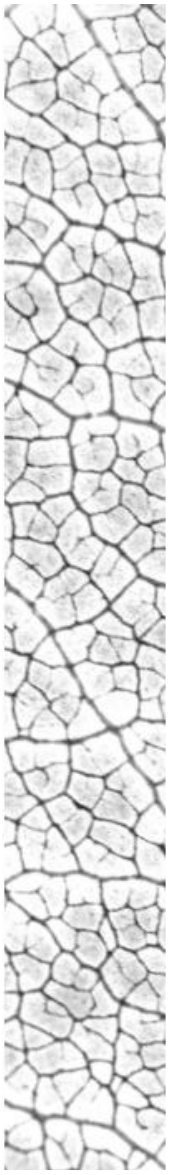

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Genetic detection of in vA, sipB, SopB and sseC genes in Salmonella spp isolated from diarrheic children patients

Maryam Sadiq Obayes [1*], Oruba K. Al-Bermani [1], Shaima Ahmed Rahim [1]

[1] College of science for Women, University of Babylon, Babil, IRAQ
*Corresponding author: Maryam Sadiq Obayes

Abstract
Salmonella Gram-negative bacteria infect human and animals and cause gastroenteritis and typhoid fever. Type III secretion system are important virulence factor of salmonella it basically and functionally associated with flagella meeting systems and typically comprise more than 20 proteins subunits that are found in the internal and external membrane of the bacterial cell. The current study aims to conduct an detection the candidates genes of type III secretion system as important virulence factor of a clinical Salmonella spp. The Patient and methods: current study includes 120 stool samples collected from 120 diarrheic children, age range from (2 to 3 years) for the period from November (2018) to December (2018) from Babylon province Iraq. After culturing samples, Salmonella spp.diagnosed. DNA extraction and PCR were achieved for detection type III secretion system genes of Salmonella spp. The results: out of 120 stool sample, 58 samples were positive for Salmonella spp. we revealed that Salmonella typhi, Salmonella typhimurium, Salmonella arizonae, and Salmonella paratyphi, the most common serovar of Salmonella enterica, was Salmonella typhi at 29.3%. Genetic detection of type III secretion system by PCR technique explains that Inva A was found about at all isolates except one isolate at percentage (93.1%) and SipB occurrence was (18.9%) finally SseC occurrence was (1.7%) whereas SopB was not detected in all salmonella isolates. inva A gene is most reliable gene in the diagnosis of Salmonella spp. and not all isolates contain a different genes of type III secretion system.

Keywords: Salmonella Type III secretion system, invA, sipB, sseC, sopB

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INTRODUCTION

Salmonella is classified into two species Salmonella bongori and Salmonella enteric. Salmonella enterica subspecies are more divided into about 2500 serovars based on the flagellar antigen and lipopolysaccharide (LPS) antigen example of these serovars, typhimurium, typhi, enteritidis, and paratyphi additionally divided into three classes (i) typhoidal salmonella causing typhoid and paratyphoid fever in men; (ii) non-typhoidal salmonella leading to gastroenteritis of human and animals and (iii) salmonella- dependent host like salmonella dublin restricted with cattle host. Serovar of virulence genes were characterized in salmonella, these are found in a clusters called salmonella pathogenicity islands (SPIs). Which are recently recorded 23 of pathogenicity islands of salmonella spp (Espinoza, et al. 2017; Sabir, & Sabir,2018). Salmonella Pathogenicity Islands -1(SPI-1) encodes as specialized needle-like structure that mediates the discharge of Salmonella invasion proteins into the host cytoplasm. Which cause inflammatory response, Inva A as an inner membrane protein is a component of the basal body f T3SS, it is widely used for diagnosis of Salmonella spp from clinical samples. The SipB as a translocon protein play important role for translocation of next effector protein additionally it induces programmed death for macrophages in vitro. Salmonella within phagocytic and non-phagocytic cells is rounded by a membrane called salmonella containing vacuole in this condition other proteins are needed by salmonella that encoded by SPI-2, prevent the fusion of the phagosome-lysosome and defense of salmonella from lysozyme action (Gerlach, & Hensel, 2007). The Salmonella Secreted Effecter (Sse) proteins like SseC type encoded by SPI-2 is a translocon for the effector proteins and the stains that mutants for his genes lack the virulence factor for surviving in the phagocytic cells (Boonyom, et al. 2010). The salmonella outer protein B (SopB) other protein that encoded by SPI-5 of salmonella that play important role in development enteritis the presence of this gene is more variable in the salmonella strains (Rahman, 2006). The

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Table 1. Details of Primers Sequencing Used In the Study of Genes

Gene details	Sequencing	Sizing	Reference
invA Forward	5-ACAGTGCTCGTTTACGACCTGAAT-3	284bp	(Smith *et al*., 2015)
invA Reverse	5-AGACGACTGGTACTGATCGATAAT-3		
sipB Forward	5-GGACGCCGCCCGGGAAAAACTCTC-3	875bp	(Tarabees *etal*.,2017)
sipB Reverse	5-ACACTCCCGTCGCCGCCTTCACAA-3		
sopB Forward	5-GATGTGATTAATGAAGAAATGCC-3	1170bp	(Soto *et al*., 2006)
sopB Reverse	5-GCAAACCATAAAAACTACACTCA-3		
sseC Forward	5-ATGAATCGAATTCACAGTAA-3	1455bp	(Bhowmick *et al* .,2011)
sseC Reverse	5-TTAAGCGCGATAGCCAGCTA-3		

Table 2. The occurrence of *salmonella* spp from stool sample

Salmonella spp	Number of isolates	Percentage %
Salmonella entericaserovar typhi	22	37.9%
Salmonella enterica serovar typhimurium	17	29.3%
Salmonella enterica serovar arizonae	10	17.2%
Salmonella enterica serovar paratyphi	9	15.5%
Total	58	99.9%

current research is designed to investigate the presence of specific virulence factor genes (type III secretion system genes)in a clinical isolates of Salmonella species.

MATERIAL AND METHODS

Patients and specimens collection

The diarrheic stool samples were collected for the period from November 2018 to December 2018 from children age range (1-3 years) who attend to Al-Noor hospital and Maternity hospital in Babylon province.These specimens seeded on the Salmonella *Shigella* ager and XLD ager for primary isolation and characterization of *Salmonella* species then incubated for 24 h at 37^{o}C.

Salmonella diagnose

The *salmonella* spp isolates were diagnosed by using Enterosystem Biochemical Kit (Liofichen, Italy)

Bacterial Genomic DNA extraction

Bacterial Genomic DNA of *salmonella* was separated according to the guidelines of the manufactured company (Promega, USA).

Primers Preparation

Primers dissolved and prepared according to manufactured company (Alpha-USA).

Duplicate reaction set was done.the reaction comprises forward primer 1µl and the reverse primer 1µl, 5µl of DNA specimen, 12.5µl of master mix (Promega-USA) and 5.5µl of Nuclease free water. All the elements was collected in a special tube called micro centrifuge tube PCR then these enter into Thermo cycle apparatus with cycling conditions for *invaA* gene as following a hot start cycle of 94^{o}C for 5 min, then 35 cycles of 94^{o}C for 1 min, 56^{o}C for 1 min and72^{o}C for 2 min, ending with a final extension step of 72^{o}C for 5 min. while for *sipB* gene were the following a hot start cycle of 95^{o}C for 5 min, then 30 cycles of 94^{o}C for 30 sec, 66.5^{o}C for 30 sec and72^{o}C for 2 min, ending with a final extension step of 72^{o}C for 10 min. whereas *sseC* gene as following a hot start cycle of 95^{o}C for 5 min, then 35 cycles of 95^{o}C for 1 min, 43^{o}C for 50 Sec and72^{o}C for 1.5 min, ending with a final extension step of 72^{o}C for 2 min. finally *sopB* gene as following a hot start cycle of 94^{o}C for 5 min, then 30 cycles of 94^{o}C for 1 min, 60^{o}C for 1min and72^{o}C for 2 min, ending With a final extension step of 72^{o}C for 5 min. the PCR amplified products Were determined on 1.5% agarose gel at 80 Volts, with DNA marker (100-2000bp).

Statistical analysis

The analyses was achieved by using Chi-square, *P* value at 0.05 and calculating odds ratios (OR) and 95% confidence intervals (CI). in order to determine whether any significant in distribution of genes among the isolate. (P value less than 0.05 was considered statistically significant)

Ethical statement

Stool samples were collected from infected children with the consent of their parents after explaining the cause. The work was also carried out with the support by Department of Biology, College of Science for women, Babylon University, Iraq.

RESULTS

Salmonella occurrence

Out of one hundred and twenty diarrheic stool specimens, 58 sample were positive culture for *Salmonella* spp. The highest isolation rate at 37.93% of *salmonella typhi*, then *salmonella typhimurium* at 29.3%, followed 17.2% of *salmonella arizonae*, finally 15.5% of *salmonella paratyphi*. The data are shown below in the **Table 2**.

Molecular Investigation

In correctness study was mainly focused on 4 genes (InvaA, SipB, SseC and SopB,) that are known to have a role in *Salmonella* infection in faces. Of 4 examined virulence genes, only 3 genes were successfully amplified using polymerase chain reaction technique.

a- *invaA* gene of *Salmonella* spp (inner membrane protein gene)

According to the PCR reaction which carried out for amplification *inva A* of the recovered *salmonella* spp in our study found that, high prevalence rate of *invaA* gene

Table 3. Distribution *invaA* gene according to the *salmonella* spp

Salmonella spp	Gene presence	Percentage %
Salmonella typhi	21	95.4%
Salmonella typhimurium	17	100%
Salmonella arizonae	9	90%
Salmonella paratyphi	7	77.7%
Total	54	93.1%

Table 4. Occurrence of *invaA* gene in *salmonella typhimurium* determination with *salmonella typhi* (reference strain)

Gene name	Serovar	Positive N (%)	Negative N (%)	*P*-Value	Chi- square	OR (95%)
invaA	*Salmonella-typhi*	21(36.20%)	37(63.79%)	0.27	0.62	1.36(0.62-2.98)
	Salmonella typhimurium	17(29.31%)	41(70.68%)			

P ≤ 0 .05 , OR= (95%CI). N(number), OR(odds ratio) , CI(confidence intervals)

Table 5. Occurrence of *invaA* gene in *salmonella paratyphi* determination with *salmonella typhi* (reference strain)

Gene Name	Serovar	Positive N (%)	Negative N (%)	*P*-Value	Chi- square	OR (95%)
invaA	*Salmonella typhi*	21(36.20%)	37(63.79%)	0.009*	5.47	3.09 (1.26-7.52)
	Salmonella paratyphi	9(15.51%)	49(84.48%)			

P ≤ 0 .05, OR= (95%CI). N(number), OR(odds ratio) , CI(confidence intervals)

Table 6. Occurrence of *invaA* gene in *salmonella arizonae* determination with *salmonella typhi* (referencestrain)

Gene Name	Serovar	Positive N (%)	Negative N (%)	*P*-Value	Chi- square	OR (95%)
invaA	*Salmonella typhi*	21(36.20%)	37(63.79%)	0.017	5.32	2.27(1.45-6.48)
	Salmonella arizonae	10(17.24%)	48(82.26%)			

P ≤ 0 .05 , OR= (95%CI). N(number), OR(odds ratio) , CI(confidence intervals)

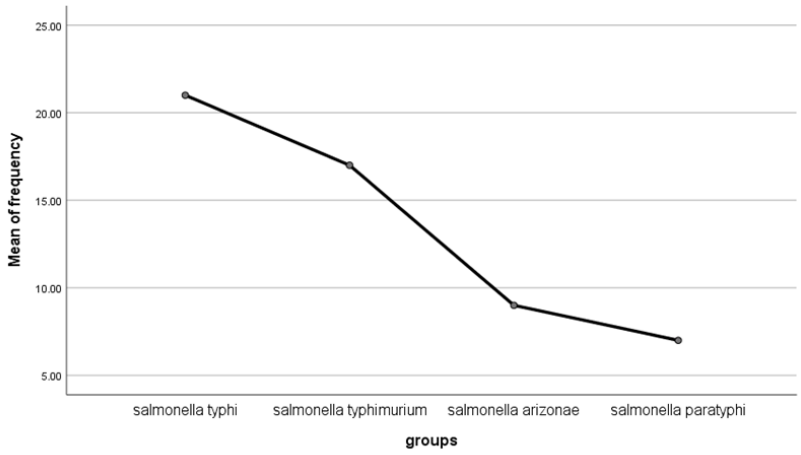

Fig. 1. Distribution of Inva A gene in *salmonella enterica* serovars

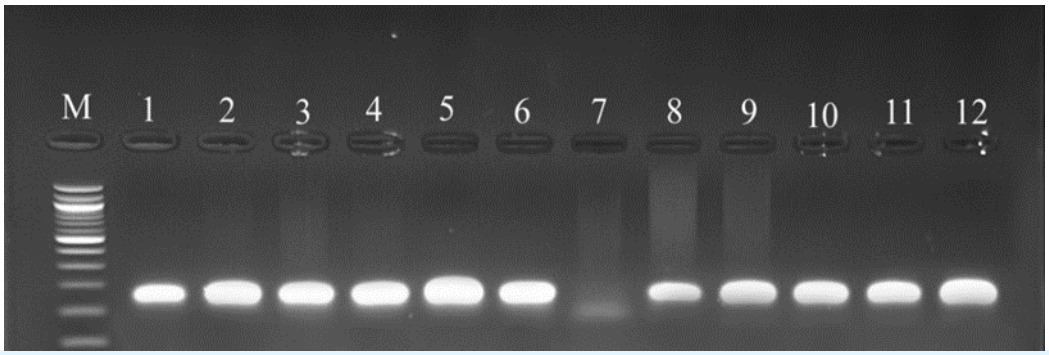

Fig. 2. Agarose gel electrophoresis of PCR assay showed *invaA* gene of *salmonella* spp .Lane (M) DNA Marker (100-1500bp); lane (1-12) *invaA* gene at 284bp PCR product size

was 95.4% in *salmonella typhi*, followed 100% for *salmonella typhimurium*, followed 90% for *salomnella arizonae* and 77.7% for *salmonella paratyphi*. **Table 3, Fig. 2**. The difference in a distribution of *invaA* gene in the the *salmonella* spp comparison with reference strain (*Salmonella typhi*) was statically significant P≤0.05, **Tables 4**, **5**, **6**; **Fig. 1**.

b- *SipB* gene of *salmonella* spp (translocation machinery component)

We found that, great prevalence rate of *sipB* gene was (36.6%) in *salmonella typhi*, followed9% for

Table 7. Distribution *sipB* gene according to the *salmonella* spp

Salmonella spp	Gene presence	Percentage %
Salmonella typhi	8	36.6%
Salmonella typhimurium	2	9.0%
Salmonella arizonae	0	0%
Salmonella paratyphi	1	4.5%
Total	11	18.9%

Table 8. Occurrence of sipB gene in salmonella typhimurium determination with salmonella typhi (reference strain)

Gene Name	Serovar	Positive N (%)	Negative N (%)	P-Value	Chi- square	OR (95%)
sipB	Salmonella typhi	8(13.79%)	50(86.20%)	0.04	3.94	4.48(0.90-22.09)
	Salmonella typhimurium	2(3.44%)	56(96.55%)			

P ≤ 0 .05 , OR= (95%CI). N(number), OR(odds ratio) , CI(confidence intervals)

Table 9. Occurrence of *sipB* gene in *salmonella arizonae* determination with *salmonella typhi* (reference strain)

Gene Name	Serovar	Positive N (%)	Negative N (%)	*P*-Value	Chi- square	OR (95%)
sipB	*Salmonella typhi*	8(13.79%)	50(86.20%)	0.0	0.00	0.00(0.00-00.00)
	Salmonella arizonae	00(00%)	00(00%)			

P ≤ 0 .05 , OR= (95%CI). N(number), OR(odds ratio) , CI(confidence intervals)

Table 10. Occurrence of *sipB* gene in *salmonella paratyphi* comparation with *salmonella typhi* (reference strain)

Gene Name	Serovar	Positive N (%)	Negative N (%)	*P*-Value	Chi- square	OR (95%)
sipB	*Salmonella typhi*	8(13.79%)	50(86.20%)	0.016	5.90	9.12 (1.10-75.47)
	Salmonella paratyphi	1(1.72%)	57(98.27%)			

P ≤ 0 .05 , OR= (95%CI). N(number), OR(odds ratio) , CI(confidence intervals)

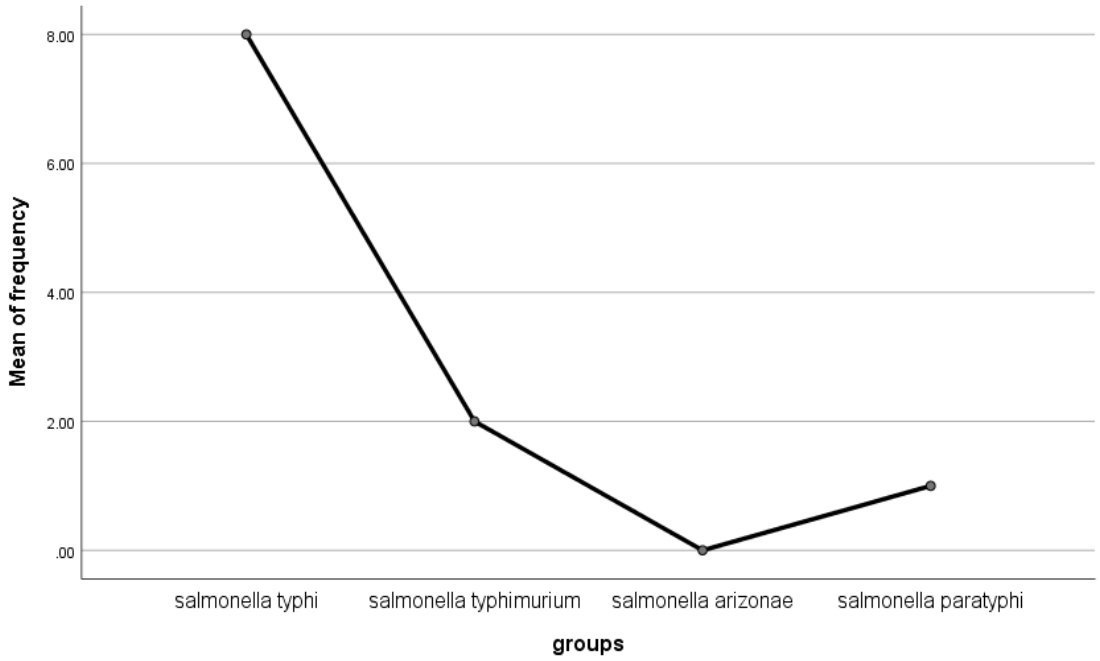

Fig. 3. Distribution of *sipB* gene in *salmonella enterica* serovars

*salmonella typhimurium***,** followed 0% for *salmonella arizonae* and 4.5% for *salmonella paratyphi*, **Table 7** and **Fig. 4**. The difference in a distribution of *sipB* gene in the the *salmonella* spp comparison with reference strain (*Salmenella typhi*) was statically significant P ≤ 0.05, **Tables 8**, **9**, **10**; **Fig. 3**.

c- *sseC* gene of *salmonella* spp (translocon protein gene)

We found that, low prevalence rate of SseC gene in all the isolated Salmonella spp, only one isolate gave positive for *sseC* gene at a percentage (4.5%) in *salmonella typhi*, **Table 11**.

d- *sopB* gene of *salmonella* spp (The *salmonella* outer protein B)

The gene represented a *sopB* that present in SPI-5 revealed negative result for all depicted bacteria. These result corresponding with those of [18] how found that about fifty *salmonella* spp.10 ten isolates were give positive for *sopB*. Soto et al.(2006)(Soto, et al. 2006)., mentioned that SpI-5 genes are variable present in different salmonella species.

DISCUSSION

The present conducted that diversity in Salmonella spp.isolated from diarrheic stool samples, similar studies were achieved by other authors like Abd ElSeed *et al*. (2015)(Abd Elseed, 2015). Comparison between the

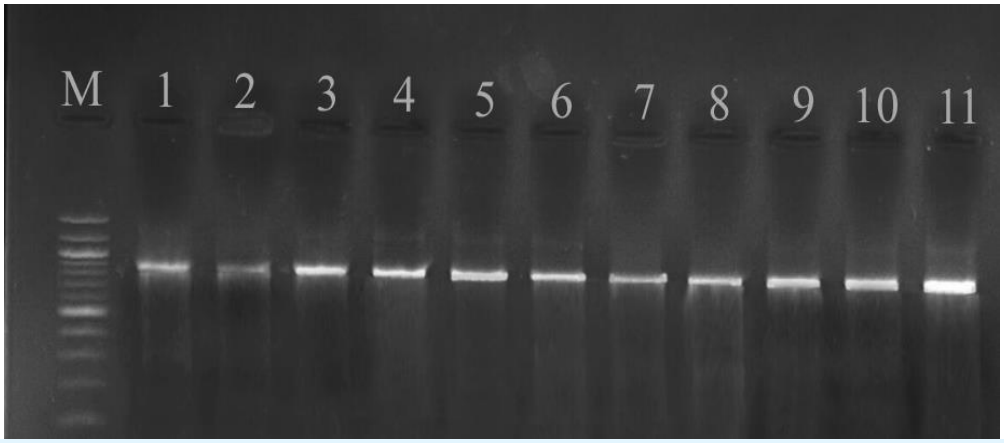

Fig. 4. Agarose gel electrophoresis of PCR assay showed Sip B gene of *salmonella* spp: lane (M) DNA Marker (100-2000bp); lane (1-11) *sipB* gene at 875bp by PCR product size

Table 11. Distribution *sseC* gene according to the *salmonella* spp

Salmonella spp	Gene presence	Percentage%
Salmonella typhi	1	4.5%
Salmonella typhimurium	0	0
Salmonella arizonae	0	0
Salmonella paratyphi	0	0
Total	1	1.7%

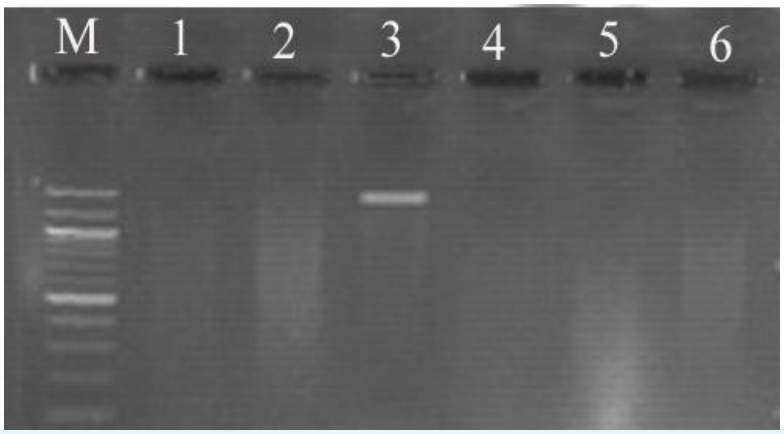

Fig. 5. Agarose gel electrophoresis of PCR assay showed SseC gene of *salmonella* spp lane (M) DNA Marker (100-2000 bp); line (1-6) *sseC* gene at 1455 bp by PCR product size

Widal test and culturing technique in the diagnosis of enteric fever in Khartoum State, Sudan. *African Journal of Bacteriology Research*, *7*(5), 56-59., conducted that only 7.5% of stool samples were positive culture of *salmonella typhi* compared with widal test, which was positive for all the suspected patients with typhoid fever. *Salmonella typhimurium* is causes gastroenteritis results from consumption of food and water which contaminated with these bacteria, sometime causes septicemia, mainly in infants (Diamond, et al. 2017). *Salmonella typhimurium* is non-typhoidal salmonellosis (NTS) and have ability to withstand the Unfavorable environmental condition makes it so hard for eradication from the food (Won, & Lee, (2017). Previous study by Tarabees et al.(2017), recorded that 3% of chicken meets were positive culture for *salmonella typhimurium.* The infection with *salmonella arizonae* was common characterized in the southern states of USA, whereas rare in Europa (Di Bella, et al. 2011). Mahajan et al.(2003)(Mahajan, et al. 2003)., found that out of seventeen patients with *salmonella arizonae*. Eleven were children, including 4 infants. The paratyphoid fever result from infection with *salmonella paratyphi* A, B, C by the paratyphoid organism is transmitted by horizontal rout (fecal-oral rout). The carrier patients elaborate high quantities of the organism in leading to contaminate the handling food causing paratyphoid infection(Maskey, et al. 2006).

In genetic investigation we found that invaA gene approximately was found in all the Salmonella spp. In Nigeria study found that *invaA* gene (284bp) was more reliable in diagnosis *salmonella* spp from cow row milk and milk products (Ramya, Madhavarao, & Rao, 2012). In Korea study had the same prevalence with the *invaA* gene (284bp) of 96%(Mezal, Stefanova, & Khan, 2013).. Caudhary and coauthors.(2015), Found that All 37

Salmonella isolates 13 of *salmonella Enteritidis* and 24 isolates of *Salmonella typhimurium* showed were positive for *invaA* gene. The other studied gene was *sipB*. It is a gene settled in SPI-1, help the *salmonella* for entrance into non-phagocytic cells. and induce apotosis for macrophages and stimulate the inflammatory cytokines secretion like TNF-α-IL-8(Kim, & ju Lee, 2017). Kagirita et al.(2017)(Kagirita, et al. 2017)., found that same distribution *salmonella* spp about (85.5%) of SipB gene that isolated from different sources humans and animals.Hu et al. (2008), hypothesized that certain deletion may be occurs in SPI-1 genes leading to weak in virulence ability of *salmonella*. The type III secretion system that encoded by SPI-2 is responsible for the institution of systemic infection by providing the intracellular survival, and replication in macrophages. The roles of *sseB*, *sseC*, and *sseD* are translocons for effector proteins. The mutation in genes encoding for translocons leading in attenuation in ability of surviving within macrophages(Li, et al. 2009).. In a previous study on the *salmonellaenterica* serovar *weltevreden* by Bhowmich et al.(2011)(Li, et al. 2009). mentioned that the SseC is more susceptible for mutation this reason many be justifies our results in low prevalence rate for SseC gene.

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