Research Article

Biological Resolution of Virulence Genes of *Salmonella* Species from different Microbiomes

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Abstract

The pathogenic promiscuity of virulence associated macromolecules in *Salmonella* infection is a key driver to their wide epidemiology and curtailing such distribution is contingent upon proper clarification of these virulence genes. This study was therefore aimed at determining the virulence genes of *Salmonella species* from different microbiomes. To achieve this, a total of three hundred (300) biological specimens were aseptically collected and processed for *Salmonella* presence using the BAM USFDA technique prior to their genotypic characterization while virulence gene detection was carried out in a primer specific polymerase chain reaction. Results obtained depict the distribution of the following *Salmonella species* viz; *Salmonella gallinarum* 19(26.39%), *Salmonella heidelberg* 19(26.39%), *Salmonella enteritidis* 18(25%) and *Salmonella typhimurium* 16(22.22%) while the occurrence of the virulence genes (*InvA*, *SopE*, *AgfA* and *SpvC*) were *Salmonella enteritidis* (7(38.8), 6(33.3), 9(50), 3(16.7), *Salmonella typhimurium* (5(26.3), 3(15.8), 2(10.5), 7(36.8)), *Salmonella heidelberg* (0(0), 8(50), 4(25), 4(25), and *Salmonella gallinarum* (12(63.2), 6(31.6), 2(10.5), 7(36.8)) respectively. It was however found that the different microbiomes analyzed were ubiquitously rich in virulence genes associated *Salmonella* species.

Keywords; Virulence, Microbiomes, Biological resolution, *Salmonella* species

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Résumé

La promiscuité pathogène des macromolécules associées à la virulence dans l'infection à Salmonella est un facteur clé de leur large épidémiologie et la réduction de cette distribution dépend de la clarification appropriée de ces gènes de virulence. Cette étude visait donc à déterminer les gènes de virulence des espèces de Salmonella de différents microbiomes. Pour ce faire, un total de trois cents (300) échantillons biologiques ont été collectés et traités de manière aseptique pour la présence de Salmonella à l'aide de la technique BAM USFDA avant leur caractérisation génotypique tandis que la détection du gène de virulence a été effectuée dans une réaction en chaîne par polymérase spécifique à l'amorce. Les résultats obtenus décrivent la distribution des espèces de Salmonella suivantes, à savoir ; Salmonella gallinarum 19(26,39%), Salmonella heidelberg 19(26,39%), Salmonella enteritidis 18(25%) et Salmonella typhimurium 16(22,22%) alors que la présence des gènes de virulence (InvA, SopE, AgfA et SpvC) était Salmonella enteritidis (7(38,8), 6(33,3), 9(50), 3(16,7), Salmonella typhimurium (5(26,3), 3(15,8), 2(10,5), 7(36,8)), Salmonella heidelberg (0(0), 8(50), 4(25), 4(25) et Salmonella gallinarum (12(63.2), 6(31.6), 2(10.5), 7(36.8)) respectivement. différents microbiomes analysés étaient ubiquitairement riches en gènes de virulence associés aux espèces de Salmonella

Mots clés: Virulence, Microbiomes, Résolution biologique, Espèces de Salmonella

Introduction

The biological resolution of virulence genes in microorganisms is central to understanding the genetic diversity of these organisms (Thomas *et al.,* 2017) and this may be an important tool for circumventing the increasing trend of virulence gene-associated microorganism's infections (Borges *et al.,* 2013). Increasingly, the continuous association of these organisms with different infections including food poisoning has been long documented (Hardy, 2004; Chaudhary *et al.*, 2015). They are also of public health significance due to the number of hospitalizations and even death accompanying their presence in different environments (Boyle *et al.,* 2007; Turgeon *et al.,* 2017). *Salmonella* species, which is our subject of concern are known to be Gram negative motile rod-shaped facultative anaerobes with over 200 species and a lot of them are well adapted to different microbiomes (Maurer, 2007). The widespread epidemiology of *Salmonella* spp. coupled with their ubiquitous presence in different living and non living matter in addition to the recent elevated multi drug resistance of Gram negative bacteria including *Salmonella* species makes them more notorious, thereby eliciting a great deal of public concern (Hur *et al.,* 2012; Thomas *et al.,* 2012a; Thomas *et al.,* 2012b; Folorunsho *et al.,* 2015; Agu *et al.,* 2018; Popoola *et al.,*2018; Popoola *et al.,* 2019).

In Nigeria, several authors have described the presence of these organisms in different samples such as food (Smith *et al.,* 2012), water (Odeyemi, 2015), poultry products amongst others (Andino *et al.*, 2014), while studies concentrating on the havoc they cause on humans cannot be underestimated (Fashae *et al.,* 2010). Presently, what is of particular interest to us is the form of *Salmonella* transmission and the way they transport their virulence genes from one point to the other (Lawley *et al.,* 2008; Van Asten and Van Dijk, 2005). Even though, it is not impossible to find studies reporting how virulence genes are disseminated within *Salmonella* strains in developed countries (Lawley *et al.,* 2008; Moreno *et al.,* 2012), such studies are seldom reported in

developing countries (Moreno *et al.,* 2012) including Nigeria (Smith *et al.,* 2012; Odeyemi, 2015) . These virulence genes include *spvC, InvA, spvR, stn, fimA* (Borges *et al.,* 2013; Chaudhary *et al.,* 2015).

Generally, the dissemination of genes within and between organisms including virulence genes may be plasmid mediated and/ or chromosomal bound (Jiang et al., 2015; Pinilla-Redondo et al., 2018; Thomas et al., 2015). When it is plasmid mediated, it may infer the possibility of horizontal transfer of virulence genes within and between different microbiomes (Cruz and Davies, 2000) and this may subsequently aggravate their pathogenicity on animal and/or humans (Chaudhary et al., 2015). Hence, present study was aimed at determining the virulence genes of Salmonella species recovered from different microbiomes in order to elucidate the role of mobility of virulence genes in Salmonella species in the generation of increased pathogenecity in a typical African setting.

Materials and Methods

Sample Source and Sampling

A total of three hundred (300) samples were collected according to the statistical scheme of International Commission for Microbiological Specification for Foods (ICMSF, 2002) with a slight modification. These samples which were aseptically collected from different microbiomes in pre-sterilized aluminum pan, were transported to the Medical Microbiology unit of the Department of Microbiology, Olabisi Onabanjo University, Ago Iwoye, Ogun State, Nigeria on ice block for further analysis prior to isolation of *Salmonella* spp. The details of the collected samples are appropriately recorded in table 1.

Isolation and Characterization of *Salmonella spp.*

Each of the sample collected was appropriately prepared and processed for *Salmonella* identification following the recommended technique of BAM USFDA with slight modifications (Andrews *et al.,* 2011). Briefly, toilet bowl, abattoir table, knife and butcher's hand samples were collected with sterile swabs and then immersed in different McCartney bottles containing Dey-Engley broth (Casein enzymatic hydrolysate 5.00g/L, Yeast extract 2.50g/L, Dextrose10.00 g/L, Sodium thiosulfate 6.00 g/L, Sodium thioglycollate1.00 g/L, Sodium bisulfate 2.50 g/L, Lecithin 7.00 g/L, Polysorbate 805.00 g/L, Bromocresol purple 0.02 g/L). For cockroaches, they were disinfected with 70% alcohol prior to dissection to obtain the gut specimen. The gut specimen obtained and the legs of houseflies were aseptically transferred into different *Salmonella-Shigella* broth (Oxoid, UK). Food samples including water and fish as well as poultry droppings were weighed differently to obtain 25g each in a wide mouthed container containing 225ml tetrathionate broth without brilliant green dye (Oxoid, UK). The mixtures were gently shaken to enhance homogeneity and then incubated at 37°C for an initial period of 1hour which served as inoculum for subsequent analyses. The inoculums (**0.1** ml mixture) were subsequently inoculated onto *Salmonella-Shigella* Agar (SSA) (Oxoid, UK) by streaking method after which the plates were incubated at 37°C for 24 hours. The emerging colonies were subsequently sub-cultured on nutrient agar for purity plating prior to identification with molecular technique. The molecular characterization of the *Salmonella* spp was done polyphasically. Firstly, each of the isolates was inoculated directly into 200 ml sterile saline and extracted using a QIAamp DNA mini kit (Qiagen) according to the manufacturer's protocol. The extracted DNA was then amplified by PCR using a pair of universal bacterial primers 27F (52 -AGAGTTTGATCCTGGCTCAG-32) and 926R

(52 -CCGTCAATTCACTTTAGAGTTT-32) to amplify the internal fragments of the 16S rRNA genes in the genomic DNA obtained from the samples (Frank *et al.,* 2001) encompassing highly conserved regions. PCRs were performed in 0.2 ml reaction tubes in a final volume of 50ìl containing 10ng of DNA, 1.5 ì Platinum Taq DNA polymerase (In vitrogen), 200 mM each of dNTPs, 20 mM Tris/HCl (pH 8.4), 50 mM $MgCl_2$. The amplification reaction included incubation at 95°C for 5 min for *Taq* activation, followed by 35 cycles at 95°C for 30s, 62°C for 1 min and 72°C for 2 min, with a final extension step at 72°C for 5 min. The amplified products were separated by electrophoresis in 2% agarose gels and visualized under UV light; purified bands of DNA were sequenced using a 310 auto Genetic Analyzer (PerkinElmer, Applied Biosystems Div., Waltham, USA) with the same primers. DNA sequences were then analyzed using the BLAST database and assigned to the reference isolate sequences with the highest bit score.

Table 1: Sources of samples used in this study

S/N	Type of sample	Number of samples	Time of collection
1	Toilet bowl	30	07/05/2021
2	Food	30	03/06/2021
3	Water	30	04/08/2021
4	Poultry droppings	30	13/04/2021
5	Abbattoir table swab	30	08/05/2021
6	Knife swab	30	02/05/2018
7	Butcher hand swab	30	07/05/2018
8	Cockroach gut	30	07/09/2018
9	Housefly leg	30	15/10/2018
10	Fish	30	27/02/2021

Molecular characterization of virulence genes of *Salmonella spp.*

This was carried out as described by Chaudhary *et al.* (2015) with slight modifications. Briefly, the DNA of *Salmonella* was prepared by using the commercial kit (Qiagen) according to manufacturer's instruction. Amplification of virulence genes (*InvA, SopE, AgfA and SpvC*) were done with primer specific to each of the virulence gene listed earlier. The complete details of the primers used is listed in table 2. The PCR protocol involved was performed in a final volume of 25 µl containing DNA template (3 µl), X2 PCR Mastermix (12.5 µl), 10 pmol/ µl of each primer (MWG-Biotech AG, Germany) (1 µl) and 5.5 µl nuclease-free water. The reaction conditions involved initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 63°C for 30 s and 72°C for 30 s. A final extension of 5 min at 72°C was employed. The resulting amplicon were separated by electrophoresis on 2% agarose gel stained with 5 µl/ml of ethidium bromide with a 100 bp DNA ladder as molecular weight marker.

Table 2: *Salmonella* virulence gene primers used for the detection of *InvA*, *spvC*, *sopE* and *agfA* genes in the isolates.

Primer Target	Primer Sequence (5'-3')	Annealing Temp.	Length (bp)	Reference(s)
InvA	F: GTG AAA TTA TCG CCA CGT TCG GGC AA	63	289	chaundhary *et al.,* 2015
	R: TCA TCG CAC CGT CAA AGG AAC C			chaundhary *et al.,* 2015
SpvC	F: ACT CCT TGC ACA ACC AAA TGC GGA	63	669	chaundhary *et al.,* 2015
	R; TGT CTT CTG CAT TTC GCC ACC ATC A			chaundhary *et al.,* 2015
	F: ACA CAC TTT CCA CGA GGA AGC G			
SopE	R: GGA TGC CTT CTG ATG TTG ACT GG	63	398	Rahman *et al.,* 2005
	F: TCC GGC CCG GAC TCA ACG			
AgfA	R: CAG CGC GGC GTT ATA CCG	63	350	Rahman *et al.,* 2005

Results

Table 3 depicts sample specific distribution of *Salmonella spp* from different microbiomes. As shown in this study, all the samples examined harbored different loads of *Salmonella spp*. The highest number of *Salmonella spp* was found in the water samples analyzed (11(15.3%), followed by toilet bowl specimens (9(12.5%) while abattoir table, butcher hand swabs and housefly legs occupied the third position with a predominant rate of 8(11.11%). In total, a universal set of 72 *Salmonella* isolates were recovered from the different microbiomes examined. The specie-specific distribution of *Salmonella* was consequently reported in table 4. In this table, four (4) different serovars of *Salmonella spp* were identified with following prevalence: *Salmonella gallinarum* 19(26.39%), *Salmonella heidelberg* 19(26.39%), *Salmonella enteritidis* 18(25%) and *Salmonella typhimurium* 16(22.22%). Of all the samples analyzed, water specimens obtained from the abattoir yielded more *Salmonella* growth than other specimens. We, however, observed that except for the food samples that show no growth of *Salmonella gallinarum*, *Salmonella heidelberg* and *Salmonella typhimurium*, abattoir samples that reveals no *Salmonella typhimurium* growth and both knife swabs and cockroach guts that connote no *Salmonella enteritidis* growth, no other sample yielded no growth of all the recovered *Salmonella spp*. These *Salmonella spp* harbor varying rates of virulence genes with the following distribution pattern *InvA* 24(33.3%), *SopE* 23(31.9%), *AgfA* 17(23.6%) and *SpvC* 21(29.1%). A total of nine (9) *Salmonella spp* (4 *Salmonella enteritidis*, 3 *Salmonella typhimurium,* 2 *Salmonella heidelberg* and 0 *Salmonella gallinarum* were found not to harbor any of the genes. The PCR amplification of some of the virulence genes are shown in plate A to plate D.

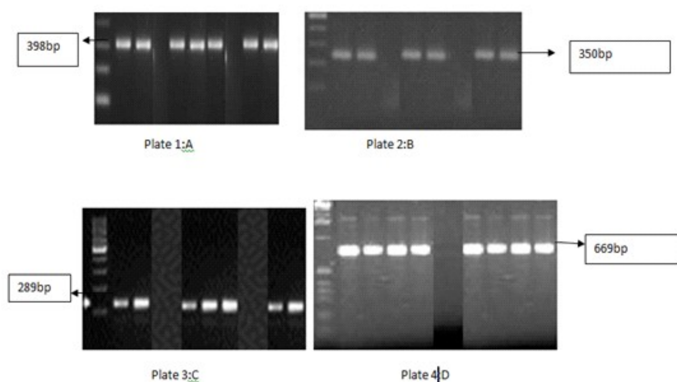

Plate 1: A, Plate2: B, Plate3: C, Plate4: D = Gel electrophoresis result of *sopE* gene (398bp), *agfA* gene (350bp), *InvA* gene (289bp), and *spvC* gene (669 bp)

Table 3: Sample specific distribution of Salmonella spp.

Type of Sample	N	(%)
Toilet bowl	9	(12.5)
Food	3	(4.17)
Water	11	(15.3)
Poultry dropping	7	(9.72)
Abbattoir table swab	8	(11.11)
Knife swab	6	(8.33)
Butcher hand swab	8	(11.11)
Cockroach gut	5	(6.94)
Housefly legs	8	(11.11)
Fish	7	(9.72)
N	72	(100)

Table 4: Specie-specific distribution of *Salmonella spp* in different samples

Type of sample	SE	SG	ST	SH	Total
Toilet bowl	2	2	3	2	9
Food	3	-	-	-	3
Water	4	3	3	1	11
Poultry droppings	2	1	1	3	7
Abbattoir table swab	3	4	-	1	8
Knife swab	-	2	1	3	6
Butcher hand swab	1	3	2	2	8
Cockroach gut	-	1	2	2	5
Housefly leg	1	2	1	4	8
Fish	2	1	3	1	7
N	18	19	16	19	72

Key: SE= *Salmonella enteritidis*, SG= *Salmonella gallinarum*, ST= *Salmonella typhimurium,* SH= *Salmonella heidelberg*, N= Total number of isolates.

Table 5: Occurrence of virulence genes in different species of *Salmonella*

		Virulence genes				
Serotype	N	*InvA*	*SopE*	*AgfA*	*SpvC*	None
Salmonella enteritidis	18	7(38.8)	6(33.3)	9(50)	3(16.7)	4
Salmonella typhimurium	19	5(26.3)	3(15.8)	2(10.5)	7(36.8)	3
Salmonella heidelberg	16	0(0)	8(50)	4(25)	4(25)	2
Salmonella gallinarum	19	12(63.2)	6(31.6)	2(10.5)	7(36.8)	0
Total	72	24(33.3)	23(31.9)	17(23.6)	21(29.1)	9(12.5)

Discussion

The importance of virulence genes in the modulation of bacterial pathogenicity through acquisition of mobile genetic elements has been documented (Pettersson *et al*., 1996; Chakravarty and Masse, 2019). These virulence genes are highly promiscuous and can easily be transferred through plasmid, bacteriophages and genomic islands (Schmidt and Hensel, 2004; Hochhut *et al*., 2006), thereby converting harmless organisms into pathogenic organisms and hence aggravating outbreaks (Chaudhary *et al.,* 2015). *Salmonella species* which are our major focus in this study was isolated from different microbiomes with the highest prevalence found in water, toilet bowl, poultry droppings, butchers' hands and housefly legs. This observation is not unexpected as *Salmonella spp* have been previously reported in water (Haley *et al.,* 2009), toilet bowl (Otokunefor *et al.,* 2020), poultry droppings (Orji *et al*., 2004), butcher hand (Chaudhary *et al*., 2015), housefly legs (Popoola *et al*., 2019) among other sources. The fact that almost all the food samples and objects that have association with food in this study were contaminated with different species of *Salmonella* is in agreement with the earlier report that documented this organism as one of the most frequently isolated food-borne pathogens (Pai *et al*., 2015). The specie-specific characterization of recovered Salmonella *species* depicts four *Salmonella* including *Salmonella enteritidis*, *Salmonella gallinarum*, *Salmonella typhimurium, Salmonella Heidelberg*; which further emphasizes the ubiquitous presence of these organisms across several microbiomes (Khan, 2014). The fact that all these organisms were recovered in almost all the specimens indicate the need for us to pay more attention to other primary sources of *Salmonella* infections than to the secondary sources in order to devise means of controlling their wide epidemiology (Eng *et al*., 2015). *Salmonella species*/serovars cause different infections ranging from non-typhoid *Salmonellosis* for *Salmonella enteritidis* (Alli *et al*., 2020), through fowl typhoid caused by *Salmonella gallinarum* (Kumari *et al*., 2013), systemic typhoid-like infection in mice caused by *Salmonella typhimurium* (Gulig *et al*., 1997) to *Salmonella Heidelberg* that has the capability of causing multiple outbreaks of Salmonellosis in animal and even human (Stefani *et al*., 2018). Our observations in this study suggest the possibility of zoonosis (Drozdz *et al*., 2021) and reverse zoonosis (Messenger *et al*., 2014) occurring from these bacterial isolates. This is because some of the *Salmonella species* known to be adapted to animals were frequently isolated from humans, the environment, objects used by humans, as well as, from some readily consumed foods and water by man. Four different virulence genes (*InvA, SopE, AgfA and SpvC*) were found in these *Salmonella* to varying percentages. Our findings further attest to the conservation of these virulence genes in *Salmonella spp*. *InvA* which was recovered in 24(33.3%) of the total *Salmonella spp* in this study is a gene that usually codes for protein that is responsible for invasion of intestinal cells of the host and has also been regarded to as the commonest of the virulence genes and a potent genetic marker for serovars that causes Salmonellosis globally (Amini *et al*., 2010). The *SopE* which is the next most predominant is a type III secretion protein that also plays important role in the invasion of *Salmonella* by stimulating the membrane ruffling (Humphrey *et al*., 2012) while the *AgfA* in *Salmonella* is involved in surface adherence and biofilm aggregation (Oliveira *et al*., 2014). Consequently, *SpvC* is *Salmonella* plasmid virulence region that plays a key role in the pathogenesis of *Salmonella* through exchange between harmless and pathogenic *Salmonella spp* (Guiney and Fierer, 2011). We however found discrepancies in the percentage distribution pattern of virulence genes in our work and some done elsewhere (Fardsanei *et al*., 2018; Mashayekh *et al*., 2021). However, this observation may be

attributable to differences in samples used for the experiment and geographical location. Also, of particular importance to note is the possibility of some of the highlighted genes to mutate through several mechanisms and therefore leading to evolution of more pathogenic strains in future (Thomas *et al.,* 2019). Our study, therefore documents a non microbiome specific distribution of *Salmonella spp* and further shows that most of the food, human and environmentally distributed *Salmonella spp* could cause outbreak as a result of the presence of different virulence genes that are potent genetic markers of pathogenicity. We therefore recommend better hygiene practice among the populace so as not to predispose the larger population to Salmonellosis outbreaks in future.

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