Original Article

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Study of the Expression of Helicobacter Pylori Urei Gene by RT-PCR

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ABSTRACT

BACKGROUND AND OBJECTIVE: Helicobacter pylori is a spiral shaped and gram negative bacterium which causes peptic ulcer and has an important role in gastric carcinoma. Different components of urease are the important factor to stimulate the immune system. There is no effective vaccine against this bacteria and research on finding an effective vaccine is necessary. The aim of present study was to produce ureI gene construct and evaluation of the expression of this gene.

METHODS: In this experimental study, the amplification of ureI gene was performed using PCR on standard strains of H. pylori genome that obtained from Pasteur institute. 612 bp fragment of ureI gene was cloned by T/A cloning technique into pTZ plasmid, and sub-cloning was done in PIRES2-EGFP vector. PIRES2-EGFP-ureI recombinant vector was transformed using electroporation into CHO cells and ureI gene expression was evaluated by RT-PCR.

FINDINGS: Cloning of 612 bp fragment for ureI gene in pTZ and PIRES2-EGFP vectors had confirmed using PCR, digestion by SacI/EcoRI restriction enzymes and sequencing. After the RT-PCR on transformed CHO cells, the ureI gene fragment was observed.

CONCLUSION: The PIRES2-EGFP-ureI recombinant vector can express the ureI gene. The successful gene expression of this target gene in animal cells can be used to evaluate the immunogenicity as a vaccine in laboratory animals. Also, this generated recombinant vector has the potential for assay as DNA vaccine in future experiments.

KEY WORDS: *Helicobacter pylori, cloning, ureI, Electroporation, Gene Expression.*

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Introduction

Helicobacter pylori cause gastrointestinal inflammation, gastric ulcer, duodenal ulcer and one of the main causes of gastric cancer (1). This microorganism is a gram negative, spiral, moving and flagellate bacteria and lives on the human stomach wall. Researches have shown that the human race has been involved with this infection for about 58,000 years (2). About half of the world's population is infected. In some parts of the world, pollution has been reported above 80%, including 83% in Chinese elders, 86% in Native children of Alaska, 80% in children and adults in Bolivia, 84% in adult of Portugal. In general terms, most polluted areas of the world include Central America, North America and Asia (3).

Between 4.7% to 65% of children globally have Helicobacter pylori infection, and the infection usually occurs during childhood and its effects persist for life (4, 5). The prevalence of Helicobacter Pylori in 2016 is reported to be 54% in the total population of Iran. So that 42% of children and 62% of adults in Iran are infected (6). Helicobacter pylori pathogenic factors are several factors such as urease, flagellum, adhesin, cytotoxin manufacturer vacuole, and the pathogenic cag island that may be involved in the disease (7). Helicobacter pylori can produce a large amount of urease and hydrolyze urea. The urease enzyme has several subunits, most notably UreA and UreB (8). The urease enzyme gene complex are ureA, ureB, ureI, ureE, ureF, ureG and ureH, respectively, in the helicobacter pylori genome (9).

Recent studies have shown that various components of this gene complex are considered as recombinant vaccines (gene vaccines or peptide vaccines). In 2001, with the aim of creating a recombinant vaccine, the urea and ureB components were cloned into prokaryotic plasmid pET and expressed in Escherichia coli (10). In several other studies, the activity of stimulating the immune system and the serum response of some components of this gene set has been proven. In a study that has done in Malaysia in 2014, meanwhile cloning and expressing ureG, its reactivity with the serum of human who infected by Helicobacter pylori has been shown, but in the control samples (healthy subjects), such a reaction has not seen (11). In 2013, research on an ureI-based vaccine was found to excite a high level of cellular and humoral immune system (12). UreI is a membrane protein with a molecular weight of 21.7 kDa, which is essential for the survival of Helicobacter pylori in acidic environments. This gene exists in a variety of Helicobacter species (5,13). In most Helicobacter pylori strains, ureI plays a protective role and is used as a detector molecule to detect Helicobacter Pylori (14). Some studies have shown that ureI may play a role in the transport of bacteria in the body. The gene also plays a role in protecting the acid and regulating the activity of cytoplasmic urease in microorganisms, and according to available studies, the bacterial urease enzyme is one of the best candidates for vaccine against Helicobacter pylori (8). Today, plasmids carrying foreign gene fragments are used as immunoassays. In animal models, it has been observed that vaccination with DNA has caused immunization against many infectious agents (15) Compared to other vaccines, the gene vaccine has the benefits of: simpler production, product purity, storage, and easier maintenance (16). As noted above, most components of the urease gene complex are suitable for stimulating the host immune system against Helicobacter Pylori. On the other hand, an effective vaccine against this bacterium has not yet been produced.

Given the high potency of the antigenic component of the Helicobacter Pylori urease enzyme, they can be used as candidates for a recombinant vaccine. The creation of a dual-purpose mechanism that can be used as a vaccine in the pathway for the production of recombinant protein for use as a peptide vaccine or direct injection of recombinant vector into a host. The aim of this study was to clone ureI gene in eukaryotic expression vector (PIRES2-EGFP) and study its expression at the level of RNA in the Chinese hamster ovary (CHO) cell line.

Methods

This is an experimental study conducted in the spring of 1395. Plasmids and bacterial strains and animal cells: The plasmids used in this study include a special T/A cloning plasmid called pTZ57R / T (Thermo Fischer Company, USA) that is designed to ease the insertion of pTZ57R / T from the abbreviation that has been used as pTZ. The eukaryotic expression vector PIRES2-EGFP (Clone Toc Company, USA) was also used to express the target gene. Due to the specific nature of this research, which involves the isolation, cloning and expression of the gene, it is not necessary to collect the sample and use a specific bacterial strain or cell line. In order to obtain the ureI gene, standard strains of Helicobacter pylori were used. In order to transform and replication recombinant vectors, E. coli strain Top10F was used. This bacterium was obtained from Biotechnology Research Center of Islamic Azad

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University of Shahrekord. Chinese chamomile ovarian cells (CHO) were used to evaluate the function of cloned gene in animal cells. DNA extraction and proliferation of ureI gene: Purification of genomic DNA from Helicobacter pylori using a DNA extraction kit (Sinagan Co., Iran) was performed according to Kit's method. The quality of the purified DNA was analyzed on agarose gel 1%, with Etidium Bromide staining.

The concentration of DNA purified by Nanodrop (PACKAGING COMPANY, USA) was measured. PCR reaction to replicate the ureI gene using a recurrence primer 5'-TTGGAGCTCAAGGATAAGGCAATGCTAGGAC-3 'and 5'- GAGAATTCCACACCCAGTGTTGGATAAAG-3'. In the 5', each of these primers was considered through the cut-off site of the two SacI and EcoRI enzymes. The PCR reaction was performed using Master Cycler Gradient (Eppendorf, Germany) and the PCR reaction in a final volume of 25 µL was performed as 20 ng DNA pattern, 2 mM MgCl2, 25 pikomol of each primer, 1 unit DNA polymerase Taq and 200 µM dNTP Mix (all materials manufactured by Sinagen Company, Iran). Then 2 to 3 drops of sterile mineral oil were added to the PCR mixture to prevent contamination and evaporation. Also, the temperature conditions for gene amplification include a 95 ° C cycle for 5 minutes, 32 repetitive cycles of 94 ° C for 1 minute, 60 ° C for 1 minute, 72 ° C for 1 minute and one The ending cycle was 72 ° C for 5 minutes.

In addition to the samples, deionized water was used twice for negative control and the obtained PCR products were electrophoresed on 1% agarose gel containing ethidium bromide and examined by ultraviolet light. The DNA fragment of the ureI gene was isolated from the gel using the scalpel razor and purified from the gel extraction kit (Bionir, South Korea) in accordance with the kit's instructions. To ensure the accuracy of the purified gene and its quality, 3 µL of it was detected on 1% agarose gel and along with electrophoresis marker. T/A cloning: T/A cloning is a technique used to clone PCR products. In this study, a T-vector called pTZ (Thermo Fisher, USA) was used to carry out T / A cloning. T-PCR products were legated according to the kits working method. Then, the product of Ligation in E. coli strain TOP10 F was chemically transformed using cold-sterile 0.1 M Calcium Chloride. The bacteria were cultured in an agar medium of LB (Luria bertani agar) containing 50 µg per milliliter of ampicillin. Plasmid purification was performed from the colonies using Kit (Kiagen Co., USA) and then the cloning accuracy was verified by PCR and using specific primers for ureI gene. The final confirmation of the formation was evaluated by enzymatic digestion and sequencing. Gene Cloning in an Expression Vector: The PIRES-EGFP expression vector was first cut off by the SacI and EcoRI enzyme (Thermo Fisher, USA) in the presence of Y / Tango buffer. After 4 hours, the solution was electrophoresed on agarose gel.

The observed single-band showed the PIRES-EGFP vector cut by these enzymes. The bands were purified by a DNA extraction kit from the gel (Bionir, South Korea). The ureI gene cloned in the PTZ vector was also cut in the same way by two SacI and EcoRI enzymes. The ureI gene was isolated and purified from pTZ vector with PIRES-EGFP vector in the presence of the T4-ligase enzyme (Thermo Fisher, USA) and the binding reaction between the ureI gene and the PIRES-EGFP expression vector was performed. The reaction product was transferred to E. coli strain TOP10F and cultured in LB Agar culture medium containing kanamycin at a concentration of 100 µg / ml. After extracting the plasmid from the obtained colonies, the accuracy of cloning and formation of the recombinant vector PIRES-EGFP-ureI were investigated by PCR methods, enzyme digestion with two SacI and EcoRI enzymes and, finally, sequencing (15). Transformation of Animal Cells with PIRES-EGFP-uerI Ultimate Organism: In order to study the expression of ureI gene in animal cells, Chinese hamster ovary cell (CHO) was used and electroporation was used for the transformation of these cells. The Gene Pulser Xcell (Bioread Company, USA) was used to conduct electroporation.

The number of 2 × 106 cells were counted from CHO cells and poured into 400 volumes in a special 0.4 ° cuvette for electroporation. Then, the amount of 800 ng / µl of the recombinant PIRES-EGFP-ureI vector was added to the cells inside the cuvette under sterile conditions (below the hood) and the cuvette was placed on ice for 5 minutes. The outer walls of the cuvette containing cells were dried with sterilized gas and placed in an electroporation device. Electric pulses were given with optimized conditions of 0.174 kV and 400 microfarads to the cells and the cells were immediately placed on ice for 2 minutes. Electroporated cells were cultured in a culture flask containing RPMI medium (Gibco, USA) with 10% FBS and penicillin and streptomycin antibiotics, and incubated at 37 °C and 5% CO2 for 6 hours. Then to each culture flask was added 100 µg / ml of neomycin antibiotics (a selective marker

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for recombinant vector-transfected cells) and the cells were kept in an incubator for 72 hours. All of the above steps (electroporation) were also carried out for another series of CHO cells that were considered as controls, with the exception that no DNA was added to the control group cells. Gene expression analysis at RNA level: Reverse transcription technique was used to study the expression of uerI gene cloned in PIRES-EGFP vector. For this purpose, RNA extraction was performed from the CHO cells transfected with the PIRES-EGFP-uerI recombinant plasmid and the control group (no recombinant vector) using a kit (Kiagen, USA). The quality and concentration of the obtained RNAs were evaluated using Nanodrop (Peck Lip Company, USA). The kit (Thermo Fisher, USA) was used to prepare the cDNA. PCR reaction on the obtained cDNAs was performed using specific primers of the uerI gene Helicobacter pylori. Considering that most of the stages of this research lead to the production of the product at each stage, therefore, the criterion of measuring variables is the observation or non-observance of the product in each step.

Results

Isolation of the ureI gene Helicobacter pylori: In this study, genomic DNA extraction from Helicobacter pylori bacteria was successfully performed and the DNA concentration after reading with nanodrop was shown to be 235 ng/µl. The results of the electrophoresis of the purified DNA on the agarose gel indicated that it was suitable for molecular testing. The length of the fragment resulting from the proliferation of the ureI gene was determined, with respect to the designed primers, after a PCR reaction was 612 bp. In this experiment, the DNA-free specimen was used as a negative control. Since the template DNA for the replication of the ureI gene is in fact the standard strain of the Helicobacter pylori genome, this standard pattern, in addition to acting as a DNA pattern, is also a positive control (Fig 1). T/A cloning and subcloning: PCR product cloning by the T/A method in the pTZ vector resulted in the production of the pTZ-ureI construct in order to create a cluster of ureI gene. The confirmatory tests used to verify the accuracy of ureI gene cloning, including PCR and enzyme digestion, showed that a large percentage of the resulting clones possessed the pTZ-ureI construct. Also, the dual enzyme digestion with SacI and EcoRI enzymes on purified plasmids revealed the presence of a 612 bp fragment related to the ureI gene in the pTZ vector.

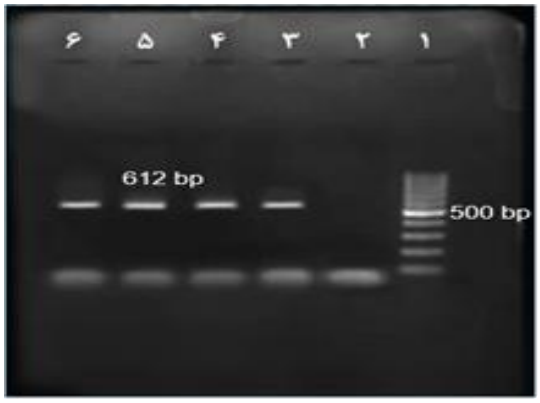


Figure 1. Reproduction of ureI gene by PCR method, Row 1: Marker 100 bp of the Formentaz Formation Production Lot, Row 2: Negative Sample (DNA without PCR Sample), Row 3-6: 612 bp sequence of ureI gene replication.

The enzyme digestion of this organism with two SacI and EcoRI enzymes and then agarose gel electrophoresis led to the formation of 5308 and 612 bp of the PIRES2-EGFP vector and ureI gene, respectively. The results of the enzyme digestion of the final PIRES2-EGFP-ureI system with two SacI and EcoRI enzymes are shown in Fig. 2. The results of the sequencing of the cloned ureI gene in the final system were BLAST in the global gene database, and the results showed no nucleotide mutations or alterations in the sequence of this gene and its sequence was confirmed.

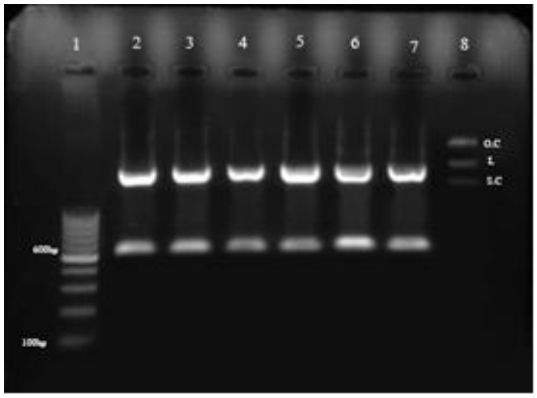


Figure 2. Enzymatic digestion of the final PIRES2-EGFP-ureI system with two SacI and EcoRI enzymes. Row 1: The 100-bp marker of the Firentaz Company Formation, line 2-7: The 612 bp gene portion of the ureI gene and the other portion of 5308 bp related to the PIRES2-EGFP vector, Row 8: The recombinant plasmid PIRES2-EGFP –ureI

Electroporation and gene expression: The results showed that the cells were resistant to this antibiotic during the culture stages in the presence of neomycin,

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which confirmed the release of the recombinant plasmid. The results of the RT-PCR reaction were positive for confirmation of the expression of the ureI gene in animal cells. So, after performing the PCR reaction on reverse transcriptase-cDNAs for the target recombinant plasmid receptor cells, the 612 bp of the ureI gene was formed. But the same experiment was not positive for cells that did not have a recombinant plasmid. The results of this study confirm the successful expression of ureI gene in Chinese hamster ovary cells.

Discussion

In this research, the ureI gene was expressed as one of the most important Helicobacter pylori antigens in the eukaryotic system. This gene was cloned in the expressive eukaryotic vector PIRES2-EGFP and its successful expression was seen on the RNA level by RT-PCR in CHO animal cells or Chinese hamster ovary. The urease gene complex contains the most important and effective genes that directly relate to the life and survival of Helicobacter Pylori. This gene set consisting of sub-major units and auxiliary genes collectively contains seven genes called ureA, ureB, ureI, ureE, ureF, ureG and ureH (9).

In numerous studies, most of the components of the urease complex have been cloned and studied for various purposes, and most studies have shown antigenicity in other components of this gene cluster. In 2013, Jie and colleagues replicated and cloned ureI from the urease genes. The researchers introduced the ureI gene in the expression plasmid pCDNA3.1 (+) and produced the recombinant vector pCDNA3.1 (+) - ureI. As a result of their work, the researchers announced that the gene is capable of successfully expressing the protein product UreI and stimulating the immune system of animal animals as a vaccine, and the overall conclusion is that the success of this mechanism is to produce a very strong Cellular and humoral immune response. Research from Jie and colleagues resembles our view from a number of perspectives.

First of all, they have used ureI gene like our research. Second, they have inserted this gene into an eukaryotic expression vector and have obtained good results (12). However, the type of eukaryotic vector used in our research has varied with recent research. In another study, the researchers replicated the subunits of ureA and ureB from the genome of helicobacter pylori isolated in Iran. These genetic fragments were first inserted into the Proctoroid vector pET and their expression in E. coli was investigated (10). This research was similar to our research in terms of the techniques used, but in the present study, we have attempted to isolate another part of the urease gene and reproduce and introduce the ureI gene. The study of ureI expression was also performed in the eukaryote cell, unlike the work of these researchers. The importance of the issue of eukaryotic expression, which is emphasized in our study, is due to the potential for this structure. As it is able to express the gene in the eukaryotic system in the laboratory environment, it is also able to produce its own product if injected into the muscle of the animal carcinoma (as a vaccine). Gu et al, cloned UreB pylori gene for expression in the tobacco mosaic plants. In this study, the ureB gene was cloned into pBI121 vector and after cloning by RT-PCR and western blotting methods, they confirmed the expression of this gene. This is the first report of H. pylori gene expression system in plants.

In the study of Gu and colleagues, as in our research, the expression of the gene was measured in the RNA expression stage by RT-PCR (17). Strugastky et al. Stated that Helicobacter pylori is associated with digestive diseases such as chronic gastritis, gastric ulcer, stomach lymphoma, and gastric cancer, and also stated that ureI is a highly protected membrane of Helicobacter pylori and is a key factor in the colonization of Helicobacter pylori in the stomach of mammals and ureI can be used as antigens and a good molecular marker for Helicobacter pylori (18).

In another study, Wang and colleagues cloned the ureI gene of the Helicobacter pylori bacteria in Escherichia coli bacteria that ultimately led to the production of the BL21 +/ureI constraint. They also showed by SDS PAGE and Western Blot method that the recombinant ureI protein was produced in an inclusion bodies and was able to work against helicobacter. They examined the expression of this protein in the prokaryotic plasmid and concluded that the expression of rUreI / his protein in the inclusion bodies was capable of acting against the anti-Helicobacter and Hys-tag antibodies (19).

Considering the relatively high prevalence of Helicobacter Pylori in human societies around the world, and especially its introduction as one of the most important factors in human gastrointestinal cancers, it is necessary to pay attention to ways of preventing this infectious agent. One of the best ways to prevent the infection is vaccination. So far, no effective vaccine against Helicobacter Pylori has been produced, and

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study on recombinant vaccines can be a good preventive measure and a major step in inhibiting Helicobacter pylori infection. According to the results of this experimental study, the ureI gene of Helicobacter pylori was first cloned by T/A method and recombinant vector pTZ-ureI was produced. This recombinant plasmid as a reliable source of the ureI gene can be shared with other researchers in the country and added to the gene pool's research centers at research centers. On the other hand, the final recombinant vector PIRES2-EGFP-ureI, in which the ureI gene (which is an important property of the host immune system stimulation) was expressed in animal cells in a cell culture medium, is important from two perspectives. One of its applications in future researches as a producer of ureI product and use as a candidate for a recombinant peptide vaccine, and on the other hand, can be used directly as a vaccine in laboratory animals.

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