



Molecular Typing of *Salmonella* Species Isolated from Stool Samples

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ABSTRACT This study aimed at comparing the biochemical characterization of *Salmonella* spp with the molecular typing method. A total of 57 stool samples were collected from three different health institutions in Nigeria over a period of 3 months. Twenty (35%) *Salmonella* species consisting of 14 (70%) *S. Typhi* and 6 (30%) *S. Choleraesuis* were identified using standard methods. The isolates were then typed using randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) and the enterobacteriaceae repetitive intergenic consensus PCR (ERIC-PCR). The ERIC-PCR differentiated the *S. Typhi* into 14 different sub-types with four of them (2s and 6s) and (7s and 11s) belonging to the same sub-types. The *S. Choleraesuis* showed no band with the ERIC-PCR while the RAPD-PCR differentiated the isolates into nine sub-types and the remaining isolates showed no visible band. The ERIC-PCR was shown to be more a discriminatory and type-able tool for *Salmonella Typhi* isolates.

INTRODUCTION

Salmonella infections occur worldwide in both developed and developing countries and are a major contributor to morbidity and economic loss (Antonie et al. 2008). *Salmonella* species have been the leading cause of food borne outbreaks and are considered one of the major causes of human gastroenteritis worldwide (Rasschaert et al. 2005). *Salmonella Typhi* causes enteric fever also known as typhoid fever majorly. This disease is endemic in the tropic and subtropics (Asia and Africa) and has become a serious public health problem in developing countries of the world, especially in Nigeria due to poor hygiene and lack of or inadequate potable water. In 2010, an estimated 26.7 million cases of typhoid fever were recorded (Buckle et al. 2012). Several cases of mortalities and morbidities associated with typhoid fever

have been recorded in Nigeria (Ibekwe et al. 2008). Other serotypes such as *Salmonella* paratyphoid A and B, *Salmonella Choleraesuis* also cause paratyphoid fever. *Salmonella Choleraesuis* is a highly host-adapted pathogen that causes swine paratyphoid in pigs of all ages. The infected animals, their products and manure pose serious health hazards to other healthy pigs and humans who take care of them or consume their meat. This organism is notorious for its extreme invasiveness and pathogenic nature in humans frequently causing septicemic disorders with scarce involvement of the gastrointestinal tract (primary bacteremia) (Taylor 1983). There is an upsurge in the incidence of *Salmonella Choleraesuis* bacteremia over the years due to increase in ciprofloxacin-resistant isolates (Wang 2006). Effective management of affected patients requires rapid diagnosis of the pathogens in clinical samples. Clinical manifestations are not reliable enough to ascertain the presence of these pathogens or active infection in particular patients (Mc Pherson et al. 2006). Standard methods commonly used in detection of these pathogens are biotyping, phage typing, serotyping, and antibiotic resistance patterns. These methods have been shown to lack the credibility to

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differentiate isolates and strains of the same serotype and are also time consuming. In most countries, serotyping is expensive and also restricted to national reference laboratories. Furthermore, some of the human and animal isolates are not identified by the serologic method (Rasschaert et al. 2005). Hence, to investigate the source and relatedness among different strains more accurate tools than serotyping are needed. Polymerase chain reactions (PCR) not only identify the isolate to species level, but also serve as an epidemiological tool by providing information on the circulating strain in an environment especially during epidemic conditions.

Molecular typing methods such as random amplified polymorphic DNA (RAPD-PCR) (Khodoo et al. 2002), repetitive extragenic palindromic (REP-PCR) (Johnson et al. 2001; Rasschaert et al. 2005; Weigel et al. 2004) pulsed field gel electrophoresis, plasmid profiling has been used for differentiation and characterization of *Salmonella* and to trace the clonality of strains (Akinyemi et al. 2005; Weigel et al. 2004).

The REP-PCR relies on primers that are complementary to the bacterial genome, to generate DNA fingerprints that allow discrimination between strains (Versalovic et al. 1991). These repetitive elements include the repetitive extragenic palindromic (REP) elements (Stern et al. 1984), the enterobacterial repetitive intergenic consensus (ERIC) sequences (Hulton et al. 1991; Yu et al. 2011) and the BOX sequences (Martin et al. 1992).

In Nigeria, 4 sub-types of *Salmonella* were defined in patients from 26 clinical isolates by RAPD and they were, *S. Typhi*, *S. Paratyphi*, *S. Choleraesuis* and *S. Enteritidis* (Smith et al. 2011). Having knowledge of the circulating strains, guides therapy, especially in species with multiple-strains and serotypes.

Objectives

The purpose of this study was to compare the conventional biochemical and molecular method of identifying *Salmonella* species and to determine the strains circulating in Nigerian population.

METHODOLOGY

Study Population

A total of 57 stool samples were collected from Federal Medical Centre Hospital, Ebute-

Metta, Mushin Health Centre and Surulere General Hospital, Ojuelegba for a period of 3 months. The samples were collected from patients who reported symptoms of typhoid fever. The samples were collected in sterile universal leak proof containers and were transported to the Molecular Biology and Biotechnology Laboratory of Nigeria Institute of Medical Research, Yaba, Lagos (NIMR) within an hour of collection for processing.

Isolation of *Salmonella* Species

For the isolation of *Salmonella* species, a loop of stool sample was inoculated aseptically into a McCartney bottle containing 9 ml of Selenite F-broth and incubated at 37°C for 24 hours.

After incubation, the stool samples were sub-cultured from the Selenite F-broth onto *Salmonella-Shigella* agar (SSA). Identification of isolates was done using *Salmonella* chromogenic agar and characterized using the method of Cowan et al. (2003).

Identification of Isolates by PCR

Deoxyribonucleic acid extraction was performed using the method of Bimboim and Doly (1979). The identity of the isolates was confirmed by PCR using species-specific oligonucleotide primer set (5'-AGC CAA CCA TTG CTA AAT TGG CGCA-3') as described by Smith et al. (2012).

RAPD-PCR

Randomly amplified polymorphic DNA polymerase chain reaction (RAPD/PCR) was performed according to the method described by Hilton et al. (1997) using primer 1254 with the sequences (5'- CCG CAG CCA A 3'). A 1 Kbp DNA ladder was used as a DNA size standard. The reaction was carried out in a 25µl mixtures containing 2mM magnesium chloride, 200µM (each) of the four deoxyribonucleotide triphosphates, 4.0 pmol of primer, 1µl of Taq DNA polymerase, 20ng of bacteria DNA culture and distilled water was added to make a total volume of 25µl. The mixture was subjected to 40 PCR cycles in a programmable 96 wells Master cycler gradient Eppendorf (Harburg, Germany). The parameters for the amplification cycles were as follows: initial denaturation at (94°C for 5 min-

utes), and the 40 cycles of (94°C for 30 sec, 20°C for 2 minutes, 72°C for 2 minutes) and final denaturation of (94°C for 30 seconds, 72°C for 2 minutes and final extension of 72°C for 5 minutes).

ERIC-PCR

Enterobacteriaceae repetitive intergenic consensus polymerase reaction (ERIC-PCR) was performed as described by (Beyer et al. 1998) using Eric 2 primer with the oligonucleotide sequences (5'-AAG TAA GTG ACT GGGG TGAGCG 3'). A 100bp DNA ladder was used as DNA size standard. The reaction was performed in a 25µl volume containing 200 µl (each) of the four deoxyribonucleotides triphosphates, 2.5mM MgCl₂, 40pmol of primer, 1µl of Taq polymerase, 1µl of bacterial culture and distilled water was added to make a total volume of 25µl. The mixture was subjected to 40 PCR cycles in a programmable 96 wells Master cycler gradient Eppendorf (Harburg, Germany). The parameters for the amplification cycles were as follows: initial denaturation of (94°C for 5 minutes), followed by 35 cycles consisting of denaturation (at 94°C for 1 minutes) and annealing (52°C for 1 minute), initial extension (72°C for 2 minutes) and final extension (72°C for 5 minutes).

The PCR amplicon were separated by gel electrophoresis in one percent agarose containing ethidium bromide (0.5µg/ml) and visualized with a photo documentation system (Clinix 1500 Japan) using a digital camera under UV illumination.

RESULTS

Out of the 57 stool samples screened, *Salmonella* species was isolated from 20 (35.1%) while the remaining 37 samples (64.9%) yielded no *Salmonella* growth. The two species of the *Salmonella* isolated are *S. Typhi* (70%) and *S. Choleraesuis* (30%).

The ERIC-PCR was able to sub-type seventy percent (14) of the *S. Typhi* isolates into 12 (A, B1, B2, C1, D1, D2, E, F, G, H, I, J, K, L) groups, one distinct isolate falling into each group. However, group two and four have two distinct isolates (B1, B2 and D1, D2) The remaining thirty percent (6) isolates of *S. Choleraesuis* showed no visible bands lane 4, 5, 12, 16, 19 and 20 as shown in Table 1.

The RAPD-PCR, on the other hand, was able to sub-typed forty-five percent (9) different

types and the remaining fifty-five percent (11) showed no visible bands as shown in Tables 1 and 2.

Table 1: Molecular subtyping of *Salmonella Typhi* and *Salmonella Choleraesuis* by ERIC and RAPD PCR

Isolates	Species	ERIC-Typing	RAPD-Typing
1s	ST	A	NT
2S	ST	B1	NT
3S	ST	C	NT
4S	ST	NT	A
5S	ST	NT	NT
6S	ST	B2	B
7S	ST	D1	C
8S	ST	E	D
9S	ST	F	E
10S	ST	G	F
11S	ST	D2	G
12S	ST	NT	H
13S	ST	H	NT
14S	ST	I	NT
15S	SC	J	NT
16S	SC	NT	NT
17S	SC	K	NT
18S	SC	L	NT
19S	SC	NT	NT
20S	SC	NT	I

NB:

ST- *Salmonella Typhi*

SC-*Salmonella Choleraesuis*

NT- Non typeable

Table 2: Sub-typing by ERIC and RAPD PCR

Molecular typing		Salmonella Choleraesuis n=20	S. Typhi	Total
ERIC	Type able strains	14	0	14
	Untypeable strains	0	6	6
RAPD	Type able strains	6	3	9
	Untypeable strains	13	3	11

DISCUSSION

The prevalence of *Salmonella Typhi* obtained in this paper is in accordance with the findings of Akinyemi et al. (2007), Okonko et al. (2010), Smith et al. (2009) and Umeh and Agbulu (2010) who reported that *S. Typhi* was the most prevalent serotype in Nigeria.

The ERIC-PCR used in this paper possess good discriminatory power and is more typeable compared to the RAPD-PCR. It was able to sub-type the *Salmonella Typhi* isolates into 14 different types while the remaining 6(30%) *S.*

Choleraesuis isolates showed no visible bands. This method has the advantage of being simple, accurate, fast and powerful for the genomic typing of bacterial strains

The most prevalent isolates are the type B and D sub-type B1, B2 and D1, and D2 line, 2, 6, 7 and 11 having 28.6 percent of the type able isolates Table 1. The remaining (71.4%) belong to different type of *Salmonella* strains.

The ERIC-PCR has been analyzed in several studies with success. ERIC-PCR has been successfully used to discriminate closely related strains of *Enterobacter aerogenes* and *Escherichia coli* (Georghiou et al. 1995; Lipman et al. 1995). There are earlier reports of ERIC-PCR by (Lim et al. 2005), wherein 57 strains of *Salmonella* were differentiated into 50 different patterns in their paper. The paper by Rasschaert et al. (2005), also confirmed the suitability of REP-PCR using five primers to differentiate *Salmonella* isolates at the serogroup level. Fingerprints were generated for the isolates that were not differentiated by serotyping. Also, in the report by Shabarath et al. (2007), ERIC-PCR differentiated the twelve *S. Weltevreden* isolates into four types. The fish isolates, which were discriminated into two by RAPD types, were discriminated into three by ERIC-PCR. The reports of Sanae et al. (2009) in Morocco also supported this claim, the ERIC-PCR was showed to be more discriminatory in typing 16 *Salmonella* strains isolated from human stools and food into 8 types. All-Shirodii et al. (2014), also remarked on the suitability of ERIC-PCR in assessing the diversity and discriminating the 64 isolates of *S. Enteritidis* reported in their work. The ERIC primer gave 8-13 bands set with approximate sizes between 100 to 2000 bp for the 64 isolates of the *S. Enteritidis*.

The RAPD-PCR on the other hand was able to type the isolates into 9 different types with the remaining (55%) isolates showing no visible bands (Tables 1 and 2). Although this technique is fairly discriminatory, simple and easy to interpret like the ERIC-PCR, it appears that the primer 1254 used in our paper is not suitable in typing the *Salmonella Typhi* isolates in Nigeria. This was also observed in the paper by Smith et al. (2011), where the primer 1254 used were dropped because it did not produce discriminatory patterns. In accordance to the results obtained in

this paper, is the work of Dos Santos et al. (2008) in Brazil, the RAPD-PCR method was used with primer 1254 and OPB 17 to type 238 of the *Salmonella Enteritidis* isolated from poultry, food samples and human in their study. Twenty-one isolates (8.8%) out of the 238 isolates were differentiated into four patterns and into seven sub-types with primer 1254, and into four patterns and ten sub-types using primer OPB 17. Un-Ho et al. (2005) in Korea, used primer OPB-18 and was able to type four 4(98%) out of the five 5 (100%) *S. Typhimurium* species isolated in their work.

There is need for documentation of molecular typing techniques of *Salmonella* species in Nigeria. Only few reports of RAPD-PCR of *S. Typhi* by Smith et al. (2006), Smith et al. (2012), and Smith et al. (2011) and plasmid profile analysis by Akinyemi et al. (2005) have been reported.

The molecular genotypic typing method has more discriminatory power and is more type able compared to the phenotypic method like the biochemical method used which was unable to differentiate further the 14 isolates of *S. Typhi* and the (6) isolates of *S. Choleraesuis*.

CONCLUSION

It can be deduced from this study that the ERIC-PCR offers high discrimination amongst the *S. Typhi* in our environment. Thus, the ERIC-PCR apart from being simple, accurate and fast will be a reliable technique in typing the isolates in Nigeria.

FUTURE RESEARCH

The future research will focus on the use of the ERIC-PCR in typing more species of *Salmonella* in the environment to further confirm its applicability in typing the isolates.

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