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Research Article

Molecular Study about some Genes among Salmonella Typhi Isolates un Hilla / Iraq

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ABSTRACT

In this study, (100) blood sample were collected from patient suspected with typhoid fever how were visits the Al-hashimiya, Hilla Teaching Hospital and Morjan Teaching Hospital in Babylon province (Iraq) during the period November (2018) to February (2019). Identification was done by cultural and biochemical tests, and finally identification by Vitek2. Results showed that positive were (16) isolates of *Salmonella enteric serovar typhi* by Vitek2 (16%) from the (100) blood. Molecular study was accomplished first by the isolation of DNA from *Salmonella typhi* isolates, they used method had succeeded in extraction of genomic DNA from all isolates. Second by using PCR technique to detection four gene by using a specific PCR primer were done by comparison with allelic ladder which gave a (578bp) it was found that (*flicA*) gene present in (15) isolates (93.75%) of the positive sample. *viaB* gene was also detected in *S. typhi* sample and found that in (15) isolates (93.75%) of the positive sample which gave molecular length (438bp) band. Molecular detection of *sipA* gene in *S. typhi* present of this gene in all (16) isolated (100%) positive, which gave molecular length (1126bp). Molecular detection of *sdiA* gene was done for (16) isolated of *S. typhi*, and the results showed that (15) isolates (93.75%) the positive results for *sdiA* virulence were detected by the presence of (274bp) band compared with allelic ladder. This study was conducted to identify the presence of *Salmonella enterica serovar typhi* within five isolated bacterial samples. One genetic locus covering a particular coding portion within the *sdiA* gene, which is responsible on the encoding of cell-division regulatory protein, was selected for amplification.

Keywords: *Salmonella enteric serovar typhi*, PCR technique, *flicA* gene, *sdiA*.

INTRODUCTION

Typhoid fever is an acute, potentially fatal systemic illness caused by *Salmonella enterica serovar typhi* and *paratyphi*, pathogens only specific to humans. *S. typhi* is a genus of rod-shaped gram-negative enterobacteriaceae that cause typhoid fever[1]. The *Salmonella typhi* bacteria are an obligate parasite with no other known reservoir outside of humans. A (100,000) organisms of *Salmonella typhi* make up an infectious dose, and the disease is typically spread through feces and urine of infected people in contaminated food and water. Typhoid fever is still a major health concern in the developing world as it is found endemically throughout Africa, South America, and East Asia and particularly in South Asia. In a broader sense, typhoid fever is categorized under enteric fever, which is described as a systemic illness characterized by fever, abdominal pain, and nonspecific symptoms including nausea, vomiting, headache, and anorexia[2]. Typhoid fever is an illness caused by bacterium *Salmonella*, is worldwide transmitted by ingestions of food or water contaminated with feces from an infected person, without treatment, the illness may, last for (3 to 4) weeks and death rates range between (12%) and (30%). Following ingestion, the bacteria spread from the intestine via the bloodstream to the intestinal lymph nodes, liver, and spleen via the blood where they multiply[3]. Flagellin is the main structural protein of the flagella of many pathogens including *Salmonella typhi*. It is a potent trigger of innate immune responses that enhance adaptive immune responses to a variety of protein antigens. Flagellin has intrinsic adjuvant activity mediated through toll-like receptor (TLR) 5 and is an attractive candidate for highly effective vaccine adjuvant conferring enhanced antibody and cellular immune responses to proteins or peptides[4]. There are 2463 servers of *Salmonella*, which comprises of (46) groups of O antigen and (114) groups of H antigen. The O antigens exists as several groups namely (A, B, C1, C2, D and E) whereas the H antigens can be separated into two flagellin loci (*fliC* and *fliB*). This loci gene function unfettered of each other and is controlled by the hin switch mechanism. However, not all *Salmonella* has this switching mechanism as some *Salmonella* such as *S. enterica* sub spp. arizonae has only *fliC* gene[5]. The surface antigens influences the virulence of the bacteria, provides motility for the bacteria through the flagella and illicit inflammation response. The O and H antigens are also the chief antigenic composition in *Salmonella*[6]. The Vi antigen is a capsular polysaccharide expressed by *Salmonella typhi*, the agent of human typhoid fever. Expression of this antigen is controlled by the *viaA* and *viaB* chromosomal loci. The *viaB* locus is composed of

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Table 1: primer sequence that used in present study.

Type of gene	Sequ. of gene	Product	Reference
FlicA	F:GCTTAATGTCCAAGATGCCTC R:GAGCAACGCCAGTACCATCTG	587bp	Kumar *et al*., 2006
ViaB	F:TTATTTCAGCATAAGGAG R:CTTCCATACCACTTTCCG	438 bp	Yasuhiro *et al.*, 1994
Sipa	F:CGGCTTCACATTCACAA R:CGGGCTCTTTCGTTCA	1126bp	Woogjae *et al*., 2017
SdiA	AATATCGCTTCGTACCAC GTAGGTAAACGAGGAGCAG	274bp	Reza *et al*., 2018

Table 2: Amplification Conditions of genes were used by PCR.

Gene	Initial Denaturation	No. of cycles	Denaturation	Annealing	Extension	Final extinction
Flic a	94Cº for 5 min	35	94Cº for 1 min	55Cºfor 1min	72Cºfor 1min	72Cºfor 5min
ViaB	94 Cº for 5min	35	94 Cº for 30 sec	55 Cº for 40 sec	72 Cº 1 for min	72 Cº for 5 min
Sipa	94 Cº for 5 min	35	94 Cº for 30 sec	52 Cº for 40 sec	72 Cº for2 min	7 Cº 2 for 5 min
SdiA	94 Cº for 5 min	35	94 Cº for 30 sec	60 Cº for 30 sec	72 Cº for 30sec	72 Cº for 30 sec

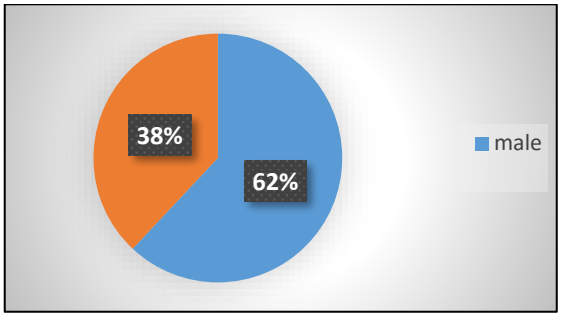

Figure 1: Distribution of infected patients according to sex.

(11) genes designated tviA-tviE (typhi Vi), vexA-vexE (Vi antigen export) and ORF11[7]. The Vi capsular polysaccharide (CPS) of *Salmonella enterica serovar typhi*, the cause of human typhoid, is important for infectivity and virulence. The Vi biosynthetic machinery is encoded within the *viaB* locus composed of (10) genes involved in regulation of expression (tviA), polymer synthesis (tviB-tviE), and cell surface localization of the CPS (vexA-vexE)[8]. *Salmonella enterica serovar typhi* depends on type III secretion systems to inject effector proteins into host cells to promote bacterial invasion and to induce intestinal inflammation. *SipA*, a type III effector, is known to play important roles in both the invasion and the elicitation of intestinal inflammation. The actin-modulating activity of *SipA* has been shown to promote *Salmonella* entry into epithelial cells[9]. *SipA*, a type III effector, is known to play important roles in the promotion of bacterial invasion and in the elicitation of intestinal inflammation. Its delivery across the host plasma membrane was shown to be swift after docking in a programmed orderly manner. The expression of CXC chemokines through phosphorylation of interleukin-8 transcription regulatory proteins JUN and p38MAK was found to be induced by *SipA*[10]. The secretion from the bacterial cell, the *SipA*, protein are thought to form a complex in the eukaryotic membrane that is required for translocation of the remaining effectors into the host cell cytoplasm[11]. The hallmark of *Salmonella* entry into host cells is the profuse host actin cytoskeletal rearrangement at the site of *Salmonella* contact with intestinal epithelial cells. *Salmonella* entry results from a series of highly coordinated cellular responses that are triggered by a panel of bacterial proteins delivered into the host cell by the type III secretion system (TTSS)[9]. The quorum-sensing system in bacteria is a well-known regulatory system that controls gene expression in a cell density-dependent manner. A transcriptional regulator (LuxR homologue), signal synthase (LuxI homologue) and autoinducer (acyl homoserine lactone) are indispensable for this system in most Gram-negative bacteria[12]. Bacteria have mechanisms by which they sense their own population density and regulate their behavior accordingly, termed quorum sensing (QS)[13]. QS System controls a diverse range of cell density-dependent factor, such as antibiotic production, biofilm formation, and pathogenicity[14].

Aim of study

To detect some virulence encoding genes of S. typhi isolated by PCR from typhoid patients such as *viaB*, *sipA* and *sdiA* genes via the following objectives:

Isolation and identification of *S. typhi* by used of current conventional methods, Vitek system and molecular technique by using *flic* (flagellin) gene.

Molecular detection of some virulence genes related to the pathogenicity of *S. typhi*.

MATERIALS AND METHODS

Patients and clinical specimens

A total of (100) samples were collected from blood with typhoid fever who attended different hospitals during the period from November (2018) to February (2019). in Al-Babylon. Blood sample was collected from patients, (10) ml of fresh venous blood samples were collected from suspected typhoid patients by sterile syringes which delivered into special screw cupped of culture bottle containing (100) ml of brain heart infusion broth and incubated at (37°C) for at least (3) days placed in

Table 3: Biochemical test to *S. typhi*.

Test	Result
Oxidase test	-
Indole production	-
Methyl red	+
Voges –proskauer	-
Simmons Citrate	
H2 S production +	+
Urea hydrolysis -	-
Catalase test +	+
TSI	alk/acid
Motility	+

bact/alert 3D apparatus for a week. If positive sample, each specimen was inoculated using direct method of inoculation on culture of selective media namely MacConkey, Blood, XLD and SS agar then inoculated at (37°C) for (18-24) hours. After incubation period, the culture performed idols, catalase, oxidase, kligler, uearase biochemical test and final diagnosis by vitek compact 2. After complete diagnosis, storage the pure colony in brain heart broth with glycerol in deep freezing.

Ethical Approval

A valid consent was achieved from each patients before their inclusion in the study.

Identification of bacteria

Colonial morphology and microscopic examination

A single colony from each primary positive culture on blood, MacConkey and nutrient agar and identify it depending on its morphological properties (colony shape, size, color, borders, and texture) and exam it by light microscope after being stained with Gram's stain. After examination it, biochemical tests were done on each isolates to complete the final identification according to[15-17].

Serological characterization of isolates

Widal test: It was used for detection of typhoid antibodies in blood samples. The fresh blood samples were tested for presence of typhoid antibodies by using O and H antigens as follows:

One ml of blood was centrifuged at (10,000) rpm for (5) min and serum was separated for serology.

A single drop (50 µl) of each O and H antigen was applied separately on a clean glass slide.

One drop of serum separated from blood was mixed well with each antigen.

The observations were made after (2) minutes.

Clear agglutination was recorded as positive according to[18].

GN-ID with VITEK-2 Compact

This system consists of personal computer, reader/incubator made up of multiple internal components including: card cassette, card filler mechanism, cassette loading processing mechanism, card sealer, bar code reader, cassette carousel and incubator, in addition to transmittance optics, waste processing, instruments control electronics and firmware. The system was equipped with an extended identification database for all routine identification tests that provide an improved efficiency in microbial diagnosis, which reduces the need to perform any additional tests, so that safety for both test and user will be improved. All the following steps are prepared according to the manufacturer's instructions. Three ml of normal saline were placed in plane test tube and inoculated with a lopefull-isolated colony. Insert the test tube into dens check machine for standardization of colony to McFarland is standard solution (1.5 x 108 cell/ml). The standardized inoculums were placed into the cassette and a sample identification number entered into the computer software via barcode. The VITEK-2 card type then is read from barcode placed on the card during manufacture, and the card, thus, connected to the sample ID number. Then the cassette was placed in the filler module. When the cards were filled, the cassette was transferred to the reader/ incubator module. All subsequent steps were handled by the instrument, the instrument; controls the incubation temperature, optical reading of the cards and continually monitors and transfers test data to the computer for analysis.

Molecular study

Extraction of Genomic DNA

Genomic DNA was extracted by using a commercial extraction system (Genomic DNA Favorgen Kit).

Transfer bacterial cells (up to 1 x 109) to a 1.5 ml microcentrifuge tube and Centrifuge for (1) min. at 14-16,000 x g then discard the supernatant.

Add (200) µl of FAGT Buffer then re-suspend the cell pellet by vortex or pipette. Incubate for 5 minute at room temperature.

Add (200) µl of FAGB Buffer to the sample and vortex for (5) seconds and incubated at (70ºC) or until the sample lysate is clear, and during the incubation, invert the tube every (3) min. At this time, pre-heat the required Elution Buffer (for step 5DNA Elution) in a (70ºC) water bath.

Add (200) µl of absolute ethanol (96-100%) to the sample and mixed by vortex for (10) seconds. (If precipitate is appears, break it up as much as possible with a pipette). Then place the FAGB Column in a (2) ml collection tube and transfer mixture (including any insoluble precipitate) to the FAGB column and centrifuge at (14000rpm or 10,000 x g). Discard the (2) ml Collection tube containing the flow-through and then place the FAGB Column in a new (2) ml collection tube.

Wash FAGB column with 400 µl of W1 Buffer. Centrifuge at (14000 rpm or 10000 x g for 30 seconds); discard the flow-through then place the FAGB column back in the (2) ml collection tube.

Add (600) µl of Wash Buffer (with ethanol) to the FAGB column then centrifuge at (14,000 rmp or 10000 x g) for 30 seconds, discard the flow-through. Place the FAGB Column back in the (2) ml collection tube and centrifuge again for (3) min. at (14,000 rmp or 10000 x g) to dry the column matrix.

Place the dry FAGB Column to new (1.5) ml micro centrifuge tube, and add (100) µl of pre-heated Elution Buffer or TE to the membrane center of FAGB column. Stand FAGB column for 3-5 min or until the buffer is absorbed by the membrane and centrifuge at (14,000 rmp

or 10000 x g) for (30) seconds to elute the purified DNA. Store the DNA fragment at (4 ºC) or – (20 ºC).

Polymerase Chain Reaction Assay

Detection of fliC, viaB, sipA, sdiA1, sdiA2 genes by PCR assay

The composition of the PCR mixture was prepared in total volume (20) µl for each gene which done separately as in Table (1)

PCR Thermocycling Conditions

The PCR tubes were placed on the PCR machine and the right PCR cycling program parameters conditions were installed as in Table (2)[19,20].

RESULTS AND DISCUSSION

This study was conducted on (100) specimens from blood samples, during the period from November (2018) to February (2019). The results indicated that clinical

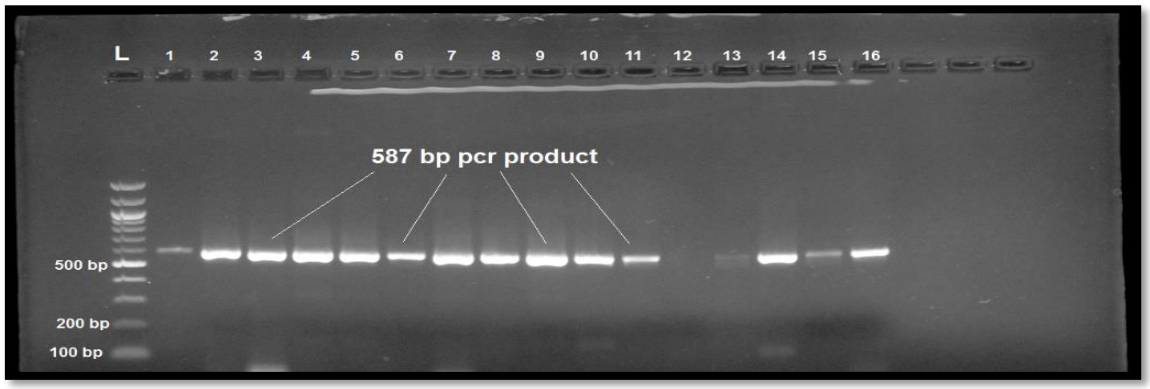

Figure 2: agarose gel electrophoresis (1.5%) of PCR amplified of *FlicA* gene (587) of *Salmonella typhi* for (60) min at (100) volt. This figure showed (1,2,3,4,5,6,7,8,9,10,11,13,14,15,16) isolates were positive results except the (12) isolates was negative result for *Flic* gene. L: ladder (DNA marker).

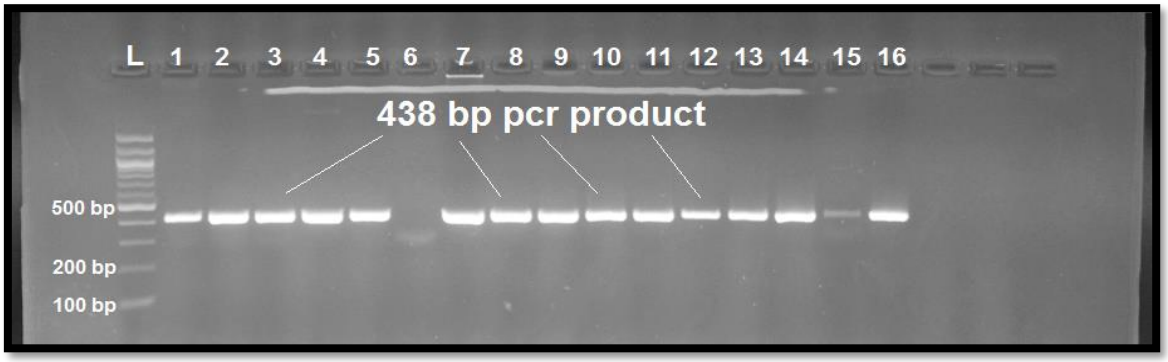

Figure 3: agarose gel electrophoresis (1.5%) of PCR amplified of *ViaB* gene (438) of *Salmonella typhi* for (60) min at (100) volt. in this Figure show (1,2,3,4,5,7,8,9,10,11,13,14,15,16) isolates was positive results except the (6) isolates was negative result for *ViaB* gene.

Figure 4: agarose gel electrophoresis (1.5%) of PCR amplified of *sipA* gene (1126)bp of *Salmonella Typhi* for (60) min at(100) volt. in this Figure show (1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16) isolates was positive results for *SipA* gene.

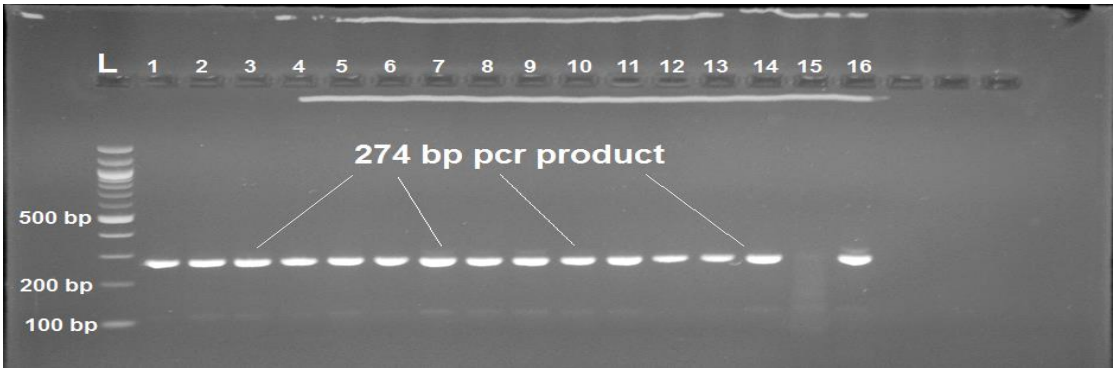

Figure 5: agarose gel electrophoresis (1.5%) of PCR amplified of *sdiA* gene (274)bp of *Salmonella Typhi* for (60) min at (100) volt. in this Figure show (1,2,3,4,5,6,7,8,9,10,11,13,14,16) isolates was positive results except the (15) isolates was negative result for *SdiA* gene.

specimens were distributed into (23) specimens as *S. typhi* and (77) specimens as other bacteria, all these specimens were cultured on different media. The clinical diagnosis depended on the presence of some symptoms such as fever, headache, anorexia, nausea and vomiting, abdominal discomfort with diarrhea or constipation for (6-18) days. Out of (100) patients, 62(62%) were male and 38(38%) were female as shown in Figure (1). Males were found to be more infected than females, these results is similar to other results of studies in AL-Musaib[21]. The reasons may due to most males were out-doored and from this point of view they could be regarded as food eating and handing or contact with other patients[22,23] expressed his opinion that greater exposure of male to contaminated food and water out-side the home might be region of higher rate of infection among this population. Typhoid fever is an infection caused primarily by *S. typhi* and is transmitted through the fecal oral route by the consumption of contaminated meat, poultry, milk and eggs.

Identification of Salmonella typhi

Serologically test

The blood specimens were positive by using Widal test. The Widal test, which measures antibody responses ≥160 titer to *S. Typhi* H and *S. Typhi* O antigens occur in the end of the first week of illness. The Widal test gave (100%) positive results for *S. typhi* while in blood culture gave (23%) positive result. misleading results using Widal test may keep one away from the true diagnosis because of cross-reaction of antigen from other infections with Salmonella antibody[24]. An erroneous interpretation of rapid diagnostic tests delays the treatment of actual infection and increases morbidity. Increased request for Widal test as a quick diagnosis of typhoid fever has produced exaggerated results, since typhoid fever and malaria often show mimicking symptoms even in laboratory diagnosis. It is therefore recommended that the assumingly high incidence of the disease using Widal test will be greatly reduced if blood culture technique is routinely adopted as a baseline for the diagnosis of typhoid fever[25]. Widal used to measure agglutinating antibodies against H and O antigens of Salmonella typhi. However, the major drawback of the Widal test is its cross reactivity with some other bacteria of the same genus. Blood culture, a more practical but less sensitive alternative is often used. This has its own lapses as it takes (2-3) days opposed to the quick diagnosis using Widal. As a result, diagnosis may appear delayed or over looked and patients without typhoid may receive unnecessary and inappropriate antimicrobial treatment[26].

Morphological characterization

The bacterial isolates obtained from clinical samples were identified initially according to cultural morphology, microscopic characteristics and biochemical tests. From those isolates, the cultural identification of *S. typhi* was depended on the colonial morphology. Since the colonies of *S. typhi* were grown on blood agar appears smooth white colonies and pale like shaped and smooth colonies when grown on the MacConkey agar, indicated that *S. typhi* is unable to ferment lactose sugar and showed pink colour colonies with black centre on XLD agar. Microscopically S. *typhi* appeared Gram-negative bacilli. The results of biochemical tests that recorded in Table (3) were considered as a complementary of the initial identification of *S. typhi* isolates. The isolates confirm to general characteristics, isolates were negative for oxidase test, indole production, urease production and Voges Proskauer, simmon citrate utilization and positive to, H_2S production and catalase test and methyl red test. The *S. typhi* isolates showed production of hydrogen sulphide and no gas production in TSI. In triple sugar iron slants, both the slant and bottom turned into red and yellow colour respectively (indicating the fermentation of glucose alone and no production of acid in the butt. All the results (morphology and cultural) were identical with[16,17,6]. The final identification was performed with the automated VITEK-2 compact system using GN-ID cards, which contained (47) biochemical tests and one negative control well. The results demonstrate that only (16) isolates from blood as *S. typhi* with ID message confidence level ranging between (95-99%).

Molecular study of Salmonella typhi

Detection of (fliC) gene

Polymerase chain reaction technique of the *S. typhi* clinical isolates revealed the *fliC* gene with product size (578pb), as shown in Figure (2). In the present study, PCR was used to detect *fliC* gene among the (16) isolates showed that PCR 15 isolates positive for this gene

(93.75%). Our results similar to that referred by[27] in Iraq were PCR positive in (100%). This result is associated with[28] who found out of (80%) suspected typhoid fever cases, flagellin gene (*fliC*) was detected by study done in Bangladesh where PCR was positive in (88.7%) of suspected typhoid fever cases. A flagellum has three basic parts: the outmost and longest part is a filament, which consists of around (20,000) protein subunits of a single protein called flagellin (*Flic*) with a molecular weight of (50 to 60) KDa. The gene *fliC-d* encode for the synthesis of H (flagellar) antigen. This antigen form the basis of classification for *Salmonella* by[29]. The *fliC* gene is present in over (100) *Salmonella serovars*. The low culture-positivity rate of blood culture for *S. typhi* in patients diagnosed clinically as suffering from typhoid fever in the community prompted us to look for an additional or alternative diagnostic tool. PCR is another test that has shown high sensitivity and specificity for diagnosis of typhoid fever in several studies depending on present *fliC* gene[30]. Several investigators have used different genes like flagellin gene, *viaB* region and no single method has yet been standardized for use in the clinical setting. Most of the studies used flagellin gene as a molecular technique for detection of *S. typhi* in clinical specimens. The alternative method where *ViaB* region is targeted can give false positive result due to the presence of this sequence in *S. Paratyphi*. Flagella are thin, rigid appendages of bacteria, and are bacterial locomotive structures[31].

Detection of (ViaB) gene

Molecular detection of *ViaB* gene was done for isolates that previously detected as *salmonella typhi.* The results showed that (15) isolates (93.75%) gave positive results for this virulence gene. Positive results were detected by the presence of (438)bp bands when compared with allelic ladder as shown Figure (3). This study is similarty with[32]. The Vi antigen is a capsular polysaccharide expressed by *Salmonella typhi*. Vi-Capsular formation has long been recognized as a protective mechanism for bacteria. Encapsulated strains of many bacteria are more virulent and more resistant to phagocytosis and intracellular killing than are none capsulated strains[33]. The *viaB* locus is composed of (11) genes designated *tviA-tviE* (*typhi* Vi), *vexA-vexE* (Vi antigen export) and ORF11. The Vi capsular polysaccharide (CPS) of *Salmonella enterica* serovar *typhi*, the cause of human typhoid, is important for infectivity and virulence. The causative agent of the human systemic infection typhoid fever, *Salmonella enterica* subspecies I serotype *typhi* (*S. typhi*), expresses a capsular polysaccharide (CPS) known as Vi antigen (Vi)[34]. The Vi is also a protective antigen and a vaccine based on purified Vi polysaccharide has been developed and licensed for use as a parenteral vaccine against typhoid fever[35].

Detection of (sipA) gene

Molecular studies of virulence gene (*sipA*) were done for all isolates of *Salmonella typhi* by using specific PCR method is used to detect the virulent gene encoding *sipA* using specific primer PCR technique clearly identified the virulent gene as *sipA* gene of *Salmonella typhi*. The genomic DNA of the sample was extracted and bands were observed by performing agarose gel electrophoresis, when PCR was performed, results clearly indicate that all isolated organisms contained *sipA* gene and all the amplified products produced a band at the level of (1126bp), when compared with the allelic ladder as showed in Figure (4). The *sipA* gene play important roles in both the invasion and the elicitation of intestinal inflammation, specially type III secretion system that inject effector proteins into host cells to promote bacterial invasion and to induce intestinal inflammation. The *sipA, protein* are thought to form a complex in the eukaryotic membrane that is required for translocation of the remaining effectors into the host cell cytoplasm[11].

Detection of (sdiA) gene

Molecular detection of Vi gene (*sdiA***)** was done for (16) *Salmonella typhi* isolates and the results showed that (15) isolates have this gene (93.75%). The positive results for (*sdiA*) virulence were detected by the presence of (274) bp band compared with allelic ladder as shown in Figure (5). *SdiA* is the first example of a bacterial receptor that exclusively detects the signals AHLs (N-acylhomoserine lactone) of other microbial species. The *sdiA* gene lies in centisome (42) region of the *Salmonella* chromosome (far enough from the unstable centisome (63) region) just upstream of *uvrC* and *sirA*[12]. *sdiA* does not regulate the virulence associated type III secretion systems of *Salmonella*. Therefore, *sirA* appears to be a major regulator of *Salmonella* intestinal virulence, whereas *sdiA* regulates accessory factors that may contribute to intestinal survival or colonization and other genes of unknown function. The *sirA* gene has also been used for PCR detection of *Salmonella* ssp. but yielded nonspecific bands for non-*Salmonella* strains[36]. *sdiA* is only known to activate genes that would help a single bacterium to adhere to host tissues, presumably because the presence of AHL producing bacteria indicates that a particular host environment has been reached[12]. Consequently, *sdiA* as a response regulator of gene transcription has very conservative domains. Each element in a quorum sensing system has the freedom to evolve greater complexity or specificity without compromising the overall system function. These characteristics (conservation and specificity) render the quorum sensing genes ideal targets for primers design[36].

CONCLUSIONS

PCR technique a useful and rapid diagnostic tool for the detection of *S. typhi* (*flic*) gene and could be employed by the diagnostic laboratories or clinics for the clinical diagnosis of typhoidial fever from patients. Genotypic detection by PCR revealed the presence of *viaB, sipA* and *sidA* genes were have relationships with their pathogenicity.

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