Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.47750/jptcp.2023.30.04.060

Molecular detection of some virulence genes in Salmonella typhi isolated from patients in Al-Diwaniyah city- Iraq

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Submitted: 18 January 2023; Accepted: 17 February 2023; Published: 26 March 2023

ABSTRACT

Salmonella typhi is an enteric serotype belonging, the Enterobacteriacaea family, as they inhabit the lymphatic tissues of the small intestine, liver, spleen, and the bloodstream of the infected human. The samples were collected distributed between ages (1-≤ 50) years and for different samples (stool, urine, blood) and for both sexes .Pathogenicity markers were also detected by Vitek test and 16 SrRNA using PCR technology. The results of isolation during the study period 14/8/2022 to 14/12/2022 showed that 51 samples out of 120 isolates were positive with the ratio of 27% that belonged to S. typhi and 16% belonged to other bacterial species**.** The molecular detection of some virulence genes (ViaB, InvA, Flic-d) was carried out for 32 S. typhi isolates using PCR technique. It was found that the DNA of all isolates contained these genes with 100%**.** The results of some isolated S. typhi strains showed the presence of genetic mutations of the non-conservative type in the amino acids of the InvA gene in the bacterial strain OQ6968441.1, where the amino acid Arginine R replaced the L-Lucene. For the Flic-d gene of the bacterial strain OQ703101.1, three non-conservative genetic mutations were found within this bacterial strain for each of the amino acid Aspartate D at one site and the amino acid Threonine T at two sites. For the T sites, they were replaced by the amino acid Asparagine N and Proline P, respectively.

Keywords: *Salmonella typhi, Virulence factor, ViaB, InvA, Flic-d*

INTRODUCTION

Salmonella bacteria are Gram-negative, bacilli that infect and colonize humans and cause infection with various clinical symptoms such as gastroenteritis, intestinal fever and bacteremia. Enteric fever is caused by Salmonella typhi, while other strains of salmonella are known as non-typhoidal salmonella. Salmonella bacteria belong to the Enterobacteriaceae family. The current classification divides these bacteria into two main types, Salmonella enterica and Salmonella bongori, with approval pending for the third type, Salmonella subterranean (Ajmera and Shabbir, 2022). The S. enterica is a major species, which is divided into six subspecies (Su and Chiu, 2007). Which includes more than 2,600 serotypes (Gal-Mor et al., 2014). Typhoid fever or enteric fever is a life-threatening bacterial infection, and it is one of the oldest diseases that have accompanied the existence of human on this planet. It is still a global health concern. Scientists have proven that human contracted typhoid fever several centuries ago and that they are the only host for this disease.

In addition, the conditions of poverty that found in most parts of the world have increased the incidence of this disease. Typhoid fever is known as a systemic disease associated with Gram-negative bacteria of the type S. enterica serovar typhi (S. typhi) (Najib et al., 2021). Also, all strains of Salmonella are pathogenic that can invade human cells and survive, thus, showing a prominent feature while attacking non-phagocytic human cells (Yadav et al., 2020). This property stimulates and exploits phagocytosis in order to reach the host cell, and the genes behind this prominent strategy have been found in Salmonella Pathogenicity Island (SPIs), as well as, genes in the DNA region (Grassl and Finlay, 2008).

Therefore, the spread of S. typhi infection is due to its ability to escape from the immune system and enter the gallbladder and form a biofilm inside it and pass into a state of stagnation, which enables it to evade the body's defenses without showing any symptoms (WHO, 2006). It should also be noted that one of the main characteristics that S. typhi possesses is its possession of vi antigen (capsule), which plays an important role in resistance to ingestion (Hart et al., 2016).

The majority of pathogenic bacteria are able to invade cells and cause infection, and for this process to occur, pathogenic bacteria must have the ability to attach to the host cell, which does not happen unless they possess adhesion proteins or the mechanisms required for this process. There are special proteins in S. typhi that enable it to adhere to the host's epithelial cells are pili and fimbrae (Clouthier et al., 1993). Penetrating into epithelial cells lining the intestine, SPIs stimulate type three secretion systems that are special proteins that form channels that allow Salmonella to inject its effectors into the epithelial cell cytoplasm across the cell membrane (Olana, 2018).

Once the salmonella bacteria are ingested in the host cell, they are encapsulated and a vacuole is formed around them from the components of the host cell membrane, and the presence of the bacteria inside the cell stimulates the immune response of the host cells (Kumar and Valdivia, 2009). This gap prevents the fusion of lysosomes, and this enables the bacteria to survive and multiply inside the host cells. It also enables the bacteria to survive inside the phagocytic cells that carry them to the parts of the immune system (Azimi et al., 2020; Thao et al., 2008).

Clinically, symptoms appear after an incubation period of 10-14 days, which include the common symptoms of malaise, loss of appetite, and headache that appear during the first two days. There is vomiting as well. Constipation occurs more frequently than diarrhea that is free of blood, which is an important point. There may be cases of enlargement of the liver, which is common in typhoid infections, while diarrhea and vomiting are not. For gastroenteritis, they are common and rose spots often appear on the abdomen and trunk for a few days, which are caused by the accumulation of bacteria that are surrounded by small round cells on the skin (Black, 1999; De Jong et al., 2012).

There are a range of microbiological and serological techniques that are used to diagnose this infection, such as cultures, antigen detection, and DNA intensification (PCR). As for the clinical signs, it is somewhat difficult to diagnose because the symptoms of the disease are overlapping and different (Ayub, 2015). The PCR is the most sensitive and rapid method for detecting microbial pathogens in clinical specimens. The studies have shown that the PCR examination of S. typhi specimens was the most accurate of all diagnostic tests such as the widal test of blood and urine samples and urine cultures (Ambati et al., 2007; Prakash et al., 2005).This study aimed to isolate and identify S. typhi from people with typhoid fever in Al-Diwaniyah city; and detect the virulence gene including ViaB, InvA and Flic-d gene.

MATERIALS AND METHODS

Samples Collection

120 different clinical samples (blood, urination and stool) were collected in equal percentages from the inpatients and patients in Al-Diwaniyah General Hospital and some patients for internal medicine doctors for both sexes and ages. The patient's information was recorded in a special form. Samples were collected from 14-8 2022 to 14-12-2022. Each sample (blood, urine and stool) was cultured on blood agar and MacConkey agar media and incubated at 37°C for 24-48 hs.

Then, one colony was taken from each positive culture and planted on selective culture media specific for Salmonella Shigella agar, Xylose-Lysine Deoxchoelate agar and Bismoth sulphite. They are incubated at a temperature of 37°C for a period of 24-48 hs. Bacterial isolates were diagnosed using the Vitek 2 test. All isolates of

S. typhi were identified using the vitek 2 system produced by the French BioMérieux company according to the steps that approved by the company.

Molecular study

Extraction of Genomic DNA

The DNA of the studied bacteria was extracted using the Genomic Extraction Kit prepared by the American company Geneaid. The extraction process was carried out according to the company's instructions.The purity of the extracted nucleic acid was checked using a Nano-drop spectrophotometer, which measures the concentration of nucleic acid (ng/µL) by reading the absorbance at a wavelength between 260-280 nm.

Agarose gel electrophoresis of extracted DNA

The 1.5g of agarose gel was dissolved in 100 ml of TBE buffer at a concentration of 1× by placing it on a heat plate for 15 mins. After that, it was left to cool at 50°C, then 3 µL of ethidium bromide was added and mixed. The gel was poured into the electrophoresis tray and left to solidify at room temperature for 15 mins. Then, the comb was carefully removed from the gel to leave the wells needed in the gel to inject the samples inside.

Primer pairs preparation

The primers and the DNA sequence initiator were designed using the genetic sequence in the GenBank according to the aforementioned sources on the National Biological Information website NCBI using the primer 3 plus primer design program (Table 1). These primers were prepared by the Canadian company IDT and were used according to the manufacturing instructions by adding deionized distilled water (dd H2O) to the dried tube containing the primers according to the size fixed on the tube. Then, they were mixed well with a vortex device to obtain a solution. Finally, they were stored until use at a temperature of -20°C.

TABLE 1: primers and the size that were manufactured by the Canadian company IDT

Primers type	Primer sequence (5'-3')		Size pb	Reference
FliC-d	ACTCAGGCTTCCCGTAACGC	F	**763**	(Levy et al.,2008)
	GGCTAGTATTGTCCTTATCGG	R		
ViaB	TGTCGAGCAGATGGATGAGCAT	F	**516**	(Wain et al, 2005)
	ACGGCTGAAGGTTACGGACCGA	R		
InvA	ACAGTGCTCGTTTACGACCTGAAT	F	**243**	(Chia-Ling et al., 2007)
	AGACGACTGGTACTGATCGATAAT	R		

Prepare the PCR master mix

The PCR mix was prepared using the kit of the Korean company Bioneer and supplemented with the AccupowerR PCR premix kit. After the mixture was prepared, the tubes were closed and mixed with a vortex machine for 10 secs. The tubes were then transferred to a PCR thermocycler to amplify the target genes. The polymerase chain reaction was carried out using a thermocycler, where the device is programmed according to the target genes, and these reactions go through several stages (Table 2).

TABLE 2: Thermal cycles for DNA amplification

16 SrRNA gene			
Steps	Temp.°C	Time	Cycle
Initial denaturation	95	5 min	1
Denaturation	95	1 min	35
Annealing	55	1 min	
Extension	72	1.30 min	
Final extension	72	10 min	1
InvA gene			
Steps	Temp.°C	Time	Cycle
Initial denaturation	94	5 min	1

Denaturation	95	30 sec	35
Annealing	52	30 sec	
Extension	72	1 min	
Final extension	72	5 min	1
Flic-d gene			
Steps	Temp.°C	Time	Cycle
Initial denaturation	95	5 min	1
Denaturation	94	30 sec	35
Annealing	53	30 sec	
Extension	72	45 sec	
Final extension	72	5 min	1
Via B gene			
Steps	Temp.°C	Time	Cycle
Initial denaturation	95	5 min	1
Denaturation	95	30 sec	35
Annealing	56	30 sec	
Extension	72	45 sec	
Final extension	72	5 min	1

DNA sequencing

The samples were sent to the Korean company (Macrogen) to conduct a DNA sequencing reaction using the DNA sequencing system. The sent results were received through a company report. The analysis of the results of the target genes (16 SrRNA, InvA, Flic-d, ViaB) was performed using the BLAST program from the National Center for Bioinformatics and Technologies, after that the scientific registration was done using the NCB-Gen Bank Submission.The Molecular Evolution Genetic Analysis (MEGA) program was used to analyze the genetic tree to measure the genetic dimension of the target genes (16 SrRNA, InvA, Flic-d, ViaB), then the genetic tree was drawn using the Neighbor-Joining Method.

RESULTS AND DISCUSSION

The results of collecting 120 samples from people suffering from symptoms of enteric fever, their aged between (1 - < 50) years, showed that 51 out of 120 samples were positive, distributed between 27% for S. typhi and 16% for other bacterial species. The results also showed that the 69 isolates were negative without growth rate of 57% (Figure 1).

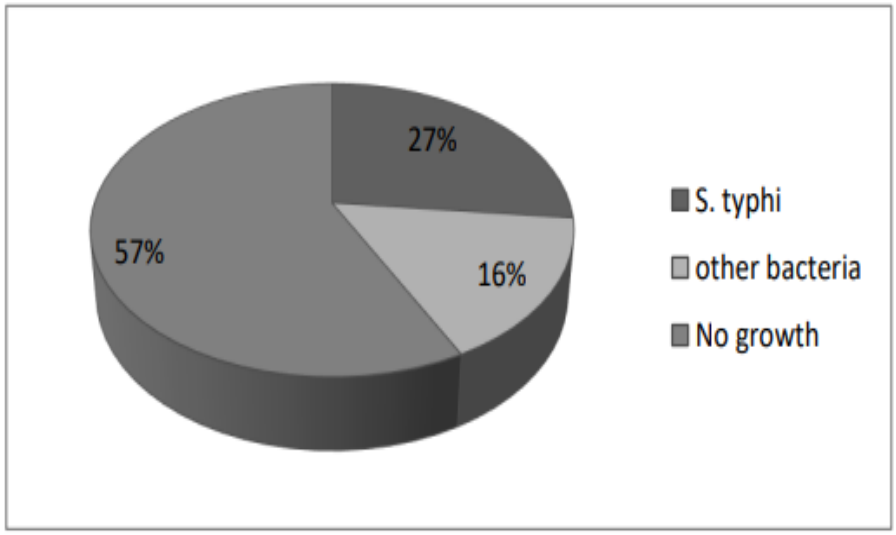

FIGURE 1: Distribution of the results of isolation of bacterial samples for enteric fever patients (chi-square value = 50.47)

The blood samples media have been showed that the growth rate was 42.5%, and the exit samples showed a growth rate of 27.5%, while the urine samples had a growth rate of 10%. The results of the statistical analysis indicated that there were significant differences between the types of samples (Table 3).

TABLE 3: Distribution of S. typhi bacteria samples according to sources, numbers and percentages

Sample type	**Number of samples**	**S. typhi**	
		number	**percentage**
Blood	40	17	42.5 %
Stool	40	11	27.5 %
Urine	40	4	10 %
Total	120	32	80 %
Chi-square value	10.82		
Sig. value	0.004		

*There are significant differences at 0.05 levels

The results of the isolation of S. typhi showed that the blood cultures recorded the highest percentage of the presence of bacteria in the isolates compared to the stool and urine cultures. These results agreed with (Ali, 2020). The results of using the rapid identification of the developing isolates by the Vitek 2 device showed to confirm the positive results obtained from the microscopic and culture characteristics. The 32 isolates belonging to the bacteria S. typhi were obtained from a total of 51 isolates, noting that all isolates were taken from people with symptoms of fever intestinal.

Molecular Study

The S. typhi DNA was successfully extracted using the commercial Genomic Extraction Kit technology .The concentration and purity of the extracted nucleic acid was determined directly by the Nano drop equipment. The purity of the extracted nucleic acid ranged (1.7-1.9) with a concentration between (30-100) ng, and the analysis of the extracted nucleic acid was confirmed by electrophoresis.

The tested samples were confirmed to contain the 16S rRNA gene, which is about (1500 base pairs) of prokaryotic DNA found in all types of bacteria (Figure 2). Determining the presence of the 16S rRNA gene in bacteria is also more important for classification based solely on phenotypic characteristics (Clarridge, 2004). In addition, the 16S rRNA gene sequence has become one of the indispensable pillars in the classification, reclassification and naming of bacteria, determining the evolutionary relationship and facilitating the classification of non-cultivable bacteria (Woo et al., 2008).

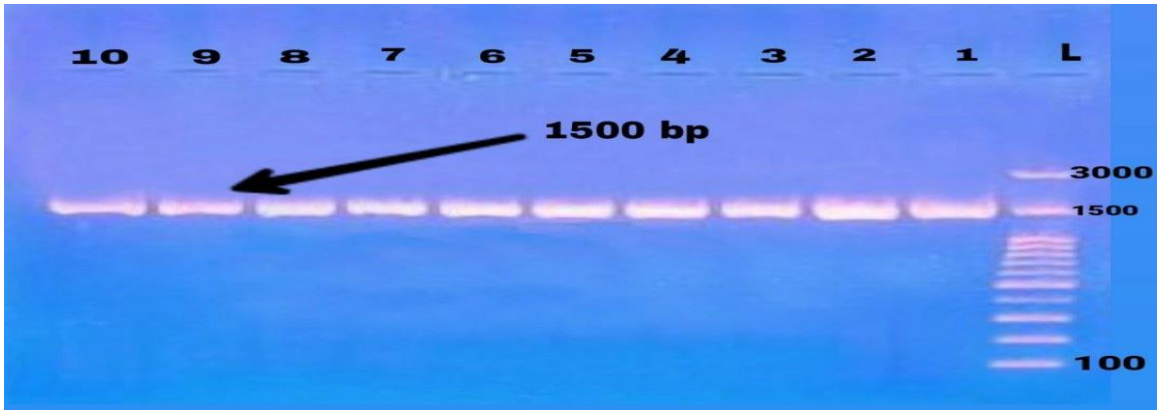

FIGURE 2: Electrophoresis of agarose gel (1.5%) stained with ethidium bromide resulting from the PCR reaction of S. typhi isolates amplified with genetic primers of the 16S rRNA gene, 1500 bp under a voltage difference of 100 volts and a current of 80 mA for 60 mins

The results of the sequence alignment of multiple nitrogenous bases are an important step for phylogenetic analysis of the affinity of strains with different groups arranged according to Goujon et al. (2010). The results of sequencing the DNA of 10 Iraqi isolates of S. typhi bacteria from the 16S rRNA gene. They were compared with other international references selected for the same 16S rRNA gene showed great similarity according to special sites for sequencing of nitrogenous bases according to the (BLAST n) algorithm from NCBI to detect the percentage of closeness between strains of isolates. They were numbered between (100-95) strains of Iraqi isolates and other international strains.

After the comparison, it was recorded in the International Gene Bank and given an international number for each isolate, which was (OQ357572, OQ357573, OQ357574, OQ357575, OQ357576, OQ326505, OQ326506, OQ326507, OQ326508, OQ326509) (Figure 3).

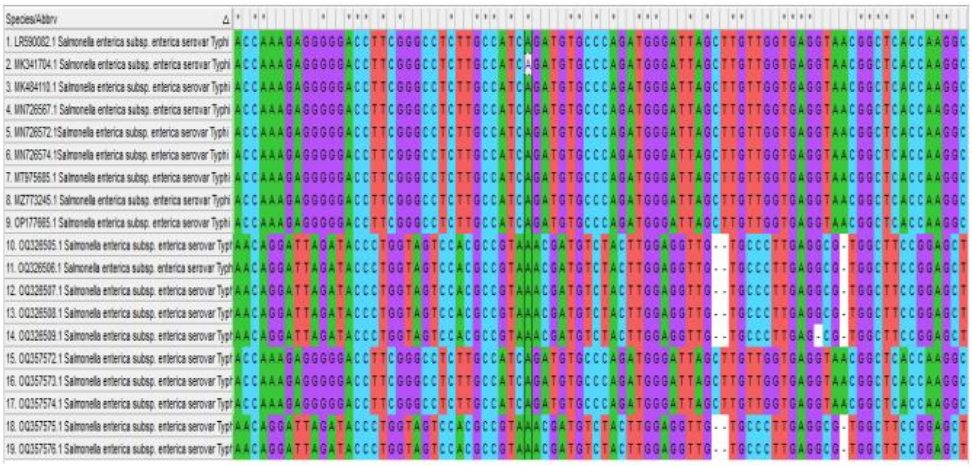

FIGURE 3: Alignment of the nitrogen base sequences of the 16S rRNA gene from the Iraqi S. typhi isolates with the international isolates recorded in the NCBI Gen Bank. * indicates the match in the sequences of the nitrogenous bases of the global isolates.

Results of phylogenetic tree analysis

The phylogenetic tree analysis was based on the molecular sequence of the 16S rRNA gene. It was used to detect S. typhi using (MEGA 11.1) program, and then plotted using the Neighbor-Joining Method (Figure 4)

FIGURE 4: Phylogenetic tree analysis based on the 16S rRNA gene used for S. typhi species, which showed the closeness of local isolates and global isolates

J Popul Ther Clin Pharmacol Vol 30(4):e593–e603; 26 March 2023.

Genetic detection of S. typhi virulence factors

The PCR test was performed for some virulence factors genes of 20 samples of S. typhi bacteria using the forward and reverse primers of overlapping genes (InvA, ViaB, Flic-d) carrying the plasmid and DNA of the selected samples. The host cell adhesion factor is a major factor in causing infection with pathogenic bacteria. There are several genes involved in encoding this factor, some of these genes have been studied including InvA, ViaB and Flic-d.

The results showed that the studied isolates of S. typhi bacteria had 100% of these genes for each gene by a two-way PCR reaction (Figures 5, 7, and 9). These results agreed with Angham, (2015), which reported that all studied samples had the ViaB gene, as well as agreed with Hirose et al. (2002) that possessing all studied S. typhi samples for the ViaB gene. Several studies have reported that a specific PCR reaction for S. typhi genes such as flagellum or capsular genes is more sensitive than an assay of serum Vi antigen antibodies for the detection of S. typhi (Das et al., 2012).

The expression of Vi antigen is controlled by two separate sites ViaA and ViaB which are located at 92 and 43 on the chromosome of S. typhi, respectively. ViaA genes are not only found in the Vi site, which is expressed in Salmonella strains, but also in E. coli bacteria. In contrast, the ViaB site is specific to Vi-expressing strains. The ViaB site of S. typhi contains at least two regions, one involved in antigen VI biosynthesis for cell surface polysaccharide transport (Virlogeux et al., 1995).

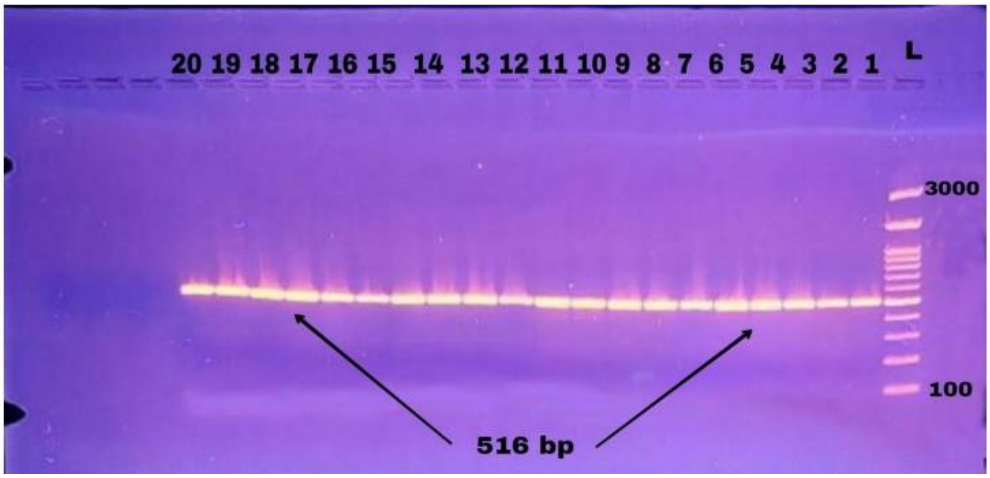

FIGURE 5: Electrophoresis of agarose gel (1.5%) stained with ethidium bromide resulting from the PCR reaction of S. typhi isolates amplified with genetic primers of the ViaB gene, 516 bp under a voltage difference of 100 volts and a current of 80 mA for 60 mins.

Many modern and rapid techniques have been used in the field of detecting mutations and genetic relationships between bacterial isolates. One of these techniques is the sequencing technique, which is of great importance in molecular biology, through which genes and mutations of bacterial isolates can be detected (Ranjbar et al., 2014). The sequencing technique was used to determine the sequence of amino acids, where an analysis of the amino acids of the ViaB gene of S. typhi bacteria of the local strain OQ696847.1 was performed to find out the possibility of important mutations of the amino acid group according to the (BLAST P) algorithm from NCBI.

The results showed that the group of amino acids of the ViaB gene is completely identical to the international strains that were compared with it, except for one strain, CIL90181.1 registered in the name of the United Kingdom, which recorded a mutation of the amino acid (E-glutamate). It was replaced by the amino acid (K-Lysine), which occurred between the sequences (60-50) of the amino acid sequences (Figure 6).

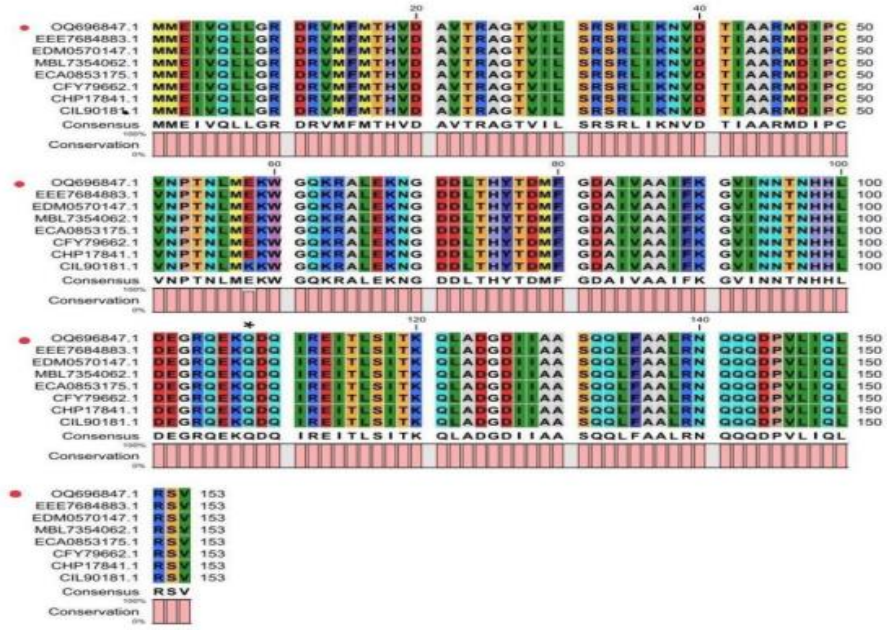

FIGURE 6: comparison of the sequences of the amino acids of the ViaB gene of S. typhi strain OQ696847.1 with other global strains . * indicates the presence of a mutation

S. typhi has many virulence factors, but the role of the InvA gene is the most important. This gene has a role in adhesion and invasion of the epithelial cells of the host. It is also responsible for encoding third type secretion systems (Suez et al., 2013). It is a diagnostic gene for the rapid detection of S. typhi isolates from different samples. It is not present in any other bacterial species (Lin et al., 2007; Kumar et al., 2010). The results of this study agreed with (Angham, 2015) and (Ligaa, 2022), which indicated that all studied samples possessed the InvA gene with 100%.

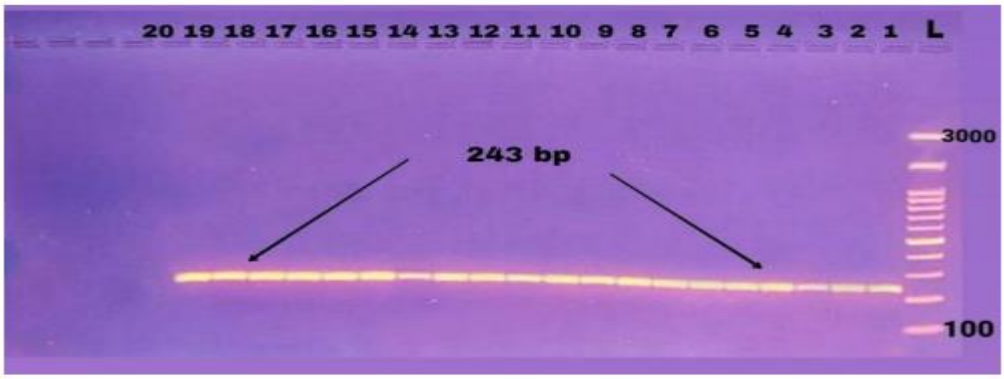

FIGURE 7: Electrophoresis of agarose gel (1.5%) stained with ethidium bromide resulting from the PCR reaction of S. typhi isolates amplified with genetic primers of the InvA gene, 243 bp under a voltage difference of 100 volts and a current of 80 mA for 60 mins.

The presence of mutations of the InvA gene was detected by sequencing technique for amino acid sequencing of isolate OQ696844.1 of S. typhi. The results revealed the presence of a mutation of the amino acid (L-Lucene). It was replaced by the amino acid (R-Arginine), which occurred between the sequences (40-20) of the amino acid sequences compared with a group of amino acid sequences of the global strains (Figure 8).

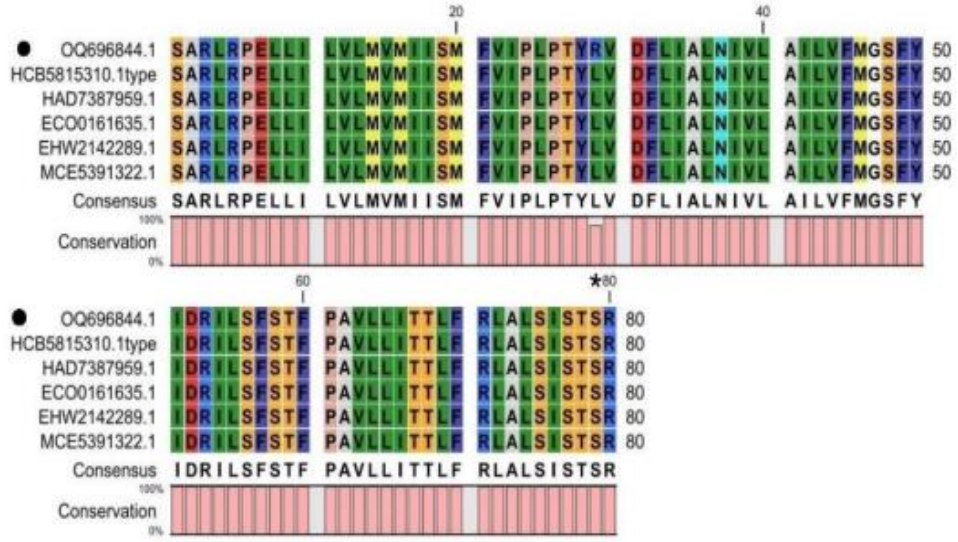

FIGURE 8: comparison of the sequences of the amino acids of the InvA gene of S. typhi of the bacterial strain OQ696844.1 with other international strains. * indicates the presence of a mutation.

The PCR results of the study for the Flic-d gene (fagellin gene) showed that this gene was present in all studied samples with 100%. It is a way to confirm the identification of S. typhi genes. These results were associated with the Ligaa (2022) study, which indicated that the results of the PCR reaction for this gene were 100% for the studied samples. The Flic-d gene was detected in various studies in 80 suspected cases of S. typhi. The PCR results showed that 70% of these cases had this gene (Khan et al., 2012).

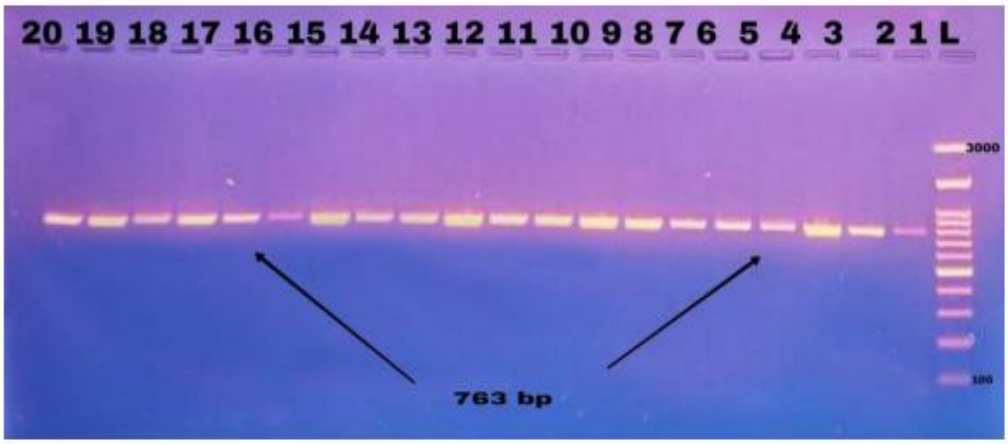

FIGURE 9: Electrophoresis of agarose gel (1.5%) stained with ethidium bromide resulting from the PCR reaction of S. typhi isolates amplified with genetic primers of the Flic-d gene, 763 bp under a voltage difference of 100 volts and a current of 80 mA for a period of 60 mins.

For the detection of mutations in this gene, it also used the technique of sequencing the amino acids of the local strain OQ703101.1. It is revealed the presence of three mutations of amino acids, all of which met within the sequences (100-120) of the amino acids within this strain, which included the amino acid (D-aspartate) in the site. The amino acid (T-Threonine) in two sites, which was replaced by the amino acid (N-Asparagine) and (P-Proline), respectively, for the two T sites (Figure 10).

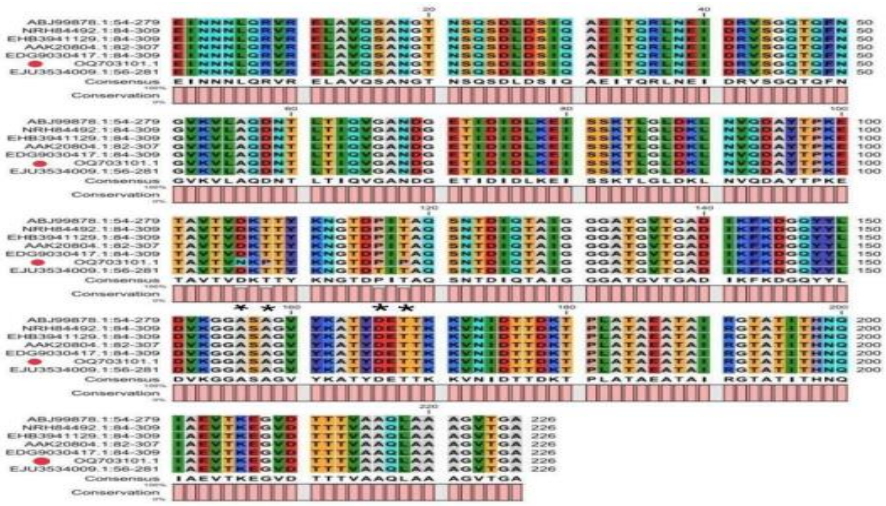

FIGURE 10: comparison of the sequences of the amino acids of the Flic-d gene of S. typhi of the bacterial strain OQ703101.1 with other international strains. * indicates the presence of a mutation

CONCLUSION

The study is concluded that S. typhi isolated from patients suffering from typhoid fever possess many virulence factors such as invasion factor and capsule. The InvA gene and Flic-d gene are specific genes for detecting S. typhi in patients with enteric fever. The genotyping of isolates was determined based on the protein-coding genes including ViaB, InvA, Flic-d by determining the genetic tree and the amino acid sequence of each gene.

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