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 gene, to determine whether the invA gene is specific for Salmonella detection as well as virulence genotyping of some genes present in Salmonella spp (invA, sitC and spvA, spvB and spvC) from food samples in Lagos, Nigeria. All 76 isolates tested positive for 16S rRNA gene while 53 (69.7%) were positive for salm3 and salm4 (389 bp) gene. PCR analysis of the invA gene (284bp) showed that 73 (96.1%) were positive, 38 (50%) of the isolates were positive for sitC gene while none were positive for spvA and spvB and with the multiplex –PCR of invA/spvC gene 25 (33%) were positive for invA (244 bp) gene and none positive for spvC 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 environment (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, spvA, spvB 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 virulence 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 (sitC and spvA, spvBand spvC).

2. Materials and Methods

A total of 189 isolates suspected to be Salmonella spp (from previous work on the identification of Salmonella spp 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), invA gene (284 bp), sitC (768 bp), spvA (604 bp) and spvB (1063 bp) and multiplex PCR (invA/spvC 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°C, followed by 30 cycles of 30 s at 95°C, 30s at 54.1°C and 1 min at 72°C and final extension for 10 min at 72°C. 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 invA 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 invA gene primers (salm3 and salm4, with invA) 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 invA gene that could be used to detect Salmonella species from our food samples. As for the sitC primer, the PCR conditions were 3 min at 94°C, followed by 30 cycles of 2 min at 94°C, 1 min at 55°C and extension for 1min at 72°C for 1min and final extension for 5 min at 72°C. As for the multiplex PCR (invA/spvC) primer, the PCR conditions were 1 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 56°C, 2 min at 72°C and final extension of 10 min at 72°C. The PCR conditions for the spvA and spvB primers were; 5 min at 94°C followed by 30 cycles of 30s at 94°C, 30s at 60°C, 1 min at 72°C and then a final extension for 5 min at 72°C. 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 salm3and salm4 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 sitC gene (Fig 4) while the multiplex PCR of
invA/spvC genes showed that only 25(33%) were positive for the invA gene and none was positive for the spvC gene.

Table 1. Primer sequences and their corresponding genes

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target gene</th>
<th>Sequence</th>
<th>Amplified fragment size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>Genus specific</td>
<td>5'-TGT TGT GGT TAA TAA CCG CA -3'</td>
<td>571</td>
</tr>
<tr>
<td>Salm3/salm4</td>
<td>invA</td>
<td>5'-GCCGCGCGGAAACGGCGAAG-3'</td>
<td>389</td>
</tr>
<tr>
<td>InvF/invAR</td>
<td>invA</td>
<td>5'-ACG CTT GTC GTC ATT CAC CTG AAT-3'</td>
<td>284</td>
</tr>
<tr>
<td>SitC 3'</td>
<td>sitC</td>
<td>5'-CGG CAC ATG TGG TTC TCC CAA ATT AAT-3'</td>
<td>578</td>
</tr>
<tr>
<td>spvA</td>
<td>spvA</td>
<td>5'-GCA CGA ATG GCC ATG GTT TCC CAA ATT-3'</td>
<td>604</td>
</tr>
<tr>
<td>spvB</td>
<td>spvB</td>
<td>5'-ACG CTT GTC GTC ATT CAC CTG AAT-3'</td>
<td>1063</td>
</tr>
<tr>
<td>Multiplex</td>
<td>invA/spvC</td>
<td>5'-ACA GTG CTC GTT TAC GAC CTG AAT-3'</td>
<td>244</td>
</tr>
<tr>
<td>invA+spvC</td>
<td></td>
<td>5'-AGA CTA CAT CTC CGA GTA ATT-3'</td>
<td>571</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salm ¾</td>
<td>70 (53/76)</td>
<td>100 (113/113)</td>
<td>100 (53/53)</td>
<td>83 (113/136)</td>
</tr>
<tr>
<td>Inv A</td>
<td>96 (73/76)</td>
<td>100 (113/113)</td>
<td>100 (73/73)</td>
<td>97 (113/116)</td>
</tr>
</tbody>
</table>

Culture is taken as the gold standard

for this gene (24–26).

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 1-10 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 invA 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 invA/invE 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 salm3 and salm4 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 invA gene (salm3 and salm4) of Salmonella spp as well as invA that amplifies 284 bp to confirm the virulence and presence of Salmonella spp isolated from food samples. The results of the PCR on invA genes showed that the primer set invAF and invAR that amplified 284 bp fragment (96.1%) was more discriminatory than the salm3 and salm4 primer set that amplified the 389bp (69.7%) fragment and the invA 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 invA gene (284 bp) of 96% (18), but their prevalence with sitC genes differed as they had higher prevalence of 96% compared to ours of 50%. Our result supports the ability of the two primer sets (salm3/salm4 and invA) to confirm the presence of virulence gene in Salmonella spp. Although the salm3 and salm4 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 salm3 and salm4 (Table1) in the detection of Salmonella spp which corroborates the earlier reported study (19, 20). Our report with invA gene (284bp) was also corroborated by a recent study from Nigeria on Salmonella spp isolated from cow raw milk and milk products in which invA (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 (sitC) and the Salmonella virulence plasmids (spvA, spvB and spvC), our results showed that whereas half of the isolates encode genes for iron transport, none of our isolates carried the spvA, spvB and spvC genes. This report corroborated with arlier the study (23) in which invA, sef genes were detected in their isolates from butcher shops in Brazil, and none of the isolates carried the spvC gene. To further corroborate the results of our study and that of Cossi et al. (23), 17 virulence genes were studied including sitC, invA and spvB, none were positive for spvB 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 spvB, cdB and pefA of which pefA and spvB were plasmid encoded (25). However, from this study only the spvB, pefA had lower prevalence (5% and 3.75% respectively) while other more common ones such as the invA had 100% prevalence (13). This suggests the possibility that our local food isolates studied did not have virulence genes (spvA, spvB and spvC) 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 spvC 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 invA 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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References


