Microbiology Research International
Vol. 12(3), pp. 57-64, July 2024
DOI: 10.30918/MRI.123.24.019
ISSN: 2354-2128
Full Length Research Paper

Probing for virulence and toxigenic genes of *Salmonella enterica* and *Listeria monocytogenes* in seafood sold in Rivers State, Nigeria

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Accepted 2 July, 2024

ABSTRACT

The presence of toxigenic and virulence genes could promote or enhance the degree of pathogenesis in *Salmonella enterica* and *Listeria monocytogenes*. This study was conducted to probe for virulence and toxigenic genes of *Salmonella enterica* and *Listeria monocytogenes* in seafood sold in Rivers State, Nigeria. A total of 126 raw and parboiled samples of *Crassostrea gasar* (Oyster) (42), *Panaeus monodon* (Prawn) (42) and *Buccinum undatum* (Whelks) (42) were purchased from 3 Local Government Area markets and subjected to standard conventional methods and molecular screening. All *Listeria monocytogenes* (25) were 100% positive for Dnase, capsule, coagulase, haemolysin and biofilm production (84%); *Salmonella enterica* (40) were 100% positive for haemolysin, capsule and biofilm production (85%). All isolates were identified molecularly as: *Salmonella enterica*-AM04528*, Salmonella bongori*-NCTC12419*, Listeria ivanovii*-PM-44*, Listeria monocytogenes*-HN1*, Listeria monocytogenes*-F6540*, Salmonella enterica*-19_85*, Salmonella enterica*-KKP1761*, Listeria monocytogenes*-NITRR/R1*, Salmonella enterica*-777SA01*, Salmonella enterica*-KKP3882*, Listeria monocytogenes*-HR27 and *Listeria monocytogenes*-FC3. The toxigenic and virulence genes were present as *stn* (100%), *plcA* (100%), *hlyA* (100%) in all *Salmonella enterica* and *Listeria monocytogenes* isolates, and *hilA* (83.33%) was present in all the *Listeria monocytogenes* and *Salmonella enterica* isolates except *Listeria monocytogenes*-HN1. This study revealed the presence of these genes, which contribute immensely to the pathogenicity of the bacteria posing severe disease conditions. It is necessary to implement regulatory measures to ensure quality control and good hygiene practices in the production, storage and handling of seafood.

Keywords: Seafood, virulence, toxigenic genes, probing, *Salmonella enterica*, *Listeria monocytogenes.*

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INTRODUCTION

Seafoods are consumed raw or parboiled without adequate cooking to kill the organisms or toxins which might pose serious risks to public health. Seafoods have been a vehicle for transmitting of *Salmonella* and *Listeria*, causing food intoxication and poisoning. Bacteria become multidrug-resistant when they acquire multiple genetic factors from different sources through various mechanisms of conjugation, transduction or transformation (Hakanen et al., 2017). Apart from the acquired resistance, bacteria have chromosomally encoded intrinsic resistance mechanisms such as the marRAB locus of *Salmonella* and *Listeria*, which confers intrinsic resistance to several antibiotics such as tetracyclines, chloramphenicol, cephalosporins, penicillins, nalidixic acid, fluoroquinolones, rifampin, organic solvents even with the aid of some virulent factors (Ayukekbong et al., 2017).

Salmonella pathogenicity islands (SPIs) are gene clusters located in certain areas of the chromosomes in the bacterial cells that are responsible for encoding the various virulence factors (adhesion, invasion, toxin genes, etc.) (Foley et al., 2013). The SPIs play different roles in the pathogenesis and virulence of *Salmonella*. The SPI-1 is required for the invasion of host cells and

induction of macrophage apoptosis. Virulent and toxigenic factors such as *hlyA*, fimbria, flagella and hemolysin have been involved in the pathogenicity of these organisms. Specifically, hemolysin is a major virulence factor originally shown to be crucial for bacterial escape from the internalization vacuole after entering the cells. However, recent studies are revisiting the role of hemolysin during infection and are revealing new insights into the action of hemolysin, in particular before bacterial entry (Foley et al., 2013).

Salmonella and *Listeria* have been involved in the production of both endotoxins and exotoxins, which is attributed to confer pathogenicity among *Salmonella* and *Listeria* serovars, leading to severe food intoxication and poisoning. The former has been found to elicit a wide range of biological responses, whereas the latter comprising enterotoxins and cytotoxins is associated with the killing of mammalian cells (Ashkenazi et al., 2010). Hence, this research aims at probing for virulence and toxigenic genes of *Salmonella enterica* and *Listeria monocytogenes* in seafood sold in Rivers State.

MATERIALS AND METHODS

Description of study area

Three markets in Rivers State, Kaa market in Khana Local Government Area, Creek Road market in Port Harcourt Local Government Area (PHALGA), and Bakana Market in Degema Local Government Area were used for the study. These markets were selected because the population density of the area is high and its easy accessibility to seafood. Sample size was calculated using the formula according to (Naing et al., 2006).

Sample collection

One hundred and twenty-six (126) samples of raw and parboiled seafood, including Whelks (*Buccinum undatum*), Oysters (*Crassostrea gasar*), and Prawns (*Penaeus monodon*), were purchased from three markets, placed in sterile polythene bags, then put in ice chests, and aseptically transported to the Department of Microbiology laboratory, Rivers State University, Port Harcourt, Nigeria, for bacteriological analysis after identification by Prof. G.C. Akani in the Department of Animal and Environmental Biology, Rivers State University.

Microbiological analysis

Bacterial isolation and identification

Salmonella and *Listeria* were isolated by picking representative or discreet colonies based on their size, margin, surface, elevation, texture, transparency and colouration (blackish and grey-green) on *Salmonella-Shigella* agar and a selective medium Polymyxin Acriflavin Lithium-chloride Ceftazidime Esculin Mannitol (PALCAM) agar supplemented with Listeria Selective Supplement II (FD063), respectively. Identification of the organism was further conducted through biochemical test procedures such as citrate utilization test, methyl red, Catalase, Coagulase, Indole test, Voges Proskauer test, sugar fermentation test and specifically hydrogen sulphide production to confirm *Salmonella enterica* (Cheesbrough, 2005).

Test for virulence factors

The hemolysis assay was done by streaking representative colonies of the isolates onto freshly prepared blood agar according to the method of Sagar (2015). DNase was carried out as described by Buchanan (2006). The motility test was carried out as described by Chakraborty and Nishith (2008). The isolates were streaked onto casein agar and incubated at 37^{o}C for 48 hours as described by Vijayaraghavan and Vincent (2013), for the protease test and biofilm formation test using congo red agar. The slide coagulase test was adopted as reported by Wemedo et al. (2016).

Molecular studies

DNA extraction and quantification

The boiling method was used for the extraction process as described by Bell et al. (1998). Pure culture of the *Salmonella* and *Listeria* isolate was put in Luria-Bertani (LB) Broth and incubated at 37^{o}C. Zero point five millilitres (0.5ml) of the broth culture of the *Salmonella* and *Listeria* in Luria Bertani (LB) were dispensed into properly labeled Eppendorf tubes and filled to mark with normal saline and was centrifuged at 14000rpm for 3 minutes, and the supernatant was decanted leaving the DNA at the base. This process was repeated 3 times. The cells were re-suspended in 500ul of normal saline and heated at 95^{o}C for 20 minutes. The heated bacterial suspension was cooled on ice (About 10 minutes) and spun for 3 min at 14000rpm. The supernatant containing the DNA was transferred to a 1.5ml micro-centrifuge tube and stored at -20^{o}C for other downstream reactions (Bell et al., 1998). The extracted DNA was quantified by using the Nanodrop 1000 Spectrophotometer as described by Olsen and Marrow (2012).

Amplification 16S rRNA

The 16srRNA amplification was carried out using an ABI

9700 Applied Biosystems thermal Cycler, as described by Srinivasan et al. (2015). The 16s rRNA region of the rRNA gene of the bacterial isolates was amplified using the forward primer: 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and Reverse primer; 1492R: 5'- CGGTTACCTTGTTACGACTT-3' and to a final volume of 50 µL for 35 cycles. The PCR mix includes (Taq polymerase, DNTPs and $MgCl_2$), the primers at a concentration of 0.5uM and the extracted DNA as template, Buffer 1X and water. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 130V for 30 minutes and visualized on a blue light trans-illuminator for 1500bpamplicons (Srinivasan et al., 2015).

Amplification stn, plcA (Toxigenic) and hilA, hlyA (Virulent) genes of Salmonella spp and Listeria spp

The *stn* and *hilA* genes from the *Salmonella enterica* and *Listeria monocytogenes* isolates were amplified using the *stn*F: 5′-GTTAATCCTGTTGTCTCGCT-3´, *stn*R: 5′-CAGAGAACTGCTTGATGCAA-3´, *hilA*F: 5′-CTGCCGCAGTGTTAAGGATA-3´, *hilA*R: 5′-CTGTCGCCTTAATCGCATGT-3´, *plcA*F: 5′-CTGCTTGAGCGTTCATGTCTCCATCC-3´, *plcA*R: 5′-CATGGGTTTCACTCTCCTTCTAC-3´, *hlyA*F: 5′-ACGCAGTAAATACATTAGTG-3´ and *hlyA*R: 5′-AATAAACTTGACGGCCATAC-3´ primers on ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 micro-litres for 35 cycles. The PCR mix included the X2 Dream Taq Master Mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs and $MgCl_2$), the primers at a concentration of 0.4M and 60ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 58ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 25 minutes and visualized on a UV trans-illuminator for a 617bp and 497bp product size (Bell et al., 1998).

DNA sequencing

The Big-Dye Terminator kit on a 3510 ABI sequencer was used for sequencing the amplified products. The sequencing was done at a final volume of 10ul; the components included 0.25ul BigDye® terminator v1.1/v3.1, 2.25ul of 5 x Big-Dye sequencing buffer, 10uM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing conditions were as follows: 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4 minutes (Srinivasan et al., 2015).

Phylogenetic analysis

Similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) database using BLASTN before the edition of the obtained sequences using the bioinformatics algorithm Trace edit. MAFFT were used to align these sequences. The evolutionary history was inferred using the Neighbor Joining method in MEGA 6.0 (Saitou and Nei, 1987). The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969).

RESULTS

A total of 25 (19.84%) *Listeria monocytogenes* and 40 (31.75%) *Salmonella enterica* were isolated from the seafood, and 6 of each *Salmonella enterica* and *Listeria monocytogenes* were identified molecularly based on their multiple antibiotic resistance profile. The virulence property results showed that all the isolates of *Listeria monocytogenes* isolates were 100% positive for Dnase, capsule, coagulase, haemolysin and 84% produced biofilm. *Salmonella enterica* isolates were 100% positive for haemolysin, capsule and 85% produced biofilm as shown in Table 1. The Agarose gel electrophoresis of the amplified 16SrRNA gene of bacterial isolates before sequencing showed that Lanes 1 – 12 represent the 16SrRNA gene bands (1500bp) while lane L represented the 100bp molecular ladder (Figure 1). The evolutionary distance between the bacterial isolates from this study and the accession numbers and their closest relatives on the phylogenetic tree is revealed in Figure 2.

The result of the electrophoresis for the amplified *stn* gene of the 6 *Salmonella* isolates revealed that Lane 1, 2, 3, 4, 5 and 6 showed the *stn* gene band at 617bp and Lane L represented the 100bp molecular ladder, and all had the toxigenic gene in their genome (Figure 3). The result of the gel electrophoresis for the amplified *hilA* gene of the 6 *Salmonella* isolates revealed that Lane 1, 2, 3, 5 and 6 showed the *hilA* gene band at 497bp and Lane L represented the 100bp molecular ladder, and 5 out of the 6 *Salmonella* isolates screened for the virulence gene (*hilA*) had them in their genome (Figure 4).

The gel electrophoresis image of the *Listeria* isolates showing the amplified *hlyA* gene of the 6 *Listeria* isolates shows that Lane 1, 2, 3, 4, 5 and 6 showing the *hlyA* gene band at 372bp and Lane L represented the 100bp molecular ladder and all the 6 *Listeria* isolates screened for the virulence gene (*hlyA*) had them in their genome (Figure 5). The electrophoresis image of the *Listeria* isolates revealing the amplified *plcA* gene of the 6 *Listeria* isolates showed that Lane 1, 2, 3, 4, 5 and 6 showing the

Table 1. Virulence potentials of *Salmonella enterica* and *Listeria monocytogenes* isolated from seafood (whelks, oyster and prawn).

Organism (n)	Dnase n(%)	Capsule stain n(%)	Coagulase n(%)	Haemolysin n(%)	Motility n(%)	Biofilm n(%)	Protease n(%)
	+ve	+ve	+ve	+ve	+ve	+ve	+ve
S. enterica (40)	0(0)	40(100)	0(0)	40(100)	40(100)	34(85)	40(100)
L. monocytogenes (25)	25(100)	0(0)	25(100)	25(100)	25(100)	21(84)	25(100)

Key: Positive (-ve); Negative (-ve).

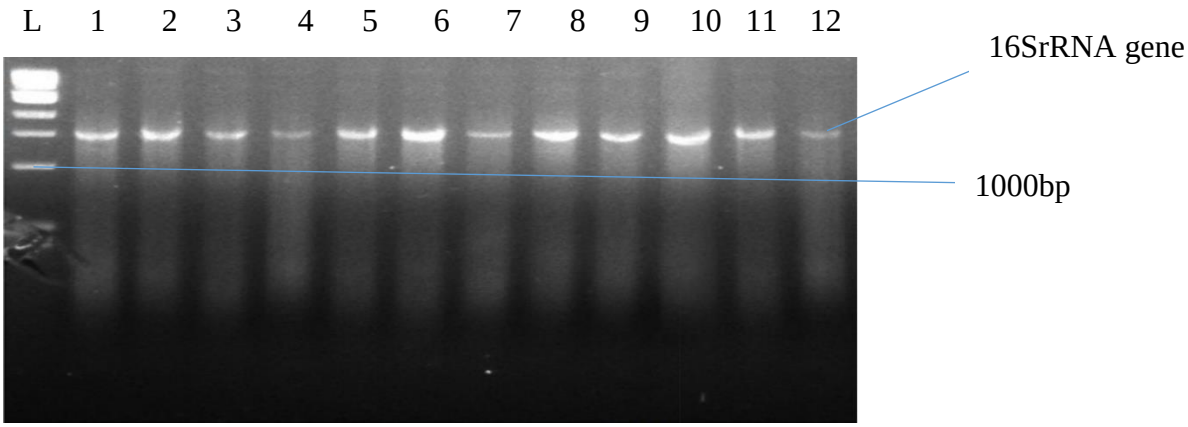

Figure 1. Amplified 16S rRNA gene of the bacteria Isolates at 1500bp.

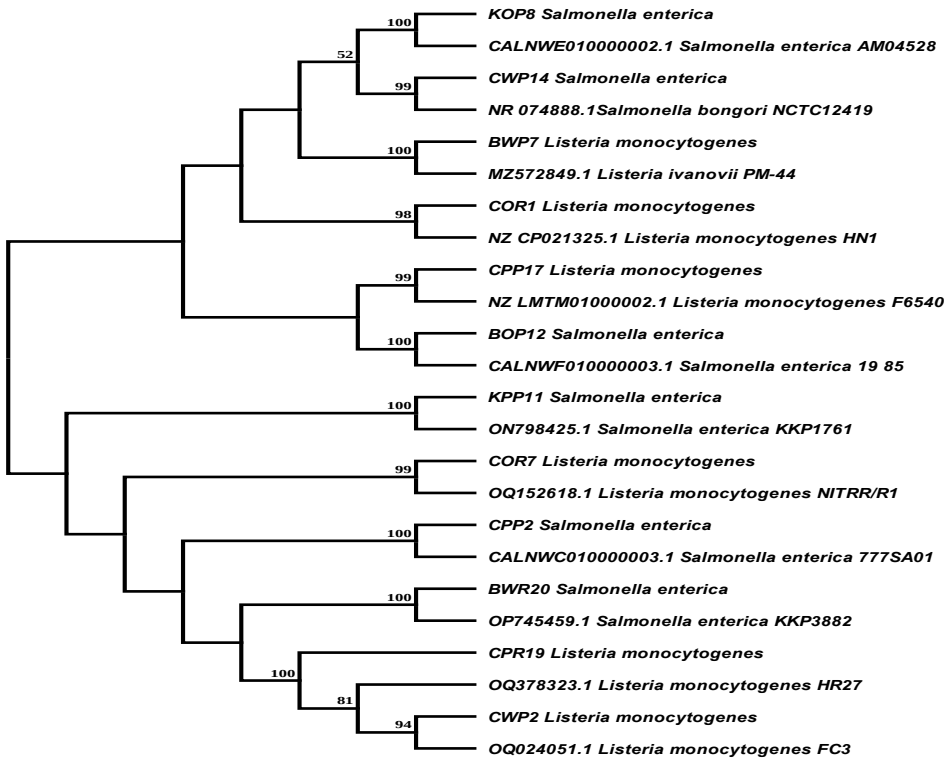

Figure 2. Phylogenetic tree showing evolutionary distance between bacterial Isolates.

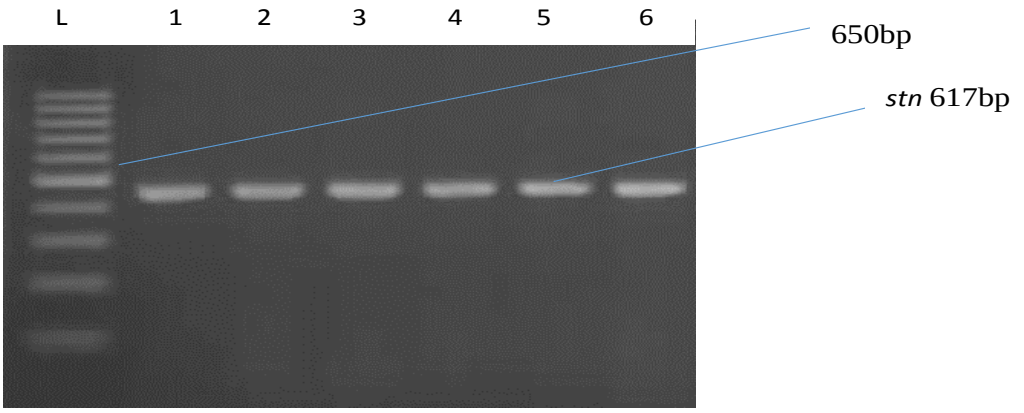

Figure 3. *stn* enterotoxin gene. Lane 1, 2, 3, 4, 5 and 6 represent the *stn* gene bands at 617bp of 6 *Salmonella* isolates while L represents the 100bp molecular ladder.

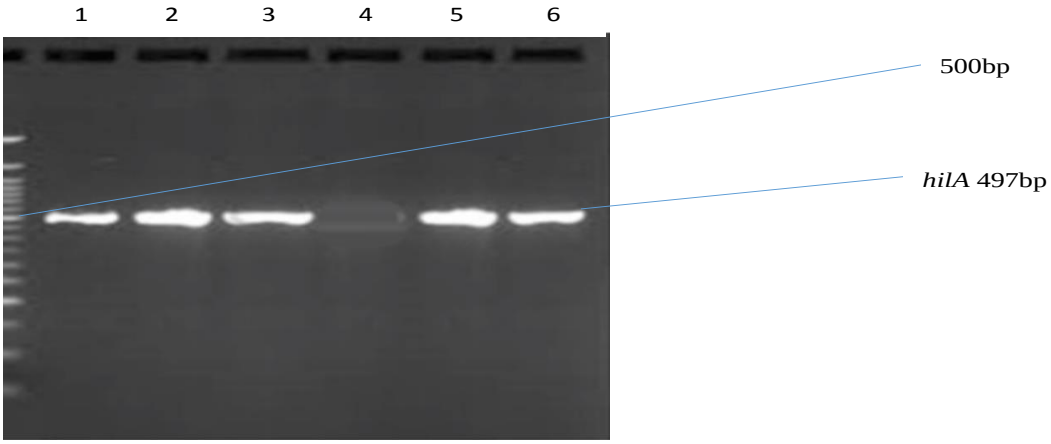

Figure 4. *hilA* bands of *Salmonella*. Lane 1, 2, 3, 5 and 6 represent the *hilA* gene bands at 497bp of the 6 *Salmonella* isolates while L represents the 100bp molecular ladder.

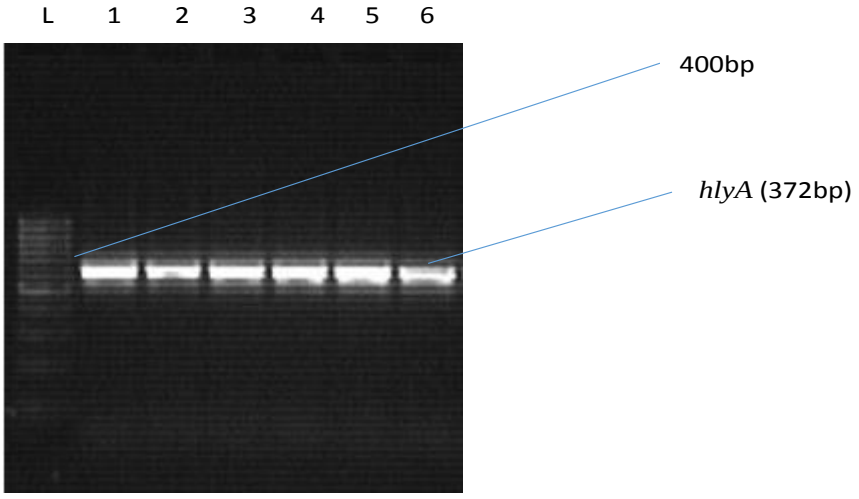

Figure 5. *hlyA* gene bands of *Listeria*. Lane 1, 2, 3, 4, 5 and 6 represent the *hlyA* gene bands at 372bp of the 6 *Listeria* isolates while L represents the 100bp molecular ladder.

plcA gene band at 1484bp and Lane L represented the 100bp molecular ladder and all the 6 *Listeria* isolates screened for the toxigenic gene (*plcA*) were present in their genome (Figure 6).

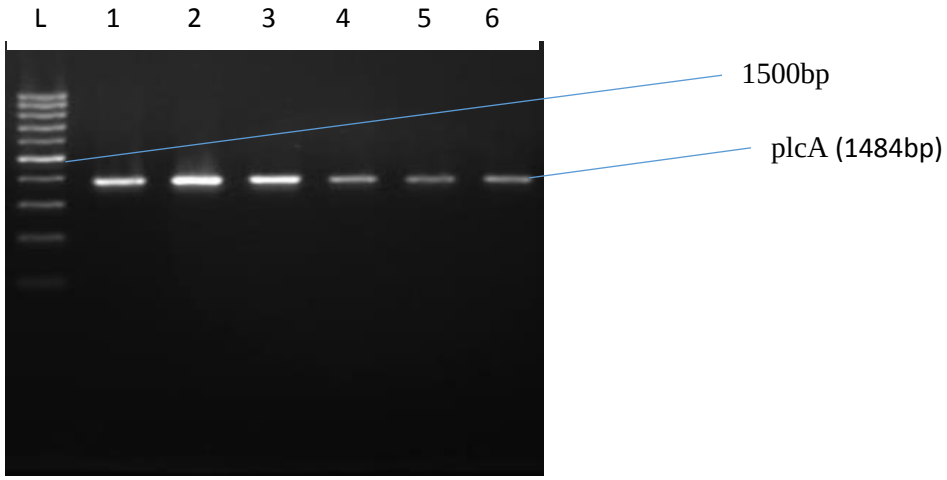

Figure 6. *plcA* gene bands of *Listeria*. Lane 1, 2, 3, 4, 5 and 6 represent the *plcA* gene bands at 1484bp of the 6 *Listeria* isolates while L represents the 100bp molecular ladder.

DISCUSSION

Salmonella enterica and *Listeria monocytogenes* possessing toxigenic and virulent properties are capable of causing human food infection and intoxication, and they play a vital role in the pathogenesis of an organism and have been implicated in food poisoning (Majowicz et al., 2010). Results of virulence potentials of *Listeria monocytogenes* and *Salmonella enterica* indicated that 100% were positive for DNase, capsule, coagulase, haemolysin (Listeriolysin O) and 84% produced biofilm. The presence of DNase helps the organisms to degrade the deoxyribonucleic acid to oligonucleotides, thereby enhancing the spread of the organisms into neighboring cells or tissues. DNase in these isolates could indicate their ability to elude the action of the immune system, thereby spreading and causing diseases within the host (Chang et al., 2011). The coagulase enzyme produced by the organisms causes blood clots by converting fibrinogen to fibrin and in turn prevents the organism from being phagocytosed by using the fibrin to coat its surface, making the organism more virulent (Majowicz et al., 2010). The presence of capsule and the ability to produce biofilm is a virulent factor because it can aid the organism to survive for a long period in a seemingly hostile environment such as even a food processing facility, partially due to its ability to endure various stress, such as sanitizers, pH and temperature and its ability to form biofilm leading to persistence in food (Chang et al., 2011).

The presence of *Salmonella enterica* and *Listeria monocytogenes* or their preformed toxin (A haemolysin) in food which colonizes the gastrointestinal tract, especially *Listeria monocytogenes* being a pathogenic species, has the unique ability to cause mammalian cells to absorb the organism into their cytoplasm. Using this ability, the pathogen penetrates the intestinal mucosa and can be disseminated by cell-to-cell spread or haematogenously (Omorodion et al., 2016).

As a result of these toxigenic and virulent factors, *Salmonella* infection remains a major public health concern worldwide, contributing to the economic burden of industrialized and undeveloped countries through the cost associated with surveillance, prevention and treatment of disease (Crump et al., 2004). The severity of *Salmonella* infections in humans depends on the virulence/toxigenic factors and varies depending on the serotype involved and the health status of the human host. *Salmonella enterica* is pathogenic as it can invade, replicate and survive in human host cells, resulting in potentially fatal disease (Hyeon et al., 2011).

An extract from the obtained 16S rRNA sequence of the isolates produced during the Basic Local Alignment Search Tool (BLASTN) in the National Centre for Biotechnology Information (NCBI) database were highly similar to the sequence from the NCBI non – redundant nucleotide (nr/nt) data base and the 16S rRNA classification of the isolates KOP8, CW14, BWP7, COR1, CPP17, BOP12, KPP11, COR7, CPP2, BWR20, CPR19 and CWP2 which identified bacterial isolates as *Salmonella enterica*-AM04528, *Salmonella bongori*-NCTC12419, *Listeria ivanovii*-PM-44, *Listeria*

monocytogenes-HN1*, Listeria monocytogenes*-F6540*, Salmonella enterica*-19_85*, Salmonella enterica*-KKP1761*, Listeria monocytogenes*-NITRR/R1*, Salmonella enterica*-777SA01*, Salmonella enterica*-KKP3882*, Listeria monocytogenes*-HR27*, Listeria monocytogenes*-FC3 with accession numbers CALNWE010000002.1, NR_074888.1, MZ572849.1, NZ_CP021325.1, NZ_LMTM01000002.1, CALNWF010000003.1, ON798425.1, OQ152618.1, CALNWC010000003.1, OP745459.1, OQ378323.1 and OQ024051.1 and relatedness of 100, 99, 100, 98, 99, 100, 100, 99, 100, 100, 81, 84(%). The phylogenic trees show the evolutionary distance or relationship tree of the 12 bacterial isolates as inferred from their nucleotide sequences using the Neighbour-Joining method.

The 6 isolates of *Salmonella enterica* were screened for the presence of toxigenic (*stn*) and virulent (*hilA*) genes and revealed that all the isolates of *Salmonella* (*Salmonella enterica*-AM04528, *Salmonella bongori*-NCTC12419, *Salmonella enterica*-19_85, *Salmonella enterica*-777SA01 and *Salmonella enterica*-KKP3882) had the toxigenic (stn) and virulent (*hilA*) genes in their genome except *Salmonella enterica*-KKP1761 that lack the *hilA* gene in its genome. These genes have contributed immensely to the pathogenicity of *Salmonella* during a disease condition (Bedasa et al., 2018). These bacteria possessing toxigenic and virulence potentials are capable of causing food infection as well as food intoxication in humans across the globe.

The *hilA* gene is required for the regulation of type 3 secretion apparatus genes, which secrete proteins that are related to cell invasion (FAO, 2017). *Salmonella* utilize the type III secretion system encoded within *Salmonella* pathogenicity island 1 (SPI1) to gain access to the mucosa of the small intestine as mediated by the *hilA* gene. This gene is essential for full virulence in *Salmonella* and is thought to trigger the internalization required for the invasion of deeper tissue (Bedasa et al., 2018).

Toxigenic factors in *Salmonella* are involved in the various stages of infection, which include the production of toxins (LP) endotoxin, enterotoxin, cytotoxin, colonization, adhesion and invasion, as well as survival inside the host cells (Nakano et al., 2012). The *Salmonella* isolates were found to carry an enterotoxin *stn* gene in this study, indicating that the *stn* gene is widely distributed among the *Salmonella* isolates irrespective of the source of the sample, species, serovars and location. The enterotoxin (*stn*) gene in *Salmonella* was highly conserved. Thus, the *stn* gene may be used as a target gene for the detection of *Salmonella* in different seafood samples because this enterotoxin is released in food after invasion of the food samples (Nakano et al., 2012). The enterotoxin *stn* can regulate OmpA membrane localization and functions in the maintenance of membrane composition and integrity. The research of Nikiema et al. (2021) proposed that *Salmonella* enterotoxin *stn* is a putative virulence factor and the causative agent of diarrhea, which are all essential for the virulence of *Salmonella.* The screening of the 6 isolates of *Listeria* to ascertain the presence of toxigenic (*plcA*) and virulence (*hlyA*) genes revealed that all the isolates of *Listeria* (*Listeria ivanovii*-PM-44*, Listeria monocytogenes*-HN1*, Listeria monocytogenes*-F6540*, Listeria monocytogenes*-NITRR/R1*, Listeria monocytogenes*-HR27*, Listeria monocytogenes*-FC3) possessed the toxigenic (*plcA*) and virulent (*hlyA*) genes in their genome.

The presence of toxigenic (*plcA*) and virulent (*hlyA*) genes in *Listeria* revealed that these genes play vital roles in the pathogenesis of diseases caused by *Listeria* (Jenkins et al., 2020). Okorie-Kanu et al. (2020) in their research, stated that the presence of toxigenic and virulence factor genes which express the toxigenic and virulent properties of the bacteria, is an important component of the bacteria that reveals the degree of pathogenicity of the organism. In vivo, the toxigenic gene (*plcA*) is required for full expression of the positive regulatory factor *prfA*, which regulates genes necessary for efficient cell-to-cell spread in the host (Mitchell et al., 2016). Another important role of the *plcA* gene is to encode PI-PLC, which is required for bacteria growth in the liver due to its toxigenic nature which may reflect its role in the lysis of macrophage phagosomes (Mitchell et al., 2016). Thus, *Listeria monocytogenes* have used this gene (*plcA*) to adopt strategies for coupling the expression of one virulent factor to that of a positive regulatory factor, which is necessary for the expression of other genes to induce severe disease conditions in the host as earlier reported by Tarr et al. (2020).

Listeria monocytogenes use the *hlyA* gene for the processes of cell invasion, intracellular survival, and cell-to-cell spreading, as this Gram-positive bacterium has evolved elaborate molecular strategies to subvert host cell functions. The *hlyA* gene as expressed in Listeriolysin O is a major virulence factor originally shown to be crucial for bacterial escape from the internalization vacuole after entry into cells. In recent studies, it is observed that Listeriolysin is expressed at high levels within the host cell phagosome, which they used to create pores within the phagosome, releasing the bacteria into the host cell cytoplasm thereby promoting survival in the host tissues (Disson and Lecuit, 2012). This work revealed the presence of these genes (p*lcA*, *hly*) and corroborates with Caixia et al. (2020), who observed the presence of this gene in *Listeria monocytogenes* when working with mice. It is also a potent antigen and is presented to the T-lymphocytes by the major histocompatibility complex class I and class II molecules to stimulate cell-mediated immune response. Listeriolysin functions as a hemolytic factor of blood, and the *hlyA* gene doubles as a promoter of gene expression

(Chen, 2019).

CONCLUSION AND RECOMMENDATION

The presence of toxigenic (*stn* and *plcA*) and virulence genes (*hlyA* and *hilA*) has been established in this study, and it contributes to the pathogenicity of *Salmonella enterica* and *Listeria monocytogenes*. Proper cooking of this seafood is highly recommended for the destruction of the preformed toxins and virulence factors in the bacteria.

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Citation: Barika PN, Akani NP, Sampson T, Robinson VK, 2024. Probing for virulence and toxigenic genes of *Salmonella enterica* and *Listeria monocytogenes* in seafood sold in Rivers State, Nigeria. Microbiol Res Int, 12(3): 57-64.