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Serotype Distribution and Virulence Profile of *Salmonella enterica* Serovars Isolated from Food Animals and Humans in Lagos Nigeria

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Distribution of *Salmonella enterica* serovars and their associated virulence determinants is wide-spread among food animals, which are continuously implicated in periodic salmonellosis outbreaks globally. The aim of this study was to determine and evaluate the diversity of five *Salmonella* serovar virulence genes (*invA, pefA, cdtB, spvC* and *iroN*) isolated from food animals and humans. Using standard microbiological techniques, *Salmonella* spp. were isolated from the feces of humans and three major food animals. Virulence determinants of the isolates were assayed using PCR. Clonal relatedness of the dominant serovar was determined via pulsed-field gel electrophoresis (PFGE) using the restriction enzyme, Xbal. Seventy one *Salmonella* spp. were isolated and serotyped into 44 serovars. Non-typhoidal *Salmonella* (NTS; 68) accounted for majority (95.8%) of the *Salmonella* serovars. Isolates from chicken (34) accounted for 47.9% of all isolates, out of which *S.* Budapest (14) was predominant (34.8%). However, the dominant *S.* Budapest serovars showed no genetic relatedness. The *invA* gene located on SPI-1 was detected in all isolates. Furthermore, 94% of the isolates from sheep harbored the *spvC* genes. The *iroN* gene was present in 50%, 100%, 88%, and 91% of isolates from human, chicken, sheep, and cattle, respectively. The *pefA* gene was detected in 18 isolates from chicken and a single isolate from sheep. Notably, having diverse *Salmonella* serovars containing plasmid encoded virulence genes circulating the food chain is of public health significance; hence, surveillance is required.

Keywords: **Pathogenicity Islands, PFGE, *Salmonella,* serotype, virulence factors**

Introduction

Salmonella remains a pathogen of concern in public health as it is a leading cause of intestinal illness around the world. *Salmonella* causes febrile illness and acute gastroenteritis in individuals of all ages depending on the serotype responsible for the infection [1, 2]. Non-typhoidal *Salmonella* (NTS) serovars are the major cause of *Salmonella* gastroenteritis which is usually self-limiting, however this could result in invasive systemic infection in infants, the elderly and immunocompromised people most especially in sub-Saharan Africa [3, 4]. Though *Salmonella* serovars are genetically related, they display wide variation in host specificity, disease manifestation and virulence. *Salmonella* comprise of two major species, *Salmonella enterica* and *Salmonella bongori* with the

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former divided into six subspecies comprising *S. enterica* subsp. enterica (I), *S. enterica* subsp. salamae (II), *S. enterica* subsp. arizonae (IIIa), *S. enterica* subsp. diarizonae (IIIb), *S. enterica* subsp. houtenae (IV) and *S. enterica* subsp. indica (VI). Subspecies I consist of over 1,367 serovars, majorly infecting warm blooded animals including humans [5, 6]. Virulence in *Salmonella* is associated with a combination of plasmid and chromosomal factors. The *invA* gene which is said to facilitate attachment and invasion of M-cells is chromosomally located. Hence it is presumed to be present in most, if not all *Salmonella enterica* serovars. The *invA* gene belong to *Salmonella* pathogenicity island one (SPI-1) which is associated with the type three secretion system (TIIIS) [7, 8]. Certain *Salmonella* serovars in subspecies I carry a vast low-copy number plasmids that contain virulence genes. spv (RABCD) essential for bacterial multiplication in the reticuloendothelial system is located on a 7.8 kb region of *Salmonella* virulence plasmid. Also plasmid borne are the fimbriae (*pef*) genes that mediate *Salmonella* intestinal adhesion. The *pef* locus accommodates four genes *pef* (BACDI) [9–11]. The food chain plays a significant role in *Salmonella* infections thus several foods could serve as a vehicle of transmission [12]. Major outbreaks of Salmonellosis have been linked to the consumption of contaminated foods mainly foods of animal origin such as beef, pork, poultry and milk [13–15]. Serotyping and virulotyping are useful tools in *Salmonella* epidemiology [16], hence this study explores the serovar distribution and virulence gene relationship of *Salmonella enterica* serovars isolated from three major food animals and humans in Lagos.

Materials and Methods

Study design and sample collection

This study comprised of two groups: (1) apparently healthy food handlers and (2) three major food animals (Chicken, sheep and cattle). Three hundred and fifty eight five stool samples were collected from group one, while three hundred and six stool samples were collected from the viscera (intestine) of the food animals that make up group two at the point of slaughter. Samples were collected in sterile universal bottles and transported in a thermo box at 4℃ to the laboratory for immediate processing.

Isolation and identification of isolates

Five grams of each faecal sample were enriched in 25 ml of selenite F broth (Oxoid, UK) and incubated at 37℃ for 18–24 h. This was followed by plating unto *Salmonella-Shigella* (SS) agar (Oxoid) and incubated at 37℃ for 24 h. Presumptive colonies were further purified by subculturing on nutrient agar (UK). Pure colonies were identified using biochemical tests [17].

Serotyping

Serotyping was done by slide agglutination test to characterize O and H antigens according to White Kauffmann-Le Minor scheme [18] using commercially available antisera (Bio-Rad F-92430 Marnes La Coquette, France).

Pulse field gel electrophoresis (PFGE)

Pulse field gel electrophoresis was carried out in line with PulseNet standardized laboratory protocol with slight modification. Isolates of *Salmonella* Budapest with the control strain Braenderup [H9812] were inoculated into Brain Heart Infusion (BHI) broth and incubated at 37℃ for 6 h and then cultured unto Tryptic Soy Agar (Oxoid) with 5% defibrinated sheep blood and incubated overnight at 37℃. Cell suspension was made by transferring colonies of isolates with a sterile cotton swab into cell suspension buffer (100 mM Tris: 100 mM EDTA, pH 8.0). Cell suspensions were adjusted to 6.8 and 7.0 using a DENSIMAT (Biomerieux SA, France) and kept in ice. Agarose plugs were prepared by adding 20 µl of 20 mg/ml proteinase K (Promaga Madison, USA) to 200 µl of cell suspension and 280 µl of a 1% agarose LF^{TM} (Amresco, USA)/1% Sodium Dodecyl Sulphate (SDS) ultrapure grade (Amresco) mixture held at 55℃ was added and mixed gently then applied carefully to plug molds. Lysing was accomplished by treating agarose plugs in cell lysis buffer (50 mM Tris: 50 mM EDTA, pH 8.0 + 1% sarcosyl) with 30 µl proteinase K (20 mg/ml) for 2 h at 55℃ and then temperature was reduced to 37℃ overnight. Plugs were washed twice with pre-heated sterile deionized water and six times with pre-heated TE buffer. All washing was done for 15 min at 55℃ in a shaker. Restriction digest was done by 25 µl of Xbal 10 U/µl (Thermo Fisher Scientific Inc., USA) at 37℃ overnight. One percent agarose (LF^{TM} Amresco) in 0.5 X Tris-Borate

EDTA buffer was used in casting agarose gel and PFGE was performed in a CHEF DR III system (Bio-Rad, USA) with running parameter set as initial switch time 2.2 s, final switch time 63.8 s, Voltage, 6 V, included angle 120° and running time 19 h. After electrophoresis gel was stained with ethidium bromide for 30 min and de-stained for 25 min then viewed using Gel Doc™ EZ imager (Bio-Rad) and analyzed with GelJ .v.2.0 [19] using the Dice coefficient and unweighted pair group method (UPGMA) a dendrogram was generated.

DNA extraction

Phenol-chloroform-isoamyl alcohol method of DNA extraction according to Adi *et al*. [20] was adapted with slight modifications.

Detection of virulence genes by polymerase chain reaction (PCR)

Five virulence genes were assayed for in all the 71 *Salmonella* isolates using primers (Table 1) targeting *invA*, *spvC*, *iroN*, *pefA* and *cdtB*. A 50 µl PCR reaction was used which contained 28.8 µl of nuclease free water, 3.5 µl $MgCl_2$ (25 mM), 10 µl 5x PCR buffer, 1 µl dNTPs (10 mM), 0.75 µl of each forward and reverse primers (10 µM), 0.2 µl One Taq DNA polymerase (5,000 U/ml) (New England Biolabs) and 5 µl DNA template. Multiplex PCR was used for *invA*/*spvC* and *pefA*/*iroN* while simplex PCR was used for *cdtB*. PCR programming conditions included:

invA/*spvC*

Thirty five cycles of Initial denaturation 94℃ for 2 min, denaturation 94℃ for 30 s, annealing at 56℃ for 30 s, elongation 72℃ for 2 min and final elongation as 72℃ for 10 min.

pefA/*iroN*

Thirty five cycles of Initial denaturation 94℃ for 2 min, denaturation 94℃ for 30 s, annealing at 65℃ for 30 s, elongation 72℃ for 1 min and final elongation as 72℃ for 10 min.

cdtB

Thirty five cycles of Initial denaturation 94℃ for 2 min, denaturation 94℃ for 30 s, annealing at 60℃ for 30 s, elongation 72℃ for 1 min and final elongation as 72℃ for 10 min.

PCR products were separated on a 1.5% agarose gel at 120 V and a 100 bp DNA ladder (New England Biolabs) was used as molecular weight maker.

Ethical approval

Ethical approval for this study was obtained from the Human Research and Ethical Committee (HREC) of the Lagos University Teaching Hospital with code number ADM/DCST/HREC/APP/1118 and Nigerian Institute of Medical Research- Institutional Review Board, with project number IRB/12/180.

Results

Isolation and identification

A total of 71 *Salmonella* isolates were recovered from human, cattle, sheep and chicken faeces which was serotyped into 44 serovars. *Salmonella* Budapest had the highest level of occurrence (31.8%) followed by *Salmonella*

Table 1. Primers used in the detection of virulence genes.

Primer	Sequence	Size (bp)	Reference
invA F	5'-ACAGTGCTCGTTTACGACCTGAAT-3′	260	Chiu and Ou [21]
invA R	5'-AGACGACTGGTACTGATCGATAAT-3'		
spvC F	5'-ACAGTGCTCGTTTACGACCTGAAT-3'	589	Chiu and Ou [21]
spvC R	5'-AGACGACTGGTACTGATCGATAAT-3'		
cdtB F	5'-GAAGCCGTTTATTTTTGTAGAGGAGATGTT-3'	268	Mezal *et al.* [22]
cdtB R	5'-ACAACTGTCGCATCTCGCCCCGTCATT-3'		
pefA F	5'-AGGGAATTCTTCTTGCTTCCATTCCATTATTGCACTGGG-3'	506	Mkrtchyan *et al.* [6]
pefA R	5'-TCTGTCGACGGGGGATTATTTGTAAGCCACT-3'		
iroN F	5'-ACTGGCACGGCTCGCTGTCGCTCTAT-3'	1205	Mezal *et al.* [22]
iroN R	5'-CGCTTTACCGCCGTTCTGCCACTGC-3'		

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Table 2. *Salmonella* enterica serotypes isolated from humans and food animals.

SN	Serotype	Frequency of occurrence	Source
1	*Salmonella* Limete	1	Human
2	*Salmonella* Portland	1	Human
3	*Salmonella* Huettwillen	1	Human
4	*Salmonella* Mowanjum	1	Human
5	*Salmonella* Paratyphi C	2	Human
6	*Salmonella* Tyhpimurium	1	Human
7	*Salmonella* Takoradi	1	Human
8	*Salmonella* Paratyphi B	1	Human
9	*Salmonella* Chagoua	1	Human
10	*Salmonella* Onireke	1	Cattle
11	*Salmonella* Somone	1	Cattle
12	*Salmonella* Farmsen	4	Cattle
13	*Salmonella* Ketheabarny	1	Cattle
14	*Salmonella* Vom	1	Cattle
15	*Salmonella* II (43,:g,z_{62}:enx)	1	Cattle
16	*Salmonella* Sandiego	1	Cattle
17	*Salmonella* Budapest	14	Cattle/Chicken
18	*Salmonella* Carno	1	Chicken
19	*Salmonella* Anecho	3	Chicken
20	*Salmonella* Muenster	1	Chicken
21	*Salmonella* Kaapstad	1	Chicken
22	*Salmonella* Agodi	2	Chicken
23	*Salmonella* Dabou	1	Chicken
24	*Salmonella* Tennyson	1	Chicken
25	*Salmonella* Goldcoast	1	Chicken
26	*Salmonella* Ebrie	1	Chicken
27	*Salmonella* Brandenburg	1	Chicken
28	*Salmonella* Alfort	1	Chicken
29	*Salmonella* Minna	1	Chicken
30	*Salmonella* Linton	1	Chicken
31	*Salmonella* Wichita	1	Chicken
32	*Salmonella* Ealing	1	Chicken
33	*Salmonella* Essen	7	Chicken/Sheep
34	*Salmonella* Livingstone	1	Sheep
35	*Salmonella* Kivu	1	Sheep
36	*Salmonella* Berlin	1	Sheep
37	*Salmonella* Dahra	2	Sheep
38	*Salmonella* Mono	1	Sheep
39	*Salmonella* Mura	1	Sheep
40	*Salmonella* Chomedey	1	Sheep
41	*Salmonella* Yovokome	1	Sheep
42	*Salmonella* Sculcoates	1	Sheep
43	*Salmonella* Wien	1	Sheep
44	*Salmonella* Orion	1	Sheep
Total	44	71	Sheep

Essen (15.9%). Thirteen *Salmonella* Budapest were isolated from chicken and one was isolated from cattle. Also three *Salmonella* Essen isolates were isolated from chicken and four from sheep. Non-typhoidal *Salmonella* serovars dominated with only two typhoidal (*Salmonella* Paratyphi B and *Salmonella* Paratyphi C) serovars isolated from humans (Table 2). Isolates from chicken accounted for 47.9% of all isolates.

Pulse field gel electrophoresis (PFGE)

A total of four clusters and three single isolates were obtained from PFGE analysis of *S.* Budapest as shown in Fig. 1. Isolates that had more than three DNA fragment difference with similarity less than 80% were considered to have originated from different clones, thus were not the same.

Detection of virulence genes by polymerase chain reaction (PCR)

Of the five virulence genes (*invA, spvC, iroN, pefA* and *cdtB*) assayed for in all 71 *Salmonella* isolates, only *cdtB* was not detected. *invA* gene was detected in all *Salmonella* isolates from humans, chicken, cattle, and sheep. Ninety four percent (94%) of isolates from sheep harbored *spvC* genes closely followed by isolates from cattle as shown in Table 3.

Discussion

Salmonella enterica serovars isolated displayed high level of diversity with 44 serovars. Chicken harbored the highest number of isolates (48%) and serovars. This is to be expected as poultry remains a major source of *Salmonella* enterica serovars [23]. Fagbamila *et al.* [24] reported 82 different serotypes of 370 *Salmonella* isolated from poultry farms in Nigeria, with *Salmonella* Kentucky having the highest rate of occurrence. However in this study *Salmonella* Budapest had the highest rate of occurrence with 13 *S.* Budapest isolated from chicken and one isolated from cattle. PFGE analysis to determine clonal relatedness of *S.* Budapest isolates revealed that they were not from the same clone indicating that *S.* Budapest could be wide spread among food animals. NTS serovars accounted for majority of *Salmonella* serovars from all three food animals with two Paratyphi C and one

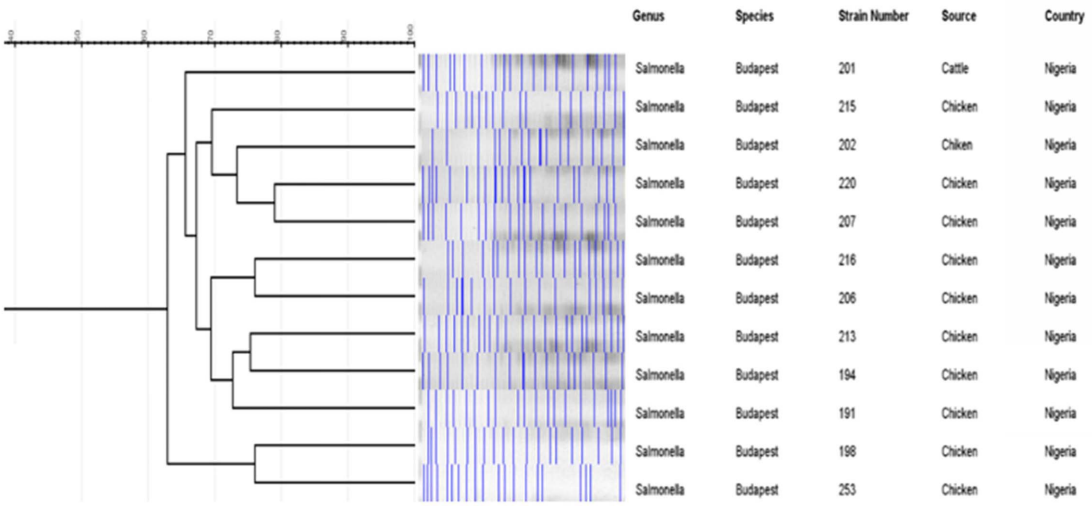

Fig. 1. Dendrogram obtained from DNA fragments generated by XbaI and Pulsed Field Gel Electrophoresis using GelJ. V.2.0. A total of 4 clusters were generated.

Paratyphi B isolated from human subjects. NTS serovars have a vast host range including ruminants and birds with their products acting as vehicle for the transmission of *Salmonella* infections to humans and have been implicated in bacterial bloodstream infections in children and adults in sub-Saharan Africa [22, 25, 26]. The invasiveness or pathology of *Salmonella* infection is determined by the status of the host and pathogen, which in this case are the virulence genes. Majority of these genes are clustered in regions on the chromosome called *Salmonella* Pathogenicity Islands (SPIs) [27]. In this study *invA* gene located on SPI-1, that facilitate attachment and invasion was detected in all isolates (100%) obtained from humans and all three food animals investigated. This is in line with the findings of El-Feky *et al.* [28] that detected *invA* gene in all NTS isolates from food products and clinical samples in Egypt. In a similar study, Smith *et al.* [29] reported 96.1% positive *invA Salmonella* spp. isolated from food samples in Lagos Nigeria. This points to the fact that *invA* gene could be a valuable tool in molecular epidemiological surveillance of *Salmonella* spp. in cases of outbreaks since *invA* gene is chromosomally located. Plasmid borne virulence genes code genes that are needed for systemic infection in host cells [30]. *pefA* and *spvC* genes that contribute to adhesion and systemic infection respectively [8] were detected in this study. *spvC* was detected in 94% of isolates from sheep and 91% of isolates from cattle, while isolates from chicken and humans were 56% and 40% positive for the gene respectively. This is similar with the findings of Borges *et al.* [31] that reported *spvC* in 92% of *Salmonella* isolates from chicken. Only 53% of *Salmonella* isolates from chicken had *pefA* and one isolate from sheep. The absence of *pefA* genes is expected to contribute to host adaptation and possibly lower outbreak potential [32]. On the other hand the presence of *spvC* and *pefA* genes are of public health significance as this could increase morbidity and mortality when isolates are implicated in both human and animal infections. Furthermore the circulation of these plasmid mediated genes in the food chain could result in emergence of virulent strains of otherwise non-virulent *Salmonella* serovars because the *Salmonella* plasmid virulence(spv) locus of which *spvC* is a part of confers complete virulence on non-virulent *Salmonella* strains [16]. *cdtB* gene, a gene responsible for the production of toxin which plays a role in cellular

Table 3. Percentage occurrence of virulence genes detected in isolates from humans, chicken, sheep and cattle.

Virulence gene	Human n = 10	Chicken n = 34	Sheep n = 16	Cattle n = 11
invA	10 (100%)	34 (100%)	16 (100%)	11 (100%)
spvC	4 (40%)	19 (56%)	15 (94%)	10 (91%)
iroN	5 (50%)	34 (100%)	14 (88%)	10 (91%)
pefA	0	18 (53%)	1 (6%)	0
cdtB	0	0	0	0

distension of host cells and not limited to typhoidal strains only [8, 33] was not detected in any of the isolates. Thus these strains may not cause acute gastroenteritis in human infection. However the presence or absence of a few virulence determinants does not imply that a particular *Salmonella* serovar cannot elicit disease, though the predominance of multiple virulence genes could indicate a potential for severe infections in humans [34]. *iroN*, a gene responsible for iron acquisition [22] was detected in all *Salmonella* serovars obtained from chicken and 50% of serovars isolated from humans. Results from this study will give a better insight into the epidemiology and pathogenicity of *Salmonella* enterica serovars circulating in the food chain, since there is a continuous expansion of host range in *Salmonella* through the acquisition of new genes through horizontal gene transfer of plasmid and pathogenicity islands [35]. Proactive and dynamic surveillance is recommended in order to have updated information on strain evolution, expansion and pathogenicity of *Salmonella* enterica circulating the food chain to forestall *Salmonella* infection outbreaks.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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