

Molecular detection of Salmonella serovars in Retailed Raw Meat samples using 16SrRNA, sitC and fliC Virulence Genes in Lagos, Nigeria.

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Abstract:

Introduction: The presence of virulence genes such as *sitC* and *fliC* are known among *Salmonella* serovars of clinical interest. The common sources of *Salmonella* include dairy products, beef, poultry, eggs and vegetables. This study was aimed at identifying salmonella using 16S ribosomal RNA gene (16SrRNA), and *sitC* and *fliC* virulence genes in raw meat samples commonly sold in open market place genotypically with PCR methods.

Methods: The study observed the presence of *Salmonella* isolated from 124 raw meat samples using conventional phenotypic methods and analysing for presence of *sitC* and *fliC* virulence genes. For molecular analysis, the 16S rRNAs of strains of *Salmonella* were used to confirm the result of the conventional method. DNA extraction and quantification was carried out followed by Polymerase Chain Reaction. The PCR products were subjected to agarose gel electrophoresis.

Result: A total of 124 isolates were collected from animal sources. Of the 124 samples, 30 *Salmonella* isolates were positive after repeated sub culturing, biochemical and serological tests. Nineteen (63%) of the 30 isolates were positive for 16SrRNAs, five (17%) for *sitC* while the *fliC* was negative.

Conclusion: The PCR assay has proven to an efficient method for the identification of virulence genes in *Salmonella* serovars. The presence of 16SrRNAs and *sitC* genes showed availability of the virulence genes in retailled raw meat samples commonly sold in Nigeria. More studies are required to track virulence factors among *Salmonella* isolates in Nigeria.

Keywords: Genes, Nigeria, PCR, *Salmonella*, Virulence

I. Introduction

Salmonella belong to the family Enterobacteriaceae, and responsible for most foodborne infections posing a major public health challenge worldwide [1]. Infection with *Salmonella* can lead to serious and potentially fatal infections including bacteraemia, septic arthritis, meningitis and pneumonia [2]. Faecal-oral transmission of *Salmonella* occurs most commonly through the consumption of contaminated food causing salmonellosis in humans [3].

Salmonella enterica contains about 2,600 serovars and are classified into six subspecies with high sequence resemblance [4]. The most frequently isolated serovars from foodborne outbreaks are *Salmonella enterica* serovars *S. typhimurium* and *S. enteritidis*. *Salmonella* relies on multiple virulence factors, many of which are clustered within *Salmonella* pathogenicity islands (SPIs) to cause a disease, and five (1-5) SPIs have been identified so far [5]. The 16S ribosomal RNA (16SrRNA) is one of the *Salmonella* pathogenicity island I virulence genes [6]. The use of 16SrRNA gene sequencing within the regulatory workflow may help to reduce the time and labor involved in the identification and differentiation of *Salmonella enterica* isolates. *Salmonella enterica* is subdivided into six subspecies: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI), the more commonly isolated subspecies are; (II, IIIa, IIIb, IV) [7], and Subspecies I is most responsible for infections in human and domestic animal infections. The *Salmonella* serovars are clinically recognized according to the disease they cause; typhoid or enteric are life threatening infections and non-typhoidal serovars which cause self-limiting gastroenteritis [8,9]

The serovars are known to cause majority of infection in both humans and animals resulting in major economic loss. *Salmonella* outbreaks have been implicated with improper handling of food by food handlers [10], and different *Salmonella* pathogens have various host ranges; some isolates infect animal and plant hosts, while others have specific host.

In developed countries up to 5% cases of NTS may be invasive, extra-intestinal disease leading to bacteremia, but in Sub-Saharan Africa the invasive non-typhoidal *Salmonella* (iNTS) is responsible for high morbidity and mortality in adults and children, with an estimated annual incidence of 175-388 cases per 100 000 children and 2000-7500 cases per 100 000 HIV-infected adults [11].

The remarkable feature of *fliC* alleles is the sequence conservation of distal parts of the gene, thus making the gene of any serotype suitable for easy amplification. Most *Salmonella* strains possess two structural genes (*fliC* and *fliB*) coding for flagellins [12].

The *sitC* (*Salmonella* iron transporter C) gene is a virulence gene found in *Salmonella enterica* strains which is involved in iron transport, its broad action includes invasion of macrophages and iron acquisition. The *sitC* is found in *Salmonella* Pathogenicity Island 1 (SPI-1) [13,14].

The advent of molecular methods for diagnosis of infectious diseases has improved the sensitivity, specificity and quality of treatment. PCR studies have shown the specificity of some gene primers such as 16S ribosomal RNA (*16SrRNA*) to detect and discriminate between *Salmonella* and non-*Salmonella* species [6]. The common sources of *Salmonella* include dairy products, beef, poultry, eggs and vegetables. Detection of the virulence genes is essential to understanding of the etiologic agent which may provide insight into their relative pathogenicity, treatment and develop prophylactic strategies for salmonellosis. This study is aimed at identifying *Salmonella* present among retailled raw meat samples in Nigeria using primers; *16SrRNA*, *sitC* and *fliC* virulence genes which are specific to *Salmonella* detection and diagnosis

II. Materials and Methods

2.1. *Salmonella* isolates

A total of thirty samples of raw meat were collected from Mushin market, Lagos between August and September 2013. These samples selected were suspected for possible contamination by *Salmonella* during the retail handling and storage process of raw meats. The collected samples were placed aseptically in polyethylene bags, labeled and refrigerated at -4°C for about 24 h before processing. The use of pre-enrichment broth of samples was performed as described earlier [16]. We used 25g of the meat samples to homogenize in 225mL of buffered peptone water broth, incubated at 37 °C for 24 h. The culture from pre-enriched broth was then divided into two aliquots; first aliquot was subjected to conventional cultural method. All the isolates that produced H₂S on deoxycholate agar plates were subjected to biochemical tests (IMViC tests; "I- indole test, M-methyl red test, V-Voges-Proskauer test, and C-citrate test; Kligler Iron Agar (KIA) test, Motility Indole Urea (MIU) test and Simmons' Citrate test), while the second aliquot was used for DNA extraction by boiling method followed by agarose gel electrophoresis and PCR to confirm the presence of *Salmonella*.

2.2 Serological test

The Serological testing was performed using the *Salmonella* test kit (Oxoid), with 25 mg samples were homogenized with 225 mL buffered peptone water medium and incubated for 15-18 h at 37 °C. 1 mL of pre-enriched broth culture were transferred to 9 mL of selenite cysteine enrichment broth culture and incubated at 37 °C for 24 h. One drop of the selenite cysteine enrichment broth culture using a Pasteur pipette were dropped within one circle on the reaction card and mixed with 1 drop of the Oxoid *Salmonella* Latex Reagent to the broth on the card. The suspension was mixed thoroughly using a clean mixing stick or inoculating loop, and then rocked the reaction card gently for 2 or 3 times. The agglutination was examined within a maximum of 2 min.

2.3 Genomic DNA extraction

Three colonies of each isolate were picked and suspended in 200 µl of distilled water and mixed by vortexing. Genomic DNA was extracted by the boiling method and briefly 1.5ml of the sample in broth was centrifuged at 10,000rpm for 5 minutes. The supernatant was discarded and the pellets were washed twice with sterile water. After this, 200µl of sterile water was added to the pellets, the pellets were vortexed to homogenize and boiled in a dry bath at 100°C for 10 minutes. This was followed by vortexing and centrifugation at 12,000rpm for 5 minutes. The supernatant was transferred into another pre-labelled eppendorf tube by gentle aspiration using a micropipette. The supernatant contains the DNA needed for PCR assay, and was stored at -20°C. The concentration and purity of the extracted DNA was estimated using a nanodrop spectrophotometer and the integrity of the DNA was assessed by 1% agarose gel electrophoresis.

2.5 Primers: *16SrRNA* (F-5' TGT TGT GGT TAA TAA CCG CA 3' and R- 5' CAC AAA TCC ATC TCT GGA 3'), *sitC* (F-5' CAG TAT ATG CTC AAC GCG ATG TGG GTC TCC 3' and R-5' CGG GGC GAA AAT AAA GGC TGT GAT GAA C 3') and Flagellin (F-5' AAG GAA AAG ATC ATC WAY 3' and R-5' TTA ACC CAG TAA AGA GAG 3').

2.5 Polymerase chain reaction (PCR) procedure using 16SrRNA, sitC and fliC primers PCR was conducted in a volume of 25µl containing 2 µl of genomic DNA from the salmonella isolates, 12.5µl mastermix, 2 µl of each primers specific for *16SrRNA*, *sitC* and *fliC* gene and 8.5 µl of deionized distilled water (DDW). The cycling

conditions for amplification in a Gene Amp PCR System 9700 thermal cycler (Applied Biosystem, India) was as follows; *16SrRNA* primer-initial denaturation step of 95°C for 3mins followed by 30 cycles consisting of 30sec at 95°C, 30s at 54.1°C, and a final extension step of 1 min at 72°C, *sitC* - 94°C for 3mins followed by 30cycles consisting of; a denaturation step of 2 mins at 94°C, 1mi at 55°C and final extension step of 1 min at 72°C, *fliC* primer: 94°C for 2mins followed by 35cycles each consisting of 30s at 94°C, 30s at 55°C and an extension step of 72°C for 1min. the above PCR cycling conditions were based on the preliminary experiments performed to optimize amplification of *salmonella* target sequences. Following electrophoresis, the gel was stained with ethidium bromide and photographed under transilluminator ultraviolet (UV) light. A 100 bp DNA ladder (MBI Fermentas USA) were used as markers for determining the molecular weight of PCR products ([17]. The amplified products were electrophoresed for the presence of desired bands on 1.5% agarose gel.

III. Result 3.1 PCR identification of *Salmonella* using the *16SrRNA* primer

Nineteen (63%) isolates of the thirty (30) samples showed amplification for the *16SrRNA* gene using the primer set *16SrRNA* (F-5' TGT TGT GGT TAA TAA CCG CA 3' and R- 5' CAC AAA TCC ATC TCT GGA 3'). The molecular weight of the amplicon was 574bp as shown in plate 1. The positive control (*Salmonella typhimurium*) also contained the amplified 574bp DNA fragment. Plate 1 which showed Lanes 1 to 4 is a representative of the amplification result for the *16SrRNA* gene.

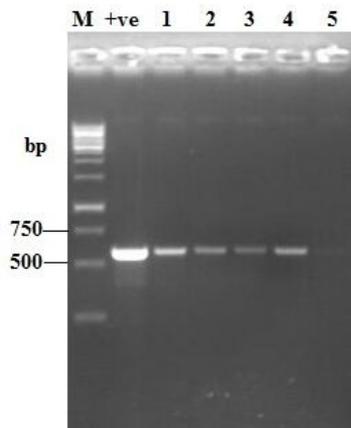


Plate 1: Representative sample of amplified 574-bp DNA fragment of the *16SrRNA* gene. Lane M = 1 kilobase pair ladder, Lane + = positive control, Lane 5 = Negative, Lane 1 to 4 positive.

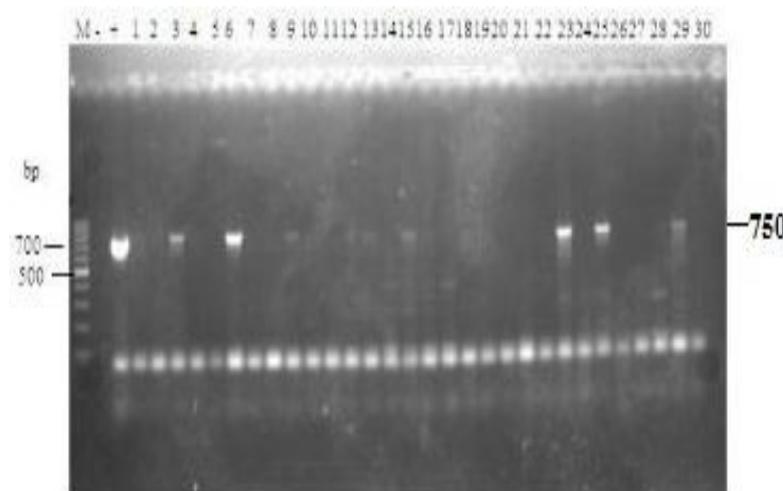


Plate 2: Agarose gel electrophoresis of amplified 750-bp DNA fragment of the *sitC* gene. Lane M = 100 base pair marker, Lane - = negative control, Lane + = positive control, Lanes 3, 6, 23, 25, 28 = positive (5).

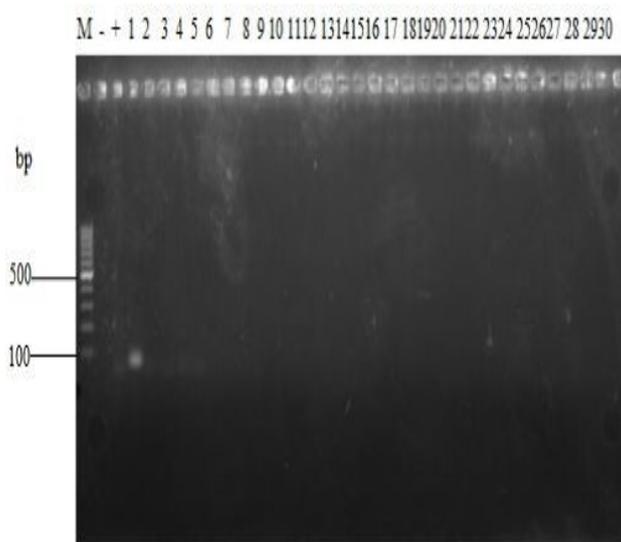


Plate 3: Agarose gel electrophoresis of *flagellin* gene using *flagellin/fliC* primer. Lane M = 100 base pair marker, Lane -ve = negative control, Lane +ve = positive control (which showed no amplification for the gene), Lanes 1 to 30 = negative.

IV. Discussion

Strains of *Salmonella* are considered as important pathogens and a leading cause of bacterial food-borne illness popularly known as Salmonellosis and is a major public health problem. Genomic variations play an important role in bacterial identification, it is necessary to use molecular techniques known to be sensitive and specific to identify suspected *Salmonella* serovars [18].

In this study, PCR method based on the 16SrRNA *sitC* and *fliC* targeting *Salmonella* virulence genes were used to determine 30 isolates of *Salmonella* in Nigeria. The results showed that 63% of 16SrRNA and 17% of the isolates matched with the *Salmonella typhimurium*. The PCR analyses of the virulence genes demonstrated suitable target genes for discriminating among *Salmonella* serotypes. Studies have also shown that this method is rapid and reproducible in identification of isolates [19]. These results showed that these methods can potentially be applied for identification of *Salmonella typhimurium*.

Various primers sets targeting *Salmonella* 16SrRNA, *sitC* and *fliC* genes have been designed for research purposes [20, 21]. 16SrRNA primer can be used for the detection of *Salmonella* in food samples (Lin and Tsen, 1996). The 16SrRNA primer amplified DNA from most of the *Salmonella* strains successfully. Recent study on the molecular epidemiology and in vitro antimicrobial susceptibility of *Salmonella* isolated from poultry in Kashmir valley, India showed that all the isolates of *Salmonella* were tested by genus-specific polymerase chain reaction (PCR), using the 16S ribosomal RNA (rRNA) primers [22,23].

Out of a total of 30 suspected *Salmonella* isolate samples used for this study, the 16SrRNA primer set amplified 19 samples as positives with an amplicon size of 574bp, and this corroborated earlier work done [13] that non-typhoidal *Salmonella* isolates from human, animal and food products in The Gambia and Senegal for the presence of twelve virulence genes including *sitC*. The *sitC* gene was found in at least 70% of the isolates tested [13], and this showed a significant association between the *sitC* virulence gene and resistance to commonly used antibiotics in West African region. Our work on the other hand showed 17% of *sitC* gene with amplicon size of 750bp. This also corroborated earlier reported use of *sitC* gene in differentiation and detection of *Salmonella typhi* in range of animal and human origin, and this gene was found to be associated with severity of salmonellosis in humans [24, 20].

We did not observe *fliC* gene amplification in any of the samples suggesting that the identified *Salmonella* species may lack *fliC* gene or may be due to inadequate primer amplification.

Adequate sanitary measures have led to a decrease in cases of typhoidal *Salmonella* in developed countries and most cases are associated with travellers returning from endemic areas in developing countries. In sub-Saharan Africa such as the Gambia and Senegal, non-typhoidal *Salmonella* (NTS) becoming a major cause of invasive disease in infants, young children, and HIV-infected adults and are associated with substantial mortality [13].

In Nigeria, morbidity associated with illnesses due to *Salmonella* continues to be on the increase and, in some cases, resulting in death [25]. Proper characterization of *Salmonella* isolates is essential for investigations of *Salmonella* outbreaks as non-typhoidal *Salmonella* strains have reached epidemic proportions in many countries despite improvements in sanitation and hygiene [26]. In view of limited adherence to high standards of hygiene,

the probability of bacterial contamination of food at various stages of processing is always very high [27]. *Salmonella* species used for research purposes are usually isolated from contaminated food and clinical samples which are mostly due to faecal-oral routes [4].

This result showed a recognisable distribution of *16SrRNA* and *sitC* virulence genes in *Salmonella* species gotten from meat samples sourced from Nigeria. The most prevalent gene was *16SrRNA* (63%), and this suggests that *16SrRNA* can be used for the identification and confirmation of *Salmonella* in food samples using the PCR method.

V. Conclusion

Infection caused by *Salmonella* species remains an important public health problem especially in developing countries. This study showed that virulence genes are present in non-typhoidal *Salmonella* isolates found in Nigeria. The *16SrRNA* is a suitable molecular tool to diagnose *Salmonella* in animal products. Identification of *Salmonella* serovars through screening of virulence genes by PCR methods can have benefits in public health specifically for rapid diagnosis, development of treatment and epidemiological investigations. More studies are required to track availability and emergence of virulence factors in *Salmonella* isolates in Nigeria.

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Conflict of Interest Authors declare that they have no conflict of interest.

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