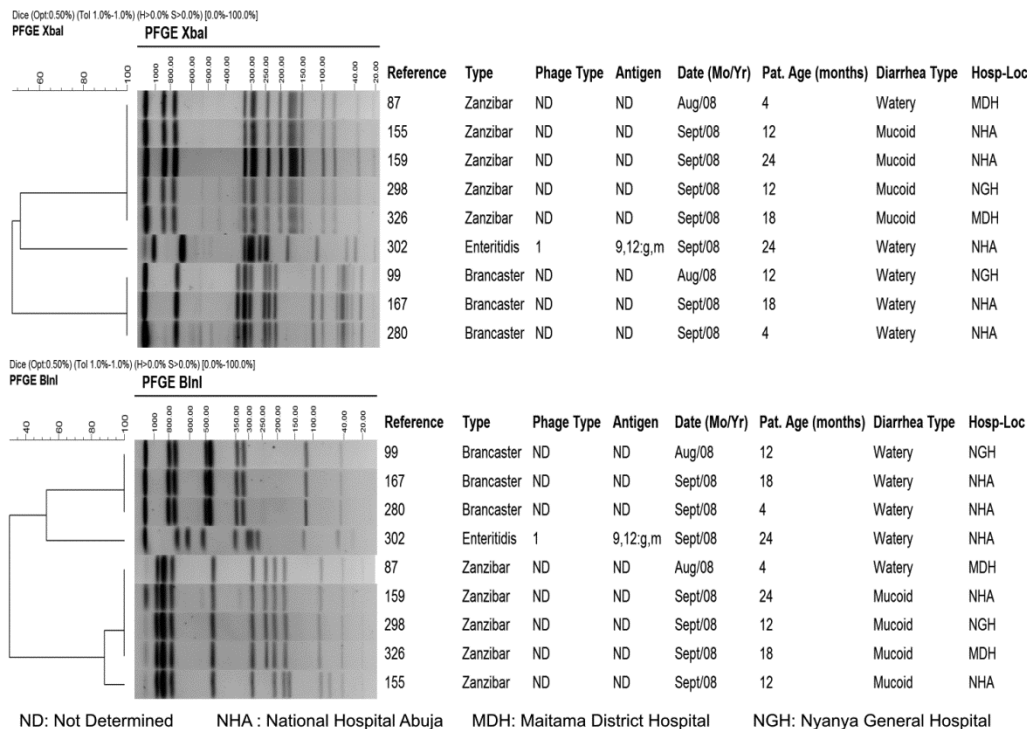
Comparatively, one isolate out the five *S. Zanzibar* isolates had a different *BlnI* restriction pattern than the other four (relating to a single band shift). The three isolates of *S. Brancaster* had the same restriction patterns for *XbaI* and *BlnI* digested chromosomal DNA, respectively. No comparison could be made for serovar Enteritidis clonality based on PFGE, as only a single patient isolate was recovered. The PFGE patterns of the five strains of *S. Zanzibar* isolated appeared identical and hence genetically related, as did the three strains of *S. Brancaster* isolates (Table 3).

The following levels of resistance were found among the *Salmonella* strains: amoxicillin, five strains (55.6%); augmentin, two strains (22.2%); cephalixin, five strains (55.6%); and cefuroxime, five strains (55.6%). Intermediate resistance was found in five strains (55.6%) only to amoxicillin-clavulanic acid (Augmentin). All the serotypes isolated were susceptible to nalidixic acid, ciprofloxacin, and ceftriaxone (Table 4a). In all, seven antimicrobial resistant phenotypes were observed among the three *Salmonella* serovars. All the *Salmonella* serovars with the exception of one *S. Zanzibar* strain were resistant to two or more antibiotics. Pan susceptibility was detected in only one strain each of *S. Zanzibar* and *S. Brancaster*. Similarly, a multidrug-resistant pattern to

four antibiotics (AMX, AUG, CEPH, and CEFU) was detected in only one strain each of these serovars (*S. Zanzibar* and *S. Brancaster*). Among the three *Salmonella* serovars, common resistant profile patterns to three antibiotics (AMX, CEPH, and CEFU) were observed (Table 4b). Although among the *Salmonella* serovars, multidrug resistance was observed in five (55.6%) of the *Salmonella* serovars (two *S. Zanzibar*, two *S. Brancaster*) and in the only *S. Enteritidis* serovar, none exhibited resistance MICs to tested antimicrobial agents according to the current CLSI breakpoints. In addition, all the serotypes exhibited susceptible zone inhibition diameters ranging from 32-34 mm (minimum and maximum) to ceftazidime, cefotaxime, and cefpodoxime alone or in combination with clavulanic acid. No ESBL-producing *Salmonella* isolates identified as either TEM or CTX-M-type ESBLs genes were detected in any of the isolates. Screening of isolates for *bla_{SHV}* and AmpC beta-lactamase production was not performed in this study.

Figure 1 illustrates phylogenetic dendrogram of PFGE patterns obtained using the restriction enzymes *XbaI* and *BlnI* showing percent similarity calculated by the Dice similarity index of PFGE restriction endonuclease digestion profiles among the nine *Salmonella* isolates (*S. Zanzibar*, *S. Brancaster*, *S.*

Figure 1. Dendrogram showing the cluster analysis on the basis of *XbaI*-PFGE and *BlnI*-PFGE of the three *Salmonella* serovars. ND: Not determined;NHA: National Hospital Abuja; MDH: Maitama Distict Hospital; NGH: Nyanya General Hospital



Enteritidis). The different patterns, sources, year of isolation, and number of strains are indicated. The Zanzibar and Brancaster isolates were the first from north-central Nigeria to be ever typed using PFGE. The five *S. Zanzibar* isolates all had the same restriction patterns using *XbaI* (no genetic diversity was identified), but one isolate had a different *BlnI* restriction pattern than the other four. Four Zanzibar isolates were from mucoid-type diarrhea. All three *S. Brancaster* isolates assigned to the same PFGE pattern had the same *XbaI* and *BlnI* patterns. Of the three Brancaster isolates, two isolates were from the municipal area and one was from the peri-urban area. No comparative assessment of *S. Enteritidis* was possible, but the lone isolate was recovered from Abuja Municipality and was phenotypically phage type 1.

Discussion

This study characterized *Salmonella* species recovered from children with acute gastroenteritis, and determined antibiotic susceptibility patterns and extended-spectrum beta-lactamase production using polymerase chain reaction, pulse field gel electrophoresis, micro dilution, and double disk diffusion techniques. Notably, a rare serotype incriminated in human *Salmonella* infections, *S. Zanzibar*, had the highest prevalence (55.6%) in this study. The first reported human infection with the serotype *S. Zanzibar* was in 1976 from the stool culture of an eight-year-old boy with gastroenteritis in Ankara [19]. Thereafter, it has been documented to be associated with adult infections in North Scotland [20], and more recently in Tunis [21]. Notably, multidrug resistant *S. Brancaster* isolated from poultry has been reported in Dakar, Senegal [22]. The reported epidemiology of *S. Brancaster* in the literature is rather limited [23]. However, its occurrence in our study is in consonance with reported isolation from human cases in the Toscana region of Italy [24], in the United States [25], and in Senegal, on the West African coast [24]. Recently, resistance to spectinomycin and streptomycin by *S. Zanzibar* isolated from chicken muscle was observed in Dakar, Senegal [26].

The choice of antimicrobial agents evaluated in this study was based on reported resistance of *Salmonella* isolates to commonly used antimicrobials [27]. The results of this study showed that 77.8% of the *Salmonella* isolates tested for antimicrobial susceptibility were resistant to two or more antibiotics. Although a previous study in Nigeria had reported a resistance rate of 61.0% [28], other authors from

Ethiopia [29] recently reported a resistance rate of 83.3%, which is, comparatively, a higher proportion of multidrug resistance. The finding of good antimicrobial activity for ciprofloxacin is in line with earlier reports from Lagos, Nigeria [28].

The identification of no ESBL-producing *Salmonella* serovars is consistent with the findings of some previous studies in developed and developing countries. No ESBLs were detected among human *Salmonella* isolates in multicenter surveys conducted by a France hospital-based network in 1994 (n= 2,622) and 1997 (n=2,464) [30]. Similarly, in China, no ESBL-producing isolates were identified among human diarrheal *Salmonella* isolates from 28 hospitals in Henan Province in 2006 [31]. More recently, non-ESBL-producing *Salmonella* isolates were identified in another multicenter study conducted in India that monitored *in vitro* susceptibility of community- or hospital-acquired Gram-negative bacilli in intra-abdominal infections to identify early changes in susceptibility patterns, with a focus on ESBL producers. [32]. Therefore, the antibiotic resistance exhibited by the *Salmonella* serotypes isolated as observed from the disk diffusion susceptibility tests may have been conferred by a mechanism for β -lactam resistance other than those analyzed in this study. Conversely, the identification of non-ESBL-producing *Salmonella* isolates may be inadvertent, but not altogether unusual.

Conclusions

Molecular characterization helps scientists to understand the nature of the epidemiology of infections caused the different *Salmonella* serotypes. Our findings have demonstrated the involvement of three *Salmonella* strains in acute gastroenteritis; ciprofloxacin, ceftriaxone, and nalidixic acid remain the antibiotics of choice in the management of acute gastroenteritis caused by the three strains of *Salmonella* isolates. The study further demonstrated the absence of extended-spectrum beta-lactamase producing *Salmonellae*.

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