Prevalence of *Salmonella* Pathogenicity Island (SPI1, SPI2, SPI3 and SPI5) Genes in *Salmonella* species Isolated from fresh Broiler Chicken Meat in Sri Lanka

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**ABSTRACT**

**Purpose:** Salmonella is a significant pathogen affecting wider range of animals and one of the main causes of diarrhoeal diseases leading to millions of human cases globally. Pathogenicity islands of Salmonella (SPI) are imperative in invasion of host cell and pathogenesis within the cell. It is found that SPI from 1 to 5 are present in every serovars of *S. enterica* and these are laterally acquired virulence regions.

**Research Method:** This study investigated the presence of some virulence genes that belong to the SPI1, SPI2, SPI3 and SPI5 within 23 Salmonella isolates from broiler chicken meat in Sri Lanka using PCR method and further some virulence genes (gene encoding the invasion-associated protein, structural gene for attachment and invasion, regulatory gene for invasion and like nucleoid structuring gene) were quantified using RT-PCR.

**Findings:** The study revealed the presence of Pathogenicity Island 1 genes such as hilA, invH, invF, invA and hns in all the isolates. Among the SPI 2 genes investigated, all the isolates of Salmonella showed the presence of ssaO, ssaQ, ssaP and ssaS (genes encoding for T3SS apparatus proteins), sscB and sscA (gene for secretion system chaperon proteins), sseF, sseD (genes coding for secreted effector proteins). In all isolates, SPI 3 genes, responsible in magnesium transport (mgtB and mgtC) were found. The rhuM gene (that supposed to code for a cytoplasmic protein) was absent in four isolates whereas the gene cigR (which predicted as codes for membrane protein) was absent in two isolates. The SPI 5 genes pipB was present in all the isolates except in one isolate whereas sopB was absent in one isolate.

**Originality/Value:** This study found that some SPI genes are conserved in most of the Salmonella isolates from fresh broiler chicken meat in Sri Lanka and the expression of different virulence genes vary with the isolate. This molecular basic will pave the way to explore more on local isolates of Salmonella, detection methods and control methods.

**Keywords:** Broiler chicken, Expression, Salmonella spp., SPI, Virulent genes

**INTRODUCTION**

*Salmonella* is an intracellular pathogen with public health significance, infecting a vast range of animals. Many foodborne and waterborne diseases are caused by this organism and depending on the host, some serotypes cause gastroenteritis whereas other serotypes cause typhoid fever (Mambu et al., 2017; Banda et al., 2018). It is categorized as gram (-) ve, facultative anaerobe belongs to the family Enterobacteriaceae, which comprises of two species refered as *S. bongori* and *S. enterica*. Over 2600 serovars of *Salmonella* have been reported to be the cause of gastroenteritis in human and animals (Bhowmick et al., 2011; ²Department of Livestock Production, Faculty of Agricultural Sciences, Sabaragamuwa University of Sri Lanka, Belihuloya, Sri Lanka

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Pathogenicity of the bacterium is attributed by the ability of the organism to invade and replicate within eukaryotic host cells (Viegas et al., 2013). *Salmonella* enters into the digestive tract via contaminated water or food and then penetrates lining of the epithelial cells of the intestinal wall and thereafter stimulates a strong inflammatory tissue response (Feng et al., 2018).

The evading ability of the host environment by the pathogen is due to virulence of the strain. The virulence of most pathogenic organisms is known to bestow by a single region of the genome whereas in pathogenesis of *Salmonella* is a complex phenomenon and multifactorial which uses many virulence factors (Wallis and Galyov, 2000; Skyberg et al., 2006). Many virulence associated genes encode these virulence factors and these genes are located throughout the whole genome including plasmids, but are mostly clustered on specific genetic regions, called *Salmonella* pathogenicity islands (SPI) (Wallis and Galyov, 2000).

Clusters of virulence gene of *Salmonella* are located in 24 SPI which are crucial in pathogenesis (Blondel et al., 2009; Haneda et al., 2009; Desai et al., 2013; Hayward et al., 2013; Urrutia et al., 2014; Elder et al., 2016; Espinoza et al., 2017). These horizontally acquired loci code for genes that are important in virulence. Some of these mechanisms include secretory expressions, serotype conversion, expression related to flagella, fimbriae and capsules. In addition, they encode genes related to colonization of *Salmonella* in the host and their survival (Fàbrega and Vila, 2013; Chen et al., 2019).

SPIs are responsible for the invasion of the host cell by bacteria and it’s pathogenesis within the cell (Hensel, 2004). One to five SPI are general in all the serovars of *S. enterica*, whereas the rest of islands are scattered among various serovars or strains (Hayward et al., 2013; Espinoza et al., 2017). Pathogenicity Islands 1 and 2 of *Salmonella* spp bear the virulence genes responsible for intestinal phase of infection while intracellular survival, expression of fimbriae, multiple antibiotic resistance, uptake of magnesium and iron and the development of systemic infections are regulated by the remaining pathogenicity islands (Viegas et al., 2013).

As reported by Bruno et al. (2009) and some other researchers, T3SS (Type three secretion system) involves in stimulating the invasion of bacteria and inflammation in the area (Ehrbar et al., 2003, 2004; Patel and Galan, 2005; Figueira 2012; Ramos-Morales 2012; Moest and Meresse, 2013; Que et al., 2013; Cardenal-Muñoz et al., 2014).

The SPI-2 involves in facilitating the bacterial replication within the cell. T3SS of Pathogenicity Island 2 is an important part in *Salmonella* virulence (Jennings et al., 2017). SPI-3 contains mgtCB operon that encodes the MgtC (macrophage survival protein) and the MgtB (Mg\(^{2+}\) transporter) (Snávely 1991a; 1991b). Wood et al. (1998) and Cao et al. (2014) reported that the SPI-5 encodes a minimum of five genes that are responsible for enteropathogenesis and these genes are; pipA, pipD, pipB, sigE, and sigD/sopB.

Eventhough, the presence of SPI genes is a kind of virulent determinant in *Salmonella* (Sterzenbach et al., 2013) and its virulence is mainly determined by the virulence genes expression. SPI-1 decreases expression of invasion genes in *Salmonella* and thereby reduces invasion into host cells. Das et al. (2018) found that mutations created in SPI-1 genes can reduce the entry of bacteria into host epithelial cells and thereby prevent the occurrence of gastro enteritis. It is found that invA, invH, invF and hns are the most important genes in this regard (Lee and Falkow, 1990; Rhen and Dorman, 2005).

Being a developing country, financial and technological constraints limit the ability to conduct regular surveillance and because of that, there is much less understanding about the causes of foodborne infections including Salmonellosis.
in Sri Lanka. However, production and consumption of chicken meat have significantly increased in the country in the recent past and the broiler chicken meat and egg production now contribute to more than 70% of the livestock sector (DAPH, 2015). Previous studies conducted in Sri Lanka have found contamination of poultry with *Salmonella*, *Campylobacter* and *E. coli* (Dissanayake et al., 2008; Kamalika et al., 2008; Kottawatta et al., 2017). Lack of molecular level investigations on *Salmonella* can be identified as a main limitation in previous studies.

As scarcity of knowledge on SPI gene distribution and expression of virulence genes in local *Salmonella* isolates underpin the importance in search of molecular background of *Salmonella* spp., presence of some virulence genes belong to the SPI1, SPI2, SPI3 and SPI5 within the *Salmonella* spp isolated from broiler chicken meat in Sri Lanka was investigated. In addition, the expression of some of the virulence related genes was also quantified.

**MATERIALS AND METHODS**

*Salmonella Isolates*

Two hundred and sixty broiler chicken meat samples were randomly obtained from different locations of the country from 2012 August to 2013 August. Isolation of *Salmonella* spp from broiler chicken meat was done by conventional method of isolaton followed by polymerase chain reaction (PCR) confirmation as described by Jayaweera et al. (2020).

**Detection of Virulence Genes in Salmonella Isolates**

Presence of virulence genes which belong to different *Salmonella* pathogenicity islands SPI1, SPI2, SPI 3 and SPI5 genes) within 23 isolates of *Salmonella* were examined by using PCR targeting gene specific primers (Table 01). The DNA was isolated by the protocol of Ausubel et al. (1992) and concentration and also the Nanodrop (ND-1000, V3.3.0, Thermo Fisher Scientific, USA) was used to detect DNA purity of the extraction spectrophotometrically.

The PCR was performed in volume of 30 µL comprising 10X buffer (3 µL; 100 mM Tris-Hydrochloric acid (HCl; pH 9) 1.5 mM Magnesium chloride (MgCl$_2$), 500 mM Pottasium chloride (KCl), 0.1% Gelatin), 200 µM concentrations each of deoxyribonucleotide triphosphates (dATP, dGTP, dTTP and dCTP), primer (10 picomoles each) and 1.0 U of Taq DNA polymerase (GeneiTm, Merck Bioscience, Bangalore), with 2.0 µl of template DNA. A thermal cycler (BioRad, CA, USA) was used to perform the PCR reactions. Primer details are summarized in Table 01. Subsequently 1.5% agarose gel was used to resolve the the PCR products. A gel documentation system was used to visualize the bands (Herolab, Wiesloch, Germany).

**Quantifying the Expression of Virulence Gene**

*RNA extraction and cDNA synthesis:* Fourteen *Salmonella* isolates (14) and the calibrator strain, *Salmonella* Typhimurium (ATCC 14028) were grown in Luria Bertani broth in three independent cultures till it reached the exponential phase for the extraction of RNA. The density of cell was determined spectrophotometrically at 600 nm (Shimadzu UV-1601, Kyoto, Japan). Subsequenlty the bacterial cells were obtained and to increase the stability of bacterial RNA, bacterial RNA protective reagent (Qiagen) was added. Extraction of RNA was done with the use of RNasy Mini Kit (Qiagen, Hilden, Germany). Next, inorder to eliminate the remnants of DNA within the RNA extracts, they were treated with the enzyme DNase I (Fermentas, Germany), as per the instruction of manufacturer. As a next step, complete degradation of DNA in the DNase-treated RNA was checked and confirmed by PCR. The concentration of the RNA extract was detected with aid of Nano Drop spectrophotometer (ND-1000, V3.3.0, Thermo Fisher Scientific, USA) and the concentration of all samples were taken to the level of 200 ng µL$^{-1}$. 

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### Table 01: Primers detecting the virulence genes in *Salmonella* isolates

| Gene | Product size (bp) | Reference |
|------|------------------|-----------|
| **Salmonella Pathogenicity Island 1** | | |
| 1 hilA | 184 bp | Bhowmick *et al.* (2011) |
| 2 invH | 151 bp | Deekshit *et al.* (2015) |
| 3 invF | 155 bp | Deekshit *et al.* (2015) |
| **Salmonella Pathogenicity Island 2** | | |
| 4 ssaO | 378 bp | Bhowmick *et al.* (2011) |
| 5 ssaP | 375 bp | Bhowmick *et al.* (2011) |
| 6 ssaQ | 969 bp | Bhowmick *et al.* (2011) |
| 7 ssaS | 267 bp | Bhowmick *et al.* (2011) |
| **Salmonella Pathogenicity Island 3** | | |
| 8 sscB | 435 bp | Bhowmick *et al.* (2011) |
| 9 sseE | 417 bp | Bhowmick *et al.* (2011) |
| 10 sseD | 588 bp | Bhowmick *et al.* (2011) |
| 11 sscA | 474 bp | Bhowmick *et al.* (2011) |
| **Salmonella Pathogenicity Island 5** | | |
| 12 mgtB | 230 bp | NCBI gene bank (NC003197) |
| 13 mgtC | 196 bp | NCBI gene bank (NC003197) |
| 14 rhuM | 222 bp | NCBI gene bank (N (NC003197) |
| 15 cigR | 244 bp | NCBI gene bank (NC003197) |
| **Salmonella Pathogenicity Island 6** | | |
| 16 pipB | 230 bp | NCBI gene bank (NC003197) |
| 17 sopB | 1686 bp | NCBI gene bank (NC003197) |

### Table 02: Primers used in gene expression study

| Gene | Product size (bp) | Reference |
|------|------------------|-----------|
| invH | 151 | Deekshit *et al.* (2015) |
| invF | 155 | Deekshit *et al.* (2015) |
| hns | 152 | Jones *et al.* (1993) |
| invA | 284 | Rahn *et al.* (1993) |
| gyrB | 173 | Deekshit *et al.* (2015) |
In order to quantify the gene expression related to virulancy, cDNA was synthesized using reverse transcription. Briefly, the reverse transcription was performed in line with the instructions given in the product manual (Fermentas International Inc, Burlington, Onatario, Canada). Two microliters of reverse primer and 2 micro gram of RNA were kept at 70°C for a period of 5 min and immediately chilled using ice. Afterward, reaction mixture (8 µL) containing 4 µL of 5x reaction buffer (250 mM Tris-HCl, pH 8.3, 250 mM KCl, 20 mM MgCl₂, 50 mM DTT), 2 µL of 10 mM dNTP mix, ribonuclease inhibitor (20 units; Fermentas International Inc, Burlington, Onatario, Canada), RevertAidTM H minus MuLV reverse transcriptase (200 units; Fermentas International Inc, Burlington, Onatario, Canada) were added to the RNA-Primer mixture. The reaction mixture was incubated at 42°C for 60 min followed by heating at 70°C for 10 min and then cooled to 4°C. Samples of cDNA were confirmed by PCR and stored at -20°C for subsequent use in gene expression by real time polymerase chain reaction.

**Application of Real-time (RT) PCR to quantify expression:** As the first step of the real time PCR, primer concentrations were optimized. *Salmonella* gyrB (gyrase B) gene was the internal standard used (Deekshit et al., 2015). In order to enable the use of relative quantification (by 2^-ΔΔct formula), real-time PCR validation was done. Briefly, it was carried out by amplifying the serially diluted cDNA and this cDNA was synthesized from 1 µg of RNA. The absence of amplifying the untargeted fragments was confirmed by performing the analysis of dissociation curve for each gene. A reaction mix containing 12.5 µL of 2 x SYBR green master mixes, fitted volumes of reverse and forward primers and template cDNA (5 µL) were used to carry out the RT PCR. Each of the reaction mixture volume (25 µL) was fixed with sterile RNase free water and completed the reaction in a 7300 fast real-time PCR system (Applied Biosystems, Foster city, USA) with initial activation (at 50°C) for a 2-min period, initial denaturation (at 95°C) for 10 min followed by 45 cycles of denaturation (at 95°C) for 15 s, primer annealing (at 60°C) for 45 s and elongation (at 72°C) for 30 s. The data acquisition was done at the end of each elongation step by 7300 SDS software (v 1.3.1).

2^-ΔΔct method described by Livak and Schmittgen (2001) was applied in quantification of relative gene expression after validation of the method. The threshold cycle shortly given as CT is the fractional cycle number at which the quantity of amplified target reaches an appropriate threshold. Δct can be determined by substracting the average CT value of reference from the average CT value of target. For the validation, calculated ΔCT value was plotted against the cDNA concentration. As the derived slope of the graph was almost equal to 0 (approximately the value was 0.04), it could prove that the efficiency of amplification of reference and the target is nearly equal, accomplishing the prerequisite for applying the 2^-ΔΔct method.

The expression of the target genes was normalized to the endogenous control *gyrB* (gyrase B) by calculating ΔCT

\[
ΔCT = CT, \text{target} - CT, \text{gyrB}
\]

and expressed in relation to a control strain by calculating ΔΔCT:

\[
ΔΔCT = ΔCT - ΔCT, \text{Salmonella Typhimurium (ATCC 14028) was set as the control for the in vitro expression experiment. The relative expression was calculated as,}
\]

\[
\text{Relative expression} = 2^{-ΔΔCT}
\]

The data obtained from the real time PCR using 7300 SDS software (v 1.3.1) at the end of each elongation step were statistically analyzed at a 95% significant interval by using SPSS software (Version 15, IBM). Duncans Multiple Range Test was performed to compare means.

**RESULTS AND DISCUSSION**

**Presence of virulence genes**

Presence of virulence genes which belong to different SPIs (SPI1, SPI2, SPI3 and SPI5 genes) within 23 isolates of *Salmonella* were tested by PCR with the use of primers which are gene specific. Table 03 presents the presence of virulence genes in *Salmonella* isolates.
It has been well documented that the clusters of virulence genes of *Salmonella* are located in 24 SPIs and they play a major role in pathogenesis (Blondel et al., 2009; Haneda et al., 2009; Desai et al., 2013; Hayward et al., 2013; Elder et al., 2016; Espinoza et al., 2017). Virulence of an organism is governed by several factors and the SPI are important gene clusters mediated in host cell invasion as well as the intracellular pathogenesis (Hensel 2004, Hayward et al., 2013; Viegas et al., 2013; Espinoza et al., 2017).

This study revealed that all the genes related to pathogenicity island 1 (SPI-I) such as *hilA*, *invH*, *invF*, *invA* and *hns* were present in all 23 *Salmonella* isolates (Table 03, Figure 01). The virulence genes of the SPI-1 are related to the invasion ability of *Salmonella* and specially the gene *hilA* involves in coordinating the expression of several genes that are essential for invasion of *Salmonella* to the host (Mizusaki et al., 2008; Saini et al., 2010; Kaur et al., 2012). Rodriguez et al. (2002) noted that *hilA* (hyperinvasive locus A), is an OmpR/ToxR type of transcriptional regulator involved in invasion. Many other researchers also investigated the involvement of *hilA* in the process of *Salmonella* invasion (Darwin and Miller, 2000, 2001; Ellermeier and Slach, 2007; Thijs et al., 2007; Smith et al., 2016; Gaviria-Cantin et al., 2017). As this *hilA* gene was well found in all the *Salmonella* isolates of the current study, it is clear that all the *Salmonella* isolates gain a potent invasive capacity showing their high virulence. The current study has shown the presence of *invA* gene in all the isolates and the finding is in agreement with other studies that detected *invA* in all the isolates (100%) obtained from chicken samples (Abd El Tawwab et al., 2013; Cossi et al., 2013; Karmi, 2013: Karatug et al., 2018). Rahn et al. (1992) confirmed that this *invA* gene (gene encoding the invasion-associated protein) contains sequences unique to *Salmonella* and demonstrated that the *invA* gene is an appropriate PCR target, with possible diagnostic applications. By then, this gene was used for identification of *Salmonella* in many studies as with the current study (Shabarimuth et al., 2007; Bhowmick et al., 2011; Deekshit et al., 2015; Yang et al., 2016; Dmitric et al., 2018; Yang et al., 2018).

### Table 03: The presence of *Salmonella* virulence genes in *Salmonella* spp. isolated from broiler chicken meat

| Gene  | Salmonella Pathogenicity Island I | Salmonella isolate | S1 | S2 | S3 | S4 | S5 | S6 | S7 | S8 | S9 | S10 | S11 | S12 | S13 | S14 | S15 | S16 | S17 | S18 | S19 | S20 | S21 | S22 | S23 |
|-------|----------------------------------|--------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| hilA  |                                  |                    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| invH  |                                  |                    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| invF  |                                  |                    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| invA  |                                  |                    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| hns   |                                  |                    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|       |                                  |                    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |

| Gene  | Salmonella Pathogenicity Island II | Salmonella isolate | S1 | S2 | S3 | S4 | S5 | S6 | S7 | S8 | S9 | S10 | S11 | S12 | S13 | S14 | S15 | S16 | S17 | S18 | S19 | S20 | S21 | S22 | S23 |
|-------|----------------------------------|--------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| ssaO  |                                  |                    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| ssaP  |                                  |                    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| ssaQ  |                                  |                    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| ssaS  |                                  |                    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| sscB  |                                  |                    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| sseF  |                                  |                    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| sseD  |                                  |                    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| sscA  |                                  |                    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |

| Gene  | Salmonella Pathogenicity Island III | Salmonella isolate | S1 | S2 | S3 | S4 | S5 | S6 | S7 | S8 | S9 | S10 | S11 | S12 | S13 | S14 | S15 | S16 | S17 | S18 | S19 | S20 | S21 | S22 | S23 |
|-------|----------------------------------|--------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| mgIB  |                                  |                    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| mgIC  |                                  |                    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| rhuM  |                                  |                    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| cigR  |                                  |                    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |

| Gene  | Salmonella Pathogenicity Island V | Salmonella isolate | S1 | S2 | S3 | S4 | S5 | S6 | S7 | S8 | S9 | S10 | S11 | S12 | S13 | S14 | S15 | S16 | S17 | S18 | S19 | S20 | S21 | S22 | S23 |
|-------|----------------------------------|--------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| pipB  |                                  |                    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| sapB  |                                  |                    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
All the *Salmonella* isolates of the present study harbor the *invH* gene as it was shown by positive PCR (Table 03, Figure 01). The gene, *invH* encodes for outer membrane lipoprotein present in *Salmonella* spp. which aids the translocation of InvG from the cytoplasm onto the membrane (Crago and Koronakis, 1998). Pati et al. (2013) investigated the role of *invH* gene in mice colitis model during the early cecal inflammation induced by *Salmonella* Typhimurium. Further, many investigations revealed that *invH* is a commonly present gene in all *Salmonella* strain except *S. enterica* subspecies arizonae (Altmeyer et al., 1993; Dehghani et al., 2013). There were no reported homologous sequences for *invH* in *Shigella*, *Proteus*, *Yersinia* and several strains of enteroinvasive and enteropathogenic *E. coli* (Altmeyer et al., 1993) confirming its specificity.

Additionally, the present study has shown the presence of *invF* gene in all *Salmonella* isolates (Table 03). The gene, *invF* was identified by sequence analysis of the SPI-1 region and was predicted to encode an AraC-type transcriptional activator which facilitates the efficient entry of these organisms into cultured epithelial cell (Kaniga et al., 1994). Therefore, the current data are in agreement with other studies done in many parts of the world (Darwin and Miller, 1999; 2000; Altier et al., 2000; Deekshit et al., 2015).

All the investigated *Salmonella* SPI-2 genes, ssaP, ssaO, ssaS, sseF, sscA, ssaQ, sseD and sscB were present in all 23 isolates of *Salmonella* of the present study. Recent work on SPI-2 indicated that the genetic elements of this pathogenicity island have a vital role in systemic infections by *Salmonella* spp. as well in intracellular pathogenesis (Hacker et al., 1997; Hensel et al., 1997). SPI-2 encoded some effector proteins (Cirillo et al., 1998; Hensel et al., 1998; Szeto et al., 2009) are involved in replication of *Salmonella* spp within the host cells (intracellular). Some *Salmonella* strains harbored 516 genes of SPI-2 (Lamas et al., 2018). Bhowmick et al. (2011) showed the presence of SPI-2 genes such as, sscB, ssaO, ssaS, ssaQ, ssaP, sseF, sscA and sseD. Many researchers have also studied the SPI-2 genes of *Salmonella* and obtained similar kind of results (Hensel et al., 1999; Hansen and Hensel 2001; 2002; Klein and Jones, 2001; Amavisit et al., 2003; Waterman and holden, 2003; Schmidt and Hensel 2004; Fazl et al., 2013; Mc Whorter and Chousalkar, 2015; Jennings 2017). The present study is also in agreement with all those previous results, showing the availability of all the identified virulence genes in all 23 *Salmonella* samples isolated from broiler chicken in Sri Lanka.
Among the investigated genes of SPI-3 (\textit{mgtB, mgtC, rhuM and cigR}), only the genes accountable for magnesium transport i.e. \textit{mgtC} and \textit{mgtB} were available in all the isolates. The gene \textit{rhuM}, which is predicted as codes for a cytoplasmic protein was absent in four isolates (S3, S5, S7, S18) whereas the gene \textit{cigR}, which is predicted as codes for membrane protein was absent in two isolates (S14 and S18).

The data of the present study also revealed the presence of SPI-3 genes namely \textit{mgtC, mgtB, cigR} and \textit{rhuM} in the isolates of this study (Table 03; Figure 03) and that is in accordance with the findings of Blanc-Potard et al. (1999). Further, Bertelloni et al. (2017) found the existence of genes \textit{mgtC} and \textit{rhuM} in paratyphoid \textit{Salmonella enterica} strains isolated from poultry with the prevalence of 13/23 (56.52%) and 4/23 (17.39%), respectively. Zou et al. (2011) investigated the virulence genes profile in \textit{Salmonella} directly harvested from food or the environment of food animal and revealed that \textit{mgtB} and \textit{mgtC} genes of SPI-3, which are important for intracellular \textit{Salmonella} replication, were present in all serotypes while the \textit{rhuM} gene was rare. That result has been clearly reproduced in the present study having the \textit{mgtB} and \textit{mgtC} genes in all the isolates while \textit{rhuM} was absent in four isolates (Table 03). In addition, Niemann et al. (2011) and Yin et al. (2016) have shown the existence of \textit{cigR} in \textit{Salmonella} isolates and it is comparable with the findings of the current study.

![Figure 02: Presence of \textit{sseD} (A) and \textit{sscA} (B) genes of SPI-2 in different \textit{Salmonella} isolates](image)

![Figure 03: Presence of \textit{cigR} (A) and \textit{mgtB} (B) genes of SPI-3 in different \textit{Salmonella} isolates](image)
The presence of sopB and pipB genes of Salmonella pathogenicity island 5 (SPI-5) were checked in the present study and found that pipB was present in all the isolates except isolate S18 whereas sopB was absent only in isolate S2 (Table 03, Figure 04). Salmonella pathogenicity island 5 (SPI-5) has genes encoding for effector proteins for SPI-1 and SPI-2 and they are essential for the development of intestinal symptoms and for intracellular surviving (Roer et al., 2016) and promisingly they are involved in secretion of fluid as well in recruitment of neutrophil (Fierer and Guiney, 2001). SPI-5 is known to encode a minimum of five genes namely, sigD/sopB, pipD, sigE, pipB and pipA. They all are important in enteropathogenesis and it is studied in a calf model of infection by Wood et al. (1998) and Pfeifer et al. (1999). Bertelloni et al. (2017) found the presence of sopB and pipB in poultry isolates of Salmonella. Bhowmick et al. (2011) revealed that sopB was present in 90.34% Salmonella isolates of seafood and it is in accordance with the findings of the current study where sopB was found in all the isolates except S2 (Table 03). According to Knodler et al. (2002) regions of SPI-5 are not preserved in all Salmonella spp. and stated that pipB is not found in some Salmonella spp. Proving the findings of Knodler et al. (2002) this study has also shown the absence of pipB in one Salmonella isolates (S18) used in the study (Table 03).

Since the current study provides the evidence on the presence of different genes belong to the different pathogenicity islands, the data would help in understanding the virulence of the isolates as most of the SPI genes are regulating the virulence of the organism. This knowledge is crucial to be used in planning strategies to overcome outbreaks of Salmonella that could arise in the future.

**Virulence genes expression in Salmonella isolates**

Expression of invA (gene encoding the invasion-associated protein), invH (structural gene for attachment and invasion), invF (regulatory gene for invasion) and hns (histone like nucleoid structuring gene) in randomly selected 14 isolates out of the 23 Salmonella isolates were quantified using relative quantification method taking the Salmonella Typhimurium (ATCC 14028) as the control strain because it has been well defined, well characterized and has also been originally isolated from broiler chicken.

The expressions of the genes checked are given in Table 04 and it was expected to understand the level of virulence of the isolate by investigating the intensity of the expression of virulence genes in isolates.
The expression of major virulence genes have been investigated and found that the isolate S6 showed a significantly higher expression of invA gene (77.23 ± 46.66) than those in other isolates. The expression of invH gene (103.41 ± 35.03) was also significantly higher in isolate S6 than those of other isolates. Isolate S17 exhibited a significantly higher expression of invF gene than those other isolates with the values of 19.17 ± 3.52. The second highest expression of invF gene was shown by isolate S6. The expression of hns gene was the highest in the isolate S23 (14.61 ± 2.71). Isolates S21, S20, S18, and S9 had a significantly lower expression for all four genes (invA, invH, invF, and hns). The results showed that all invA, invH, invF and hns genes were present in all the tested isolates, but the virulence gene expression levels were differed among the isolates (p≤ 0.05). Thus, it was clear that the virulence of the tested isolates was different from each other. Isolate S6 showed the highest expressions for two (invA and invH) out of four tested virulence genes and the second highest expression for invF gene signaling the high virulence of that isolate. Apart from that, isolates S23 and S17 also carried virulence genes with significantly higher expression levels than those in the rest of the isolates.

Laughlin et al. (2014) revealed that it is essential to have precise regulation of the virulence gene expression by the pathogen both temporally and spatially. Suez et al. (2013) also studied the virulence genes profile and pathogenisity characterization of non-Typhoidal Salmonella. It has been shown that HilD regulates many genes either directly or indirectly through HilA, InvF or FlhDC (Martínez-Flores et al., 2016).

As non-typhoidal Salmonella infections are leading to serious threats to human health globally, there is a necessity of identifying the virulence genes and their expression patterns involved in pathogenesis. Deletion of genes encoding virulence in bacteria can be an approach to attenuate the bacterial virulence and subsequently for the effective therapeutic intervention. The genes invA, invH, invF and hns are considered as the most important genes in regulating the virulence of Salmonella (Lee and Falkow, 1990; Rhen and Dorman, 2005). The findings thus indicated that Salmonella isolated from broiler chicken meat in Sri Lanka also contained high expression of virulence genes showing the possibility of causing severe health problems.

| Isolate | invA Gene expression | invH Gene expression | invF Gene expression | hns Gene expression |
|---------|----------------------|----------------------|----------------------|----------------------|
| ST      | 1.02 ± 0.23          | 1.19±0.87            | 1.16±0.69            | 1.01±0.19            |
| S1      | 3.43 ± 0.27          | 1.83±0.37            | 4.41±1.66            | 1.81±0.15            |
| S3      | 2.11 ± 0.46          | 1.46±0.20            | 2.01±0.77            | 1.93±0.10            |
| S6      | 77.23 ± 46.66        | 103.41±35.03         | 10.98± 2.36          | 4.17±0.77            |
| S8      | 0.07 ± 0.02          | 0.11±0.04            | 0.19±0.13            | 1.65±0.31            |
| S9      | 0.07± 0.02           | 0.04±0.04            | 0.10±0.02            | 0.15±0.02            |
| S12     | 4.33± 0.07           | 17.28±7.43           | 4.80±0.26            | 8.56±0.72            |
| S13     | 6.82± 6.20           | 11.33±9.45           | 6.47±4.97            | 2.67±2.18            |
| S15     | 2.88± 0.70           | 12.64±2.07           | 1.42±0.77            | 2.87±0.36            |
| S17     | 1.81± 0.23           | 1.65±0.12            | 19.17±3.52           | 0.37±0.02            |
| S18     | 1.02± 0.33           | 0.64±0.22            | 0.12±0.03            | 0.69±0.21            |
| S20     | 0.52± 0.19           | 0.61±0.36            | 0.21±0.09            | 0.09±0.05            |
| S21     | 0.62± 0.15           | 0.55±0.23            | 0.50±0.22            | 1.14±0.29            |
| S22     | 7.08± 2.76           | 42.97±8.64           | 4.32±3.00            | 11.79±4.12           |
| S23     | 14.33± 8.32          | 64.14±13.94          | 4.61±0.55            | 14.61±2.71            |

Different letters show the statistically significant differences in attachment at p<0.05 based on one way ANOVA and mean separation by Duncans Multiple Range Test. SE ST indicates the Salmonella Typhimurium (ATCC 14028)
In this study, the highest gene expression for \textit{invA}, \textit{invH}, and \textit{hns} were shown by isolates S6 and S23 that belong to serotype \textit{S. Typhimurium}, while the highest expression level of the gene \textit{invF} was warranted by isolate S17, which belongs to the serotype \textit{S. Enteritidis}. Huehn \textit{et al.} (2010) showed that the virulence determinants are known to be highly conserved among serovars and this is consistency with the current study where it found different expression levels for tested four genes among the different isolates. \textit{S. enterica} serovars have shown diverse host specificity and different capability to cause disease in the hosts and it is thought to be serovar reliant. These variations among the serovars found to be related to the availability or absence of genes and also more importantly the expression levels of virulence genes (Andino and Hanning, 2015).

CONCLUSION

This study concluded that pathogenicity Island 1 genes are present in all the isolates from fresh broiler chicken meat in Sri Lanka. Genes encoding for T3SS apparatus proteins, genes for secretion system chaperon proteins and genes coding for secreted effector proteins of SPI 2 genes are also present in all the isolates. Presence of SPI 3 genes shows variations among the isolates and majority of the isolates carry the SPI 5 genes as well. The expression of different virulence genes also vary with the isolate.

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Conflicts of Interest

The authors declare that there is no conflict of interests.

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