Original Research Article

ISOLATION AND IDENTIFICATION OF SALMONELLA SPECIES ASSOCIATED WITH FEBRILE PATIENT USING BASIC MOLECULAR TECHNIQUES

Abstract

Salmonella enterica, serotype Typhi, remains the predominant *Salmonella* species causing enteric fever. This disease threatens public health as it is associated with personal hygiene techniques and practices. This study investigates *Salmonella Typhi* using conventional bacteriological techniques as well as polymerase chain reaction (PCR). The results showed that of the four hundred and eighty-six (486) stool samples analyzed, stool cultures revealed 189 putative isolates. These isolates, after identification by conventional biochemical methods, showed 6 isolates (1.23%). The Six (6) confirmed strains were then confirmed PCR with the following set of primers. STR (F) TAT GCC GCT ACA TAT GAT GAG, STR (R) TTA ACG CAG TAA AGA GAG and STN (F) ACT GCT AAA ACC ACT ACT, STN (R) TGG AGA CTT CGG TCG CGT AG and aroC for GGCACCAGTATTGGCCTGCT and aroCs rev CATATGCGCCACAATGTGTTG. The results confirmed one (1) strain of S. Typhi and two (2) related serovars (Salmonella Typhimuirium). The isolates of Salmonella showed a high rate of antibiotic resistance to gentamicin (100%), augumentin (83.3%), cotrimoxazole (83.3%). In contrast, the isolates were very sensitive to nitrofurantoin (100%) and ofloxacin (83.3%). Others show a different resistance rate. This study confirms the need for multi-step diagnosis for enteric fever to provide appropriate and effective treatment. This finding will guide the use of antibiotics in the treatment of Salmonella infections in the study area and justify the search for new effective antibiotics against multi drug resistance.

Keywords: Typhoid fever, *Salmonella* Typhi, Multi Drug resistance, PCR and

Introduction

Typhoid fever continue to be of global concern because of its public health implications especially in low and middle income countries (LMICs) with inadequate water, sanitation and hygiene practices (Feasey *et al.*, 2015 and Schwenk, 2020). *Salmonella* enterica causes gastroenteritis, enteric fever (typhoid and paratyphoid) in humans and animals (Khadka *et al*., 2021). Humans are the only known reservoir of *S*. Typhi. Cases are eventually transmitted from person to person, but transmission can occur through direct or indirect exposure after the pathogen has been released into the environment. This is called long-cycle transmission (Akullian *et al*., 2015). The World Health Organization (WHO) estimates the annual global incidence of typhoid fever at approximately 21 million cases. (Mogasale *et al.*, 2014), is probably an underestimation because of poor diagnosis. There are several ways to diagnose

enteric fever, including clinical signs and symptoms, serological markers, bacterial culture, antigen detection, and DNA amplification. None of these approaches is completely satisfactory. The most reliable diagnostic methods are blood, bone marrow, and stool cultures, but these are expensive techniques, and the infecting organism may be dead on arrival at the hospital if the patient has taken antibiotics before clinical samples can be taken. Serological diagnosis is mainly based on the Felix Widal test in many low- and middle-income countries such as Nigeria. first standardised in the 1950s, (Abdulaziz and Hisham 2014). However, the speed and accuracy of the test allows better treatment of all diseases, including fever. Typhoid fever is broadly defined as an enteric fever characterized by fever, abdominal pain, and nonspecific symptoms including nausea, vomiting, headache, and loss of appetite. When enteric fever is caused by *Salmonella* paratyphi A, B or C, it is called paratyphoid fever. Clinical differences in signs, symptoms, and outcomes between typhoid and paratyphoid are very small (Ochiai *et al*., 2008).

Materials and methods

Location of Studies

This study was carried out at the Federal Medical Center, Yenagoa, Bayelsa State, Department of Medical Microbiology and Nigerian Institute of Medical Research, Biotechnology and Molecular Biology Unit, Yaba-Lagos, Nigeria.

Collection of Samples

A total of 486 stool samples were collected from patients with fever at health centers in yenagoa. Samples were collected according to routine laboratory procedures into universal vials. A sample loop was inoculated into selenite F medium for the preliminary isolation of *Salmonella* spp. Routine bacteriological examinations were immediately performed in the Department of Medical Microbiology at the Federal Medical Center in Yenagoa, Bayelsa State.

Stool Analysis and Biochemical Identification

Samples in selenite F medium were incubated for approximately 18–20hrs to recover the isolates, then the broths were inoculated on *Salmonella–Shigella* agar (SSA) prepared according to the manufacturer's instructions. The streak plate method was used and incubated for 24hrs to observe the black center colony. Suspected strains were subcultured on SSA and incubated for 24hrs to obtain pure strains of *Salmonella* Typh*i*, a pure strain of 189 suspected colonies obtained from the above cultures was screened by various biochemical tests for the differentiation and identification of isolated *Salmonella* species, based on hydrogen sulfide (H_2S) production, gas production, indole, urease, sugar fermentation and mobility assays.

PCR Amplification of the STR, STN and the AroC genes

Polymerase chain reaction was carried out to amplify the STR, STN (fliC gene) and the Aroc gene using the primer pairs with sequence as shown in Table 1. PCR reaction was performed using Solis Biodyne 5X FIREPol Master mix. PCR was performed in 20 µl of the reaction mixture and the reaction concentration was reduced from 5x concentration to 1X concentration containing 1X primary mixed PCR buffer (Solis Biodyne), 1.5 mM MgCl2, 200 µM of each deoxynucleoside triphosphates (dNTPs) (Solis Biodyne), 20 pMol of each primer (Jena Bioscience, Germany), 2 units of FIREPol DNA polymerase (Solis Biodyne), 2 µl DNA extracts and sterile distilled water were used to form reaction mixture. Thermal cycling was performed in an Eppendorf Vapo protected thermal cycler (Nexus series) for initial denaturation at 95°C for 5 min, followed by 35 amplification cycles for 30 s at 95°C; 30 s at (see corresponding annealing temperatures for each primer pair in Table 1) and 1 min at 72°C. This was followed by a final 10 min extension at 72°C. The amplified product was separated on a 1.5% agarose gel and electrophoresis was performed at 80V for 1 h 30 min. After electrophoresis, DNA bands were visualized by ethidium bromide staining. A 100 bp DNA ladder (Solis Biodyne) was used as a DNA molecular weight marker.

Table.1; Primers for detection of *Salmonella* spp

Primers	Oligonucleotide Sequence (5/ - 3/)	Annealing Temperature
STR (F) STR (R)	TAT GCC GCT ACA TAT GAT GAG TTA ACG CAG TAA AGA GAG	50°C
STN (F) STN (R)	ACT GCT AAA ACC ACT ACT TGG AGA CTT CGG TCG CGT AG	50°C
aroCs for aroCs rev	GGCACCAGTATTGGCCTGCT CATATGCGCCACAATGTGTTG	57°C

Antibiotic Susceptibility

With the aid of the Kirby-Bauer disc diffusion method, the susceptibility of the bacterial isolates to various antibiotics was ascertained. Ofloxacin (5 mg), Ciprofloxacin (5 mg), Gentamicin (10 mg), Ceftazidine (30 mg), Nitrofuratoin (300 mg), Augmentin (30 mg), Cefixime (5 mg), and Cefuroxime (30 mg) were among the antibacterial substances present on the discs. Oxoid sensitivity test agar plates were swabbed with bacteria stock solution cells that had already been calibrated to 0.5 McFarland's turbidity. After that, the discs were carefully positioned on the agar with sterile forceps before being incubated for 24 hours at 37°C. A meter rule was used to measure the zones of sensitivity.

Result

Salmonella Typhi was detected in approximately 189 presumptive isolates of *Salmonella* spp. using routine biochemical tests and stool culture. As shown in Table 2, the results of biochemical screening of the presumed *Salmonella* species isolate revealed a total of six Salmonella typhi (6, 3.17%), which were subsequently subjected to molecular identification.

Table 2: Results of *Salmonella* obtained during isolations of *Salmonella* Typhi

Methodology		Number of isolates (N=189) (Cultural)	Percentage (%)
Cultural and Biochemical test		6	3.17%
Molecular (PCR)	*S*. Typhi	1	0.53
	S. Typhimuirium	2	1.06
Total		9	4.76

The outcomes of the polymerase chain reaction-based identification of *Salmonellae* using the AroC gene sequence for the identification of the six (6) presumed *S*. Typhi are shown in Figure 1 below. Only two of the six isolates were confirmed to be Salmonella typhimurium, according to the results, which showed that the amplification product of 639 bps from the six isolates is indicative of the bacterium.

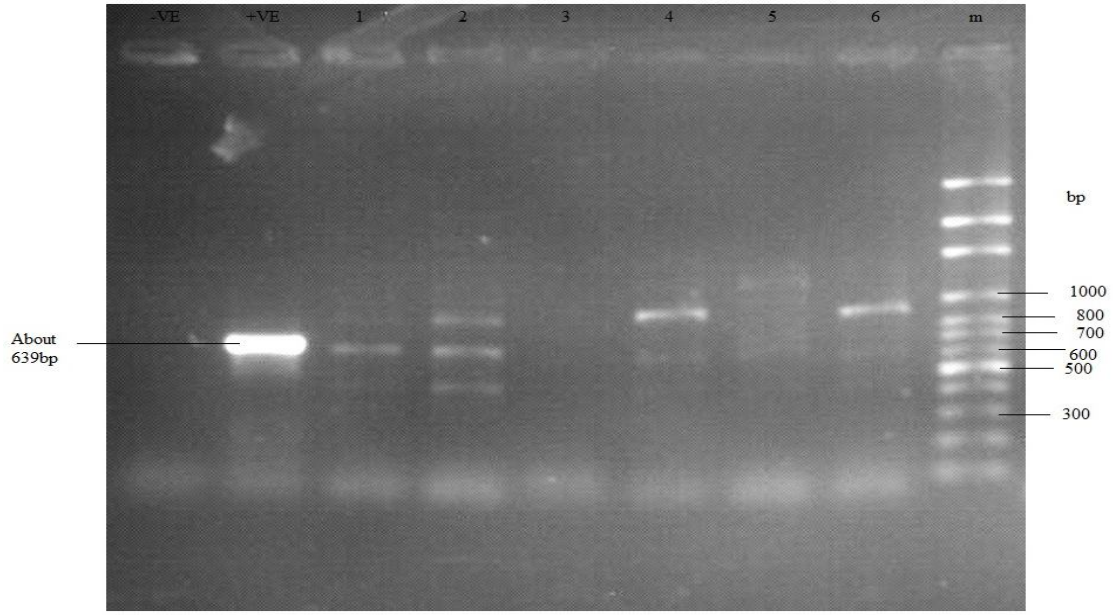

Figure.1: Confirmation of *Salmonella* genus by *aroC* gene amplification
+ve control *Salmonella* Typhimurium. (639bp)

Figure 2 shows the final identification of *S.* Typhi using the fliC gene sequence. Only one of the six tested isolates produced an amplification product of 495 bp, according to the results. Despite the difficulty in isolating and identifying *S.* Typhi, the AroC detected in isolates

demonstrated the close relatedness of the isolates to *S*. Typhi, and as a result, the primer sequence specific for the flagella unique to *S*. Typhi reveals its specificity.

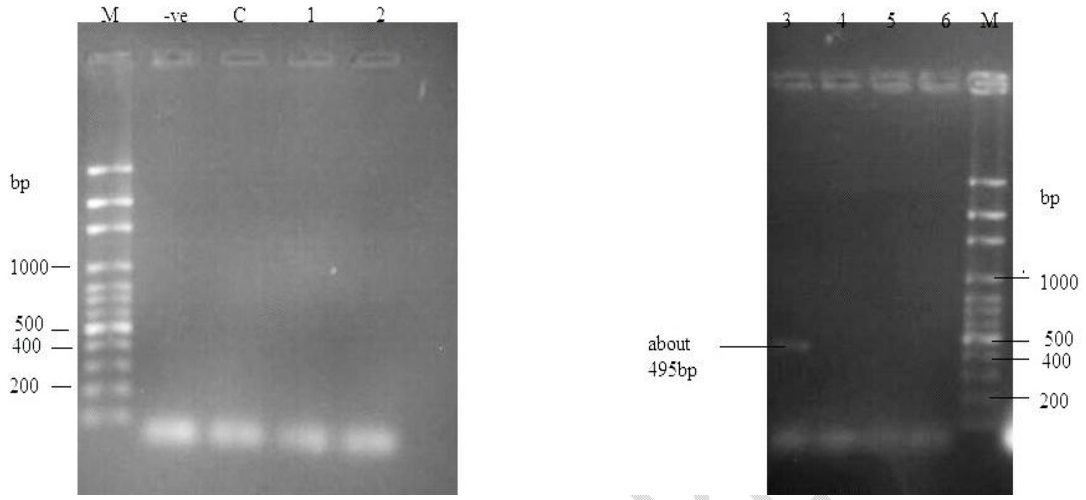

Figure. 2: Confirmation of *Salmonellae enterica serovar* Typhi by amplification of *fliC* gene

The antibiotic profile of *Salmonella* spp. against the following antibiotics is shown on Figure 3 below. Ofloxacin (16.7%-resistance and 83.3% sensitivity), Augmentin (83.3%-resistance and 16.7% sensitivity), Nitrofuratoin (100% resistance), Ciprofloxacin (66.7% resistance and 33.3% sensitivity), Ceftazidine (33.3% resistance and 66.7% sensitivity), Cefuroxime (50% resistance and 50% sensitive), Gentamicin (100% resistance), Cefixime (66.7% resistance and 33 *Salmonella* had a 100% resistance to gentamicin and nitrofurantoin. On the other hand, ofloxacin and ceftazidine showed the highest susceptibilities, with values of 83.3% and 66.7%, respectively. There were different levels of susceptibility and resistance in the other

organisms.

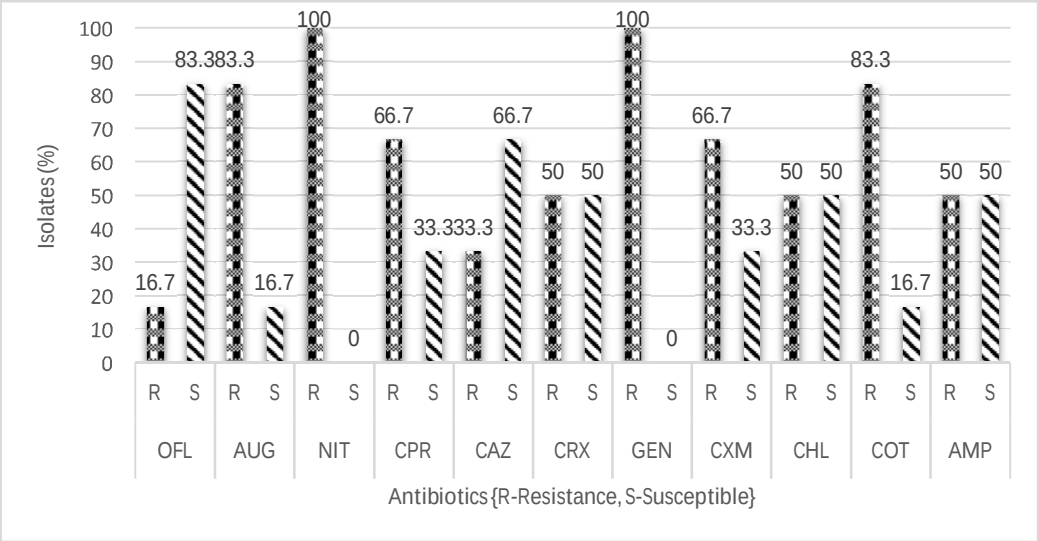

Figure 3 Percentage of *Salmonelae* resistance to antibiotics

In Figure 4 below the comparative percentage of antibiotics susceptibilty-resistance pattern of *Salmonellae*, shows that 55% of isolates were resistant to all antibiotics, while 45% were susceptible.

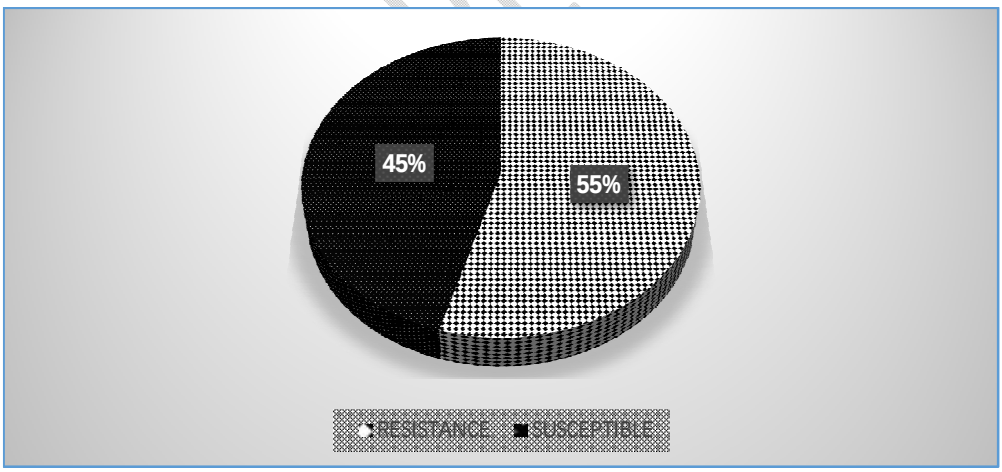

Figure 4 Results showing the percentage resistance of *Salmonelae* to antibiotics

4.2. Discussion

This study demonstrated the reliability of the isolation of *S.* Typhi by polymerase chain reaction. The identification process revealed that two strains of *S.* Tyhimurium were

closely related to *S*. Typhi, but *S* typhi and *S*. Typhimurium using the same procedure gave similar results until molecular means were used to identify and differentiate them.

Although bacteriological culture is still the gold standard for determining typhoid fever, its use has been constrained by the acute febrile illness because it is not always readily available. When a clinical picture for an acute febrile illness in an area with an endemic typhoid outbreak is unclear, a quick, precise, sensitive test that can distinguish between typhoid and non-typhoid febrile illnesses should be used. The majority of the time, clinicians choose to treat rather than wait for the results of stool or blood cultures, which could take 3-5 days. While this strategy may have some value, it would be more beneficial to use rapid antigen screening directly from the suspected patient's stool, especially in places where culture facilities are scarce or nonexistent and Widal testing is the norm. (Olopoenia *et al*., 2000).

This study also noted the high percentage of *Salmonella* species isolates that were resistant to antibiotics like Gentamicin (100%), Augumentin (83.3%), and Cotrimoxazole (83.3%), as well as Ampicillin, resistance to floroquinolones, and reduced susceptibility to those antibiotics as reported by Adeshina *et al*. (2009). However, the country's widespread use of quinolones in animal feeds as well as the use of animal waste to improve the soil for fruits that are consumed raw may be to blame for the rise in resistant *Salmonella* species. The two antibiotics to which bacteria were most susceptible were nitrofurantoin (100%) and ofloxacin (83.3%). There have been previous reports of the ineffectiveness of ampicillin, chloramphenicol, gentamicine, ofloxacin, ceflaximide, polymixin B, carbencillin, and tetracycline against *Salmonella* spp. (Adachi *et al*., 2005; Filioussis *et al*., 2008). The different patterns of microbial isolates across studies highlight the necessity of surveillance to assess and periodically monitor the shifting patterns of the microflora, particularly in a hospital setting (Mordi and Momoh, 2009). Typhoid diagnosis and early drug resistance pattern identification are now of utmost importance due to the emergence and ongoing

growth of multiple drug resistance. Typhoid severity is directly correlated with multidrug resistance. According to Mutai *et al*. (2018) and Britto *et al*. (2020), the association has been attributed to incorrect initial treatment, which led to further disease progression and subsequent hospital presentations. The widespread use of antimicrobial agents, which also caused the emergence of multidrug-resistant (MDR) strains, is likely to blame for the sharp rise in the frequency of resistance in *S*. Typhi. The prevalence of MDR *S*. Typhi strains, which are resistant to chloramphenicol, ampicillin, and trimethoprim, has raised concerns in recent years (Mourad *et al*., 1993; Accou-Demartin *et al*., 2011). Drug resistance was found to be independently correlated with higher bacteremias in a multivariate analysis. This suggests that *S*. Typhi virulence and the multidrug-resistant phenotype may be related (Accou-Demartin *et al*., 2011).

Plasmid-mediated multidrug resistance in *S*. typhi is frequently caused by genes carried on large transferable plasmids. There are many different plasmids present in MDR *S*. Typhi, but those of the incHI1 incompatibility type seem to be particularly prevalent. In particular, plasmids from the incompatibility complex group incHI1 are frequently responsible for encoding resistance to drugs like tetracycline, ampicillin, trimethoprim, chloramphenicol, and trimethoprim (Hampton *et al.*, 1998). Their frequent occurrence may be explained by an enhanced plasmid-related transmission potential compared to drug-sensitive strains (Accou-Demartin *et al.*, 2011).

Conclusion

Enteric fever is a significant public health issue, particularly in developing nations. *S*. Typhi strains that are resistant to the majority of useful antibiotics emerge and spread globally, morbidity and mortality linked to typhoid fever are rising. Because typhoid fever has an insidious onset and no specific symptoms, the clinical diagnosis is frequently incorrect. In Nigeria, stool culture is the method that is frequently used to identify drug resistance in

typhoid patients. It has a high positive predictive value, but it also has some drawbacks. For instance, the results are not available for nearly four to five days, making it impossible to detect disease early on. Since its detection rate is only about 30%, its insensitivity is another significant drawback. It is blamed on a number of things, most notably the patient's prior antibiotic administration. In addition to being a waste of the limited resources available for healthcare, missed alternative diagnoses also contribute to the rise in antimicrobial resistance. As a result, there is a pressing need for the creation of an efficient method that can not only detect typhoid at an early stage but also give medical professionals the information they need to begin targeted treatment as soon as the disease manifests itself by assessing the level of drug resistance. Another crucial aspect is the procedure's cost effectiveness, which needs to be on par with current approaches.

A significant development in this area has been made possible by molecular techniques because they focus on the pathogen itself. In addition to being more specific, these are also quicker and more accurate. The Polymerase Chain Reaction (PCR), a straightforward, quick, and effective method of gene amplification, has quickly grown in popularity among molecular biologists and has a wide range of potential uses in the detection and treatment of infectious diseases. Typhoid can already be diagnosed quickly, sensitively, and specifically using PCR (Haque *et al.*, 2001). Therefore, it was thought that the development of a multiplex PCR that targeted particular genes associated with both drug resistance and diagnosis would be quite beneficial. The speed makes it easier to characterize isolates and measure drug resistance genes, and it allows for quicker implementation. There were a few instances that were also ciprofloxacin resistant (Figure 3). Its much higher sensitivity suggested the molecular identification method over stool culture. Biochemical identification yielded six positive results, whereas molecular confirmation yielded just one case of *S.* Typhi and two related strains of *S.* Typhimurium, as opposed to 189 positive stool

cultures. Better sensitivity is one thing, but the amount of time required is another. While stool culture followed by drug sensitivity testing using the disc diffusion method requires at least five days, multiplex PCR can produce results in as little as 24 hours. These are the characteristics that demonstrate this method's superiority to all others and that will allow the clinician to make a quick, accurate diagnosis.

The study demonstrates the need for a multistep typhoid and fever diagnosis in clinical settings because it will allow for a more accurate identification of the underlying causes rather than just making assumptions based on symptoms. However, open, efficient, and timely communication between those providing laboratory services and those in the public health and food safety domains charged with surveillance and intervention is the most fundamental requirement in the application of laboratory characterisation of isolates to the protection of public health, not the sophistication of the laboratory methods. The patient will recover quickly with the help of prompt and targeted treatment, and money that would have been spent on hit-and-miss care will also be saved. Typhoid bacteria must be eradicated quickly and completely to reduce the carrier

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