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Molecular detection of some virulence genes in salmonella spp isolated from food samples in Lagos, Nigeria

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Abstract: Food-borne salmonellosis is the most prevalent disease and major source of *Salmonella* spp in humans and its detection particularly in developing countries is quite cumbersome and time consuming. Molecular methods for its detection as well as the genotypic diversity of some of the genes responsible for *Salmonella* virulence are necessary. The aim of the study was to screen for *Salmonella* spp using the 16S rRNA, to determine whether the *inv*A gene is specific for *Salmonella* detection as well as virulence genotyping of some genes present in *Salmonella* spp (*inv*A, *sit*C and *spv*A, *spv*B and *spv*C) from food samples in Lagos, Nigeria. All 76 isolates tested positive for 16S rRNA gene while 53 (69.7%) were positive for *salm*3 *and salm*4 (389 bp) gene. PCR analysis of the *inv*A gene (284bp) showed that 73 (96.1%) were positive, 38 (50%) of the isolates were positive for *sit*C gene while none were positive for *spv*A and *spv*B and with the multiplex –PCR of *inv*A/*spv*C gene 25 (33%) were positive for *inv*A (244 bp) gene and none positive for *spv*C gene. The use of *invA* gene for *Salmonella* detection in our food samples is recommended however for most of our isolates the virulence genes were not detected.

Keywords: Detection, Food Samples, PCR, *Salmonella*, Virulence Genes

1. Introduction

Salmonella spp are bacteria that cause salmonellosis, and are also common causes of human foodborne outbreaks and diseases in developed and developing countries with attendant public health problem (1, 2). Salmonellosis affects 1.3 billion people worldwide each year with an estimated 3 million annual deaths from non-typhoidal salmonelloses (NTS) (3). The disease outcomes is due to exposure to *Salmonella* spp which ranged from mild symptoms to severe disease cases and sometimes fatal. *Salmonella* spp are carried by a range of domestic animals including birds and some wild animals. *Salmonella* spp have been widely isolated from raw meats, poultry and poultry products, milk and milk products and the enviroment (4),In addition outbreaks have also been associated with poor cooking, reheating of foods, and improper handling of food by food preparers. Representing 30.4% of all *Salmonella* strains isolated from humans, *Salmonella enterica* serotype Typhimurium was the second most commonly isolated *Salmonella* serotype in the Republic of Ireland (5).

Although *Salmonella* gastroenteritis is generally a self-limiting illness, severe cases may require antimicrobial therapy. Food contamination with antibiotic-resistant bacteria can be a major threat to public health, as the antibiotic resistance determinants can be transferred to other pathogenic bacteria, potentially compromising the treatment of severe bacterial infections. The prevalence of antimicrobial resistance among foodborne pathogens has increased during recent decades (6). This increase is attributed to the selection pressure created by using antimicrobials in food-producing animals, in addition to the

unregulated use of antibiotics by humans in developing countries (7). The pathogenicity of NTS is attributed to identified virulence genes which has an association with antibiotic resistance (8).

In developing countries poor sanitary conditions appear to be the main risk factor for the transmission of *Salmonella* spp (9). Majority of Nigerians live below poverty level and patronize low cost foods such as those sold in bukas. However, the hygiene conditions of those bukas are not near standard hygiene practices, and this facilitates transmission of NTS infections among the food consumers. The conventional cultural method of detection of *Salmonella* in food is problematic, cumbersome and time consuming. To overcome this problem, molecular based PCR technique which is rapid, specific and more sensitive has been developed (10, 11). The use of amplification of DNA by PCR method is a revolutionary tool in diagnosis of pathogenic organisms (12, 11). Several virulence genes which are target genes for PCR amplification of *Salmonella* species including *invA, sitC, spv*A, *spv*B and *spvC,* have been used to detect as well as screen for genotypic virulence in *Salmonella* isolates from environmental and food samples (13, 8). Information on the use of PCR target genes to identifity the NTS from foods and food samples is lacking in Nigeria. The study is aimed at using PCR *inv*A virulenes genes to confirm the presence of *Salmonella* spp isolated from food samples as well as to screen our local isolates for some of the virulence genes (*sit*C and *spv*A*, spv*Band *spv*C).

2. Materials and Methods

A total of 189 isolates suspected to be *Salmonella* spp (from previous work on the identification of *Salmonella* from food samples using the REVEAL Kit (Neogen Corp. US), were cultured from meat samples on Salmonella-Shigella agar for 24 hours and pure colonies were obtained and identified using biochemical test as earlier described (4).

2.1. DNA Extraction

From the isolates, 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 containing the DNA was transferred to another tube and stored at -20°C. The concentration and purity of the extracted DNA was estimated using a Nanodrop spectrophotometer.

2.2. DNA Primers

The primers consist of 16S rRNA (574 bp), *salm* 3 and *salm* 4 (389 bp), *inv*A gene (284 bp), *sit*C (768 bp), *spv*A (604 bp) and *spv*B (1063 bp) and multiplex PCR (*inv*A/*spv*C gene: 244 bp and 571 bp). The primer sequences and their corresponding genes are shown in Table 1.

2.3. DNA Amplification

The reaction for all the PCR was carried out in a 25µl reaction mixture containing 1x PCR buffer (Promega, UK), 1.5mM Magnesium Chloride, 200µM of each dNTP, 20pMol of each primer, 1.25U Taq DNA polymerase (Promega, UK). The DNA was diluted to give a final concentration of between 10- 200ng/µl and 1µl of this was used in the PCR and the amplification was carried out in an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg). The 16S rRNA gene, the PCR conditions were 3 min at 95^0C, followed by 30 cycles of 30 s at 95^0C, 30s at 54.1^0C and 1 min at 72^0C and final extension for 10 min at 72^0C. For the *salm* 3 and *salm* 4 primer, the PCR conditions were initial denaturation of 5 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 65°C and 1.5 min at 72°C and final extension 10 min at 72°C. The *inv*A gene, the PCR conditions were initial denaturation of 1 min at 95°C, followed by 35 cycles of 1 min at 94°C, 1 min at 56°C and 1 min at 72°C and final extension of 10 min at 72°C.The results of the two *inv*A gene primers (*salm*3 and *salm*4, with *inv*A) were tested using the Chi-square analysis (one-tailed) as well as checking for the positive predictive value (PPV), negative predictive value (NPV), sensitivity and specificity with culture as the gold standard. The idea was to actually predict the best *inv*A gene that could be used to detect *Salmonella* species from our food samples. AS for the *sit*C primer, the PCR conditions were 3 min at 94^0C, followed by 30 cycles of 2 min at 94^0C, 1 min at 55^0C and extension for 1min at 72^0C for 1min and final extension for 5 min at 72^0C. As for the multiplex PCR (*inv*A/*spv*C) primer, the PCR conditions were 1 min at 94 ^{0}C, followed by 30 cycles of 30 s at 94 ^{0}C, 30 s at 56 ^{0}C, 2 min at 72^0C and final extension of 10 min at 72^0C. The PCR conditions for the *spv*A and *spv*B primers were; 5 min at 94^0C followed by 30 cycles of 30s at 94^0C, 30s at 60^0C, 1 min at 72^0C and then a final extension for 5 min at 72^0C. The PCR products were separated on a 1.5% agarose gel at 80 Volts and 50bp DNA ladder was used as molecular weight marker. The primer sequences and their corresponding genes are shown in Table 1.

3. Results

Only 76 (40.2%) of the 189 isolates were confirmed to be *Salmonella* spp using biochemical testing, out of these 53 (69.7%) of the isolates were confirmed as *Salmonella* spp using the *salm*3and *salm*4 primer set (Fig1). A total of 73 (96.1%) of the isolates were confirmed to be *Salmonella* spp using the *invA* primer set (Fig 2). Table 2 shows a comparison between the sensitivity, specificity, positive and negative predictive values of *invAF and invAR* with *salm* 3 and *salm* 4 primer sets. Chi square analysis between the two primers show there is statistical significance with a p-value of 0.019, $P<0.05$. Fig 3 shows the PCR amplification of 16S rRNA *Salmonella* spp. A total of 38 (50%) isolates were positive for the *sit*C gene (Fig 4) while the multiplex PCR of

*inv*A/*spv*C genes showed that only 25(33%) were positive for the *inv*A gene and none was positive for the *spv*C gene.

Table 1. *Primer sequences and their corresponding genes*

Primer	Target gene	Sequence	Amplified fragment size (bp)
16S rRNA	Genus specific	5'-TGT TGT GGT TAA TAA CCG CA -3' 5'-CAC AAA TCC ATC TCT GGA -3'	571
Salm3/salm4	*inv*A	5'-GCTGCGCGCGAACGGCGAAG-3' 5'-TCCCGCCAGAGTTCCCATT-3	389
InvF/invAR	*inv*A	5' -ACA GTG CTC GTT TAC GAC CTG AAT -3' 5' -AGA CGA CTG GTA CTG ATC GAT AAT-3'	284
*Sit*C 3'	*sit*C	5'-CAG TAT ATG CTC AAC GCG ATG TGG GTC TCC- 5'- CGG GGC GAA AAT AAA GGC TGT GAT GAA C-3'	578
spvA	*spv*A	5'-GTC AGA CCC GTA AAC AGT-3' 5'-GCA CGC AGA GTA CCC GCA-3'	604
spvB	s*pv*B	5'- ACG CCT CAG CGA TCC GCA -3' 5' – GTA CAA CAT CTC CGA GTA -3'	1063
Multiplex			
*inv*A/*spv*C		5'-ACA GTG CTC GTT TAC GAC CTG AAT-3' 5'-AGA CGA CTG GTA CTG ATC TAT -3'	244
*inv*A+*spv*C		5' GTC CTT GCT CGT TTA CGA CCT GAA T 3' 5' TCT CTT CTG CAT TTC GTC A 3'	571

Table 2. *Comparison between salm3/4 and invA genes in detection of Salmonella spp from foods*

	Sensitivity	Specificity	PPV	NPV
Salm ¾	70 (53/76)	100 (113/113)	100 (53/53)	83 (113/136)
Inv A	96 (73/76)	100 (113/113)	100 (73/73)	97 (113/116)

Culture is taken as the gold standard

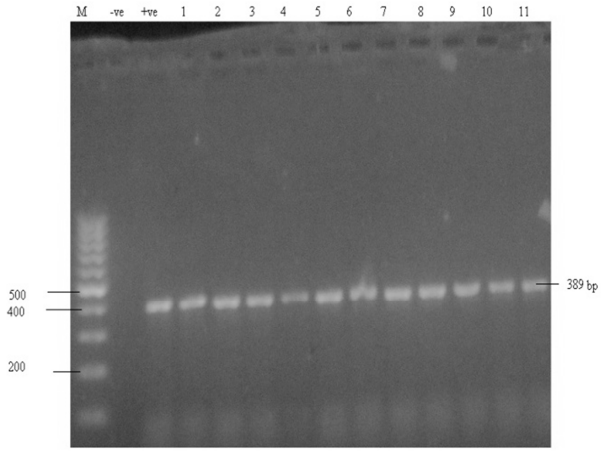

Fig 1. *PCR amplification of invA genes of Salmonella spp using salm3 and salm4 gene primer Lanes M: 100 bp ladder; Lanes 1-11 PCR products (389 bp) of salm3/4 from Salmonella spp, lane –ve and +ve is the negative and positive controls respectively.*

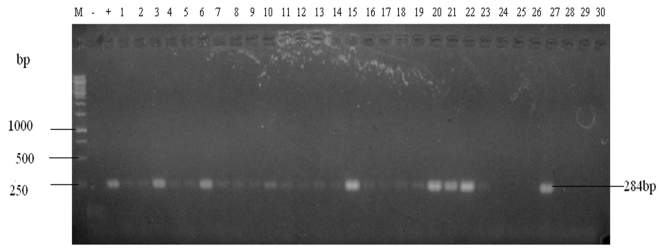

Fig 2. *PCR amplification of invA genes of Salmonella spp using invA primers Lanes M: 100 bp ladder; Lanes 1-30 PCR products (284 bp) of invA primers from Salmonella spp, lane – and + is the negative and positive controls respectively. Some lanes show no amplification and were negative for this gene (24 – 26).*

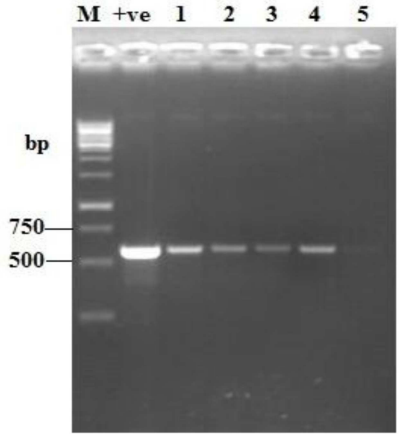

Fig 3. *PCR amplification products of 574-bp DNA of 16Sr RNA gene of Salmonella spp. Lane M = 1 kb ladder, Lane (+) = positive control, Lane 5(-) =Negative, 1-4 positive.*

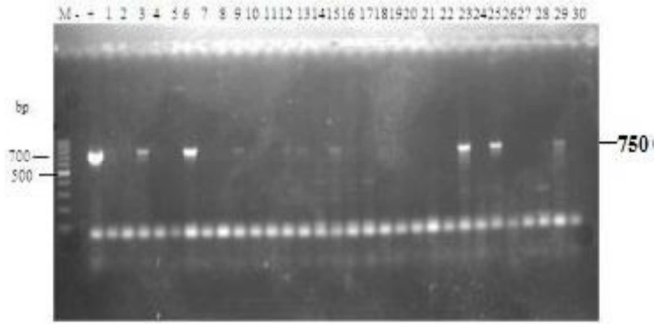

Fig 4. *PCR amplification products of 750-bp DNA fragment of the sitC gene in Salmonella spp from food samples .Lane M = 100 base pair marker, Lane – = negative control, Lane + = positive control*

4. Discussion

The prevalence of the virulence gene *inv*A from *Salmonella* spp in food samples presents a public health risks and often times result to food industry losses. The detection of *Salmonella* spp in the developing countries is usually difficult involving cultivation and identification of the organism from humans, food samples and environmental samples. The traditional cultural methods of isolating *Salmonella* spp are usually time consuming and laborious. Until recently researchers tried to establish a more rapid method, which could reduce the man-hours of *Salmonella* identification procedures from different ranges of samples. The use of primer set *inv*A/*inv*E for confirmation of isolated *Salmonella* spp from turkeys came as relief from time waste on this identification procedures (14, 15). This effort was followed by the proposal of Ferretti *et al.* (16) for rapid detection method of *Salmonella* serotypes from samples within 12 hours using primers *salm*3and*salm*4 and *invA* gene. The *Salmonella invA* gene codes for the protein in the inner membrane which helps the organism to invade the host epithelial cell. The *invA* gene contains sequences unique to *Salmonella* spp and has proved appropriate for specific targets in various diagnostic and research laboratories (17). The use of *Salmonella* specific PCR with primers *invA* is rapid, sensitive and more specific for detection of *Salmonella* in many food samples. The amplification of *invA* gene has been validated as a standard for detection of invasion gene from *Salmonella* spp (12).

This study involves the use of PCR virulence genes which amplifies a 389 bp fragment within the conserved *inv*A gene (*salm*3and *salm* 4) of *Salmonella* spp as well as *inv*A that amplifies 284 bp to confirm the virulence and presence of *Salmonella* spp isolated from food samples. The results of the PCR on *inv*A genes showed that the primer set *inv*AF and *inv*AR that amplified 284 bp fragment (96.1%) was more discriminatory than the *salm* 3 and *salm* 4 primer set that amplified the 389bp (69.7%) fragment and the *inv*A gene was more sensitive than salm3/4 as well as its ability to detect more negative results in comparison to the latter, although both showed 100% PPV and specificity. A recent study from Korea had the same prevalence with the *inv*A gene (284 bp) of 96% (18), but their prevalence with *sit*C genes differed as they had higher prevalence of 96% compared to ours of 50%. Our result supports the ability of the two primer sets (*salm* 3/*salm* 4 and invA) to confirm the presence of virulence gene in *Salmonella* spp. Although the *salm*3 and *salm*4 primer set has been suggested to be useful for direct detection of *Salmonella* spp as well as excellent correlation with the conventional method (16) our results do not confirm that. Our findings however suggest that the primers are specific for detection of *Salmonella* spp, but *invA and invA* primers was found to be more sensitive (96.1%) compared to *salm*3 and *salm*4 (Table1) in the detection of *Salmonella spp* which corroborates the earlier reported study (19, 20). Our report with *inv*A gene (284bp) was also corroborated by a recent study from Nigeria on *Salmonella* spp isolated from cow raw milk and milk products in which *inv*A (284 bp) was prevalent in 100% of their isolates (21). This means that the use of *invA* gene (host invasion) can greatly reduce the reports of false-negative experienced in most laboratories (22). In monitoring some virulence genes such as those that code for iron transport (*sit*C) and the *Salmonella* virulence plasmids (*spv*A, *spv*B and *spv*C), our results showed that whereas half of the isolates encode genes for iron transport, none of our isolates carried the *spv*A, *spv*B and *spv*C genes. This report corroborated with arlier the study (23) in which *inv*A, *sef* genes were detected in their isolates from butcher shops in Brazil, and none of the isolates carried the *spv*C gene. To further corroborate the results of our study and that of Cossi *et al*. (23), 17 virulence genes were studied including *sit*C, *inv*A and *spv*B, none were positive for *spv*B in both food and environmental isolates (24). In a related study out of 17 virulence genes tested, 14 *Salmonella* isolates were positive with the exception of *spv*B, *cdt*B and *pef*A of which *pef*A and *spv*B were plasmid encoded (25). However, from this study only the *spv*B, *pef*A had lower prevalence (5% and 3.75% respectively) while other more common ones such as the *inv*A had 100% prevalence (13). This suggests the possibility that our local food isolates studied did not have virulence genes (*spv*A, *spv*B and *spv*C) located on the plasmids but could be chromosomally located as the latter three genes are for *Salmonella* virulence plasmids. The other possibility could be due to the fact that not all isolates contain virulence plasmids as lower proportions were reported in some reports for the plasmid virulence genes (26, 27, 13,18). In contrast Amini *et al.* (8) reported that the *spv* genes were prevalent in 88.6% for poultry, 90% for humans and 100% for bovine, although all isolates were S. Enteritidis, while other study reported moderate prevalence (42.85%) of the *spv*C gene in their commercial food stuffs (28). However, other reports have confirmed the ubiquitous distribution of virulence genes amongst *Salmonella* spp irrespective of the host (25). The PCR method using target gene remains a suitable molecular tool to diagnose *Salmonella* in human, animal and plant products (8, 19). These findings have important health implications to the entire populace considering the high prevalence of virulence genes in food samples studied and it also underscores the need for rapid identification of *Salmonella* virulence genes using the PCR method.

In conclusion, most of our food isolates harbored *invA* virulence gene and this has its economic and public significance for the country. Consequently, it is necessary to consider food hygiene programs to prevent health hazards and economic loss due to increasing morbidity and mortality associated with salmonellosis with possible antibiotic resistance. Rapid detection of *Salmonella* spp by PCR of *inv*A genes is recommended, although all our isolates did not harbor the *spv* genes. Further analysis on accurate distribution of virulence genes is important to help us develop accurate preventive measures against *Salmonella* spp in Nigeria.

Abbreviations

NTS: Non Typhoidal Salmonella, invA: invasion gene A, sitC: salmonella iron transport, spv: Salmonella plasmid virulence, salm: Salmonella, pefA: plasmid encoded fimbriae A

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