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Prevalence of *Salmonella* Pathogenisity 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 diarroeal 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 magenisum 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;

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Issenhuth-Jeanjean *et al.,* 2014; EFSA 2015; Ryan *et al.,* 2017). Subsp. *enterica*, *arizonae*, *salamae*, *houtenae*, *diarizonae* and *indica* are the six subspecies of *S. enterica. Salmonella enterica* subspecies *enterica* is composed of more than 1500 serovars with some of great clinical relevance, such as *S*. Typhimurium and *S*. Enteritidis isolated predominantly from poultry (Lamas *et al.,* 2018).

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 organims 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). Pathogenisity 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) (Snavely 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-1genes can reduce the entry of bacteria into host epithelial cells and thereby prevent the occurance 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 Bertaini 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 RNeasy 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}.

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

	Gene	Gene description	Primer sequence	Product size	Reference
		Salmonella Pathogenicity Island 1			
1	*hilA*	Regulator protein	F: GTCCGGTCGTAGTGGTGTCT R: CGGCAGTTCTTCGTAATGGT	184 bp	Bhowmick *et al*. (2011)
2	*invH*	Structural gene for attachment and invasion	F: AACGCTGATAATTCCGCATC R: CGGTCATGAGTTGCTCTTCA	151bp	Deekshit *et al*. (2015)
3	*invF*	Regulatory gene for invasion	F: TGTCGCACCAGTATCAGGAG R: AAATAGCGCGAAACTACGGA	155 bp	Deekshit *et al*. (2015)
		Salmonella Pathogenicity Island 2			
4	*ssaO*	T3SS apparatus protein	F: ATGGAAACTTTGCTGGAGAT R: TCAACTTTGGTAATACGCAT	378 bp	Bhowmick *et al*. (2011)
5	*ssaP*	T3SS apparatus protein	F: ATGCGTATTACCAAAGTTGA R: TCATTCGCTATTCTTAACAT	375 bp	Bhowmick *et al*. (2011)
6	*ssaQ*	T3SS apparatus protein	F: ATGTTAAGAATAGCGAATGA R: TTACGCTGTATTTTTGCAAA	969bp	Bhowmick *et al*. (2011)
7	*ssaS*	T3SS apparatus protein	F: ATGAATGATTCTGAATTGAC R: TCAACCATGCTCTCCAATTC	267 bp	Bhowmick *et al*. (2011)
8	*sscB*	Secretion system chaperon protein	F: ATGATGATGAAAGAAGATCA R: TTAAGCAATAAGAGTATCAA	435 bp	Bhowmick *et al*. (2011)
9	*sseE*	Secreted effector protein	F: ATGGTGCAAGAAATAGAGCA R: TTAAAAACGTCGCTGGATAA	417 bp	Bhowmick *et al*. (2011)
10	*sseD*	Secreted effector protein	F: ATGGAAGCGAGTAACGTAGC R: TTACCTCGTTAATGCCCGGA	588 bp	Bhowmick *et al*. (2011)
11	*sscA*	Secretion system chaperon protein	F: ATGAAAAAAGACCCGACCTA R: TTAGCTCCTGTCAGAAAGTT	474 bp	Bhowmick *et al*. (2011)
		Salmonella Pathogenicity Island 3			
12	*mgtB*	Magnesium transport	F: GTGGTGCGAACCATCTTTTT R: ATTCAGCTTCTGCAGGCATT	230 bp	NCBI gene bank (NC003197)
13	*mgtC*	Magnesium transport	F: TCCAGTGAATTGCGGTGATA R: GCGGTTCGTCAGTGGTTACT	196 bp	NCBI gene bank (NC003197)
14	*rhuM*	Predicted cytoplasmic protein	F: CATCGGCTGTACCCGACTAT R: CAGCACGCTGATGAATGAGT	222 bp	NCBI gene bank (N (NC003197)
15	*cigR*	Predicted membrane protein, putative cleavable N-terminal signal sequence	F: CTGATAACCCGTCAGCCCTA R: GGCGACGTTGATTACGTTTT	244 bp	NCBI gene bank (NC003197)
		Salmonella Pathogenicity Island 5			
16	*pipB*	Translocator protein	F: AATATCGGATGGGGGAAAAG R: AACCTGACTCACGCAGACCT	230 bp	NCBI gene bank (NC003197)
17	*sopB*	Translocator protein	F: TTTCAATTGCTTCACGTTTGA R: AATTCCGCGTCTTTTCAGATT	1686 bp	NCBI gene bank (NC003197)

Table 02: Primers used in gene expression study

Gene	Gene description	Primer sequence	Product size (bp)	Reference
invH	structural gene for attachment and invasion	F:AACGCTGATAATTCCGCATC R:CGGTCATGAGTTGCTCTTCA	151	Deekshit *et al*. (2015)
invF	regulatory gene for invasion	F:TGTCGCACCAGTATCAGGAG R:AAATAGCGCGAAACTACGGA	155	Deekshit *et al*. (2015)
hns	histone like nucleoid structuring gene	F:TACCAAAGCTAAACGCGCAGCT R:TGATCAGGAAATCTTCCAGTTGC	152	Jones *et al*. (1993)
invA	gene encoding the invasion-associated protein	F:GTGAAATTATCGCCACGTTCGGGCAA R:TCATCGCACCGTCAAAGGAACC	284	Rahn *et al*. (1992)
gyrB	Gene encoding for gyrase B	F:GCGTGAACTGTCATTCCTGA R:TACCGTCTTTTTCGGTGGAG	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 perfomed 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 $MgCl_2$, 50 mM DTT), 2 µL of 10 mM dNTP mix, ribonuclease inhibitor (20 units; Fermentas International Inc, Burlington, Onatario, Canada), RevertAidTM H minus M 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 rection.

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 carriedout 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).

$2^{-\Delta\Delta ct}$ 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 $2^{-\Delta\Delta ct}$ method.

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

ΔCT = CT, target - CT, *gyrB* and expressed in relation to a control strain by calculating ΔΔCT:

ΔΔCT = ΔCT - ΔCT, *Salmonella* Typhimurium (ATCC 14028) was set as the control for the *in vitro* expression experiment. The relative expression was calculated as,

Relative expression = $2^{-\Delta\Delta Ct}$

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 a organism is goverened 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 Slauch, 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 (Shabarinath *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

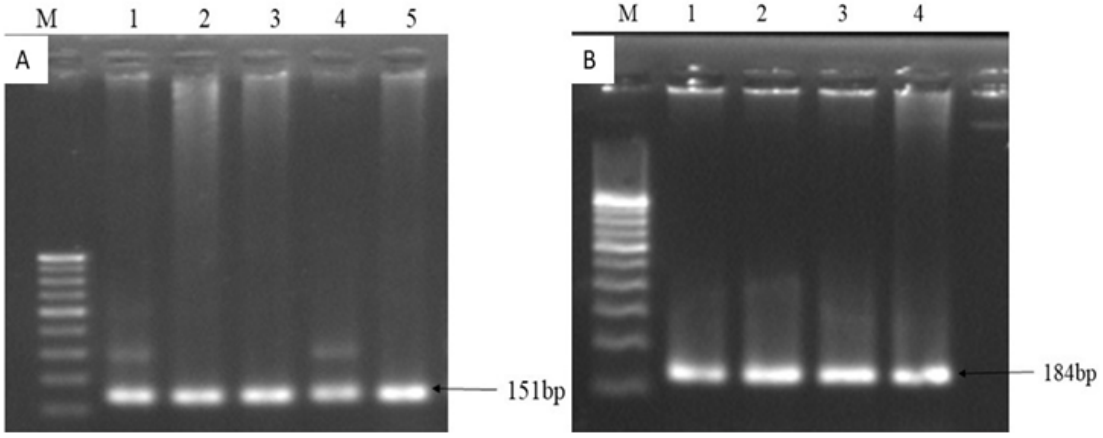
Salmonella Pathogenicity Island I																							
Gene	*Salmonella* isolate																						
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S7	S18	S19	S20	S21	S22	S23
hilA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
invH	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
invF	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
invA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
hns	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Salmonella Pathogenicity Island II																							
Gene	*Salmonella* isolate																						
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S7	S18	S19	S20	S21	S22	S23
ssaO	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ssaP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ssaQ	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ssaS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
sscB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
sseF	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
sseD	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
sscA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Salmonella Pathogenicity Island III																							
Gene	*Salmonella* isolate																						
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S7	S18	S19	S20	S21	S22	S23
mgtB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
mgtC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
rhuM	+	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
cigR	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+
Salmonella Pathogenicity Island V																							
Gene	*Salmonella* isolate																						
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S7	S18	S19	S20	S21	S22	S23
pipB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
sopB	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

hilA; Regulator protein, invH; structural gene for attachment and invasion, invF; regulatory gene for invasion, ssaO; T3SS apparatus protein, ssaP; T3SS apparatus protein, ssaQ; T3SS apparatus protein, ssaS; T3SS apparatus protein, sscB; Secretion system chaperon protein, sseF; Secreted effector protein, sseD; Secreted effector protein, sscA; Secretion system chaperon protein, mgtB; Magnesium transport, mgtC; Magnesium transport , rhuM; Predicted cytoplasmic protein, cigR; Predicted membrane protein, putative cleavable N-terminal signal sequence, pipB; Translocator protein, sopB; Translocator protein, hns; histone like nucleoid structuring gene, invA; gene encoding the invasion-associated protein.

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 hat *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.

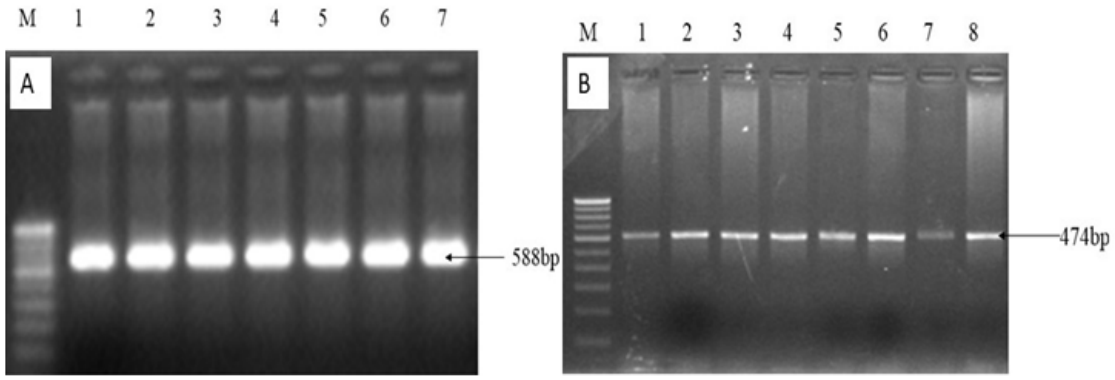
Additionally, the present study has shown the presence of *invF* gene in all *Salmonella* isolates (Table 03). The gene, *invF* was identified by sequence analysis of the SPI-1 region and was predicted to encode an AraC-type transcriptional activator which facilitates the efficient entry of these organisms 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 comparted 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 reserchers 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.



A : M-100 bp DNA Ladder GeNei ™, Bangalore, India; Lane:5 Positive control (ATCC14028) ; Lane: 1-4, S2, S3, S4 and S5
B : M-100 bp DNA Ladder GeNei ™, Bangalore, India; Lane:1 Positive control (ATCC14028) ; Lane: 2-4, S1, S2 and S3

Figure 01: The presence of *invH* (A) and *hilA* (B) genes of SPI-1 in different *Salmonella* isolates



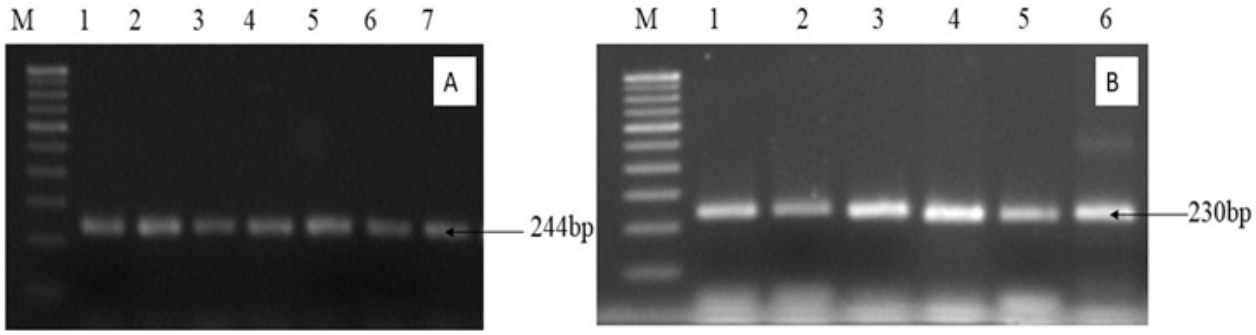
A : M-100 bp DNA Ladder GeNei ™, Bangalore, India; Lane:1 Positive control (ATCC14028) ; Lane: 2-7, S2, S3, S4, S5, S6 and S7

B : M-100 bp DNA Ladder GeNei ™, Bangalore, India; Lane:1 Positive control (ATCC14028) ; Lane:1 Positive control (ATCC14028) ; Lane: 2-4, S1, S2, S3, S4, S5, S6 and S7

Figure 02: Presence of *sseD* (A) and *sscA* (B) genes of SPI-2 in different *Salmonella* isolates

Among the investigated genes of SPI-3 (*mgtB*, *mgtC*, *rhuM* and *cigR*), only the genes accountable for magenisum transport i.e. *mgtC* and *mgtB* were available in all the isolates. The gene *rhuM,* which is predicted as codes for a cytoplasmic protein was absent in four isolates (S3, S5, S7, S18) whereas the gene *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 *mgtC*, *mgtB*, *cigR* and *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 *mgtC* and *rhuM* in paratyphoid *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 *Salmonella* directly harvested from food or the environment of food animal and revealed that *mgtB* and *mgtC* genes of SPI-3, which are important for intracellular *Salmonella* replication, were present in all serotypes while the *rhuM* gene was rare. That result has been clearly reproduced in the present study having the *mgtB* and *mgtC* genes in all the isolates while *rhuM* was absent in four isolates (Table 03). In addition, Niemann *et al*. (2011) and Yin *et al*. (2016) have shown the existence of *cigR* in *Salmonella* isolates and it is comparable with the findings of the current study.



A : M-100 bp DNA Ladder GeNei ™, Bangalore, India; Lane:1 Positive control (ATCC14028) ; Lane: 2-7, S2, S3, S4, S5, S6 and S7

B : M-100 bp DNA Ladder GeNei ™, Bangalore, India; Lane:1 Positive control (ATCC14028) ; Lane:1 Positive control (ATCC14028) ; Lane: 2-4, S1, S2, S3, S4, S5 and S6

Figure 03: Presence of *cigR* (A) and *mgtB* (B) genes of SPI-3 in different *Salmonella* isolates

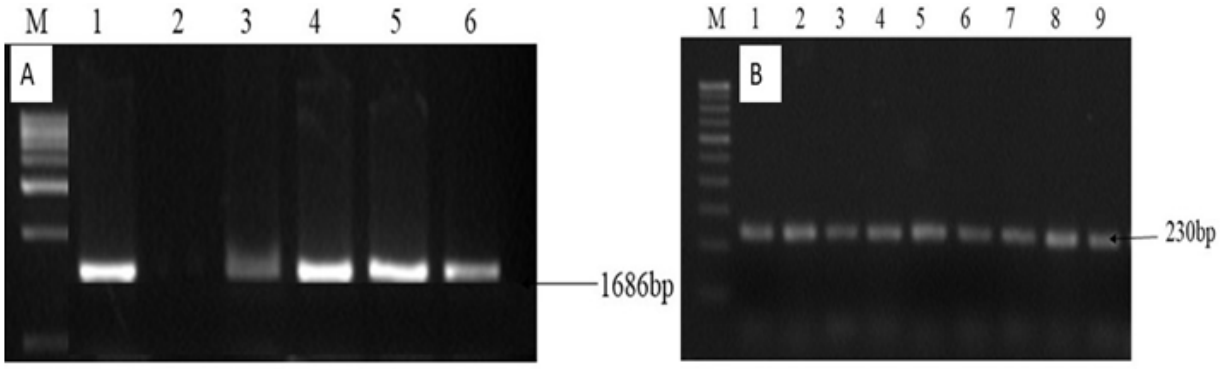
The presence of *sopB* and *pipB* genes of *Salmonella* pathogenicity island 5 (SPI-5) were checked in the present study and found that *pipB* was present in all the isolates except isolate S18 whereas *sopB* was absent only in isolate S2 (Table 03, Figure 04). *Salmonella* pathogenicity island 5 (SPI-5) 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 abscence of *pip*B 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 stratergies to overcome outbreakes 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.



A : M-100 bp DNA Ladder GeNei ™, Bangalore, India; Lane:1 Positive control (ATCC14028) ; Lane: 2-6, S2, S3, S4, S5 and S6

B : M-100 bp DNA Ladder GeNei ™, Bangalore, India; Lane:1 Positive control (ATCC14028) ; Lane:1 Positive control (ATCC14028) ; Lane: 2-9, S1, S2, S3, S4, S5, S6, S8

Figure 04: Presence of *sopB* (A) and *pipB* (B) genes of SPI-5 in different *Salmonella* isolates

Table 04: Expression of invA, invH, invF and hns genes in Salmonella isolates

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

Different letters show the statistically significant differences in attachment at p<0.05 based on one way ANOVA and mean sepration by Duncans Multiple Range Test.se ST indicates the Salmonella Typhimurium (ATCC 14028)

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 ($p \leq 0.05$). 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.

In this study, the highest gene expression for *invA*, *invH,* and *hns* were shown by isolates S6 and S23 that belong to serotype *S.* Typhimurium, while the highest expression level of the gene *invF* was warranted by isolate S17, which belongs to the serotype *S.* Enteritidis. Huehn *et al*. (2010) showed that the virulence determinants are known to be highly conserved among serovars and this is consistence with the current study where it found different expression levels for tested four genes among the different isolates. *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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