



RESEARCH PAPER

Molecular Characterization of *Salmonella* Typhi Strains Isolated from Patients Clinically Diagnosed of Typhoid Fever in Kaduna State-Nigeria

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ABSTRACT

Typhoid fever; a systemic disease caused by *Salmonella enterica* serovar Typhi (*S. Typhi*) remains a major public health problem in many parts of the world. Accurate diagnosis of the disease requires isolation of the organism. Therefore, the main objective of this study was to molecularly characterized *S. Typhi* strains isolated from patients clinically diagnosed of typhoid fever in selected hospitals within Kaduna State-Nigeria. Stool and blood samples were collected and subjected to culture test. *Salmonella* positive cultures were subjected to PCR amplification of *invA*, *tyv*, *fliC-d* genes. Virulence antigen expressing *S. Typhi* was detected by PCR amplification of *viaB* gene. *Salmonella* Typhi strains were also determined by sequencing *invA* amplicons. Genetic relatedness of the strains were determined by phylogenetic analysis. Out of the 420 patients recruited, 22(5.2%) and 01(0.2%) had their stool and blood positive for *Salmonella* infection. Out of the 22 *Salmonella* stool culture positive, 15(68.2%) were true positive while 7(31.8%) were false positive. The only blood culture positive was confirmed to be real positive. The prevalence rate of *Salmonella* infection among the 420 patients was 3.8%. The prevalence rate of *S. Typhi* among the 420 patients was 1.2%. Eighty percent of the isolated *S. Typhi* expressed virulence antigen (Vi) while 20% lack Vi. Hundred percent of the isolated *S. Typhi* were *S. Typhi* str. CT18. The phylogenetic analysis indicated that the isolated *S. Typhi* CT18 strains were more related to each other than Typhi str.Ty2 which was more than them. Phylogenetic analysis also indicated that the isolated *S. Typhi* str. CT18 emerge from Typhi str.Ty2 due to clonal expansion over time. The use of molecular method in diagnosing typhoid fever is more accurate than culture methods. The circulating strain in the study areas was *S. Typhi* str. CT18 which are of high virulence.

Keywords: *Salmonella* Typhi, Virulence, Antigen, Sequencing, CT18 Strain

INTRODUCTION

Typhoid fever, also known as enteric fever or simply as typhoid, is a disease caused by the gram negative bacterium; *Salmonella* Typhi (Wain *et al.*, 2015). In 2000, it was reported that about 21.7 million cases of typhoid fever and 217,000 related deaths occurred worldwide as a result of typhoid fever (Crump and Mintz, 2010). In 2013, it resulted in about 161,000 deaths (GBD, 2014). About 17.8 million cases of typhoid fever occur each year in lower-income and middle-income countries (LMICs). Incidence typically peaked in the 2–4 year old age group (Marina *et al.*, 2017). *S. Typhi* is generally transmitted in food and water contaminated with faeces from those excreting the bacteria either during the acute illness or during chronic asymptomatic carriage (Bhan *et al.*, 2005). Enteric fever is most common in resource-poor settings where

the provision of clean drinking water and sewage disposal facilities are inadequate (Bhan *et al.*, 2005). The clinical presentations of typhoid fever are diarrhea, abdominal discomfort, constipation, headache and fever (Clark *et al.*, 2010). If the disease is not treated, it can lead to complications such as intestinal perforation or neurological problems (Azmatullah *et al.*, 2015). Widely used methods for the diagnosing typhoid fever include bacterial culture, microscopy and serological assays, specifically the Widal test (Parry *et al.*, 2011). Molecular diagnostics of enteric fever, in particular nucleic acid amplification by polymerase chain reaction (PCR), have been growing rapidly in the last

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decade. The targets for *S. Typhi* in PCR-based assays include the *S. Typhi* flagellin (*fliC-d*) gene (Nath *et al.*, 2010), the *Salmonella* invasion (*invA*) gene (Cocolin *et al.*, 1998), the tyvelose epimerase (*tyv*; previously *rfbE*) gene and the paratose synthase (*prt*; previously *rfbS*) gene (Ali *et al.*, 2009).

S. Typhi expresses a capsular polysaccharide antigen known as virulence antigen (Vi). Studies have shown that Vi is antiopsonic and antiphagocytic (Looney and Steigbigel 1986), reduces the level of secretion of *Salmonella* Typhi induced tumor necrosis factor alpha (a marker of activation) by human macrophages (Hirose *et al.*, 1997), and increases the level of resistance of the organism to oxidative killing (Looney and Steigbigel 1986). The Vi capsular antigen is reported as a significant virulence factor for typhoid fever, as strains positive for Vi production have higher rates of infection (Hone *et al.*, 1988), and it continues to be the focus for improvement in current treatment and prophylaxis for this disease. *viaB* gene was found to be encoding for the Vi antigen of *S. Typhi* (Hashimoto *et al.*, 1995).

The main aim of this study was to molecularly characterized *S. Typhi* isolated from patients clinically diagnosed of typhoid fever in selected hospitals within Kaduna State-Nigeria, and determines the strains in circulation.

METHODOLOGY

Sampling Sites

Six Hospitals were selected from three Senatorial Zone in Kaduna States of Nigeria. These Hospitals were: Ahmadu Bello University Medical Centre, Samaru, Zaria; ST. Luke's Anglican Hospital Wusasa, Zaria; General Hospital, Kawo, Kaduna; Yusuf Dantsoho Specialist Hospital, Kaduna; General Hospital, Kwai and General Hospital Kafanchan.

Ethical Consideration and Patients' Consents

Approvals for the study were obtained from Kaduna State Ministry of Health, Nigeria. Patients' consents were obtained prior to collection of samples.

Inclusion and Exclusion Criteria

The inclusion criteria were consented outpatients clinically diagnosed by clinicians

of typhoid fever. The exclusion criteria include non consented patients and anyone that does not satisfy the inclusion criteria.

Sample Size and Demographic Data

Four hundred and twenty each of blood and stool samples were collected. This amounted to 840. Seventy of each blood and stool samples were collected from each hospital. Demographic data of consented outpatients such as age and sex were recorded during the collection of specimens.

Cultural Tests

Five milliliters of blood and 10-30g of stool samples were collected from each consented patient that was clinically diagnosed of typhoid fever. The samples were transported to Department of Microbiology Laboratory, Ahmadu Bello University, Zaria-Nigeria. Substantial amount of stool and 3ml of blood collected were subjected to enrichment by inoculating them into 9ml of Selenite-F Broth and Tryptic Soy Broth contained in test tubes respectively. These were incubated at 37°C for 18hrs. A loopful of the culture from Tryptic Soy Broth and Selinite-F Broth were streaked on *Salmonella-Shigella* Agar (SSA) separately and incubated overnight at 37°C. The plates of colourless colonies with or without black centers were suspected to be *Salmonella*. The suspected culture were subjected to biochemical confirmation. Five biochemical testes were employed. These were: urease production, sugar fermentation (triple sugar iron), indole production, methyl red and voges-proskauer tests.

DNA Extraction

DNA of the *Salmonella* positive cultures were extracted using lysis buffer, proteinase, phenol-chloroform, ethanol and sodium acetate through various stages of vortexing, incubation, centrifugation and decantation. The resulting pellets (DNAs) were used for PCR amplification.

Multiplex PCR Amplification of *invA*, *tyv* and *fliC-d* Genes

A set of primer pair *invA* was used to confirm *Salmonella* species. Primers for detecting *tyv* and *fliC-d* genes which encoded for O-serogrouping 9 and flagella (Hd) respectively

were used for determining *Salmonella* Typhi as they were specific for Typhi (Bee and Kwai 2009). The sequences of the primers used in the multiplex PCR detecting and typing of *Salmonella* Typhi and their expected amplicon sizes are represented in table 1. Five microlitre of each F and R primers of *invA* were added together in a tube and 40µl of sterile water was added. *tyv* and *fliC-d* primers were also prepared as in *invA*. One microlitre of each of the mixture was then transferred to mPCR tube which contained mPCR premix (premix contain all the PCR reagents). Two microlitre of extracted DNA was added to the tube. It was then spine to mix them together. After the spinning, the tubes were then put in thermal cycler (PCR Machine) for genes amplification. The Machine was then set at the following PCR conditions: Pre-denaturation = 95°C for 5mins; Denaturation = 95°C for 30s; Cycles = 35; Annealing = 55°C for 30s; Extension = 72°C for 30s and Final Extension = 72°C for 5mins.

PCR Amplification of *viaB*

PCR was used in amplifying *viaB* gene which encoded for Vi (Virulence antigen) in *Salmonella* Typhi. A set of primer pair of *viaB*, their sequences and amplicon size is represented in table 1. Five microlitre of each F and R primers of *viaB* were added together in a tube and 40µl of sterile water was added. One microlitre each of the mixture was then transferred to each PCR tube which contained monoplex PCR premix. Two microlitre of extracted DNA of confirmed *Salmonella* Typhi was added to each of the tube. The tubes were then spine to mix the mixture together. After the spinning, the tubes were then put in thermal cycler (PCR Machine) for gene amplification. The Machine was then set at the following PCR conditions: Pre-denaturation = 95°C for 5mins; Denaturation = 95°C for 40s; Cycles = 35; Annealing = 50°C for 30s; Extension = 68°C for 30s and Final Extension = 68°C for 7mins.

Table 1: Primers used in Confirming *Salmonella* species, Determining *Salmonella* Typhi and the Virulence Antigen

Target Antigens	Genes	Sequence (5' to 3')	Amplicon Size (bp)
Invasive <i>Salmonella</i> Antigen O Group 9	R- <i>invA</i>	GTGAAATTATCGCCACGTTTCGGGCAA	285
	F- <i>invA</i>	TCATCGCACCGTCAAAGGAACC	
	F- <i>tyv</i>	GAGGAAGGGAAATGAAGCTTTT	614
	R- <i>tyv</i>	TAGCAAACGTGTCTCCCACCATAC	
Flagella (Hd)	F- <i>fliC-d</i>	ACTCAGGTTCCCGTAACGC	763
	R- <i>fliC-d</i>	GGCTAGTATTGTCCTTATCGG	
Virulence Antigen (Vi)	F- <i>viaB</i>	GTTATTCAGCATAAGGAG	439
	R- <i>viaB</i>	CTCCATACCACTTTCCG	

(Bee and Kwai 2009)

Electrophoresis of PCR Products

After the PCR amplification in thermal cycler, the PCR tubes were removed and 8µl of the each of the PCR products were transferred into well of agarose gel of 1.5% Ethidium Bromide where they were resolved by electrophoresis for 20mins. One hundred bp molecular weight marker was used. After the electrophoresis, the amplicons were then viewed under UV light. The pictures of the amplicons were then taken.

Purification of *Salmonella* Typhi *invA* Gene

The *invA* genes of the isolated S. Typhi were amplified using PCR procedure described above. The resulting amplicon sizes of 285bp were then proceed for purification and extraction of DNA for sequencing. The fragment produced by the *Salmonella* Typhi *invA* primers was isolated on agarose. TBE gel fragment was isolated with a purification kit. The PCR purification spin protocol for *Salmonella* spp. fragment was utilized at DNA LABs laboratory, Kaduna-Nigeria by using

QIAquick PCR Purification Kit protocol. This protocol was designed to purify single-or double- stranded DNA fragments from PCR and other enzymatic reaction. The purified DNA was stored at -20°C for sequencing.

Sequencing of *invA* Gene of *S. Typhi*

The concentration of *invA* fragment was measured by using NanoDrop ND-1000 UV spectrophotometer. The result for this measurement was 21.82ng/1. The fragment was diluted to required concentration for sequencing in a Sanger Sequencing Machine. About 10-12µl of the DNA was prepared at a concentration of 1.00ng/µl, transferred into sterile small ependorf and placed in Sanger Sequencing machine and sequence.

invA Nucleotide Sequence Analysis

The resulting nucleotide sequence of the *invA* gene of *S. Typhi* was compare with the ones deposited at GenBank by using BLAST (Basic Local Alignment Search Tool) 2.0 search programs, National Center for Biotechnology Information (NCBI) and the strains determined.

Phylogenetic Analysis

Alignment of the nucleotide bases of the isolated *S. Typhi* str. CT18 with that of other 3 *S. Typhi* strains and *S. Typhimurium* str. LT2 (Table 2) available at GenBank, NCBI, was done using EMBL-EBI Multiple sequence alignment. The resulting alignment was used to construct Phylogenetic tree using Clustal Omega software. The strains' relatedness and relationship were determined.

Table 3.3: The *Salmonella* Strains used in Phylogenetic Comparison

Strain Name	ACC. NO
<i>S. Typhimurium</i> LT2	NC003197.2
<i>S. Typhi</i> Ty2	AE014613.1
<i>S. Typhi</i> p-stx-12	CP003278.1
<i>S. Typhi</i> PM016/13	CP012091.1

Key: ACC.NO: Accession Number.

RESULTS AND DISCUSSION

Table 2.1: Number and Percentages of Culture Positive for *Salmonella* Species

Diagnostic Tests	N	Positive	Negative
Stool Culture	420	22(5.2)	398(94.8)
Blood Culture	420	01(0.2)	419(99.8)
Total	840	23(2.7)	817(97.3)

Key: Figures in parenthesis indicates percentages; N = Number of samples collected

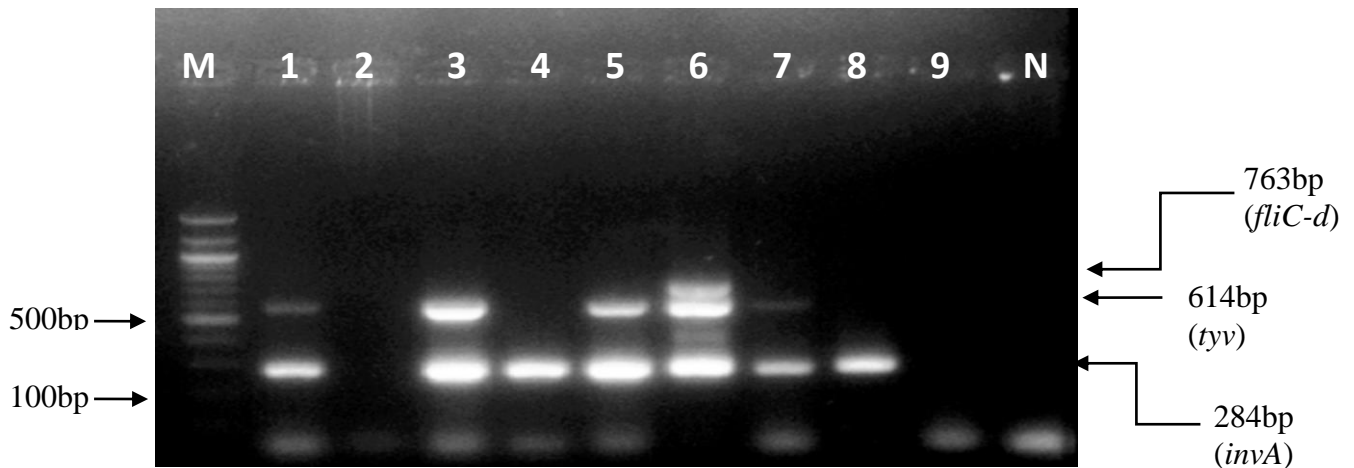


Plate I: Electrophoresis Gel Picture of mPCR Amplified *invA*, *tyv* and *fliC-d* Genes of Isolates from ABUMC and STL.

Key: Lane M: Molecular Marker (100bp); Lane 1, 3, 5 and 7: Amplicons of *invA* and *tyv* Genes; Lane 4 and 8: Amplicons of *invA* gene; Lane 6: Amplicons of *invA*, *tyv* and *fliC-d* Genes; Lane 2 and 9: No Gene Amplified; Lane N: Negative Control

ABUMC: Ahmadu Bello University Medical Centre, Zaria-Nigeria STL: ST. Luke's Anglican Hospital, Wusasa, Zaria-Nigeria



Plate II: Electrophoresis Gel Picture of mPCR Amplified *invA*, *tyv* and *fliC-d* Genes of Isolates from GK and YD.

Key: Lane M: Molecular Marker (100bp); Lane 10, 11, 12, 13 and 16: No Gene Amplified; Lane 14 and 15: Amplicons of *invA*, *tyv* and *fliC-d* Genes; Lane N: Negative Control, GK: General Hospital. Kawo. Kaduna-Nigeria YD: Yusuf Dantsoho Memorial Hospital. Kaduna-Nigeria

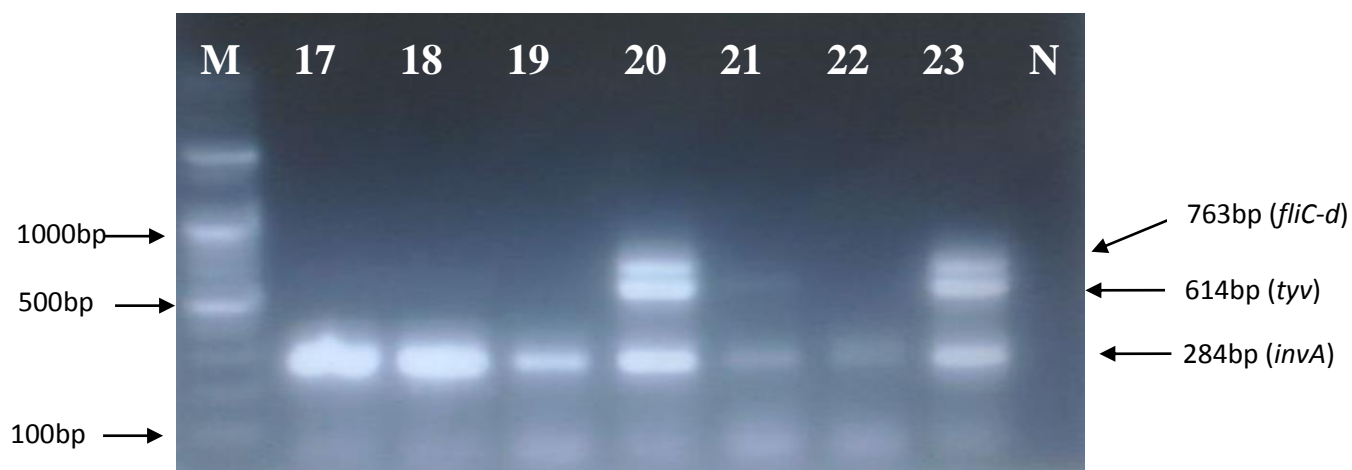


Plate III: Electrophoresis Gel Picture of mPCR Amplified *invA*, *tyv* and *fliC-d* Genes of Isolates from PK and KW.

Key: Lane M: Molecular Marker (100bp); Lane 17, 18, 19, 22: Amplicons of *invA* Gene; Lane 21: Amplicons of *invA* and *tyv* Genes; Lane 20 and 23: Amplicons of *invA*, *tyv* and *fliC-d* Genes; Lane N: Negative Control PK: Sir Partrick Yakowa Memorial Hospital, Kafanchan-Nigeria; KW: General Hospital, Kwoi, Kaduna-Nigeria

Table 2.2: Detection of *Salmonella* Typhi from Culture Positive *Salmonella* Species

Lane No	ID	<i>invA</i>	<i>tyv</i>	<i>fliC-d</i>	Inference
1	SSB3	+	+	-	<i>Salmonella</i> Species
2	SSB19	-	-	-	Other Species
3	SSB43	+	+	-	<i>Salmonella</i> Species
4	SSB66	+	-	-	<i>Salmonella</i> Species
5	SST7	+	+	-	<i>Salmonella</i> Species
6	SST10	+	+	+	<i>Salmonella</i> Typhi
7	SST31	+	+	-	<i>Salmonella</i> Species
8	SST51	+	-	-	<i>Salmonella</i> Species
9	SST56	-	-	-	Other Species
10	SGK2	-	-	-	Other Species
11	SGK11	-	-	-	Other Species
12	SGK33	-	-	-	Other Species
13	SGK41	-	-	-	Other Species
14	SYD15	+	+	+	<i>Salmonella</i> Typhi
15	SYD21	+	+	+	<i>Salmonella</i> Typhi
16	SYD66	-	-	-	Other Species
17	SPK6	+	-	-	<i>Salmonella</i> Species
18	SPK20	+	-	-	<i>Salmonella</i> Species
19	SPK25	+	-	-	<i>Salmonella</i> Species
20	SPK36	+	+	+	<i>Salmonella</i> Typhi
21	SPK42	+	+	-	<i>Salmonella</i> Species
22	SPK46	+	-	-	<i>Salmonella</i> Species
23	BKW57	+	+	+	<i>Salmonella</i> Typhi

Table 2.3: Number and Percentages of True and False *Salmonella* Positive Culture Tests

Diagnostic Tests	Culture Positive	PCR +ve (Real Positive)	PCR –ve (False Positive)
Stool Culture	22	15(68.2)	07(31.8)
Blood Culture	01	01(100)	00(0.0)
Total	23	16(69.6)	07(30.4)

Key: Figures in parenthesis indicates percentages.

Table 2.4: Prevalence of *Salmonella* Infection in the Selected Hospitals

Hospitals	NP	No. Positive (%)	No. Negative (%)
ABUMC	70	3 (4.3)	67 (95.7)
STL	70	4 (5.7)	66 (94.3)
GK	70	0 (0.0)	70 (100)
YD	70	2 (2.9)	68 (97.1)
PK	70	6 (8.6)	64 (91.4)
KW	70	1 (1.4)	69 (98.6)
Total	420	16 (3.8)	404 (96.2)

$p = 0.668$

Key: NP: Number of patients required; ABUMC: Ahmadu Bello University Medical Centre; STL: ST. Lukes Hospital; GK: General Hospital, Kawo; YD: Yusuf Dantsoho Memorial Hospital; PK: Sir Patrick Yakowa Memorial Hospital; KW: General Hospital, Kwoi;

Table 2.5: Number and Percentages of Culture Positive for *Salmonella* Typhi

Diagnostic Tests	N	S. Typhi	Other <i>Salmonella</i> Species
Stool Culture	420	4(1.0)	11(2.6)
Blood Culture	420	1(0.2)	0(0.0)
Total	840	5(0.6)	11(1.3)

Key: Figures in parenthesis indicates percentages; n = Number of samples collected

Table 2.6: Prevalence of *Salmonella* Typhi in the Selected Hospitals

Hospitals	NP	S. Typhi	Other <i>Salmonella</i> Serovars
ABUMC	70	0 (0.0)	3 (4.3)
STL	70	1 (1.4)	3 (4.3)
GK	70	0 (0.0)	0 (0.0)
YD	70	2 (2.9)	0 (0.0)
PK	70	1 (1.4)	5 (7.1)
KW	70	1 (1.4)	0 (0.0)
Total	420	5 (1.2)	11 (2.6)

$p = 0.391$

Key: NP: Number of patients recruited; ABUMC: Ahmadu Bello University Medical Centre; STL: ST. Lukes Hospital; GK: General Hospital, Kawo; YD: Yusuf Dantsoho Memorial Hospital; PK: Sir Patrick Yakowa Memorial Hospital; KW: General Hospital, Kwoi; Figures in parenthesis indicates percentages.

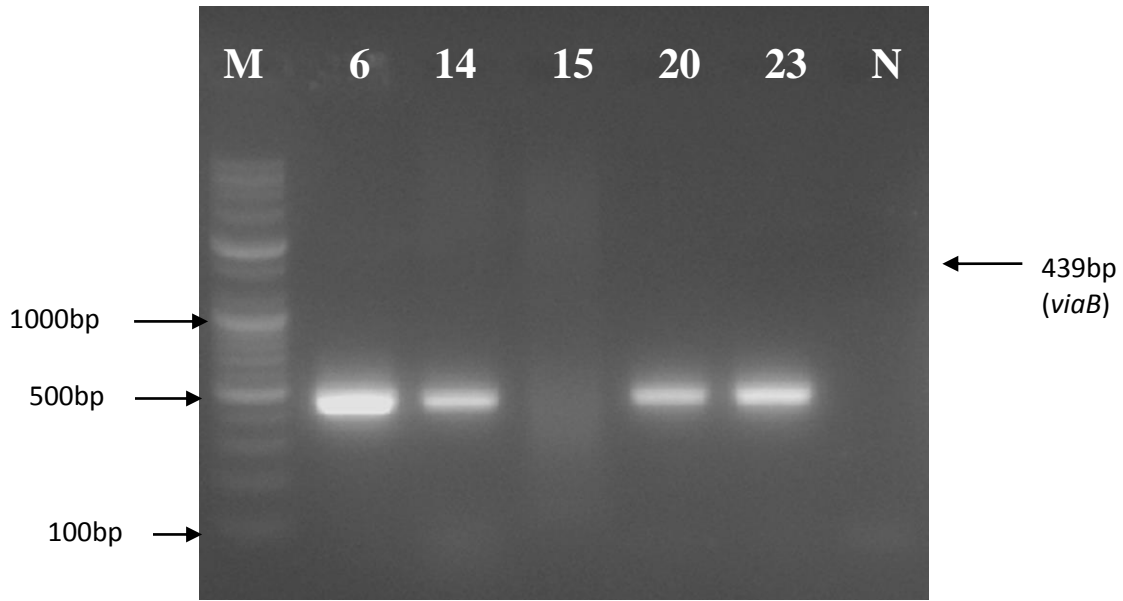


Plate IV: Electrophoresis Gel Picture of PCR Amplified *viaB* Gene of *Salmonella Typhi*

Key: Lane M: PCR Ladder Marker (100bp); Lane 6, 14, 20 and 23: Amplicons of *viaB* Gene; Lane 15: No *viaB* Detected; Lane N: Negative Control

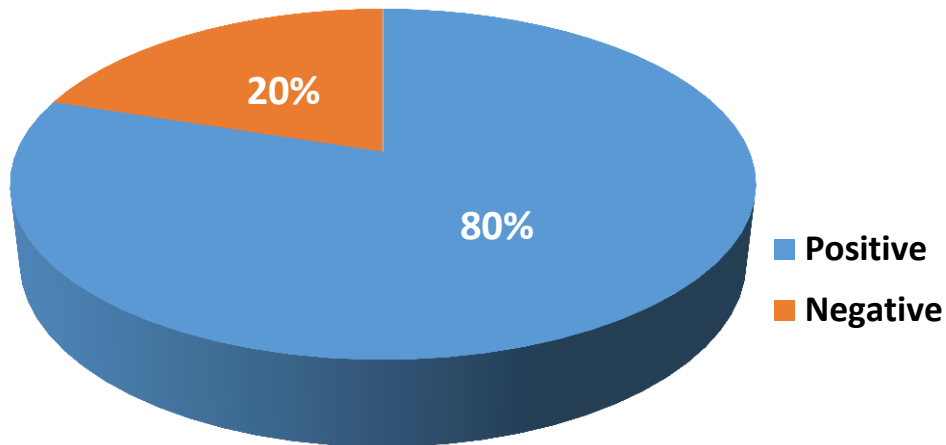


Fig. 2.1: Percentage of *S. Typhi* Expressing Virulence Antigen

Table 2.7: *Salmonella* Typhi Strains Isolated from Selected Hospitals in Kaduna State

ID	Corresponding <i>S. Typhi</i> Strains				Genus	Species	Subsp.	Serovar	Strains
	QI	QC(%)	IDT(%)	ACC. NO					
SST10	29915	99	100	NC003198.1	<i>Salmonella</i>	<i>Enterica</i>	Subsp. enterica	Typhi	CT18
SYD15	66221	99	100	NC003198.1	<i>Salmonella</i>	<i>Enterica</i>	Subsp. enterica	Typhi	CT18
SYD21	208635	98	100	NC003198.1	<i>Salmonella</i>	<i>Enterica</i>	Subsp. enterica	Typhi	CT18
SPK36	155659	99	100	NC003198.1	<i>Salmonella</i>	<i>Enterica</i>	Subsp. enterica	Typhi	CT18
BKW57	148875	79	100	NC003198.1	<i>Salmonella</i>	<i>Enterica</i>	Subsp. enterica	Typhi	CT18

Key: ID: Isolate Number; QI: Query Identity Number; QC: Query Covered; IDT: Identities; ACC.NO: Accession Number.

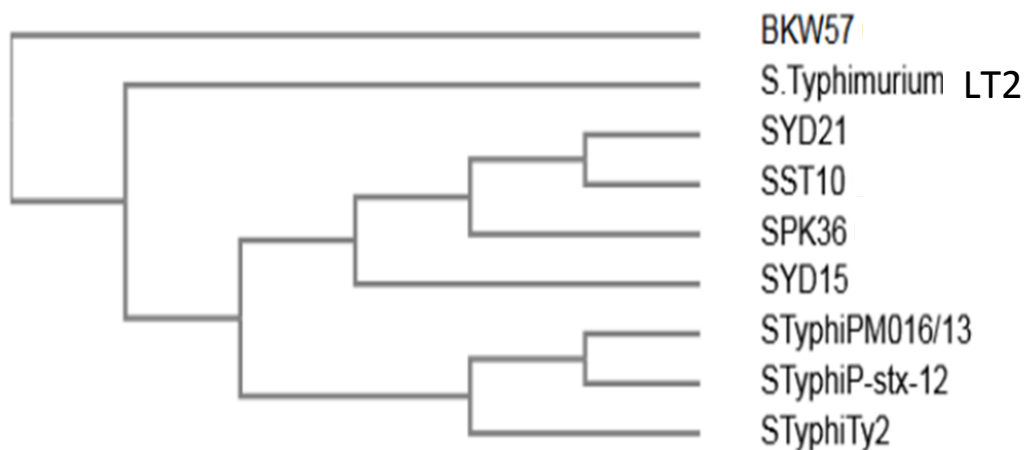


Fig. 2.2: Phylogenetic Tree of the Isolated *S. Typhi* str. CT18, Three other *S. Typhi* Strains and *S. Typhimurium* str. LT2

Twenty two (5.2%) and 01(0.2%) were stool and blood cultures positive for *Salmonella* species respectively. The findings on number and percentage of culture positive *Salmonella* species show that stool culture has a high sensitivity than blood culture. Unspecified host factors or antibiotic use prior to hospital visit may be possible explanations for why majority of the blood cultures yielded no bacterial growths (Hohman, 2011) compare to stool culture.

PCR assay was used to confirm culture positive *Salmonella* species. The assay targeted the amplification of *invA* gene (*Salmonella* invasion gene). The primer has been shown to have excellent specificity for

Salmonella, detecting 100% *Salmonella* isolates without false positives (De Clercq, 2007). Lampel *et al.* (2000) affirmed that *Salmonella* specific PCR with primer for *invA* is sensitive and specific for detection of *Salmonella* in many clinical samples.

PCR was also used to detect *S. Typhi* from the isolated *Salmonella* isolates, targeting the amplification of two genes; *tyv* (tyvelose epimerase gene) and *fliC-d*. The widely used targets for detecting *S. Typhi* in multiplex PCR technique include the *S. Typhi* flagellin (*fliC-d*) and tyvelose epimerase (*tyv*) genes as corroborated by reports of Ali *et al.* (2009), Nandagopal *et al.* (2010) and Nath *et al.* (2010).

The PCR amplification of *invA* gene of the 23 culture positive *Salmonella* species showed that 16(69.6%) were true positive while 7(30.4%) were false positive. This is not surprising as PCR diagnostic method is more specific and sensitive than culture method. The PCR method detected the *invA* gene which is highly specific for *Salmonella* species. The culture method is not specific as the PCR method because the cultural morphology and biochemical characteristics used in isolating and identifying *Salmonella* species respectively are similar to that of other bacteria. Therefore, the similarities may account for false positive culture and this may eventually lead to wrong diagnosis in typhoid fever.

The prevalence rate of *Salmonella* infection among the patients in the study area was 3.8%. This prevalence rate was lower compared to other reports elsewhere in Nigeria; in Zaria, Adeshina *et al.* (2009) reported 14.1% cases, in Katsina State, Abdullahi *et al.* (2014) reported 15% cases, in Lagos, Fashola *et al.* (2015) reported 35% cases and in North-Western Nigeria, Abdullahi *et al.* (2017) reported 6.4% cases. The decrease in the prevalence rate in this study may be due to improvement in hygiene, better awareness, taking of antibiotics prior to hospital visit and more effective diagnosis and treatment.

Four (1.0%) stool cultures and 1(0.2%) blood culture were positive for *Salmonella* Typhi. These findings indicated that stool culture probably has a high sensitivity and specificity for diagnosing typhoid fever. This finding supported an earlier study by Hohman (2011) that established that stool culture picked highest typhoid fever cases than blood. *Salmonella* Typhi an enteric microorganism and its principle habitat is in the intestine of humans, so it is easily detectable in stool even in cases of early ingestion of the bacteria (Prajapati *et al.*, 2008). Stool culture is an important adjunct for diagnosis; it may be positive even when blood culture is negative and it is also important for the monitoring of carriage of *S. Typhi* after apparent clinical cure, which is a risk factor for people involved (Prajapati *et al.*, 2008).

The prevalence rate of *Salmonella* Typhi was 1.2%. This was low compared to the ones obtained by Akinyemi *et al.* (2007) in Lagos;

Okonko *et al.* (2010) in Abeokuta; Smith *et al.* (2009) in Lagos; Umeh and Agbulu (2010) in Benue, all in Nigeria. It was also low compared to the one reported by Breiman *et al.* (2012) in Kenya, who reported 24.7% and Chandra *et al.* (2017) in Tanzania who reported 27.05%. This shows that the cases of typhoid fever is decreasing and this may be due to public awareness; improve in hygiene and probably increase in antibiotic use prior to hospital visit. Kaduna hospitals had the highest rate of *S. Typhi* with 2.9% rate. Zaria hospitals had the least prevalence rate of 0.0%. However, there was no association between the Hospitals and the prevalence rate ($p > 0.05$). The different between the prevalence rates may be due to differences in level of awareness and probably more hygiene practice is observed in Zaria than Kaduna. It may also be that patients in Zaria indulged more in self medication than Kaduna patients.

The Vi (virulence antigen) capsular antigen is a significant virulence factor for typhoid fever, as strains positive for Vi production have higher rates of infection (Hornick *et al.*, 1970; Hone *et al.*, 1988). The five isolated *S. Typhi* in this study were subjected to PCR amplification of *viaB* gene which encoded for virulence (Vi) antigen. *viaB* gene was detected in 4(80%) out of the 5 *Salmonella* Typhi isolated. Therefore, 80% of the isolated *S. Typhi* in this study were of the high virulence types. Our findings was similar to that of Wain *et al.*, (2005) which show that more than 99% (2,221 of 2,222) of *Salmonella* Typhi in Pakistan were able to express the Vi capsular antigen. Mehta and Arya (2002) described high prevalence rate of Vi while Jesudason *et al.* (1994) described a low prevalence rate of it in *Salmonella* Typhi isolates. The expression of the Vi capsular antigen in *S. Typhi* infection presents a possible explanation for the different in clinical manifestation of typhoid fever compared to those infected with other *Salmonella* serotypes (Tran *et al.*, 2010). The Vi polysaccharide plays a critical role in pathogenicity of *S. Typhi* by facilitating bacterial resistance to complement-mediated killing and phagocytosis (Liaquat *et al.*, 2015; Hu *et al.*, 2016). Vi also serves as a primary protective antigen of *S. Typhi* (Wong *et al.*, 1974). The non expression of Vi in 1(20%) of the *S. Typhi* isolates in this study could be as a result of prolong culturing (Pickard *et al.*, 1994). Also, Vi negative mutants of

Salmonella Typhi are known to accumulate from previously Vi positive *Salmonella* Typhi isolates following laboratory passage. Insertion and deletion mutations in the *tviA* gene, encoded within the *viaB* locus, that can lead to the loss of Vi expression and an increase in invasive properties has been reported (Zhao *et al.*, 2001). Furthermore, Vi-negative mutants have been reported in stored isolates (Nair *et al.*, 2004; Bueno *et al.*, 2004). Thus, Vi antigen expression is encoded on an unstable and possibly mobile region of the *Salmonella* serovar Typhi genome (Pickard *et al.*, 2003). However, Vi-negative *Salmonella* Typhi can cause a typhoid fever-like illness, as can *Salmonella* Paratyphi A, which does not typically express the Vi antigen (Kapil *et al.*, 1997). Vi continues to be the focus for improvement in current treatment and prophylaxis for typhoid fever. Vi subunit vaccine is now extensively used in preventing typhoid fever (Date *et al.*, 2015).

The amplicon of 285bp PCR fragment representing the *invA* gene of *Salmonella* Typhi isolates were sequenced using Sanger Sequencing Machine at DNA LABs, Kaduna-Nigeria. The resulting nucleotide sequence of the isolates were compared with the already deposited ones in GenBank and these shows that all the isolated *S. Typhi* nucleotide sequences correspond to that of CT18 strains with accession number of NC003198.1. *S. Typhi* str. CT18 was first Isolated in December 1993 at the Mekong Delta region of Vietnam, from blood of a 9-year-old girl who was suffering from typhoid and submitted on the 10-SEP-2013 to the National Center for Biotechnology Information (Parkhill *et al.*, 2001). Akinade *et al.* (2015), in Abeokuta-Nigeria, isolated *S. Typhi* which had 100% identities with *S. Typhi* str. CT18 (with accession number NC003198.1). Ajayi *et al.* (2017), in Akure-Nigeria, reported isolation of *S. Typhi* str. CT18.

The phylogenetic tree allowed us to understand the close genetic relationship among the isolated *S. Typhi* str. CT18 and other isolates deposited in GenBank. The phylogenetic analysis of the nucleotide sequence of these isolates shows two major clusters, one representing the isolated strains and the other one representing *S. Typhi* strains deposited in GenBank. Isolate number SYD21 and SST10 co-clustered and this reveal high

similarity. They share the same root, meaning that they have recent common ancestor, whereas another isolate number BKW57 might have accumulated enough variations allowing it to cluster separately. Isolate number BKW57 shared recent root (common ancestor) with *S. Typhimurium* LT2 (NC003197.2) which is completely another *Salmonella* serovar. This might be that BKW57 emerge recently from *S. Typhimurium* LT2 due to clonal expansion of *S. Typhimurium* LT2. Isolate number BKW57 appeared to be the most recent among the *S. Typhi* strains. Isolated SYD21 and SST10 did not cluster with SPK36 but they share common root with it which is an indication that they might have emerge from the same ancestor. This observation in the phylogenetic tree corroborates with the PFGE based analysis of Thong *et al.*, (1996) where *S. Typhi* strains from Papua New Guinea showed highly similar PFGE patterns exhibiting limited genetic diversity among the strains. Obtained strains (from GenBank) of *S. Typhi* str. PM016/13 and *S. Typhi* str. P-stx-12 also co-clustered and share common ancestor with another obtained *S. Typhi* str. Ty2. The phylogenetic tree shows that the obtained *S. Typhi* strains were more primitive than the isolated *S. Typhi* str CT18. *Salmonella* Typhi str. Ty2 appears to be the common root (ancestor) of all the analyzed strains and more primitive than others. This was in agreement with Parkhill *et al.*, (2001) who stated that *S. Typhi* str CT18 was recently isolated compared to *S. Typhi* Ty2. *Salmonella* Typhi Ty2 strain was the foundation for vaccine development and was the parent of mutant strains Ty21a and CVD908 and their derivatives (Levine, 1999).

In our study, culture test has low sensitivity in diagnosing typhoid fever. Out of the 23 biochemically confirmed culture positive *Salmonella* species, 16(69.6%) were true positive and 07(30.4%) were false positive. Therefore, it is necessary to develop a highly sensitive and specific diagnostic tool (such as PCR) in Nigerian hospitals for proper diagnosis. Though, there was low prevalence rate (1.2%) of typhoid fever in our study areas compared to previous studies in other places, it is still a serious health problem, therefore, it is recommended that the public awareness on the modes of transmission, the need for proper hygiene and sanitation should be sustained.

Eighty percent of our isolated *S. Typhi* expressed virulence (Vi) antigen. This indicated that the circulating *S. Typhi* in the study areas were of high virulence type.

The results of the *invA* sequence demonstrated clearly that there is need to introduce gene sequencing method in identifying *S. Typhi* implicated in typhoid fever to strains level. This will improve typhoid diagnosis and allow specific and efficient identification of the strains involved as *S. Typhi* str. CT18 is known to carries multiple drug resistance cassettes on a large plasmid. *Salmonella* Typhi CT18 harbors 2 large plasmids, one of which encodes multidrug resistance, that are not found in Ty2 (Parkhill *et al.*, 2001). MDR *S. Typhi* str. CT18 is circulating in the study areas. Hence, an effective national *Salmonella* surveillance system should be set up for monitoring and controlling *Salmonella* as well as controlling of antimicrobial resistance by the both the National and Kaduna State Ministries of Health of Nigeria.

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