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Diversity and antimicrobial resistance of *Salmonella enterica* isolated from fresh produce and environmental samples

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ABSTRACT

In Nigeria, as in most developing countries, vegetable farmers depend largely on wastewater for irrigation and on untreated manure for soil enrichment. *Salmonella* is among the most important foodborne pathogens worldwide with fresh vegetables as major route of transmission to man. A total of 440 samples comprising vegetables and environmental samples (irrigation water and manure treated soil) were analyzed for the presence of *Salmonella*, resistance and virulence genes and diversity of isolates by genotyping. Samples were obtained from five irrigation fields in Kano and Plateau States and cultured using selective isolation with prior enrichment method. Presumptive isolates were identified and characterized using conventional biochemical methods and Microbact 24E (Oxoid, UK) identification kit. Amplification of virulence (invasive A and enterotoxin) genes by polymerase chain reaction (PCR) further confirmed *Salmonella* and its virulence potential. Enterobacterial repetitive intergenic concensus (ERIC) fingerprinting PCR showed genetic diversity of confirmed isolates. Confirmed isolates were evaluated for susceptibilities to eight commonly used antimicrobial agents. Sixty-one (13.9%) samples were positive for *Salmonella*. The distribution of serotypes included; *Salmonella typhi* (7.7%), *Salmonella paratyphi* (2.0%) and *Salmonella typhimurium* (4.1%). *S. typhi* had the highest isolation rate and was most commonly detected in vegetables. Simultaneous resistance to all antibiotics assayed was found amongst the salmonellae. Fingerprinting pattern of the *Salmonella* strains from the different samples showed marked similarities and close genetic relatedness. Cluster analysis at a coefficient of similarity of 0.82 grouped the fifteen strains of *Salmonella* assayed into five different groups. Our results indicate that irrigated vegetables are vehicles of transmission of potentially pathogenic *Salmonella* isolates that can contribute to the development of salmonellosis and other *Salmonella* related infections in Nigeria. This study provides data that support the potential transmission strains of *Salmonella* harboring virulence and resistance factors from vegetables and environmental sources to cause infections in humans.

1. Introduction

The genus *Salmonella* consists of rod shaped, Gram-negative, flagellated, facultative anaerobes, and belongs to the family Enterobacteriaceae (Salehi et al., 2005). The genus consists of two separate species; *Salmonella bongori* and *Salmonella enterica* and encompasses over 2500 known serotypes, all of which are considered potential human pathogen (Baggesen et al., 2000). *Salmonellae* causing human disease are traditionally divided into a small number of human-restricted invasive typhoidal serotypes (*S. enterica* var. Typhi and *S. enterica* var. Paratyphi A) and thousands of non typhoidal *Salmonella* serotypes commonly known as NTS serotypes, which typically have a broad vertebrate host range and cause various presentations that usually include diarrhoeal disease (Gordon et al., 2012).

Salmonella is an important cause of foodborne infections with a broad host spectrum (Wei et al., 2011). It is frequently isolated from environmental sources that serve as relay for the bacteria and play a major role in its spread between different hosts (Liljebjelke et al., 2005). *S. enterica* remains a formidable public health challenge (Cummings et al., 2012) and with a reported increase in its incidence (CDC, 2011). Salmonellosis can result in a number of

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disease symptoms including gastroenteritis, bacteremia, typhoid fever and focal infections (Darwin and Miller, 1999). Certain cases of salmonellosis are severe and often require antimicrobial therapy for treatment, thus, resistance to antimicrobial drugs is a great concern (Marrero-Ortiz et al., 2012). *Salmonella* species are becoming increasingly resistant, making it more difficult to treat patients with severe infections (Rusul et al., 2012). This makes multidrug resistant *Salmonella* an important subject area of research and a major concern for food safety (Rusul et al., 2012). An estimated 94% of *Salmonella* infections are foodborne (Scallan et al., 2011) and consistent contamination with irrigation waters has been shown to be a common route of crop contamination in produce related *Salmonella* outbreaks (Levantesi et al., 2012). Several epidemiological studies lend support to the role contaminated irrigation water and animal manure serve as transmission vehicles of enteric pathogens to fresh produce (Erickson and Doyle, 2012). Typhoid fever (enteric fever) caused by the bacterium *Salmonella* is an endemic tropic and sub tropic disease (Adabara et al., 2012). It accounts for several cases of morbidities and mortalities in Nigeria (Ibekwe et al., 2008) affecting both young children and adults (Akinyemi et al., 2005). In Lagos, Nigeria, out of 85, 187 confirmed cases of *Salmonella* associated diseases, 880 deaths were recorded between 1999 and 2008, giving a case-fatality rate of 1.03% (Akinyemi et al., 2012). The true incidence of *Salmonella*-associated diseases is difficult to evaluate because of lack of epidemiological surveillance systems especially in developing countries like Nigeria (Akinyemi et al., 2012). Many cases are either not documented or many milder cases are not diagnosed or reported (Olowe et al., 2007). The disease is systemic and is often contracted by ingestion of food or water contaminated with the pathogen usually from a feaco-oral source (Adabara et al., 2012). An increasing number of human salmonellosis cases have been linked to ingestion of contaminated irrigated vegetables (Lee et al., 2012). The present rainfall pattern in Nigeria creates prolonged dry season period during cropping season which affects crop development and compel the need for irrigation (Nwauwa and Omonona, 2010). Due to shortage of water, vegetable farmers rely greatly on available water sources for irrigation of produce such as: lettuce, cabbage, carrots, cucumber, tomatoes, green pepper and other vegetables that are commonly eaten raw. Since infection by *Salmonella* is reported to be endemic in Nigeria (Adabara et al., 2012), analysis of *Salmonella* strains genetic diversity is important for epidemiology of the infection.

Molecular subtyping of *Salmonella* isolates is an invaluable epidemiological tool that can be used to track the source of infection and to determine the epidemiological link between isolates from produce and environmental sources (Ait Melloud et al., 2011). The polymerase chain reaction (PCR) provides a new strategy for the detection of *Salmonella* (Rahn et al., 1992). Enterobacterial repetitive intergenic consensus (ERIC) is found in enterobacterial genomes non-coding conserved repetitive sequences of 126 bp long (Wei et al., 2011). ERIC–PCR has reportedly been used for studying bacterial source tracking in the field and analysis of genetic diversity (Wan et al., 2011). Casarez et al. (2007) reported *Escherichia coli* strains diversity from natural water using ERIC–PCR fingerprinting. As one of the main enterobacterial pathogenic bacteria, ERIC sequences exist in *Salmonella* genome (Wei et al., 2011). The invasion locus (*inv*) consists of at least 12 different genes required for adherence and or invasion (Tafida et al., 2013). The *inv*A gene is essential for invasion of epithelial cells by *Salmonella* (Galan and Curtiss, 1991), while, *Salmonella* enterotoxin (*stn*) is a putative virulence factor responsible for enterotoxic activity (Chopra et al., 1999).

This study was aimed at assessing the prevalence, phenotypic, antibiogram and genotypic characteristics of *Salmonella* from vegetable and environmental samples in some major irrigation fields in Nigeria.

2. Materials and methods

2.1. Study area and sampling

Kano is one of the states in Northern Nigeria with marked long dry season periods. Hence, farmers rely on irrigation for crop production. Vegetable farmers are found using irrigation pumps and watering cans to water their crops from hand dug wells, streams and rivers. In Plateau state, vegetable farmers also irrigate their vegetables from available water sources such as mine ponds, rivers and streams. In both states, abattoir, municipal and industrial waste waters are often released into surface water bodies, from where farmers derive their irrigation water. Sprinkler irrigation of vegetables is the most common form of irrigation practiced.

From May 2010 to March 2011, on a weekly basis, vegetables such as cabbage, lettuce, cucumber, tomatoes, green pepper and spinach were purchased from farmers and environmental samples such as irrigation water, manure treated soils (soil amended with cow dung) were obtained from the sites. Samples were collected in the morning. Water samples were collected according to the procedure recommended by American Public Health Association (APHA, 1992). Representative manure treated soil samples were collected aseptically using ethanol-sterilized spatula. About 100 g of sample was collected at designated site at five different points, one from the center and at four different points, on the periphery and mixed together. Samples were transported to the laboratory in sterile plastic bags. Vegetables available in the study sites during the study period were purchased and analyzed. Using 90% ethanol sterilized scissors and forceps, vegetables were cut into factory sterile polythene bags and taken to the laboratory. All samples were transported to the laboratory on ice packs and analyzed within six hours of collection. A total of 440 samples comprising; 238 vegetable samples, and environmental samples made up of 84 irrigation water and 118 manure treated soil samples were screened for *Salmonella*.

2.2. Isolation and identification of Salmonella

Salmonella was isolated from samples using Selenite F Broth enrichment and isolation on *Salmonella–Shigella* agar (Turki et al., 2012). Ten milliliters of each water sample was inoculated in 10 ml of Selenite F Broth and incubated at 37 °C for 24 h. Twenty five grams of cut vegetables was pre-enriched in 225 ml sterile peptone water and incubated at 37 °C for 24 h. One milliliter of the broth was transferred into 9 ml Selenite F Broth for selective enrichment (Okafor et al., 2003). One gram of well mixed manure treated soil was placed in 9 ml of Selenite F Broth and incubated at 37 °C for 24 h. After the enrichment, *Salmonella* was detected by plating the broth on *Salmonella–Shigella* (SS) agar and incubation at 37 °C for 24 h. Typical *Salmonella* colonies which appeared colorless with black centers were picked and confirmed as Gram negative by Gram-staining. Pure cultures of isolates was made on slants of nutrient agar, incubated at 37 °C for 24 h and stored in refrigerator at 4 °C as stock cultures of presumptive *Salmonella* for further tests.

2.3. Biochemical characterization of isolates

Biochemical identification of isolates was as described by Farmer (1999). All isolates that gave reactions typical of *Salmonella* in the tests and substrates were considered to belong to the genus *Salmonella*. Typical *Salmonella* reactions are methyl red positive, citrate positive, nitrate positive, lysine decarboxylase positive, Voges Proskauer negative, indole negative, oxidase negative, motile in motility medium, produces H_2S, ferments glucose, mannitol, dulcitol, and maltose but fail to ferment lactose, sucrose, adonitol and raffinose.

2.3.1. Testing with Microbact (Oxoid, UK) 24E Gram-negative Bacillus (GNB) rapid identification system

This is a miniaturized computer aided identification system for the identification of organisms belonging to the family Enterobacteriaceae. Organism identification is based on pH change and substrate utilization. An overnight culture of presumptive *Salmonella* colonies on selective media was obtained and the test was carried out and interpreted as recommended by the manufacturer.

An 8 digit code was then obtained which was fed into the computer identification software which immediately gave the probable identity of the organism tested in percentage. The Microbact software recommends a 75% cut-off point for a probable identification. All tests that gave less than 75% were not accepted as *Salmonella*.

2.4. Serological identification of Salmonella

Salmonella serotyping was done according to Kauffman–White Scheme (Grimont and Weill, 2007) involving the use of *Salmonella* antisera. Polyvalent *Salmonella* antisera A-G, A-S, surface antigen, phase 1 and phase 2 flagellar H antigens (Denka Seiken, Japan) were used for the serological identification of *Salmonella*. Antigenic suspension of each isolate was made by suspending about 3–5 colonies of pure culture of isolate in 0.5 ml physiological saline. A drop of each polyvalent antiserum and 30 μl of physiological saline was placed on a clean glass slide and used as control. About 10 μl of antigenic suspension was placed on a clean glass slide, serum and physiological saline was on the other end of the glass slide. The reagents were mixed by tilting the glass slide back and forth for one minute and the agglutination pattern was observed. Agglutination was observed with light through the slide. Strong agglutination observed within one minute was recorded as positive while delayed or weak agglutination was regarded as negative. About 0.2 ml antigenic suspension was dispensed in 2 ml physiological saline and heated to about 121 °C for 15 min. The heated solution was centrifuged at 900 × *g* for 20 min. The supernatant was discarded. The precipitate was suspended with 0.2 ml physiological saline and was used as heated cell suspension. A drop of the polyvalent antiserum and 30 μl physiological saline was placed on a cleaned glass slide and used as control. On another glass slide 10 μl of antigenic suspension was mixed with serum and physiological saline by tilting the glass slide back and forth for one minute and agglutination pattern was observed and results recorded.

2.5. Evaluation of susceptibility of the isolates to antimicrobial agents

All the isolates identified as *Salmonella* were tested for their susceptibility to eight (8) commonly used antimicrobial agents with the following disk contents; amoxicillin/clavulanate (30 μg), sulfamethoxazole/trimethoprim (25 μg), ciprofloxacin (5 μg), ceftriaxone (30 μg), gentamicin (10 μg), tetracycline (30 μg), kanamycin (30 μg) and cephalothin (30 μg) by the disk diffusion method described by Khan et al., (2006) and based on recommendations of Clinical and Laboratory Standards Institute guidelines (CLSI, 2010).

Briefly, three colonies of the test organism were inoculated in Mueller–Hinton (Oxoid) broth and incubated at 37 °C until the turbidity approximated 0.5 McFarland's standard. The broth suspension was made to cover the surface of the Petri dish of Mueller–Hinton agar (Oxoid) with the use of a sterile bent glass rod and allowed to dry for five minutes before the paper discs containing the antibiotics were placed on the agar plates with sterile forceps. Plates were incubated at 37 °C for 24 h and the test was carried out in duplicates and mean value recorded. *E. coli* ATCC strain 25922 which is sensitive to all the antibiotics served as quality control. Zones of inhibition were measured to the nearest millimeter and interpreted based on interpretation of zone diameter of test culture provided by (CLSI, 2010). The multiple antibiotic resistance (MAR) index was calculated and interpreted according to Krumperman, (1983).

2.6. Molecular characterization of S. enterica

Representative strains of the *S. enterica* (10 *S. typhi*, 6 *S. tyhphimurium* and 2 *S. paratyphi*) isolates were selected based on the distribution of their abundance in the study and further confirmed through the amplification of the specific *Salmonella* invasive (*inv*A) gene and the *Salmonella* enterotoxin (*stn*) gene fragments.

2.7. Template DNA preparation using extraction kit

A single colony of pure *S. enterica* was inoculated into 10 ml of Luria–Bertani (LB) broth medium and incubated at 37 °C overnight. DNA extraction was carried out using ZR Fungal/Bacterial DNA MiniPrep ZRD6005 (Zymo Research, USA). All protocols were followed and ultra-pure DNA was eluted into 100 μl DNA elution buffer.

2.8. DNA amplification and detection

A total of 18 *S. enterica* isolates, identified and characterized in the study were further confirmed by detecting the presence of the *Salmonella* invasion *inv*A (187 bp) gene according to Nucera et al. (2006). *Salmonella* enterotoxin (*stn*) gene was amplified according to Chopra et al. (1999). Strains were also assayed for antibiotic resistant genes through the amplification of the quinolone resistance-determining region (QRDR) of *gyr*A (448 bp) according to Gomez et al. (2004) and plasmids conferring multidrug resistance *pct* (428 bp) according to Cottell et al. (2011). Amplification was performed using thermal cycler (Bio-Rad Thermal cycler, California, USA). A negative control (reaction tube with nuclease-free water) was included as quality control.

3. DNA sequencing

The presence of genetic determinants (*stn* and *inv*A) was confirmed by amplicon sequence analysis. PCR products were purified using PCR clean-up kits (Qiagen, USA) according to the manufacturer's instructions. Purified PCR products were sequenced by Sanger method (3730xL, Applied Biosystems) at Firstbase, Malaysia. Nucleotide sequences were edited by BIOEdit and compared with the known sequences in Genbank, using BLASTIN of the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/). The sequence data for *Salmonella inv*A gene described in this paper have been submitted to the GenBank data library under accession number KF026356.

3.1. Genotypic determination of diversity among Salmonella enterica isolates using DNA fingerprinting assay

From the characterized *S. enterica* isolates, 15 representative isolates (selected based on ratio of serotypes obtained from samples) were analyzed using enterobacterial repetitive intergenic concensus fingerprints (ERIC–PCR) to determine their genetic relatedness. The PCR products (amplicons) were separated by electrophoresis on a 2% (w/v) agarose gel containing 2 μl florosafe. Electrophoresis was ran at 100 V for 30 min for gene detection while for DNA fingerprinting, electrophoresis was ran at 120 V for 45 min and visualized on an ultra violet (UV) transilluminator gel imaging system (Bio-Rad Gel Imaging system, Bio-Rad California, USA). Bands were photographed and band positions were determined and compared

Table 1
Primer sequences, PCR preparations and conditions used in molecular characterization and genotyping of *Salmonella enterica* obtained in the study.

Target primer sequence	PCR preparation (25 μl)	PCR conditions	Reference(s)
*Inv*A SAL1 5'-GTGAAATTATCGCCACGTTCGGGCAA-3' SAL2 5'-TCATCGCACCGTCAAAGGAACC-3'	12.5 μl Dream Taq mastermix, 8.5 μl nuclease free water, 3 μl template DNA and 0.5 μl of each primer mix.	5 min at 94 °C prior to 35 cycles of 1 min at 94 °C, annealing temperature at 55 °C for 1 min, extension at 72 °C for 1 min and a final extension of 10 min at 72 °C.	Nucera et al., 2006
Stn P15 5'-TTGTGTCGCTATCACTGGCAACC-3' Stn M13- 5'-ATTCGTAACCCGCTCTCGTCC-3'	12.5 μl Dream Taq mastermix, 8.5 μl nuclease free water, 3 μl template DNA and 0.5 μl of each primer mix.	5 min at 94 °C prior to 35 cycles of 1 min at 94 °C, annealing temperature at 60 °C for 90 min, extension at 72 °C for 1 min and a final extension of 10 min at 72 °C.	Chopra et al., 1999
$ERIC_1$ 5'-ATGTAAGCTCCTGGGGATTCAC-3' $ERIC_2$ 5'-AAGTAAGTACTGGGGTGAGCG-3'	12.5 μl Dream Taq mastermix, 8.5 μl nuclease free water, 3 μl template DNA and 0.5 μl of each primer mix.	1 min at 94 °C prior to 1 min at 95 °C, annealing temperature at 52 °C for 1 min, extension at 65 °C for 8 min and a final extension of 16 min at 65 °C.	Bayer et al., 1995
QRDR gyrAF 5'-GCGCGTGAGATGACCCGCCGT-3' gyrAR 5'-CTGGCGGTAGAAGAAGGTCAG-3'	12.5 μl Dream Taq mastermix, 8.5 μl nuclease free water, 3 μl template DNA and 0.5 μl of each primer mix.	5 min at 94 °C prior to 35 cycles of 1 min at 94 °C, annealing temperature at 54 °C for 1 min, extension at 72 °C for 1 min and a final extension of 10 min at 72 °C.	Gomez et al., 2004
pCT(008)F 5'-CATTGTATCTATCTTGTGGG-3' pCT(009)R 5'-GCATTCCAGAAGATGACGTT-3'	12.5 μl Dream Taq mastermix, 8.5 μl nuclease free water, 3 μl template DNA and 0.5 μl of each primer mix.	5 min at 94 °C prior to 35 cycles of 1 min at 94 °C, annealing temperature at 60 °C for 1 min, extension at 72 °C for 1 min and a final extension of 10 min at 72 °C.	Cottell et al., 2011

Key: ***i****nv*A = *Salmonella* invasion gene, *stn* = *Salmonella* enterotoxin gene, ERIC = Enterobacterial repetitive intergenic concensus, QRDR = Quinolone resistance determining region, pCT = Multidrug resistant plasmid.

to molecular weight markers (1 kb and 100 bp DNA ladders, First base, Malaysia). The primers, PCR preparations and conditions used are presented in Table 1.

4. Results

Of the 440 samples screened for *Salmonella*, 61(13.9%) yielded *Salmonella*. Serological classification of these isolates showed 7.7% (34/440) *S. typhi* of which 64.7% (22/34) were from vegetables (Table 2). *S. typhi* strains were most detected from vegetables irrigated with water which had point sources of contamination from abattoir wastewater sites and sewage wastewater from households represented in the study as 'domestic'. Other *Salmonella* serotypes observed were *S. paratyphi* 2.0% (9/440) and *S. typhimurium* 4.1% (18/440). Comparing the *Salmonella* spp. isolation rate during the wet season with that obtained during the dry season, it was observed that, in general, significantly less *Salmonella* spp. was isolated during the wet season (data not shown).

Table 2
Occurrence of *Salmonella* serotypes in the study samples.

Sample type	No. tested	*S. typhi*	*S. paratyphi*	*S. typhimurium*	Total
Vegetables	238	22	3	10	35 (8.0)
Manure treated soil	118	6	1	4	11 (2.5)
Water	84	6	5	4	15 (3.4)
Total	440	34 (7.7%)	9 (2.0%)	18 (4.1%)	61 (13.9%)

Table 3
Multiple antibiotic resistance indexes of *Salmonella* isolates obtained in the study.

No. of antibiotics isolates were resistant to	Antibiograms	Resistant isolates vegetables	From water	Manure treated soil	MARI (%)
1*R*	TE/CN/KF	3	1	1	0.1
2*R*	KF, TE/KF, CRO/AMC, KF/KF, *K*	17	6	1	0.3
3*R*	SXT,TE,KF/KF,TE,CIP/KF,CRO,CIP/KF,TE,K/SXT,KF,K/AMC,KF,*K*/SXT,KF,AMC/AMC,KF,TE	11	6	5	0.4
4*R*	SXT,KF,TE,K/SXT,AMC,KF,TE/KF,TE,CIP,*K*	2	–	3	0.5
5*R*	SXT,KF,CN,TEK;SXT/AMC,KF,TE,K;CN/CIP,KF,TE,K;AMC/CIP,KF,TE,K,CRO/CN,KF,TE,K,AMC/SXT,CN,KF,TE,AMC	7	3	1	0.6
6*R*	CRO,CN,CIP,KF,TE,*K*	–	–	1	0.8
8*R*	AMC,SXT,CRO,CN,CIP,KF,TE,*K*	–	–	1	1.0

Key: *R* = Resistance, KF = Cephalothin (30 μg), SXT = Sulfamethoxazole (25 μg), AMC = Amoxicillin/Clavulanic acid (10 μg), Gentamicin = CN (10 μg), CIP = Ciprofloxacin (5 μg), Ceftriaxone (30 μg), TET = Tetracycline(30 μg) and *K* = Kanamycin (30 μg).
MARI was calculated using the formula: *a*/*b* where '*a*' represents the number of antibiotics to which a particular isolate was resistant and '*b*' the total number of antibiotics tested.

Table 4
Distribution, phenotypic resistance and virulence of *Salmonella* serotypes.

Salmonella serotypes	Isolate code	Source	Site of origin/point source of contamination	Isolate resistance pattern								Virulence Genes	
				KF	SXT	AMC	K	CIP	CN	TE	CRO	*inv*A	*stn*
Typhi	KVB1	Vegetables	Abattoir	R	R	S	S	S	S	R	S	+	+
Typhi	KVB20	Vegetables	Abattoir	R	R	S	S	S	R	S	S	+	+
Typhi	KVB26	Vegetables	Abattoir	R	R	S	R	S	S	R	S	+	+
Typi	KVB27	Vegetables	Domestic	R	R	R	S	S	S	R	S	+	+
Typhi	JVB1	Vegetables	Abattoir	R	S	S	R	R	S	R	S	+	+
Typhi	JVB2	Vegetables	Abattoir	R	R	S	R	S	R	R	S	+	+
Typhi	JVB3	Vegetables	Domestic	R	R	R	R	S	S	R	S	+	+
Typhi	JVB27	Vegetables	Domestic	R	S	S	R	R	R	R	S	+	+
Typhi	JVB33	Vegetables	Industrial	R	S	R	R	R	S	R	S	+	+
Typhi	KWB 23	Irrigationwater	Abattoir	R	S	R	S	S	S	R	S	+	+
Typhimurium	KSB 16	Soil	Abattoir	R	S	S	R	R	S	R	S	+	+
Typhimurium	JSB 33	Soil	Domestic	R	S	S	R	R	R	R	R	+	+
Typhimurium	JWB 27	Irrigationwater	Abattoir	R	R	R	R	S	S	R	S	+	+
Paratyphi	JSB65	Soil	Industrial	R	R	R	R	R	R	R	R	+	+
Paratyphi	JWB4	Irrigationwater	Domestic	R	R	R	S	S	R	R	S	+	+

Key: K = Kano, J = Plateau,V = vegetables, B = *Salmonella*, R = resistant, S = susceptible, KF = cephalothin (30μg), SXT sulfamethoxazole (25μg), AMC = Amoxicillin/Clavulanic acid (10μg), Gentamicin = CN (10μg), CIP = Ciprofloxacin (5μg), Ceftriaxone (30μg), TET = Tetracycline (30μg) and K = Kanamycin (30μg), *inv*A = invasive A gene, *stn* = *Salmonella* enterotoxin gene, + = detected.
(Abattoir, domestic and industrial = wastewater sources used for irrigating vegetables in the study area and considered point sources of contamination to sites in the study apart from manure).

Antibiogram of identified *Salmonella* isolates to eight commonly used antibiotics revealed seven resistance patterns. All isolates exhibited multidrug resistance, each isolate being resistant to 3 or more antibiotics (Table 3), and with multidrug antibiotic resistance index (MARI) of 0.1–1.0. Resistance to Kanamycin was the most common resistance phenotype observed among all isolates. One isolate JSb65 from industrial waste contaminated site exhibited complete resistance to all eight antibiotics tested (Table 4). A 187 bp DNA fragment amplified for the *inv*A gene and a 284 bp amplified for the *stn* gene in all strains tested further confirmed the presence of *Salmonella*. Table 5 shows the diversity and relatedness of strains from different sources as grouped in clusters and singletons on the dendrogram. To further characterize isolates for the genetic basis of resistance, fragments of QRDR and MRDP were amplified in all isolates by PCR (Table 6).

4.1. *Genetic diversity of* S. enterica *isolates*

ERIC–PCR produced fingerprints of the fifteen representative *S. enterica* strains obtained in the study from vegetable and environmental samples. Fingerprints showed 1–12 bands with amplicons having molecular weights of 150 bp–1 kb, and a common intensive band of about 9000 bp in most of the strains. Cluster analysis at a coefficient of similarity of 0.82 grouped the fifteen strains of *S. enterica* into five different groups, designated 1–5; two clusters and three singletons (Fig. 1). Cluster 2 comprises isolates obtained from 10 samples including vegetables, irrigation water and soil with a similarity coefficient of 0.84, cluster 3 comprise strains from two different vegetable samples with a similarity coefficient of 0.85, while singletons 1, 4 and 5 had an isolate each. Some isolates grouped based on their respective serotypes and sources from which they were isolated with some exceptions. Cluster 2 is made up of strains of *S. typhi* from vegetables (KVb27), (KSb16) from manure treated soil and (KWb23) from irrigation water. Strains of *S. typhimurium* JSb65 from manure treated soil and JVb27 also clustered together. Strains of *S. paratyphi* from environmental samples also grouped together JSb33 and JWb4. Cluster 3 shows 2 strains of *typhi* obtained from two different vegetable samples. The three remaining isolates had quite divergent profiles and formed singletons.

Table 5
Diversity and relatedness of strains as shown by dendrogram.

Clusters	*Salmonella* serotypes	Isolate code	Source
Singleton 1	Typhi	KVB1	Vegetables
Cluster 2	Typhi	KVB27	Vegetables
Typhi	KSB16		M/soil
Typhi	JVB3		Vegetables
Typhi	JVB1		Vegetables
Typhi	JSB65		M/soil
Typhi	JWB27		Irrigationwater
Typhi	JVB2		M/soil
Typhi			Vegetables
Typhi	KWB23		Irrigationwater
Typhimurium			Irrigationwater
Cluster 3	Typhimurium	JVB27	Vegetables
Typhimurium	JVB33		Vegetables
Singleton 4	Paratyphi	KVB26	Vegetables
Singleton 5	Paratyphi	KVB20	Vegetables

Table 6
Genotypic and multiple drug resistant genes in *Salmonella* strains from samples.

Salmonella serotypes	Isolate code	Source	*inv*A	*stn*	QRDR	MDRP
Typhi	KVB1	Vegetables	+	+	+	+
Typhi	KVB27	Vegetables	+	+	+	+
Typhi	KSB16	M/soil	+	+	+	+
Typhi	JVB3	Vegetables	+	+	+	+
Typhi	JVB1	Vegetables	+	+	+	+
Typhi	JSB65	M/soil	+	+	+	+
Typhi	JWB27	Irrigationwater	+	+	+	+
Typhi	JSB33	M/soil	+	+	+	+
Typhi	JVB2	vegetables	+	+	+	+
Typhi	JWB4	Irrigationwater	+	+	+	+
Typhimurium	KWB23	Irrigationwater	+	+	+	+
Typhimurium	JVB27	Vegetables	+	+	+	+
Typhimurium	JVB33	Vegetables	+	+	+	+
Paratyphi	KVB26	Vegetables	+	+	+	+
Paratyphi	KVB20	Vegetables	+	+	+	+

Key: K = Kano, J = Plateau, V = Vegetables, M/Soil = manure treated soil, W = Irrigation water, *inv*A = Invasive A gene, *stn* = *Salmonella* enterotoxin gene, QRDR = Quinolone resistance determining region, MDRP = Multidrug resistance plasmid, + = Detected.

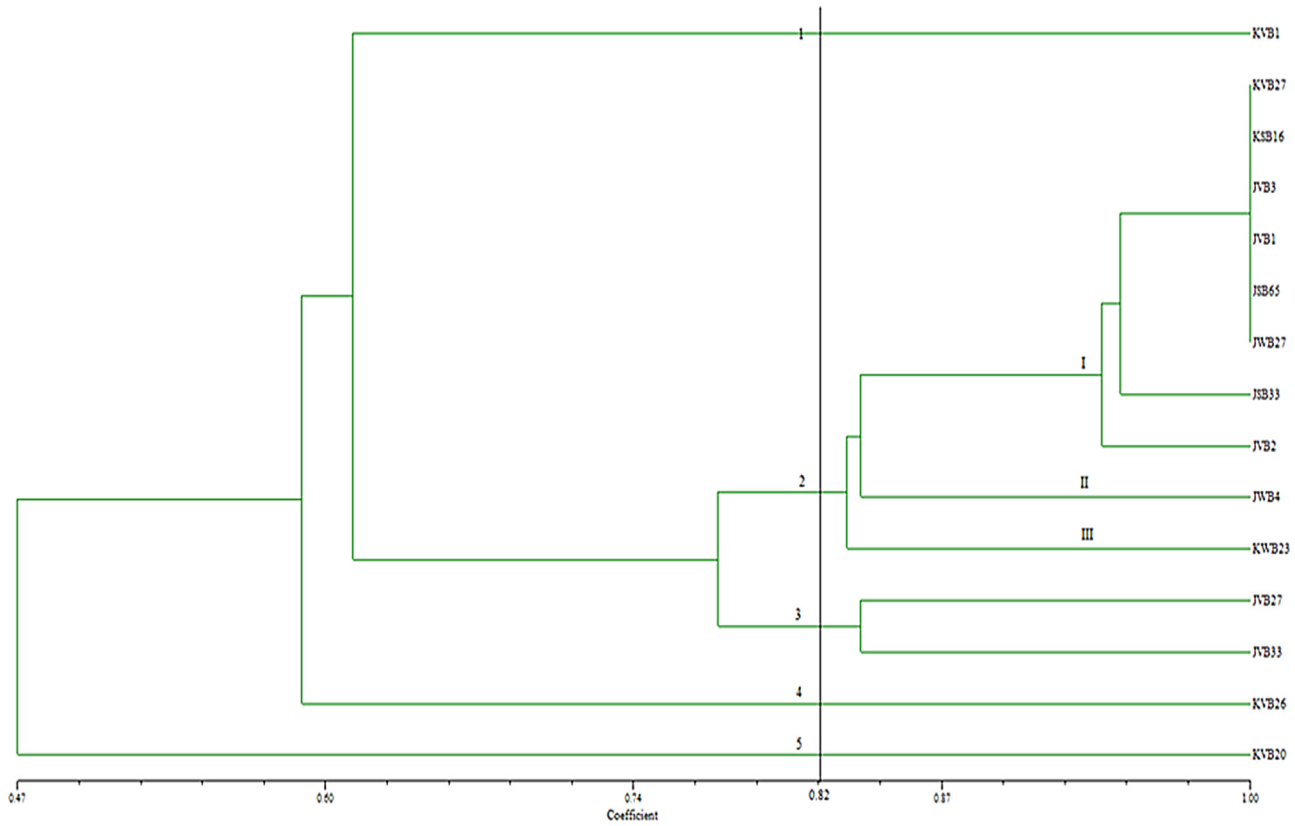

Fig. 1. Dendrogram showing genetic relatedness among strains of *Salmonella enterica* obtained in the study.

5. Discussion

Our study revealed the presence of *Salmonella* in vegetables and environmental samples analysed with an overall isolation rate of 13.9%. This finding, in itself, is not surprising since *Salmonella* is reported to be an environmentally persistent pathogen capable of surviving and proliferating in diverse environments (Winfield and Groisman, 2003). Vegetable farmers in the study locations depend largely on contaminated wastewater sources to irrigate their produce, while also using untreated animal manure as sources of nutrient supply to vegetables. Moreover, previous studies report that *Salmonella* can persist in the farm environment for extended periods of time due to movement within the farm from animals, human and livestock excrement, soil and plants (Kupriyanov et al., 2010). The 13.9% prevalence of *Salmonella* obtained in our study is however, lower than the 40% isolation rate by Ndiaye et al. (2011) from irrigation water used for vegetable production in Dakar, Senegal.

The distribution of serotypes of *Salmonella* in the studied samples comprised *S. typhi* 7.7% (34/440), *S. paratyphi* 2.0% (9/440) and *S.typhimurium* 4.1% (18/440). *S.typhi* had the highest prevalence rate of 7.7%. This result is quite worrisome as *S. typhi* is strictly a human pathogen that causes invasive fever (typhoid fever), whereas most other *Salmonella* serotypes cause mainly gastrointestinal symptoms without systemic invasion (Braden, 2006). Its high prevalence could be attributable to poor quality of wastewater used in irrigating these crops as *S.typhi* has been reported to be frequently isolated from sewers and feacally contaminated waters (Famurewa and Moro, 1989; Uzeh and Agbonlahor, 2001). Wastewater from human sewage systems also served as point source of contamination. The presence of *S.typhi* in our study is not unrelated to this human source of contamination.

Vegetables are increasingly recognized as sources of *Salmonella* outbreaks (Jacobsen and Bech, 2012). Interestingly, *S.typhi* and *S. typhimurium* which are etiologic agents of typhoid and enteric fever were remarkably higher in vegetable samples in this study. The detection of *S. typhi* from vegetables is an indication of feacal contamination and could be traced to sewage discharge into wastewater used in irrigation of vegetables. Bagudo et al. (2014) observed that increased contamination of natural water has intensified the detection, frequency and persistence of pathogenic microorganisms in areas affected by sewage discharge. *S. typhimurium* has long been reported as a common cause of foodborne gastroenteritis (Hur et al., 2012). Its detection in vegetables in the study is not unconnected with the use of untreated animal manure as a source of nutrient replenishment in the soil by farmers. Farmers use untreated manure from cattle in the study area and Wedel et al. (2005) reported that cattle are a major reservoir of *Salmonella*. In a related study, Nafaranda et al. (2006) observed a 13.2% *S. typhimurium* from vegetables irrigated with wastewater from Gwagwalada, Nigeria. Previously, Islam et al. (2004) also reported contaminated vegetables harvested from manure amended soils elsewhere. The risk presented by *S. typhimurium* as potential farm pathogen is further heightened by the increase in organic production in the country (Mustapha et al., 2012), where farmers use untreated animal manure in produce production.

Several studies have shown that *Salmonella* exhibit multidrug resistant patterns (Adley et al., 2011). Multiple drug resistance was observed in all isolates of *Salmonella* tested in this study. The emergence of *Salmonella* isolates with high multiple antibiotic resistance suggests that these isolates must have originated from environments where antibiotics are misused or often used as therapeutic measures in humans and growth promoters in livestock (Singh et al., 2010). The detection of *S. typhi is* an indication

of contamination of sewage of human origin, which was most detected in the study was also observed to be most resistant and hence implies human use/misuse of antibiotics. Although, it is possible that isolates may have acquired the genes for resistance to multiple antibiotics from other enteric bacteria. One isolate from manure treated soil was particularly observed to be resistant to all eight antibiotics tested. This represents a great public health issue as certain cases of salmonellosis are severe and often require antimicrobial therapy for treatment (Marrero-Ortiz et al., 2012). Hence, these multidrug resistant *Salmonella* strains obtained from vegetables commonly eaten raw is a major concern for food safety (Rusul et al., 2012). Studies from other parts of the world, where antibiotics are commonly employed, have revealed that routine use of these drugs is often associated with a rapid increase in the proportion of resistant strains (Weiss et al., 2002). The concern is heightened by reports that multiple drug resistant *Salmonella* isolates have been suggested to be more virulent than non-multiple drug resistant *Salmonella* isolates (Foley and Lynne, 2007). The detection of these resistant *Salmonella* strains in this study calls for attention. Our findings indicate that these isolates have the potential to develop resistance for routinely prescribed antimicrobial drugs and pose considerable health hazards to consumers, hence the need for institution of prudent control measures. We cannot however, affirm that the antimicrobial resistance genes must have been traced from a definitive source due to small number of isolates tested.

The invasion gene *inv*A, essential for full virulence in *Salmonella* (Mezal et al., 2013) was detected in all isolates studied. Other researchers also amplified this gene from *Salmonella* isolates (Nashwa et al., 2009). This further confirms our isolates as potentially pathogenic *Salmonella* strains as amplification of *inv*A gene is now recognized as an international standard procedure for the detection of *Salmonella* genus (Amini et al., 2010). The presence of both genes in the *Salmonella* strains assayed in this study is in agreement with the report of Shanmugasamy et al. (2011). The present study supports the ability of these specific primer sets to confirm the isolates as *Salmonella* and indicated that *inv*A and *stn* genes are widely distributed among *Salmonella* strains irrespective of their serovars and source of isolation (Salehi et al., 2005). The detection of the *stn* and *inv*A genes confirms that both genes contain sequences unique to *Salmonella* strains, hence, both genes are suitable PCR target for detection of *Salmonella* strains from varied sources. It has been reported that *Salmonella* strains contain both antimicrobial resistance and virulence genes as factors such as colonization and survival in the host may select for resistance (Marrero-Ortiz et al., 2012). Notably, in our study both virulence and resistance genes were detected in 100% of *Salmonella* strains assayed.

Fingerprinting patterns of *S. enterica* strains from the different samples also showed marked similarity and close genetic relatedness as well as diversity. The largest cluster, cluster 2 had isolates from vegetables, manure treated soil and irrigation water sources at a similarity coefficient above 0.82, hence highly genetically related. Consistent contamination with irrigation waters has been shown to be routes of crop contamination in produce related *Salmonella* outbreaks (Levantesi et al., 2012). This has been shown in the marked genetic similarity observed in our study. Previous studies also reported genetic similarities among *Salmonella* isolates obtained from food, environmental and clinical samples (Mezal et al., 2013). The continuous use of untreated wastewater in vegetable production in Nigeria therefore, calls for concern, as *Salmonella* is one of the leading causes of intestinal illness all over the world as well as the etiologic agent of more severe systemic diseases such as typhoid and paratyphoid fevers (Levantesi et al., 2012). All isolates in cluster two were resistant to more than one antimicrobial agent and generally isolates showed the presence of both virulence and resistance genes. The same phenomenon was noted by Marrero-Ortiz et al., 2012 who found that *Salmonella* isolates displaying multidrug resistance often clustered into distinct clusters. A number of likely factors must have led to the development of antimicrobial resistance and virulence among these *Salmonella* isolates. In this study, all isolates were positive for multidrug resistance plasmids (pCT) regardless of serovar. In a number of cases, including this study, it has been demonstrated that these MDR plasmids are able to horizontally be transferred from donor strains to recipients (Kaldhone et al., 2008), which could lead to potential treatment difficulties (Marrero-Ortiz et al., 2012). It is noteworthy that isolates from different sources clustered in a group, hence indication of possible genetic transfer among isolates.

The similarities in genotypic profiles among *Salmonella* strains from vegetables and environmental samples in our study as indicated by clusters and as previously reported elsewhere from clinical, food and environmental samples (Thong et al., 2002), are consistent with the observation that these vegetables and environmental isolates have the potential to cause human infection.

6. Conclusion

In the present study, we ascertained the presence of *Salmonella* strains in vegetables and environmental samples from irrigation sites in Nigeria, where farmers irrigate vegetables commonly eaten raw with untreated wastewater, while also using untreated animal manure in produce production. Consumption of these vegetables without proper disinfection and appropriate heating represents a serious public health risk. To our knowledge this is the first study that has genotyped *Salmonella* from these sources in this region and deposited *Salmonella* sequence in thegene bank. A better understanding of the factors that potentially contribute to the development and dissemination of resistant zoonotic pathogens can improve the tools used by public health specialists to control antimicrobial resistance, which can limit potential treatment options in severe cases of salmonellosis.

Recommendations

With the increased awareness of the health benefits of consumption of raw vegetables, the lack of access to quality water by vegetable farmers and the increase in organic production in Nigeria, we advocate strict compliance with treatment of wastewater and composting manure before use. In view of the serious implications arising from these situations, the chain of transmission of this pathogen and its mechanism of resistance should be carefully studied and monitored to reduce the spread and threat to human health. The indiscriminate use of antibiotics by man and in animal production poses a great threat to disease management and should be seriously checked and discouraged.

Limitations of study

A limited number of representative isolates were used for the molecular aspect of this study because it was undertaken outside the country of study at Universiti Putra Malaysia, which entailed restricted research isolate size.

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