Polymerase chain reaction (PCR) analysis of Salmonella typhi from patients in Lagos, Nigeria

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Abstract: **The polymerase chain reaction (PCR), is a very sensitive and specific molecular method, in the identification of microorganism. Definitive diagnosis of bacterial pathogens is a major healthcare challenge in developing countries including Nigeria. PCR profiles of *Salmonella typhi* in Lagos, Nigeria is presented in this study. Bacteriological analysis of stool samples from patient diagnosed clinically for typhoid fever was carried using selective media such as Selenite F (SF), buffered peptone water (BPW) and trypticase soy agar (TSA) which were incubated at 37ºC for 18-24 h. After incubation 0.1 ml of sample from each enrichment medium was inoculated into 10 ml of Rappaport Vassiliadis R10 broth which was incubated at 42ºCfor 24 h. One loopful of the RV10 broth was inoculated into selective media:;MacConkey agar (MA), xylose lysine deoxycholate agar (XLD), *Salmonella*- Shigella agar (SSA) and brilliant green deoxycholate agar (BGDA). Suspected colonies were identified biochemically and kept on nutrient agar slants at 4°C for further analysis. All the *S. typhi* isolates produced reproducible and distinguishable profiles from samples and were amplified and analyzed by gel electrophoresis. The chromosomal DNAs had molecular weight that ranged between 0.52kbp to 2.9Kbp. Fifty-six(83.3%) of the *S. typhi* harbored both 0.9kbp and 1.4Kbp molecular weight, 50 (83.3%) had 2.8Kbp, 42 (70%) had 1.8Kbp molecular weight while the isolate with the least molecular weight DNA were 20,(33.3%). All the isolates belong to five distinctive clones. PCR with RAPD was very discriminatory as isolates classified together by other methods were classified into fewer clones.**

Keywords: Genomic DNA, PCR, Molecular diagnosis, Salmonellae, *Salmonellatyphi*, Oligonucleotides

I. INTRODUCTION

Typhoid fever (enteric fever) is a major public health problem in the developing countries of the world with an annual incidence of 540 per 100 [1, 2]. *Salmonella typhi* is the etiology of typhoid fever with an estimated 21.7 million illnesses with greater than 600,000 [3]. Typhoid fever is a systemic disease caused by *S. typhi* and is a major cause of morbidity and mortality worldwide, which emerged as a major important infection in the early 19th century [4]. Humans are the only natural host and reservoir for fever agent, with the infection occurring in all age groups. It is transmitted by ingestion of food or water contaminated with feces [2].In Nigeria, typhoid fever is not only endemic but constitute a great socio-medical problem, being responsible for many cases of pyrexia of unknown origin, high morbidity and mortality [3]. Nigeria falls within the region of estimated high incidence of typhoid fever in the sub-Saharan Africa and the causative agent is characterized by co-endemicity with other non-bacterial febrile infections like malaria and viral diseases which make direct clinical diagnosis difficult [5, 6].

One of the greatest challenges the developing countries like Nigeria face is that of diagnosis. Some methods used include cultural, and non-cultural techniques like serotyping, plasmid profile analysis and PCR, the latter two being molecular [4].Isolation of *S. typhi* from culture of bone marrow aspirate is regarded as the gold standard for confirmation of typhoid fever but blood culture is preferred because obtaining blood specimen is easier and less invasive[7]. Blood culture is slightly less sensitive than bone marrow aspirate culture[8]. Widal test is most commonly used in the diagnosis of typhoid fever Nigeria despite its several shortcomings [1, 2, 9]. Paired sera for the demonstration of 4-fold rise in antibody titer level are hardly collected in laboratories in Nigeria[10].

Rather, laboratory confirmation is usually based on results of a single Widal test on serum samples collected during acute phase of the illness. The development of the PCR offers an extremely sensitive epidemiological tool based on the amplification of DNA from clinical isolates and are particularly useful for the detection of pathogens which are present at a low concentration, require fastidious growth conditions or cannot be cultivated. This simple technique has reproducible application to stool or possible food or environmental vehicle with potential for expanded epidemiological use[11].A rapid genomic typing method with broad application in biology on the basis of RAPD, which is a modification of the PCR has been described[12]. One or more short arbitrarily chosen primers are able to anneal and prime at multiple locations throughout the genome to produce a spectrum of amplified products characteristics of the template DNA[13]. RAPD can amplify from anonymous genomes with arbitrarily chosen oligonucleotide primer [14].The dearth of information in developing countries and particularly in Nigeria on diagnosis and molecular epidemiology of typhoid fever, further compounded by most of the few reports based on Widal with its several shortcomings necessitated this study. The objectives of the current study are to isolate *S. typhi* from patients diagnosed of typhoid fever, carry out antibiotic susceptibility testing, serotyping and molecular analysis of *S. typhi* using RAPD.

II. MATERIALS AND METHODS

2.1 Bacteriological analysis

Two hundred and sixty Salmonellae recovered from patients in two General hospitals and five private hospitals in Lagos were included in the present study. Stool samples from patients were bacteriologically investigated from patients diagnosed clinically of typhoid fever, which were inoculated into enrichment media which included Selenite F (SF), buffered peptone water (BPW), trypticase soya agar(TSA),which were incubated for 24 h at 37ºC. After incubation, 0.1ml of sample was inoculated into 10 ml of Rappaport Vassiliadis R10 broth (RV Merck, Germany) and was incubated at 42ºC for 24 h. One loopful of RV culture, which changed from blue to green or discolored was streaked onto the surface of MacConkey agar (MA), xylose lysine deoxycholate (XLD) agar (Oxoid), Salmonella-Shigella agar (SSA) (Difco, USA) and brilliant green deoxycholate agar (BGDA) plates. Suspected colonies were identified and cultured on nutrient agar slopes, and kept at 4ºC till use. Phenotypic identification was performed using VITEK, GNI+ (bioMerieux, France) and API 20E identification system[15].

2.2Antibiotic susceptibility testing

Antimicrobial susceptibility tests were applied to the *Salmonella* isolates by disk diffusion method on Mueller Hinton agar (Merck, Germany) with antibiotic disks according to the Clinical Laboratory Standards institute guidelines [16]. Fifteen different antibiotics with the following concentrations in micrograms (µg) were used: ampicillin (10), chloramphenicol (10), colistin sulphate (25), gentamycin (10), nitrofurantoin (20), cotrimoxazole (25), streptomycin (25), tetracycline (25), kanamycin (30), augmentin (30), amoxicillin (30), cefotaxime (30), perflacin (10). Ceftriazone(30) and ofloxacin (10) (Table 1).

Table 1: Antibiotics used in the study and their respective classes

Antibiotic class	Antibiotic
β-lactams	Ampicillin (10µg)
Chloramphenicol	Chloramphenicol (10µg)
Polymyxin	Colistin sulphate (25µg)
Nitrofuran	Nitrofuratoin (20µg)
Sulphonamide	Cotrimoxazole (25µg)
Quinolones	Nalidix acid(30µg)
Streptomycin	Streptomycin (30µg)
Tetracycline	Tetracycline (25µg)
Macrolide	Kanamycin (25µg)
Cephalosporin	Cefotaxime (30µg)
Cephalosporin	Cefuroxine (30µg)
Fluoroquinolone	Ofloxacin (10µg)
Fluoroquinolone	Perflacin (10µg)
Fluoroquinolone	Phenociprofloxacin (10µg)
Aminoglycoside	Gentamycin (10µg)
Macrolide	Erythromycin (15µg)
Glycopeptides	Vancomycin (30µg)
Penicillin	Amoxycillin (30µg)
Penicillin	Augmentin (30µg)

2.3 PCR Analysis

2.3.1 Preparation and purification of chromosomal DNA

Only sixty of the randomly selected pure *S. typhi* were grown on moist 5% blood agar for 24 h at 37°C aerobically and chromosomal DNA was extracted as follow: The bacteria were harvested following overnight culture and re-suspended in DNA was a buffer containing 50Mm NaCl. Washed cell was suspended in 400µl of ice-cold50 Mm Tris, pH 8.0 containing 25% sucrose and 1 mg/ml lysozyme and incubated on ice for 20 minutes. About 50 µl of 5% sterile sodium dodecyl sulphate (SDS) was then added into 50Mm Tris (pH 8.0) followed by addition of 200µl Tris EDTA buffer (10 mM Tris, 1 Mm EDTA, pH 8.0) and 50µl of 2 mg/ml proteinase K.Lysate obtained were incubated at 56ºC for 1 h and extracted once with an equal volume of phenol/chloroform/isoamylalcohol (25:24: 1) by centrifuging at 14, 000 x g for 15 minutes, at room temperature and cells were extracted once with chloroform. Nucleic acids were precipitated by adding 0.1 volumes of 3M sodium acetate, pH 6.0 and two volumes of ice-cold 100% ethanol, centrifuged at 14, 000 x g for 5 minutes at 4ºC, dried under vacuum and re-dissolved in sterile water [16].

2.3.2 Quantification of purified DNA

Sample dilutions were made by adding 10 µl of the homogenous DNA sample to 990µl of deionized water and mixed thoroughly. A fixed beam spectrophotometer was used to measure absorbancy of each sample dilution in a 10 mm path length quartz cuvette at 260 nm and 280 nm using deionized water as the control sample. The concentration formula of DNA was calculated using the formula: C=A260/20x100, C=Concentration in mg/mg, A20= absorbancy at 260, A260= DNA at 1mg/ml gives a reading of 20 optical density units.

2.3.3. RAPD Reaction

Six purified RAPD 10 mer-primers with reproducible profiles with sufficient number of fragments to be discriminatory were used (Abgene, Epsom, Surrey, U.K.) which includedOPFO8-5 ′-GGC-ATA TCG′ G-3′, OPF13-5′ GGC TGC AGA A-3′,OPHO4-5′-GGA AGT CGC C-3′, OPGO4-5′-AGC GTG TCT G-3′, OPG10-5′-AGC′ GCC GTC-3′ and W-06-5′-AGG CCC GAT G-3′ respectively. The PCR was performed according to manufacturer instruction; in 50µl volume containing 1.25 units Taq DNA polymerase 75mMTns- HCL (pH 8.8 at 25°C), 20Mm $(NH_4)2SO4$, 0.01% (W/V) Tween 20 and 0.2Mm each dATP, dCTP, Dgtp and dTTP, 2µl of water,

1µl of RAPD (10) and 2µl of DNA template (5mg/µl^{-1}). A no-DNA control was performed by adding 2µl of sterile distilled water. The PCR reaction mix was overlaid with mineral oil and the tube briefly pulsed in a bench top centrifuge to ensure separation of the pulses. Amplification was performed in a DNA MACHINE (PTC 100-60 thermocycler (M^{r}), Watertown, (MA) with maximal ramping as follows: 1 cycle at 4 mins 30 seconds at 94°C, followed by 35 cycles of 30 seconds at 94°C, 1 min at 32°C and 1 min at 72°C and the reaction products stored at 4°C until required.

2.4 Gel Electrophoresis

A 20µl portion of the RAPD reaction products was loaded onto a 2% Agarose gel containing 0.5 µgml^{-} ethidium bromide (GibcoBRi) and electrophoresed on Tris buffer. The DNA fragments were visualized by placing the gel on a UV (300nm) transillumator and the gel recovered using Polaroid camera.

III. RESULTS AND DISCUSSION

A wide spectrum of antibiotics often employs 0.9kbp employed in the treatment of salmonellosis which comprised of most of the various classes of antibiotics were used in this study. The classes, names and concentrations of the antibiotics in micrograms are shown in Table 1.

Two hundred and sixty Salmonellae were recovered from bacteriological investigation carried out on patients clinically diagnosed of typhoid fever, diarrhea and gastroenteritis. The distribution of the *Salmonella* serotypes are as shown in Table 2. *S. typhi* was most prevalent serotype with 58.5%, S. *paratyphi* B (17.3%), *S. paratyphi* A (11.5 %) and *S. typhimurium* (4.6%) as shown in Table 2.

The PCR analysis of 60 of the *Salmonella* isolates using the RAPD showed nine different types of chromosomal DNA which sizes ranged from 0.9kbp to 2.9kbp. Isolates with molecular wrights of 0.9kbp, 1.4kbp and 2.7kbp hadwith 86.7%, 86.7% and 80.0% respectively. However, isolates with molecular weight of 1.0kbp and 2.0kbp were least frequently encountered (Table 4). The result of the gel electrophoresis of the chromosomal DNA of the *Salmonella* isolates is shown in Figure 1. The bacteria analyzed into only 5 clones.Table 5 shows the distribution of the *S.typhi* using the PCR which shows high specificity and sensitivity by dividing them into only five clones while totally rejecting some isolates as *Salmonella* as determined by the other methods of diagnosis. This may also inadvertently be due to cross-reactivity of antibodies due close evolutionary relationship.

Table 2: Incidence of *Salmonella* (260) isolates from patients.

Species	Number	Percentage (%)
S. typhi	152	58.5%
S. paratyphi B	45	17.3%
S. paratyphi C	21	8.1%
S. paratyphi A	30	11.5%
S. typhimurium	12	4.6%
Total	260	100

Table 3: Percentage of antibiotic resistance of bacterial isolates from subjects

		Antibiotics (%)														
ISOLATE	NO TESTED	AMP	CHL	COL	GEN	NIT	COT	STR	TET	KAN	AUG	AMO	CEF	PEF	CFR	OFL
S. typhi	200	6.5	13	94	0.0	53	11.5	23	100	98	12.5	9.5	0.0	0.0	0.0	0.0
*Salmonel la*spp	80	40	21.3	100	93.8	62.5	82.5	77..5	85	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 3 shows that the Salmonellae were highly susceptible in-vitro to most of the antibiotics tested but as demonstrated high multiple resistance to tetracycline, colistin sulphate and nitrofurantoin (Table 3).

LEGEND - AMP- Ampicillin,CHL-Chloramphenicol, COL-Colistin sulphate, GEN-Gentamycin, NIT-Nitrofurantoin, COT-Cotrimoxazole, STR-Streptomycin, TET-Tetracycline, KAN-Kanamycin, AUG-Augmentin AMO-Amoxicillin, CEF-Cefotaxime, PEF-Perflacin, CFR-Cefuroxime, OFL Ofloxacin

Table 4: PCR profile of *S. typhi* from subjects

	Molecular weight	Number (n=60)	Percentage (%)
1	2.9kbp	36	60.0
2	2.8kbp	48	80.0
3	2.0kbp	28	46.7
4	1.8kbp	40	66.7
5	1.5kbp	32	53.3
6	1.4kbp	52	86.7
7	1.0kbp	28	46.7
8	0.9kbp	52	86.7
9	0.52kbp	16	26.7

Table 5: Clones of *S. typhi* isolated from subjects.

Clones	Isolates
A	1, 3, 6, 8, 9, 10, 14, 18, 21.
B	4, 17, 24, 27, 30.
C	12, 28, 35, 36, 37, 39, 44, 46, 52.
D	5, 22. 32, 45, 48, 51. 56, 60.
E	2, 5, 13, 15, 25, 34, 38,, 41, 43, 57.

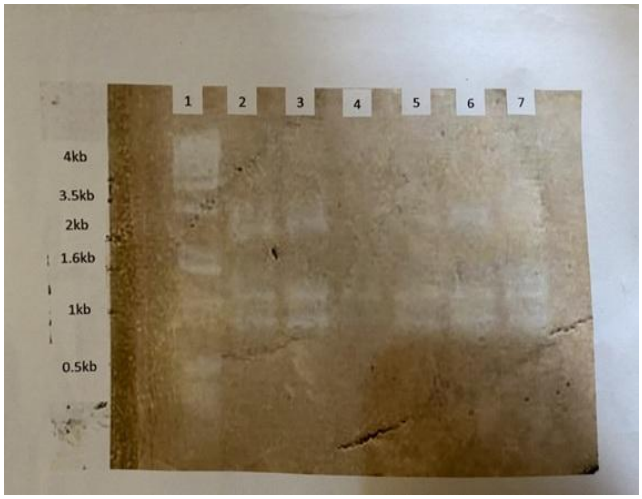

Figure 1: Patterns of *Salmonella typhi* recovered from subjects.

Lane 2 to 7 are the RAPD Profiles of *S. typhi* isolated.Lane 1 is marker (10kbp)

Typhoid fever is one of the major health problems in Nigeria as it is endemic and even assume sporadic epidemic proportion especially among slum dwellers in our cities. The high incidence of *S. typhi* is quite phenomenal, considering the high morbidity and mortality rate of the disease. Because of its protein presentation, it is often difficult to make a clinical diagnosis of salmonellosis but can easily isolated from clinical specimens are in standard microbiology laboratories but most diagnosis are based on clinical symptoms or when specimens are not sent for culture where hospitals and clinics lack such laboratories. Similar findings have been reported. However, no *S. enteritidis* was recovered in this study as against Onile and Odugbemi[17]. This study also shows that *Salmonella* spp. could be isolated with relative ease from stool samples through the pre-enrichment and enrichment methods using selective media (18).

The PCR analysis of the clinical *Salmonella* isolates using an arbitrarily selected 10-mer oligonucleotide primers under well designed and optimized conditions, is capable of being used for the reproducible amplification of random fragments of DNA from *Salmonella* genomes to differentiate between and within serotypes. Interestingly, the RAPD primers used (AB-gene, Sweden) possessed attributes like discrimination between unrelated strains but can reliably and reproducibly recognize similar strains, thus provided the required reproducible discriminatory profiles. It was observed that the *Salmonella* within the same plasmid profile fell within different RAPD grouping. This finding is consistent with Hilton *et al*(19) who reported that plasmid profile allows more differentiation with the isolates falling into five groups with RAPD, thus is more discriminatory. This study shows that no one typing scheme can readily provide all the information potentially useful in an epidemiological investigation. Thus, a variety of methods including molecular methods such as RAPD, RFLP and ribotyping where possible should be used according to some earlier studies [13, 19,20].

Prediction of banding patterns depends on the primer used but their intensity and number of RAPD products vary greatly with PCR condition. Production of only one or two weak intensity irrespective of primer is enough to disregard such isolates but when two or more share identical sets of RAPD bands for same primer, it suggests that these isolates are of the same strain. The present study is able to achieve best amplification of fragments at concentration of Taq XL DNA polymerase with 2µL as earlier reported, thus, this study is quite revealing as an epidemiological landmark in studies on salmonellosis in general and typhoid fever in particular. The observation of five different clones of *S. typhi* among the subjects from the molecular analysis of their chromosomal DNA is consistent with report by Mirza[21] with similar pulse field gel electrophoresis (PFGE) patterns. The *S. typhi* isolates from the patients studied belong to 5 clones, thus suggests that the *S. typhi* from the patients can be traced to the same source(s). This finding agrees with Tarret[22] from the study on Tajikistan involving 24,000 cases characterized by overflow of sewage contaminated municipal water and person to person spread.

The result from the molecular studies using the RAPD conforms that there was no major outbreak of typhoid fever in Lagos, Nigeria, contrary to previous reports on the media as there was considerable heterogenicity among the isolates, which belongs to five predominant clones. Similar reports was reported on *Salmonella* isolates in Hong Kong and Vietnam [23],which belong to two predominant clones. This further emphasizes the significance of molecular typing, plasmid analysis and serotyping[24]. This study also agrees with a report by Kidema and Ferson[25] from their study on *S. typhi*especially when they harbor similar plasmids among similar strains from the patients. Khan et al. (2[26] reported diagnostic efficacy of PCR with 64.5%, followed by Widal test with 26% and blood culture with only 14.5%. Among their samples that were negative with blood culture, 58.5% were successfully detected by PCR while only 13.4% were positive for the Widal test. Findings of Khan [26] agrees with our study that PCR is a fast and reliable technique in the diagnosis of typhoid fever among suspected cases. Our RAPD assay shows a positive result only with *S. typhi* perhaps because the other strains have no significant sequence homology with the primers that were used in this study, which also agrees with CDC [27]. In another study, Saha et al

[28]had sixty-six percent (38/59) of the water sources of Dhaka were contaminated with typhoidal *Salmonella* DNA, thus recommended the use of PCR-based detection as a low-cost approach to determine the presence of typhoidal *Salmonella* in the environment which successfully guide informed designs of blood culture-based surveillance. According to WHO [29], robust low-cost and sustainable methods to supplement traditional blood culture-based surveillance systems are highly desirable, not only to evaluate the burden of enteric fever but also to aid in prioritization and monitoring the impact of interventions. The study strongly suggests the need for the establishment of compatible network for the exchange of molecular data on the West African sub-region and an inter-continental scale so as to control salmonellosis globally.

IV. CONCLUSION

We showed that salmonellosis is endemic in the study area with the high prevalence of *Salmonella* isolates. The cultural method using pre-enrichment and enrichment of stool samples showed that it is effective in the diagnosis of salmonellosis. Serotyping on the hand showed high efficacy in differentiating the Salmonellae (in serotypes while the high specificity and sensitivity demonstrated by the PCR (RAPD) within very short time as against other methods was outstanding and provide additional information to the volume of literature on the diagnosis of salmonellosis. The Salmonellae showed a high susceptibility in-vitro to most of the first line drugs which suggests their effectiveness in the treatment of salmonellosis. The high resistance to tetracycline, colistin sulphate and nitrofurantoin may likely be due to abuse of the drugs. Self-medication or improper use of these over-the-counter drugs. Isolation, identification and antimicrobial susceptibility of pathogens be very helpful in optimizing antimicrobial use. It is therefore recommended that a West African regional reference laboratory be established with emphasis on the use of the various PCR techniques as against other methods that would serve as a reference laboratory for salmonellosis and *Salmonella* epidem [ologyin Africa.

REFERENCES

[1] Abuobeida AAA, Typhoid and paratyphoid fever. Africa Health, 1996; 18(5): 14-15

[2] MoroDD, Okiki PA, Grillo JA and Raheem TY. Concurrent infections of some pathogenic bacteria and parasites in patients diagnosed of gastroenteritis in Yaba, Lagos, Nigeria. J. Res. Rev. Sci. 2004; 3:215-221

[3] Ramyil SMZ, Ogundeko TO. Use of stool culture as determinant parameters of enteric fever in adults attending Bigham University teaching hospital Jos, Nigeria. Clinical Med. Res. 2014; 3(2): 31-35

[4] GemechuA,Edemew A, Berhanu K. Comparative study of Widal test against stool culture for typhoid fever suspected cases in southern Ethiopia. Pathology and Laboratory Med. Int. 2017; 9 1-7

[5] Enabulele O, Awunor, SN. Typhoid fever in a tertiary hospital in Nigeria: Another look at theWidal agglutination test as a preferred option in diagnosis. 2016; Nig.Med. J .57 (3):145-149

[6] Von Kalcreuth V, Konings F, Aaby P, Adu-Sarkodie Y, Ali M, Aseffa A et al . The typhoid fever surveillance in Africa Programme (TSAP): Clinical, diagnosis, and epidemiological methodologies. CLIN. Inf. Dis. 2016, 62 (1): S9-S16

[7] Bhutta Z. Current concepts in the diagnosis and treatment of typhoid fever Brit. Med. J., 2006; 333: 78-82

[8] Tanyigna KB., Bello CS., Okeke N., Onwukeme KE. Comparison of blood, bone marrow aspirate, stool and urine cultures in the diagnosis of enteric fever. Nig J. Med. 2001; 10: 21-24

[9] Moro DD, Salu OB, Oluduro OA, Famurewa O, Akinside KA. Theprevalence of bacterial pathogens and parasitic intestinal worms among vendors in Ajegunle, Lagos. J. Bio. Phy. Sci. 2000; 1: 129-134

[10] Agbonlahor MI, DE, Agbahowa MO, Idukpaye O, Agbonlahor, FE, Ekundayo AO, Emele FE, Osund e MI, Omoregbe R, Onyemelukwe NE, Okaa GO. Baseline typhoidal antibody levels in apparently healthy Nigerians. Nig. Quart. J. Hosp. Med. 1997; 7: 242-245

[11] Savariano SJ, Bourgeois AL. Epidemiology of diarrhoeal disease in developed countries. Trans. Roy. Soc. Trop. Med. 1993; 87:(3): 7-11

[12] Welsh J, McCellard M. Fingerprinting genomes using arbitrary primers. Nucl. Acids Res. 1990; 18: 7213-7217.

[13] Young KA, Power EGM, Dryden MS, Phillips L. RAPD typing of clinical isolates of Staphylococcus hemolyticus. Lett. Appl. Bacteriol. 1994;18: 86-89

[14] Caetano-Anoile G.Amplifying DNA with arbitrary oligonucleotide primers. PCR Methods and Applications 1993; 3: 85-94

[15] Lee KE, Lee Y. DT104 from swine in Korea Isolation of multidrug-resistant *Salmonellatyphimurium* in Korea. J. Microbiol. 2007; 45(6): 590-592

[16] CLSI. Performance standards for antimicrobial susceptibility testing twenty fifth International Supplement. CLSI document M100-S25 Wayne, PA: Clinical and Laboratory Standards Institute., 2015.

[17] Onile BA, Odugbemi T. *Salmonella* serotypes in Ilorin. West Afric. J. Med. 1989; 6(1): 7-10

[18] Yan JJ, Ko WC, Chui CH, Tsai SH, Wu HM, Wu JJ. Emergence of ceftriaxone-resistant *Salmonella* isolates and rapid spread of plasmid-encoded CMY-2-like cephalosporinase, Taiwan. Emerg. Infect. Dis. 2003; 9:323- 328.

[19] Hilton AC, Banks JG, Penn CW. Random amplification of polymorphic DNA (RAPD) of *Salmonella* strain differentiation and characterization of amplified sequences. Soc. Appl. Bact. 1996; 81: 575 584

[20] Lindsay EA, Lawson AJ, Walker RA, Ward LR, Smith HR, Scott FW, O'Brien J, Fischer IST, Grook PD, Wilson D, Brown DJ, Hardardattir H, Wannet WJB, Tschape H, Threlfall EJ. Role of electronic data exchange in international outbreak caused by *Salmonella* enterica serotype typhimurium DT204b. 2002; 8(7): 732-734.

[21] Mirza S, Kariuki S, Mamun KZ, Beeching J, Hart CA. Analysis of plasmids and chromosomal DNA of multidrug- resistant *Salmonella* enterica serovar typhi from Asia. J. Clin. Microbiol. 2000; 38(4): 1449- 1452.

[22] Tarr PE, Kuppens L, Jones TC, Ivanoff B, Aparin PG, Heymann DL. Considerations regarding mass vaccination against typhoid fever as an adjunct to sanitation and public health measures, potential use in an epidemic in Tajislistan. Am. J. Trop. Med. Hyg. 1999; 6 (11): 163-170.

[23] King IFG, Francis NJ, Kingston RE. Native and recombinant polycomb group complexes establish a selective block to template accessibility to repress transcription in vitro. 2002; 22(22): 7919-7928

[24] Bernnet AR, Greenwood D, Tennant C, Banks JG, Betts RP. Rapid and definitive detection of *Salmonella* in foods by PCR. Lett. Appl. Microbiol. 26: 437-441.

[25] Kidenya V, Ferson MJ. Typhoid and paratyphoid fever in south eastern Sydney, 1992-97. Comm. DIS. Intell. 24(8): 233-236

[26] Khan IU, Sajid, S.,Javad A., Sadiq S, Sha SU, Khan SN, Ulah, K. Comparative disgnosis of typhoid fever by Polymerase chain reaction and widal test in Southern Districts (Bannu, Lakki,

Marriot and DL-Khan} of Khyber Pakhthinkhwa, Pakistan. Acta Scientifica Malaysia {ASM}, 2017; 1{2}: 12-15
[27] Centers for Diseases Control and Preventions [CDC], 2016.
[28] Saha, S, Jammoy AM, Andrews JR, Sajib MSI, Yu AT, Baker S, Luby SP, Saha SK. Evaluating PCR-based detection of Salmonella Typhi and Paratyphi A in the environment as an enteric fever surveillance tool . Am. J. Trop. Med. Hyg. 2019;100{1}: 43-48
[29] World Health Organisation {WHO}: Typhoid vaccines: WHO positive paper, March 2018- Recommendations. Vaccines, 2018; pp 153-172.