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Quantitative Real Time PCR study on the effects of *hilA* gene on the expression of pathogenicity genes in *Salmonella enterica* ssp.

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ABSTRACT

Salmonella enterica serovar *typhimurium* is one of the important worldwide health issues. The *hilA* gene encodes a transcriptional activator that regulates expression of the majority of the genes responsible for the *Salmonella* invasive phenotype. In the present study, PCR was performed using our designed primers for amplification of upstream and downstream regions of *Salmonella typhimurium hilA* gene. Each DNA fragment was T/A-cloned into pGEM-T easy vector and then sub-cloned into pET32 expression vector together with Kanamycin resistance gene. The recombinant plasmid was transformed into bacteria (*Salmonella typhimurium*) using electroporation. The *hilA*-Knockout mutant was characterized to evaluate the predicted role of the *hilA* gene in virulence. Quantitative RT-PCR was carried out to study the impact of hilA knock out on the expression of downstream genes including *invF* and *invA*. We confirmed the successful preparation of *hilA* gene construct (pET32-up-kan-down) followed by the efficient electroporation of the construct to bacterial cells. The homologous recombination resulted in the *hilA* confirmed by PCR. We demonstrated that *hilA* knockout leads to attenuation of *invA* and *invF* genes expression. The *hilA* knockout strain may be useful for the development of efficient vaccines against the *Salmonella typhimurium*.

Key words: *Salmonella typhimurium*, *hilA* gene, cloning, Electroporation, Quantitative RT-PCR

Introduction

Salmonella are Gram-negative and facultative intracellular bacteria that cause various diseases in most of animal species, as well as humans (Ellermeier & Slauch, 2007). The *Salmonella enterica* (*S. enterica*) species contain approximately 2600 various serotypes. Among *Salmonella serotypes, Salmonella typhi, Salmonella gallinarum* and *Salmonella typhi-suis*, are restricted to humans and chickens but *Salmonella enteritidis* and *Salmonella typhimurium*, can infect a wide range of hosts from poikilothermic to homoeothermic animals (Evangelopoulou et al., 2015). Salmonellosis is a disease caused by *S. enterica* serovar *typhimurium* (*S. typhimurium*) that could cause gastroenteritis or systemic infection transmitted by water and food contamination (Srikanth et al., 2011). The virulence of *Salmonella* is dependent on genes located in *Salmonella* pathogenicity islands (SPIs) (Jacobsen et al, 2011). SPI-1 is located at the centisome 63 of the bacterial chromosome and encodes a specialized invasion proteins system, known as type III secretion system (T3SS). These specialized proteins stimulate host-cell responses for the bacteria's benefit and are essential to the beginning of the entry process to host cells (Terence et al, 2011). SPI-1 contains all of the genes essential for the establishment of systemic infection (Ellermeier & Slauch, 2007). Sip family and regulators such as *HilA, HilC, HilD* and *InvF* are examples of invasion secretion proteins. The invasion proteins and structural genes are located in *prg/org, inv/spa,* and *sic/sip* operons (Ellermeier & Slauch, 2007). The T3SS are complex molecular agents with a role in delivery of the secreted protein into host cells (Srikanth et al., 2011). The T3SS create a needle-like structure used for the infusion of bacterial secreted effectors into the epithelial cells of the host, resulting in a bacterial invasion of eukaryotic host cells (Priyadarshi & Tang, 2010).

HilA (hyper invasion locus A) gene is located on SPI1. The protein *HilA* containing a DNA binding region is a transcriptional regulator of SPI1 genes and belongs to the

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OmpR/ToxR family of regulators. The OmpR and other members of this family, have DNA binding domains that probably interact with DNA as a dimer in which any monomer faces the identical orientation (Lostroh & Lee, 2001; Hüttener et al., 2014).

It is known that *hilA* gene is a regulatory component that activates the expression of invasion genes (Jones, 2005). *HilA* appears to directly activate the *prg/org* and *inv/spa* expression operons, and indirectly activate the expression of many secreted effectors (such as *sip* operon), by binding to the *HilA* box. The *inv/spa* genes encode proteins that are necessary for introduction of the salmonella into host epithelial cells and causes activation of *sic* and *sip*, that encode chaperone and invasion proteins respectively. Thus *HilA* plays important roles in the regulation of all the components necessary for a functional T3SS and also invasion genes located at other chromosomal loci (Jones, 2005).

Recombinant DNA technology and gene cloning is a major DNA-based approach in gene manipulation. This technology permits researchers to study the role of genes and integrate them into the genome of other organisms (Maurya et al., 2014). One of the important tools in genetic engineering is the antibiotic resistance. For instance, Kanamycin is inactivated with aminophosphotransferases (APHs) by transferring γ-phosphate to the OH group in the 3' position of the pseudosaccharide. Therefore, during the knock out procedure, kanamycin will be expressed in *Escherichia coli* (Yazdani et al., 2012).

The purpose of this study was knockout of the *hilA* gene in *S. typhimurium*, as one of the pathogenicity genes, via generation of a gene construct that carries a kanamycin resistance gene replacement between the upstream and downstream areas of *hilA* gene to investigate the possible effects of knockout on the expression of virulence genes.

Materials and Methods

Bacterial collection and culture conditions

Salmonella typhimurium and *E. coli* (Top10F' strain) were cultured in Luria-Bertani (LB) broth and incubated at 37°C/200-rpm for approximately 18 h until the log growth phase (OD600 = 0.9) was reached.

Strains and media

The T/A cloning performed using pGEM T-Easy kit (Invitrogen. San Diego, CA) in Top10F' strain of *E. coli* (Invitrogen, the Netherlands). Bacterial cultures were grown at 37°C in Luria-Bertani (LB) Medium. The pET28 and pET32 plasmids were respectively used for *kan* gene amplification and sub-cloning.

Figure 1. *Salmonella Pathogenicity Island I (SPI-1). Gene map of Salmonella invasion gene located.*

Extraction of genomic DNA from S. typhimurium

Bacterial DNA was extracted from bacterial cell culture using DNPTM Kit (CinnaGen, Iran) according to the manufacturer's instructions. The quality of extracted DNA was evaluated on 1% agarose gel electrophoresis.

Amplification of kanamycin gene, and flanking regions of hilA gene

Oligonucleotide primers were designed for amplification of flanking regions of *hilA* gene of *S. typhimurium* and also for the *kan* gene (pET-28 vector were used as template), according to the published sequence (Table 1).

The amplification was done using Thermal Cycler (Eppendorf, Germany), for amplification of *kan* gene and upstream and downstream regions of *hilA* gene.

PCR reactions were performed in a total volume of 25 µL in 0.2 ml tubes, containing 2 µL of template DNA, 1 µM of each primer, 2.5 µL of 10X PCR buffer AMS, 2 mM $MgCl_2$, 200 µM dNTPs, and 1 unit of Taq DNA polymerase (Fermentas, Germany).

The following conditions were applied: initial denaturation at 95°C for 5 minutes, followed by 32 cycles; denaturation at 94°C for 1 minute, annealing at 64°C for 1 minute, elongation at 72°C for 1 minute. The program was ensued by final

Table 1. *The primer sequences used for PCR amplification.*

Gene amplified	Primers	Primer sequences (5'-3')	Amplicon size (bp)	restriction sites
Upstream	up-F	CTGTCTAGAAGCCTGAGGATGATACTGC	662	*XbaI*
of *hilA*	up-R	ATGCCATGGGATAATAGTGTATTCTCTTAC		*NcoI*
kanamycin	kan-F	ATACCATGGATGAGCCATATTCAGCGTG	835	*NcoI*
resistance	kan-R	CCAGTCGACTTAGAAAAATTCATCCAG		*SalI*
Downstrea	down-F	TATGTCGACTGGGACACAAAGCAGATTAG	480	*SalI*
m of *hilA*	down-R	CCACTCGAGACACTCGTTAATCGTTTG		*XhoI*

elongation at 72°C for 5 minutes and amplified sample was held at 15°C.

Analysis of PCR products

The PCR amplification products (10 µl) were subjected to electrophoresis in a 1% agarose gel and stained by ethidium bromide in 1X TBE buffer at 80 V for 30 min. Aliquots of 10 µl of PCR products were applied to the gel. A constant voltage of 80 V for 30 min was used for products separation. The 100bp DNA ladder (Fermentas, Germany) was used as a molecular weight marker to determine the length of the amplified fragments. After electrophoresis, the images were obtained in UVIdoc gel documentation systems (UK).

Cloning of hilA gene and plasmid construction

PCR products purified using a gel extraction kit (Bioneer Co., South-Korea) after gel electrophoresis according to the manufacturer's instructions. The amplified products were cloned into pGEM-T easy vector (Promega Co), and the recombinant vectors were transformed under heat shock and calcium chloride shock into *E. coli* TOP10F' competent cells. The presence of *kan* gene and flanking regions of *hilA* gene was confirmed by restriction enzyme and PCR analysis.

Sub-cloning of the hilA and kan genes

The *hilA*-up fragment was removed from the pGEM T-easy vector by XbaI-NcoI double digestion and sub-cloned in XbaI-NcoI linearized pET-32 to get pET-32-up fragment. Then, *kan* fragment was released from the pGEM T-easy vector by NcoI-SalI double digestion and sub-cloned into NcoI-SalI linearized pET-32-Up to create pET-32-Up-Kan. Finally, *hilA*-down fragment was released from the pGEM T-easy vector by SalI-XhoI double digestion and subcloned into SalI-XhoI linearized pET-32-Up-*kan* to construct the pET-32-Up-Kan-down recombinant vector. The final construct was confirmed by double digestion by XbaI-XhoI and PCR using *hilA*-up-F and *hilA*-down-R primers.

Electroporation

Electroporation was performed using a Gene Pulser Xcell Electroporation Systems (Biorad) as described below. A total amount of 20 ml LB medium was inoculated with *S. typhimurium* and cultured overnight at 37°C. After 12-16 hours, 2 ml of a fresh overnight culture was sub-cultured into 200 ml LB medium and cultivated for ~3 hours until OD600 reaches 0.4 to 0.6 (early log phase). The cells were centrifuged at 4200 rpm at 4°C for 5 minutes and then the cell pellet was washed twice in electroporation buffer (cold sterile 10 glycerol). For each transformation, 2 µg of the plasmid was transferred into pre-chilled micro electroporation cuvettes together with 50 µL of cell suspension (*S. typhimurium*). This suspension was incubated on ice for 10 min until electroporation. The Gene Pulser Xcell Electroporation Systems (Biorad) was set to 25 µf and 2.5 kv. After puls, cuvettes chilled again on ice for 10 min. Then 1 ml of LB broth medium was added to the electroporated suspension and incubate for 60 min on 37°C to allow the cells to recover. To obtain single colonies, 20 µl of the culture was transferred onto an agar plate and incubated overnight at 37°C. The gene construct was inserted into the bacterial genome by homologous recombination and *hilA* gene was knockout. The procedure of electroporation was confirmed by PCR using *hilA*-up-F and *hilA*-down-R primers.

Quantitative Real Time PCR

Quantitative RT-PCR analysis was carried to evaluate the expression of two pathogenic genes (*invF* and *invA*) that are downstream of *hilA* gene to investigate whether the *hilA* knockout can alter the expression of *invF* and *invA* genes. The specific primers used for real-time PCR are listed in Table 2.

Table 2. *Oligonucleotide primers used for quantitative RT-qPCR.*

Gene	Gene product	Primer (5'-3')
16SrRNA	Housekeeping gene	F: GAATGCCACGGTGAATACGTT
		R: ACCCACTCCCATGGTGTGA
invA	downstream *hilA* of gene	F: GGTGCGAGAAAGCAGACC
		R: AACAAAACCCACCGCCAGG
invF	downstream *hilA* of gene	F: GCGTCCTAATCACCTGTGCTG
		R: GTTTACGATCTTGCCAAATAGCG

Total RNA was isolated from electroporated *S. typhimorium* bacterial cells using the SV total RNA isolation system (Promega). Any genomic DNA was eliminated using RNase-Free DNase I (Takara). The Purity of the total RNA was determined with a NanoDrop-1000 spectrophotometer. The cDNAs were synthesized using the PrimeScript RT reagent kit (Takara). The PCR reactions were performed on Rotor-Gene 6000 Real time PCR instrument (Corbett Research, Australia) by SYBR Premix Ex Taq kit (Takara) in a final volume of 10 µL. Real time cycling conditions were as follows: 95 °C for 40 s; 45 cycles of 95 °C for 15 s, 58 °C for 20 s and 72 °C for 25 s. The expression levels of *hilA* gene were normalized to the transcripts of the housekeeping gene, 16s rRNA, which served as an internal control and negative control samples were used in each run. Relative expressions were calculated using $2^{-\Delta\Delta Ct}$ method. The RT-qPCR assays were performed in duplicate and the data were presented as the mean ± standard error of the mean (SEM) where applicable.

Statistical analysis

Results were expressed as mean±SE. The data were analyzed by one-way ANOVA following the Dunn's post hoc analysis, using GraphPad Prism version 7.00 (GraphPad

Software, La Jolla California USA). Differences were considered significant at P<0.05.

Results

DNA Extraction and Amplification

Total DNA was successfully extracted and PCR amplified products were run on 1% agarose gel. As shown in Figure 2, flanking regions for *hilA* and *kan* genes respectively correspond to 662 bp, 480 bp and 835 bp fragments (Figure 2).

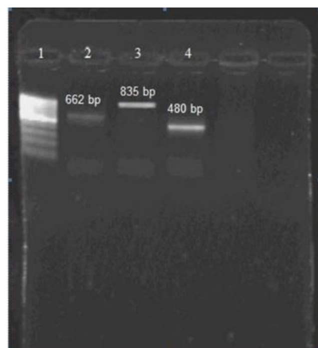

Figure 2. *Analysis of PCR amplified flanking regions of hilA and kan gene on the agarose gel electrophoresis. Lane 1 is a 100bp DNA marker (Fermentas, Germany), lanes 2 (662 bp) is the amplified fragment for hilA-upstream region, lane 3 (835 bp) is the product for kan gene and lane 4 (480 bp) is a hilA-downstream region.*

T/A Cloning, Sub-Cloning and Sequencing

In the next step, the upstream and downstream regions of *hilA* gene and *kan* gene were cloned with T/A cloning technique in pGEM T-easy vector successfully. Then, these fragments were removed from the pGEM T-easy vector using restriction endonuclease sites of XbaI, NcoI, SalI, and XhoI and finally subcloned in a polyclonal site (PCS) in pET-32 expression vector. The *E. coli* TOP10F' strain competent cells were used for transformation of pET-32-Up-Kan-Down recombinant vector.

The plasmid was purified and the sub-cloning procedure was confirmed by restriction endonuclease digestion of pET-32-Up-Kan-Down recombinant plasmid. Figure 3 shows the recombinant plasmid (pET-32-Up-Kan-Down) after digestion. A 1977 bp DNA fragment represents a large fragment yielded from the connection of to 662, 835 and 480 fragments corresponding to the upstream region of *hilA*, *kan* gene and downstream regions of *hilA* respectively. The length of pET-32 vector is 5900 bp. Ultimately the construct was further confirmed by sequencing (Sequence confirmation of the construct by chromas software).

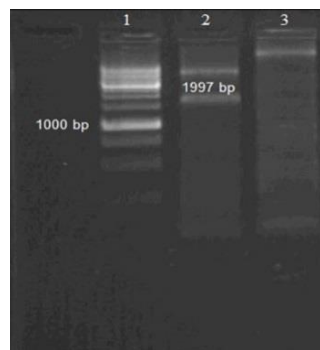

Figure 3. *Agarose gel electrophoresis analysis of digested pET32-Up-Kan-Down recombinant plasmid using* Xba*I and* Xho*I restriction endonuclease enzymes. Lane 1 is 1 kb DNA ladder (Fermentas, Germany). In lane 2, A 1977 bp DNA fragment represents a large fragment yielded from the connection of to 662, 835 and 480 fragments corresponding to upstream region of* hilA, kan *gene and downstream regions of* hilA *respectively. Lane 3 is an uncut recombinant plasmid.*

Electroporation

In the next step, Electroporation reaction was performed to insert pET-32-Up-Kan-Down recombinant plasmid to *S. typhimurium* bacterial cells by electric shock. Initial experiments were carried out using the Bio-Rad electroporation cuvettes. After two washes in 10% glycerol, approximate transformation efficiencies of 106 transformants per µg of plasmid DNA was obtained under the following conditions. The TC parameter was set to 5.1 and the voltage to 2481 V. After the homologous recombination reaction between the gene construct and bacterial genome, the cells were transferred to plates containing the kanamycin antibiotic which resulted in the appearance of kanamycin-resistant colonies (data not shown).

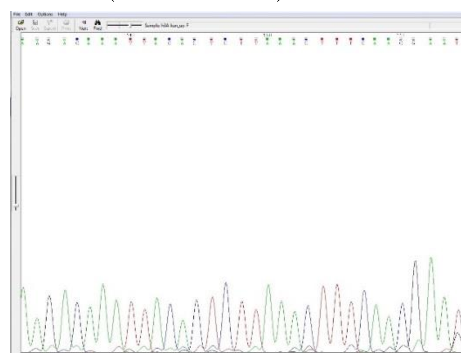

Figure 4. *Sequence confirmation of construct by chromas software.*

The Expression Level Analysis of invF and invA genes in Recombinant Bacteria

In order to evaluate whether *hilA* knockout leads to reduced virulence, RT-qPCR analyses were performed. Wild type and knockout lines of *S. typhimurium* were compared to understand the effect of knockout. Indeed, the expression levels of two of virulence genes downstream of *hilA* gene

(*invF* and *invA*) were investigated. The results demonstrated that the expression levels of these genes have been significantly reduced and therefore the procedure of gene knockout has been successful (Figure 5).

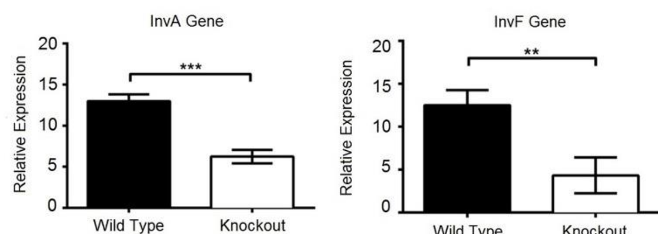

Figure 5. *Atenuation of the expression of downstream genes of hilA including InvA and InvF.*

Discussion

S. typhimurium is often found in the membrane of the intestinal lumen. *Salmonella poisoning* is due to an outer membrane mostly containing lipopolysaccharides (LPS) which are characteristic components of the cell wall and keep the bacteria safe from the environment (Sebastian et al., 2009).

The LPS contains the O-antigen, a polysaccharide core, and the lipid A. The phosphate groups of lipid A, which is attached to the outer membrane and is a conserved core oligosaccharide, specifies the bacterial toxicity.

The O-antigen that is a changeable number of extremely polymorphic carbohydrate subunits can stimulate the host immune system (Claudia et al, 2009).

On the other hand, *Salmonella* serotypes are transmitted through the ingestion of contaminated water or food. *Salmonella* crosses the intestinal tissue through M cells (Microfold cells) as an entry port to invade deeper tissues that are positioned between the epithelial cells covering lymphoid follicles (Miller et al., 2007; Tahoun et al., 2012).

After adhesion and invasion of epithelial cells and achieving the lamina propria of the intestinal cell wall, bacteria face tissue macrophages associated with the M cells (Velge et al., 2012).

During the adherence, a series of signalling events occur in the epithelial cell resulting in the conversions of the morphology of the eukaryotic plasma membrane and cytoskeletal reconfigurations that eventually lead to the entrance of *Salmonella* into the intestinal host cell (Evangelopoulou et al., 2015). *Salmonella* pathogenicity is dependent on SPI and secretory proteins. The *hilA* gene promoter is part of the SPI1 responsible for regulation of T3SS of *S. typhimurium*. Therefore, *HilA* plays a crucial role in invasion genes expression (Lucas et al., 2000; Boddicker et al., 2003).

Shi-Zhong Geng and co-workers (2009), studied the role of asd gene in *Salmonella enterica* serovar Pullorum. The *asd* gene was effectively knocked out by replacing the *asd* gene with the *CmR* (antibiotic resistance) gene and the SP Δasd mutant was performed successfully (Geng et al., 2009).

In 2015, *sipB* knock out mutant was generated to study its biological characteristics. The results showed that the virulence, adherence and invasion of the mutant were remarkably decreased in the mutants (Chen et al., 2015).

Tomoya Baba and co-workers (2006), designed a set of experiments to perform several single-gene deletions for unnecessary genes in *E. coli* K-12 using constructs to replace open-reading frame coding areas with a kanamycin cassette. Among the 4288 target genes, 3985 mutations were created. They did report that they were unable to disrupt 303 genes (Baba et al., 2006).

Wei Sheng and co-workers (2015) managed to delete the SPI-2 of *Salmonella Pullorum* via λ-red recombinant system and characterized the mutant's virulence. They showed that the virulence of knockout strain was clearly attenuated and suggested this approach as a promising method for the development of vaccines (Yin et al., 2015).

Furthermore, in a recent study in 2015, our group succeed to delete the *sipC* gene of *S. typhimurium*. We constructed the new recombinant vector which in the kanamycin resistance gene replaced the *sipC* gene of *S. typhimurium* (Safarpour Dehkordi et al., 2015).

In the present study, we demonstrated that deleting the *hilA* gene of *S. typhimurium* leads to significant attenuation in the expression levels of pathogenic genes and thus pathogenicity. We constructed the new recombinant plasmid to replace *hilA* gene with kanamycin resistance gene in *S. typhimurium*. The resulting vector was inserted into the wild type strain of *S. typhimurium* by the electroporation method. In order to evaluate the effect of *hilA* gene knockout and its effect on the pathogenicity, we performed quantitative RT-PCR analysis for downstream genes of *hilA* gene. We observed that the expression levels of these genes were clearly reduced suggesting that the pathogenicity of bacteria would be decreased. However, further characterization of these mutants must be performed in the future to confirm our hypothesis.

Conclusions

The present study clearly showed that deletion of *Salmonella enterica* serovar *typhimurium hilA* gene has changed the expression level of some pathogenic genes. Based on these results, the manipulated strain might be used for an attenuated vaccine against *S. typhimurium* in the future.

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