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Molecular Characterization of *Salmonella* Species Isolates from Some Hospitals in Jos, Nigeria

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ABSTRACT

The conventional methods of identification of Salmonella involving microbiological enrichment and successive identification mostly are tedious, time consuming and not specific. Therefore, the aim of this study was to utilize molecular techniques to characterize Salmonella species isolates from some Hospitals in Jos, Nigeria. The 10 isolates collected from some Hospitals in Jos, Nigeria were screened for Salmonella using conventional biochemical methods. The positive isolates were identified using polymerase chain reaction (PCR) for discernment of invasion A (invA) gene at explicit molecular size (284 bp) utilizing explicit primers (forward and reverse). Sequencing of the invA gene was performed and the similarities and differences between our invA gene and published sequences on GenBank were assessed. Seven out of ten confirmed Salmonella species isolates were positive to the invA gene while the remaining three were negative. The homology level of nucleotide sequence (97.746%) demonstrated high similitude between the local isolates and the other sequences on GenBank. Molecular characterization of the Salmonella isolates provides data about the virulence of the pathogen just as its relatedness to different organisms which offer data about the genome of the organisms and are helpful for epidemiological examinations. Therefore, Molecular methods which enable the detection of virulent genes are extremely important surveillance tools that are required to assist in curbing the escalation of infections caused by Salmonella.

Keywords: Invasion A gene, polymerase chain reaction, primer, *Salmonella*.

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I. Introduction

Salmonella is a Gram-negative, rod-shaped, motile facultative anaerobe that causes salmonellosis. *Salmonella typhi* and *Salmonell aparatyphi* are pathogenic absolutely for humans, causing systemic diseases and typhoid fever, whilst *Salmonella enterica* serovar *typhimurium* causes gastroenteritis [1]. The genus *Salmonella* is a member of the family of Enterobacteriaceae. The genus consists of two species, *S. bongori* and *S. enterica*, the latteris divided into six subspecies: *S. e. enterica*, *S. e. salamae*, *S. e. arizonae*, *S. e. diarizonae*, *S. e. houtenae*, and *S. e. indica*. The scientific classification group consists of more than 2500 serotypes (also serovars) characterized based on the somatic O (lipopolysaccharide) and flagellar H antigens (the Kauffman–White classification). *Salmonella* have been clinically grouped as invasive (typhoidal) or non-invasive (non-typhoidal) *Salmonella* in light of host preference and expressions in humans. Salmonellosis persists as a major public health issue in many parts of the world and endemic infection in the tropical and subtropical areas [2]. *Salmonella*, a major inhabitant of the gastrointestinal tract, is perceived as one of the most widely recognized reasons for food borne infections around the world. The disease is experienced worldwide however, it is principally found in developing nations where sanitary conditions are poor [3]. In tropical nations as well as Nigeria where the infection is frequently experienced, it provides explanation for several cases of morbidities and death [4]. The prevalence of *Salmonella* infections has continued to rise and misdiagnosis which often leads to complications in the treatment and recurrence of infections. Molecular characterization of *Salmonella* species and detection of virulent gene is critical for rapid and reliable detection and identification of *Salmonella* species which helps healthcare providers in proper treatment of infections caused by *Salmonella* as traditional methods of isolation and identification such as cultural, biochemical and serological methods have been found to be tedious, time consuming, non-specific and non-sensitive. Furthermore, little molecular analysis has been done on *Salmonella* species obtained from hospitals in Nigeria. The invA gene encodes a protein in the inner membrane of bacteria, which is essential for intrusion of epithelial cells of the host and it contains unique nucleotide sequences specific to the genus *Salmonella* which has been demonstrated as a specific PCR target [5]. The invA target gene of *Salmonella* is situated on the pathogenicity island 1 (SPI-1) and has been utilized as a significant target for identification of *Salmonella* [6]. Many reports had also affirmed the effective detection of 100% of *Salmonella* isolates from poultry utilizing specific primers for the invA gene with no false positive or negative results [7]. Therefore, results of this research will provide more information on the molecular characterization of *Salmonella* species by invasion A (invA) gene detection in *Salmonella* species isolates obtained from some Hospitals in Jos, Nigeria. The aim of this research is to use molecular techniques to detect invA gene in *Salmonella* species isolates from some hospitals in Jos, Nigeria.

II. Materials and Methods

A. Conventional Identification

1. Collection of Salmonella Isolates

A total of ten suspected *Salmonella* specie isolates labelled A-J were collected from some hospitals in Jos, Nigeria. The number of isolates collected from each hospital was the number of available suspected *Salmonella* specie isolates. The isolates were Subcultured on *Salmonella-Shigella* Agar (SSA).

2. Biochemical Identification

Gram staining and biochemical analysis were done as confirmatory tests. The following biochemical tests were carried out as recommended for the biochemical screening of *Salmonella* species; Indole, Methyl red, Voges-Proskauer, Citrate, (IMViC), Triple Sugar Fermentation (TSI), Urease, catalase, Nitrate reduction and oxidase tests [8], [9].

B. Molecular Identification

1. Salmonella Genomic DNA Extraction

Salmonella Genomic DNA was extracted using Bioneer DNA extraction kit (Accuprep). Bacterial cell cultures in broth were pelleted by centrifugation at 8000 rpm for 5 mins. The supernatant was discarded, and the bacterial cell pellets were resuspended in a mixture of 20 µl of Proteinase K and 10 µl of RNAse that was initially incubated for 2 mins at room temperature. Two hundred microlitre (200 µl) of Genomic binding (GB) Buffer was added and brief vortexing was done to yield a homogeneous solution. It was incubated at 60 °C for 10 mins. Then, 400 µl of 100% ethanol (ice-cold) was added and mixed. The lysate was transferred into the upper reservoir of binding column tube without wetting the rim. The tubes were closed, centrifuged for 1min at 8000 rpm and the solution was discarded, binding column tube was transferred to a new tube for filtration. 500 µl of Wash Buffer 1, was added and centrifuged at 8000 rpm for 1 min. The flow through was discarded and the Spin Columns transferred into new clean Collection tubes followed by the addition of 500 µl of Wash Buffer 2 and centrifuged at 8000 rpm for 1 min. The flow through was discarded and the Spin Columns transferred to clean Collection tubes and centrifuged at 12000 rpm for 1 min to completely remove ethanol. The Spin Columns were transferred to sterile 1.5 ml Eppendorf tubes for elution followed by the addition of 200 µl of Elution buffer and kept for 1min until elution buffer was completely absorbed into the glass fibre of binding column tube before it was centrifuged for 1min at 8000 rpm to elute. The elution column was removed, and the bottle closed, stored at 4 °C for short term.

2. Polymerase Chain Reaction (PCR) Amplification

The extracted DNAs of the *Salmonella* isolates were subjected to PCR amplification to detect the Inva gene. Polymerase Chain Reaction was carried out using 20 µl of pre-mix (Accupower) containing taq (*Thermusaquaticus*) polymerase, dNTPs and $MgCl_2$. Sixteen microlitre (16 µl) of deionized water was added to the pre-mix, 2 µl of template DNA, 2 µl of forward and reverse Inva gene primer (forward and reverse) was added and then centrifuged. deionized water (18 µl) and 2 µl of forward and reverse Inva gene primer was used as negative control. The PCR conditions includes pre-denaturation at 94 ℃ for 5mins, denaturation at 94 °C for 30 secs, annealing at 52 °C for 30secs and extension at 72 °C for

1 min. Final extension at 72 °C for 5 mins. The PCR was completed in 35cycles [10].

3. Analysis of Genomic DNA by Gel Electrophoresis

Agarose gel powder (3 g) was weighed and dissolved in 100 ml of Tria-acetate-EDTA (TAE) buffer. The solution was heated in a microwave to dissolve and allowed to cool in a water bath set at 50-55 °C. A comb was placed in position in a gel casting chamber and 5µl of ethidium bromide was added to the cooled gel and then the gel was poured into the tray. It was allowed to cool for 15-30 mins at room temperature. The comb was removed, and the gel placed in an electrophoresis chamber and covered with TAE buffer. A molecular ladder (100 bp) was loaded in the first well followed by the PCR amplicons and the negative control was loaded in the last well and electrophoresed for 35mins. The gel was transferred to the UV Trans-illuminator where the bands were visualized.

4. Sequencing of the invA Gene

Large reaction PCR was performed using 4 premix tubes for one isolate and the gel electrophoresis was carried out using a bigger comb for casting the gel. Surgical blade was used to cut the band after visualizing under a UV transilluminator. The gel was weighed, and the DNA extracted. Sequencing reaction was prepared in 2.0 ml tube by adding 5.0 µl of DH_20,5.5 µl of DNA template,2.0 µl of invA primer, 8.0 µl of Dideoxy terminator cycle sequencing (DTCS) quick start master mix (Beckman Coulter). The sequence reaction was set up in the PCR machine and ran for 30 cycles. 0.5 ml sterile tube was labeled and 5 µl of the stop solution/glycogen mixture was added. The sequence reaction was transferred to the tube and mixed thoroughly. Sixty microliters (60 µl) of cold 95% ethanol was added and mixed. It was centrifuged at 14,000 rpm for 15 mins. The supernatant was removed with micropipette. The pellet was rinsed with 70% ethanol and centrifuged at 14,000 rpm for 2 mins. The supernatant was removed with a micropipette and dried in a vacuum. The sample was resuspended in 40 µl of loading solution and transferred to the appropriate well of the sample plate and overlaid with a drop of mineral oil. The sample plate was loaded into the instrument and ran [11].

C. Sequence Analyses

The results of the sequencing were analyzed using Basic Local Alignment Search Tool (BLAST), a web tool of the NCBI (National Center for Biotechnology Information). The evolutionary history was inferred using the Neighbor-Joining method [12]. The evolutionary distances were computed using the p-distance method [13] and are in the units of the number of base differences per site. Evolutionary analyses were conducted in Molecular Evolutionary Genetics Analysis (MEGA X) [14].

III. Results

Microscopically all the isolates were Gram negative and motile. Biochemically all of the isolates were found to be catalase, urease, indole and oxidase negative but Citrate, methyl red and nitrate reduction test positive. *All isolates* in TSI culture typically produced alkaline (red) slant and acid (yellow) butt, with production of H_2S (blackening of agar). All the isolates were identified and confirmed as *Salmonella* based on cultural, morphological and biochemical tests. Further identification was done by molecular method using invA gene amplification and sequencing. In view of the results of PCR, there were 7 samples from 10 samples of *Salmonella* isolates which detected an invA gene with a length of 284 bp, whereas 3 samples did not show the target band (Fig. 1). The sequence produced significant alignment in the GenBank (Table II) and the relationship with 8 representative sequences retrieved from GenBank was established (Fig. 2).

TABLE I: Biochemical Reactions of *Salmonella Species* Isolates from Some Hospitals in Jos, Nigeria

Sample	Ureas	Indole	Methyl red	Citrate	Catalase	Oxidase	Voges-Proskauer	Nitrate reduction	Glucose (TSI)	H_2s Production
A	-	-	+	+	-	-	-	+	K/A	+
B	-	-	+	+	-	-	-	+	K/A	+
C	-	-	+	+	-	-	-	+	K/A	+
D	-	-	+	+	-	-	-	+	K/A	+
E	-	-	+	+	-	-	-	+	K/A	+
F	-	-	+	+	-	-	-	+	K/A	+
G	-	-	+	+	-	-	-	+	K/A	+
H	-	-	+	+	-	-	-	+	K/A	+
I	-	-	+	+	-	-	-	+	K/A	+
J	-	-	+	+	-	-	-	+	K/A	+

Keys: + = POSITIVE; - = NEGATIVE.

TABLE II: Sequences Producing Significant Alignment in GenBank

Description	Accession Number
Salmonella enterica subsp. *enterica* strain 11AIxB Invasion protein (invA). gene. partial cds	KJ71884.1
Salmonella enterica subsp. *enterica* serovar *enteritidis* strain NCCP 16206 chromosome, complete genome	CP041973.1
Salmonella enterica strain R19.2839chromosome, complete genome	CP046429.1
Salmonella enterica strain FDAARGOS.668chromosome, complete genome	CP046280.1
Salmonella enterica subsp. *enterica* strain EQAS2016S1 chromosome, complete genome	CP033360.1
Salmonella enterica subsp. *enterica* serovar *enteritidis* strain AUSMDU00010527 chromosome, complete genome	CP045956.1
Salmonella enterica subsp. *enterica* serovar *enteritidis* strain AUSMDU00010528 chromosome, complete genome	CP045955.1
Salmonella enterica strain CFSAN096147 chromosome, complete genome	CP044257.1
Salmonella enterica subsp. *enterica* serovar *senftenberg* strain AR-0405 chromosome, complete genome	CP044203.1

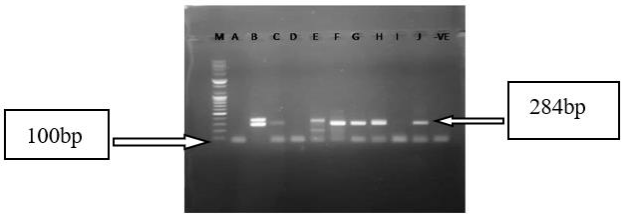

Fig. 1. Polymerase chain reaction amplification products of DNA of invA gene of *Salmonella spp*.

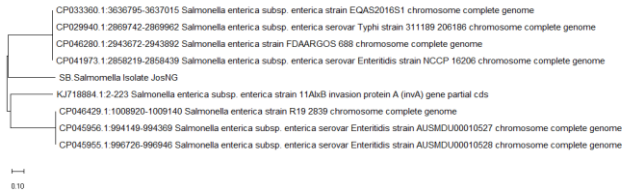

Fig. 2. SB *Salmonella* Isolate Jos NG showing the relationship with 8 representative sequences retrieved from GenBank.

The optimal tree with the sum of branch length = 1.51280337 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. This analysis involved 9 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 225 positions in the final dataset.

IV. Discussion

The nonappearance of target bands in 3 samples (Fig. 1) demonstrated that the strains of *Salmonella* bacteria identified were not invasive or may likewise utilize other invasive mechanisms like type III secretion system effectors [15]. The result showed that *Salmonella spp*. (DNA template) matched and bound with the invA gene primer at 284 bp. This value was same as stated by [16]. In a previous study, PCR using the invA primers has shown to be specific for detection of *Salmonella* spp [17]. Our result confirmed the finding of previous reports, which indicated that nearly all *Salmonella spp*. carries this gene [18]. This study additionally validates some of the previous research completed utilizing the PCR technique, where non-typhoidal *Salmonella* was isolated from animals, food, and human stool samples in developing nations [19]. Presently, *Salmonella* is identified by standard bacteriological, biochemical, and serological methods. These methods are generally tedious, non-specific, and time-consuming [20]. Identification of *Salmonella* by PCR with primers for invA gene is rapid, sensitive, and explicit to detect *Salmonella* in numerous clinical samples [21]. In a related study, [22] showed that molecular methods had higher sensitivity and specificity of detecting *Salmonella* species from fresh-frozen meat and poultry samples in Jordan using both molecular methods and conventional methods of identification. This study underpins the capacity of this explicit primer set to confirm putative *Salmonella* isolates from some Hospitals in Jos, Nigeria as *Salmonella* species. The invA gene is usually targeted for the identification of *Salmonella* bacteria at the genus level [23]. The invA primer used in this research to detect *Salmonella* species quickly and accurately were as previously reported by Darwin and Miller [24]. This is additionally in agreement with studies carried out by Pererat and Murray [25] that the results of PCR on different serotypes of *Salmonella* demonstrated positive outcomes, but PCR results on non-*Salmonella* strains, for example, *E. coli*, *Klebsiella*, *Proteus*, and *Shigella* were negative for the invA gene. DNA sequencing and bioinformatics analyses were performed to confirm the PCR results in our study and compared with related *Salmonella* sequences retrieved from the GenBank NCBI-BLAST database. Regarding the present study sequence, we found 97.76% sequence similarity to invA gene sequences of *S. enterica* strain 11AIxB.Phylogenetic analysis of nucleotide sequence of the invasion protein A (invA) gene showed that the sequence recovered from our isolate and 8 sequences from other isolates retrieved from GenBank (Table II) were grouped into two clusters. Cluster one showed that our study sequence SB *Salmonella* isolate JosNG was in a close relationship with strain EQAS2016SI, strain 311189 206186, strain FDAARGOS 688 and strain NCCP 16206 retrieved from GenBank. The second cluster showed that invA gene of strain 11AIxB is closely related to the R19 2839 strain, the strain AUSMDU00010527 and the strain AUSMDU00010528 retrieved from GenBank. The phylogenetic trees (Fig. 2) demonstrated that the invA gene of SB *Salmonella* isolate JosNG is closely related to the other *Salmonella* strains but in a separate sister cluster with invA gene of strain 11AIxB because of the presence of significant amino acid substitutions and due to some changes in nucleotides or that the SB *Salmonella* isolate JosNG is different from the compared invA gene strains 11AIxB. These results corroborate with the findings of Kingsley *et al*. [26] who documented that the isolates of DT2 formed a distinct phylogenetic cluster within *Salmonella typhimurium* serotypes. The result of invA alignment corresponds with reports of Galan and Curtiss [27] as well as Shi *et al*. [28] who documented that the invA is present and useful in almost all virulent *Salmonella* strains and its conserved gene. These discoveries can have significant advantages in public health, particularly for fast diagnosis and engineering the perfect vaccine, epidemiological investigations, and prophylactic strategies for Salmonellosis in Nigeria.

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