Original Article

Cloning tagD gene from helicobacter pylori in PFLAG-CMV-3 eukaryotic vector to generate a DNA vaccine

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Abstract

Introduction:
Helicobacter pylori is strongly associated with gastritis, stomach cancer, gastric lymphoma and peptic ulcer in human. Thiol peroxidase is encoded by tagD gene and plays a significant role in colonizing H. pylori in the stomach. The product of tagD gene stimulates the immune system in the host. This study aimed to isolate and clone tagD gene in the eukaryotic expression vector PFLAG-CMV-3 as a DNA vaccine candidate.

Materials and methods:
In this experimental research, tagD gene (537 bp) was amplified by PCR. The PCR products were cloned using cloning commercial kits (Thermo Fisher Co., COUNTRY) in pTZ vector. This gene was subcloned in the eukaryotic expression vector (PFLAG-CMV-3 vector), then transferred into CHO cells by electroporation method and was expressed.

Results:
The results indicate that amplification and cloning of tagD gene was successful, and the pTZ-tagD vector was formed. PFLAG-CMV-3 vector construction was confirmed by digestion and gene sequencing. The 19 kDa band was observed by gene expression analysis on SDS-PAGE.

Conclusions:
tagD gene in the PFLAG-CMV-3-tagD recombinant vector has the ability to produce specific protein in CHO cells. Therefore, this gene construct is useful to evaluate the immunogenicity as a DNA vaccine against H. pylori infection in animal models.

Keywords: Helicobacter pylori, tagD, cloning, electroporation

Introduction

Helicobacter pylori (H. Pylori) is a gram-negative, motile, helical and microaerophilic bacterium found on the stomach epithelial cells. The bacterium is the main cause of chronic active gastritis, and stomach and duodenal ulcers. It also causes inflammation in the mucous lining of the stomach by neutrophils, lymphocytes, and plasma cells. Since this bacterium grows in and colonizes deep layers of mucus overlying gastric epithelial cells, it does not have specific endoscopic findings and therefore its clinical diagnosis is difficult (1, 2). Studies have approved the role of H. Pylori in malignancy and gastric cancer and thus the International

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Agency for Research on Cancer (IARC) have classified the bacterium in the carcinogenic agents class I (3). There is a significant difference between the prevalence of the infection in Western countries and developing countries. The prevalence of H. Pylori seropositivity in developing countries is 90% of the population, while in developed countries, excluding Japan, it is below 40% (4). This cancer was responsible for 65,000 deaths worldwide in 2000. It is the fourth most common malignancy in the world and accounts for 10% of all cancer deaths annually. H. Pylori infection is common in Iran with a prevalence of 60%-90%. Gastric cancer has a high prevalence rate, too. This suggests that Iran is a high risk area for H. Pylori infection (5, 6). A study reported the prevalence of infection with H. Pylori in Ardabil Province 89.5%, which is the highest rate in Iran (7).

The tagD gene in the H. Pylori encodes thiol peroxidase (HpTpx) that is a polypeptide of 166 amino acids (8). Thiol peroxidase is one of the important adhesins of H. Pylori playing a critical role in its colonization in gastric mucosa (8, 9). The gene also plays an important role in activating H. Pylori to survive in oxidative stress conditions of the stomach. Thiol peroxidase is a family member of Peroxiredoxin (PRX) proteins and one of the most abundant antioxidant enzymes in H. Pylori. Although many studies have been conducted on H. Pylori, limited information is available on the biochemical properties of HpTpx. HpTpx acts as an H_2O_2 scavenger in the presence of Thioredoxin (Trx). Microorganisms that HpTpx has become ineffective in them have a reduced ability in gastric colonization of the host mice and also are more sensitive to destruction by peroxide and superoxide compared to the wild-type bacteria. HpTpx also plays a critical role in oxidative stress resistance and stimulates the immune system in the host body. Recent studies showed widespread resistance to antibiotic treatment of H. Pylori infection (9). Hence the need for an alternative treatment or prevention (such as a DNA vaccine) arises. DNA vaccines include direct injection of a specific plasmid (DNA) containing the encoding gene of the antigen which causes gene expression in cells and presents it to the immune system due to its specific promoters isolated from human viruses (such as cytomegalovirus). Hence a recombinant protein which is necessary to stimulate the immune system is produced endogenously rather than exogenously (e.g., hepatitis b recombinant vaccine). The induced protein stimulates the cellular and humoral immune system at different stages with its natural form (10).

The present study was conducted to construct a recombinant vector carrying the tagD gene and its expression in animal cells as a candidate for the design of a DNA vaccine against H. Pylori.

Materials and Methods

Bacterial strains, vectors, and animal cells

In this experimental study, the standard strain of H. Pylori (ATCC 43504) used to extract tagD gene, and E. coli strain Top10F strain used for transformation and amplification of recombinant gene were procured from Shahrekord Azad University Research Center. The PTZ T/A cloning vector was used for the cloning process and eukaryotic expression vector PFLAG-CMV-3 was used for subcloning. Chinese Hamster Ovary (CHO) animal

cells were used as the host cell for transformation and gene expression.

DNA extraction and tagD gene amplification

DNA extraction was performed using Cinnagen kit, according to manufacturer's instructions. The quantity and quality of extracted DNA were verified by 1% agarose gel electrophoresis and Nanodrop spectrophotometer (ND2000 Thermo).

The tagD gene sequence of H. Pylori (access number: AF021091) was procured from National Center for Biotechnology Information (NCBI) bank to design the primer. The sequence of used primers is presented in Table 1.

Table 1: Primers used in the different stages

Product Size	Primer sequences (3' to 5')	Primer	Gene
537 bp	AGCAGATCTATTAGAAAGGATTTAACCATGC	tagD-F	tagD
	TCCGATATCTAACTTCCTATTCCAACAC	tagD-R	

The PCR was performed with a final volume of 5.2 µl including 2.5 µl template DNA, 0.2 µmol of each primer, 200 µmol of dNTP 10 mM, 1.5 µmol of 50 mM MgCl2, 2.5 µl of PCR (10X) buffer, and 2.5 units of Taq DNA polymerase. PCR was performed in a thermocycler (Germany) with the initial denaturing 5 minutes at 95 °C, followed by 32 cycles of denaturation for 1 minute at 95 °C, bonding for 1 minute at 62 °C, elongation for 1 minute at 72 °C and the final elongation for 5 minutes at 72 °C. Final PCR product was electrophoresed in the agarose gel containing ethidium bromide, and assessed by ultraviolet radiation. The PCR product of gene was purified from the agarose gel using purification kit (Bioneer, South Korea) according to manufacturer's instructions.

T/A cloning

The PCR products purified from the gel were cloned using a PTZ T/A cloning kit (Fermentas, Germany) transformed into E. coli bacteria TOP10 F strain. Then, the above-mentioned bacteria were cultured in the LB Agar medium containing antibiotic ampicillin (100 µg per ml). in the next step, the accuracy of cloning was verified by the PCR method and plasmid purification was performed on the resulting colonies (using kits made by Bioneer, South Korea). Enzymatic digestion was used for final approval of the resulting structure (PTZ-tagD). PTZ vector used in this study was designed such that it had BglII and EcoRV restriction enzyme site on both sides of the cloned gene.

Cloning of the gene in expression vector

A tagD gene fragment was isolated from PTZ vector by enzymatic digestion with BglII and EcoRV enzymes and inserted into the PFLAG-CMV-3 expression vector. The incorporation reaction between the PFLAG-CMV-3 vector and tagD fragment was performed using T4 DNA ligase and the PFLAG-CMV-3-tagD structure was made. The resulting vectors were inserted into E. coli host cells in a

medium containing the neomycin via transformation. The accuracy of subcloning was approved through the PCR method enzymatic digestion and finally, sequencing was performed by Gen Ray Company.

Transferring final organism PFLAG-CMV-3-tagD to animal cells

In this study, CHO cells were used for tagD gene expression in animal cells and electroporation was used for their transformation. An electroporation device (Gene Pulser Xcell Construction, Bio Rad, USA) was used. A total of 106×2 CHO cells was counted (400 µl) and transferred into electroporation 0.4 cuvette along with 800 ng/µl of the PFLAG-CMV-3-tagD recombinant vector. Optimized electric pulses of 0.174 kV and 400 µF were applied to the cells and the resulting cells were cultured in the presence of neomycin (50 µg/ml).

SDS-PAGE performance

The molecular weight and the location of expressed proteins in CHO eukaryotic cells were approved by the Sodium Dodecyl Sulphate PolyacrylAmide Gel Electrophoresis (SDS-PAGE) test. The transformed CHO cells were precipitated for 3 minutes at 3000 rpm. An amount of 100 µl of PBS was added to cell deposition and the resulting cells were electrophoresed on 12% SDS-PAGE gel and stained using Coomassie Blue.

Results

Proliferation and isolation of tagD gene of H. Pylori

Electrophoresis of purified DNA on 1% agarose gel indicated the good quality of DNA. The concentration of extracted DNA was 300 ng/ml which verified the quality of the extracted DNA. The PCR product was consistent with the intended fragment size of 537 bp (Figure 1).

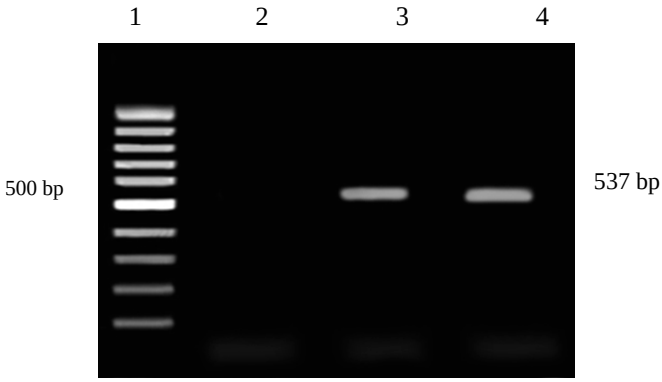

Figure 1: The PCR product of tagD gene with a size of 537 bp

Cloning T/A and subcloning

PCR product was cloned using T/A cloning method in PTZ vector and then transformed into E. coli TOP10F. PCR test and enzymatic digestion were used to verify the accuracy of cloning the gene segments. PCR showed that the resulted clones contained the mentioned gene structures. After extraction of the plasmid in the presence of ampicillin and enzymatic digestion using BglII and EcoRV enzymes, the presence of the gene fragment in the vector PTZ was verified, in that the enzymatic digestion resulted in two fragments of 2886 bp and 537 bp

related to the vector and the tagD gene, respectively.

After the enzymatic digestion of PTZ vector in the previous stage, the desired gene fragment was extracted from the agarose gel and successfully ligated in the carrier PFLAG-CMV-3. The accuracy of subcloning in the first stage was verified by PCR and then using enzymatic digestion (with BglII and EcoRV enzymes), in that vector digestion of PFLAG-CMV-3-tagD with the two enzymes resulted in the formation of two fragments, one with a size of 6331 bp related to the vector PFLAG-CMV-3, and another with a size of 537 bp related to the tagD gene which indicated the formation of the final organism (Figure 2).

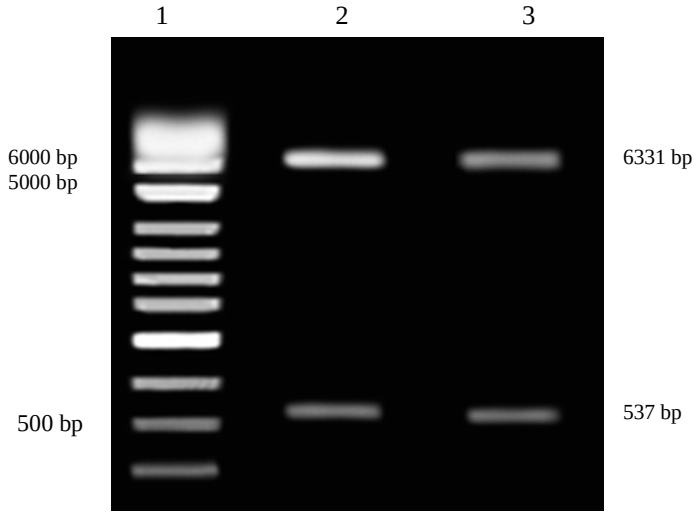

Figure 2: Enzymatic digestion of final organism PFLAG-CMV-3-tagD with BglII and EcoRV enzymes. The band of 6331 bp is related to vector PFLAG-CMV-3 and the band of 537 bp is related to the tagD gene.

Gene expression and electroporation

The electroporation of the final organism PFLAG-CMV-3-tagD was performed in CHO cells and its accuracy was verified by growth in the presence of neomycin antibiotic and PCR. SDS-PAGE reaction was used to examine the gene expression of tagD in these cells. The protein product of the tagD gene weighing 19 kDa indicated the expression of the protein in CHO eukaryotic cells.

Discussion

In this study, the tagD gene of H. Pylori was isolated using PCR method, proliferated, cloned in the pTZ vector, created in the pTZ-tagD recombinant vector, and verified. The recombinant vector can be used as a reservoir of tagD gene for later research, or presented to other researchers. Then the final organism PFLAG-CMV-3-tagD was created by transferring the gene from vector pTZ-tagD to eukaryotic plasmid PFLAG-CMV-3. Since the expression of the eukaryotic gene was observed in animal cells, the new recombinant vector appears to be useful for research such as testing DNA vaccine or peptide vaccine production against H. Pylori which exists in most human societies, yet has no vaccines. The gastric mucosal barrier is made of several layers called the pre-epithelial (mucosa), epithelial and post-epithelial (fibroblasts and immune cells) layers. Any disruption in this area leads to a variety of clinical problems including gastritis or even cancer. The bacterial agent needs to

penetrate this barrier in order to develop a disease. Among pathogenic bacteria, H. Pylori plays a crucial role in the development of inflammation, ulcers, and adenocarcinoma, which is attributed to different mechanisms for evading the barrier and the host response (11). H. Pylori infection is one of the most common bacterial infections in human societies and studies show that more than 50 percent of the world population are infected (12, 13). In 10%-15% of cases, the infection can lead to severe inflammation, peptic ulcer, lymphoma or gastric cancer (11). Studies show that people that host the bacteria are 2.26 times at higher risk of gastric cancer than others (14). H. Pylori is one of the most genetically variable microorganisms among the bacteria. Several studies have been conducted to determine the bacterial pathogens for screening tests. Mutations in the genome of the bacteria impede its detection and its chronic presence provides the ground for cancer (15). Because it is difficult to diagnose the disease at early stages and that H. Pylori is resistant to antibiotics and has a high prevalence, specific modern treatments such as a proprietary vaccine are needed. A valuable method proposed by immunologists is the administration of the third generation vaccines, that is, a plasmid containing the encoding gene of the antigen (10, 16). Highly specific and immunogenic H. Pylori antigens are used due to the increased specificity, prevention of the degradation of normal flora of the digestive system and reduction of the side effects (17) among which the tagD gene is potentially suitable for the production of specific antibodies. The tagD gene that encodes the protein thiol peroxidase in H. Pylori (HpTpx), is one of the most important adhesins in H. Pylori and an antioxidant that empowers H. Pylori to survive under oxidative stress of the stomach and has a critical role in colonization of the bacteria in gastric mucosa (9, 18). Despite extensive studies on H. Pylori, no specific study has yet been conducted on the tagD gene, while similar studies have been conducted on other pathogenic genes. Iranian researchers conducted a study in 2009 on H. Pylori hpaA gene. In that study, the hpaA gene was cloned inside the prokaryotic expression vector pET28a, transformed into E.coli Bl21De3 and the expression of the HpaA recombinant proteins was verified by SDS-PAGE reaction inside the host (19). In a similar study in 2011, the pathogenic gene ureB122 of H. Pylori was cloned into the pET32a vector and transformed into E. coli Bl21De3 bacterial cells. The amount of the protein expression was assessed by the SDS-PAGE reaction and after approval, it was introduced as a vaccine candidate (20). Also in 2013, Gonzalez et al., isolated cagA gene of H. Pylori and cloned it into a prokaryotic expression vector. The expression of 80 kDa protein of CagA verified its accuracy (21). In 2014, ureA gene of H. Pylori was cloned into pTZ57R/T vector by Iranian researchers, transformed into the DH5 α host, and after verification, subcloned into the expression vector and the expression of the corresponding protein was examined. The high purity of expressed protein was verified and a protein-based vaccine against this bacterium was proposed (22). In all of the above, the coding genes of H. Pylori antigens were used to clone the gene. The present study also considered one of the most important H. Pylori antigens, but its difference was in the selected eukaryotic vector to create the

final organism of tagD gene, which was not employed in any of the above-mentioned studies. We aimed to produce a protein from some bacterial genes to produce a peptide vaccine. The final produced and verified organism in this study had a dual potential for either the production of recombinant proteins or direct application in laboratory animals as a DNA vaccine. If the recombinant vector is injected into the muscles of laboratory animals, it might face the common limitations of all DNA vaccines, that is, insufficient absorption of the injected DNA into the target cells. However, the use of nanoparticles or tissue electroporation has increased the absorption of injected DNA vaccines.

Conclusion

Since the diagnosis of gastric cancer at an early stage is difficult and in most cases it is diagnosed at advanced stages, which make the treatment difficult, the main way to control this cancer, like gastritis and gastric ulcers, is to control H. Pylori. It is difficult to fight H. Pylori; therefore, researchers are inclined to use innovative genetic engineering techniques to control this infection including the production of a DNA vaccine against H. Pylori. Creating a gene organism using tagD gene of H. Pylori is a good candidate for producing a DNA vaccine against this bacterium. However, there is a long way to achieve an optimal vaccine.

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Conflict of interest

There is no conflict of interest.

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