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Original Article

Molecular identification of gene of the *Helicobacter pylori* neutrophil activating protein effective in controlling allergic asthma by PCR method

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ABSTRACT

Helicobacter pylori neutrophil activating protein (HP-NAP) is one of the important proteins of *Helicobacter pylori* (*H. pylori*). HP-NAP has immunomodulatory properties and directs allergic responses from Th2 to Th1. The aim of this study is the molecular identification of the HP-NAP gene native to Iran. The native strain of *H. pylori* was purchased from the Pasteur Institute. A specific urease test was performed, along with gram staining and microscopic observation. To identify HP-NAP in native bacteria, the PCR method was used. Finally, the PCR product was electrophoresed to detect the HP-NAP gene. After bacterial staining and microscopic observation, bent gram-negative bacteria were observed. The urease test showed a change in the urea reagent (phenol red), indicating *H. pylori*. The PCR test and electrophoresis revealed a 260 bp band related to the HP-NAP gene, compared with ladder 100. According to the study conducted and the confirmation of the presence of this protein in the structure of native bacteria, as well as previous studies in different regions of the world, observing the effects of this protein as an immunomodulator of the immune system, we can hope that in the future this protein will be used as a potential means to control and treat allergic diseases, especially allergic asthma.

شناسایی مولکولی ژن پروتئین فعال کننده نوتروفیل *هلیکوباکتر پیلوری* موثر در کنترل آسم آلرژیک به روش PCR

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چکیده

پروتئین فعال کننده نوتروفیل *هلیکوباکتر پیلوری* (HP-NAP) یکی از پروتئین های مهم *هلیکوباکتر پیلوری* (*H. pylori*) است.HP-NAP دارای خواص تعدیل کننده ایمنی است و پاسخ های آلرژیک را از Th2 به Th1 هدایت می کند. هدف از این مطالعه شناسایی مولکولی ژن HP-NAP بومی ایران است .سویه بومی *هلیکوباکتر پیلوری* از انستیتو پاستور خریداری شد. آزمایش اوره آز اختصاصی، رنگ‌آمیزی گرم و مشاهده میکروسکوپی انجام شد. برای شناسایی HP-NAP در باکتری های بومی از روش PCR استفاده شد. در نهایت، محصول PCR برای تشخیص ژن HP-NAP الکتروفورز شد. پس از رنگ‌آمیزی باکتریایی و نیز مشاهده میکروسکوپی، باکتری‌های گرم منفی خمیده مشاهده شدند. آزمایش اوره آز تغییر در معرف اوره (فنل قرمز) را نشان داد که نشان دهنده *هلیکوباکتر پیلوری* است. آزمایش PCR و الکتروفورز یک باند ۲۶۰ جفت باز مربوط به ژن HP-NAP را در مقایسه با نردبان (لدر) ۱۰۰ نشان داد. با توجه به مطالعه انجام شده و تایید وجود این پروتئین در ساختار باکتری های بومی و همچنین مطالعات قبلی در در مناطق مختلف جهان، با مشاهده اثرات این پروتئین به عنوان تعدیل کننده ایمنی سیستم ایمنی، می توان امیدوار بود که در آینده از این پروتئین به عنوان وسیله ای بالقوه برای کنترل و درمان بیماری های آلرژیک به ویژه آسم آلرژیک استفاده شود.

واژه های کلیدی: *هلیکوباکتر پیلوری*، آسم آلرژیک، پروتئین فعال کننده نوتروفیل

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INTRODUCTION

H. pylori is a spiral-shaped, microaerophilic gram-negative bacterium that has evolved with humans for approximately 50,000 years and can be considered an important risk to human health [1-3]. This bacterium infects the stomach of about 45% of the world's population and can cause stomach ulcers, gastric lymphoma, and stomach cancer [4-7]. *H. pylori* infection in children can also lead to iron and vitamin B12 deficiency. According to findings, the infection rate of *H. pylori* is higher in developing countries than in developed countries, likely due to differences in health standards [8-10]. Additionally, findings suggest that H. pylori infection may be related to the development of bronchitis and lung cancer; however, the direct effect of *H. pylori* on respiratory diseases has not been fully determined to date [10-12]. HP-NAP is an important protein of H. pylori, released during bacterial growth, with some remaining attached to the outer membrane. This protein is considered an immunomodulator. If the neutrophil activating factor can activate and increase the efficiency of macrophages, it can be considered a key factor for infection prevention and treatment [13]. This protein can strongly stimulate neutrophils, monocytes, and dendritic cells, increase the production of IFN-γ, and decrease IL-4, thereby directing the Th2 immune response to the Th1 response. Therefore, it is possible that HP-NAP can reduce the incidence of allergic asthma in people infected with *Helicobacter pylori* [14-17]. Allergic asthma is a chronic inflammatory disease of the bronchi characterized by cough, shortness of breath, and wheezing. It is caused by an excessive Th2 response to environmental allergens. In allergic asthma, Th2 cytokines cause the production and release of cytokines (IL-4, IL-5, and IL-13) and stimulate B cells to produce IgE, resulting in eosinophilic inflammation in the bronchi. On the other hand, they inhibit the secretion of Th1-related cytokines (IL-12, TNF-α, and INF-γ), leading to asthma attacks when atopy symptoms are observed [18-22]. Recognizing, controlling, preventing, and treating asthma is crucial. An asthma attack results from the immune system's reaction against allergens. Some infectious diseases can affect the development of allergic disorders [23]. Certain bacteria can direct allergic responses from Th2 to Th1 by producing IFN-γ and IL-12 [24, 25]. Therefore, using some bacteria or their components that induce a Th1 response can prevent the development of allergic asthma [26]. In allergic asthma patients, stimulation of a Th1-mediated immune response can suppress Th2 responses. If the Th1 response is insufficient, the Th2 response is elevated, ultimately leading to allergic asthma in the bronchi [19]. Microbial products can help maintain the balance between Th1 and Th2 responses. Therefore, bacteria are effective in controlling and treating asthma and can be used as a potential therapeutic approach in managing allergic asthma [9, 19, 27]. Recent studies have shown an association between the presence of *H. pylori* containing HP-NAP and a reduced risk of asthma [28-30]. The aim of this study is to identify the molecular gene of HP-NAP effective in controlling allergic asthma using the PCR method. The native bacteria of Iran were prepared, and molecular tests were performed to confirm the presence of this peptide in native *H. pylori*. If the presence of this peptide is proven, it can be extracted for further research and clinical research as a potential therapeutic strategy and as a new tool for the prevention and treatment of allergic asthma.

MATERIALS AND METHODS

Preparation of bacteria

Since this study was not conducted on native bacteria, the native strain of Helicobacter pylori was ordered and purchased from the Pasteur Institute of Iran. After preparation, the bacteria were transferred to the laboratory for further studies using an anaerobic jar and gas pack type C.

Diagnostic tests

First, warm staining was used to observe and identify the bacteria under the microscope. To confirm, the bacteria were cultured in urea agar medium to observe the fermentation of urea by the urease enzyme of this bacterium, resulting in a color change from yellow to pink.

Identification of HP-NAP by PCR method

To determine HP-NAP in indigenous bacteria, the PCR method was employed. The genome sequence of H. pylori containing the HP-NAP gene is available under registration number AF227081.1 (from the World Gene Bank in the NCBI database), and primer design was done using Gene Runner software. The sequences of the designed primers are as follows:

Forward primer $^{5'}$GTTTGCGGACATGTTTGACG$^{3'}$

Reverse primer $^{5'}$GCAACTTGGCCAATTGATCG$^{3'}$

These primers were received from Pishgam after ordering. DNA extraction was performed first to conduct the PCR test. A sufficient amount of the bacterial colony was removed using a sterile swab and placed in a microtube containing sterile distilled water with a comparative turbidity of half McFarland. DNA extraction was done using the boiling method, and the success of the genome extraction process was checked using a NanoDrop device made by ABI, USA, with an appropriate DNA concentration measured at 700 nanograms. Following this, DNA was extracted to determine the HP-NAP gene using a thermocycler made by Gradient Analytica Gina. The PCR reaction was carried out in a final volume of 25 microliters containing 2.5 microliters of Master Mix 2x (Pishgam, Tehran, Iran) and 1 microliter of a solution containing 1 microliter primer (10 picograms) and 1.4 ng DNA template. The temperature program included an initial temperature of 95°C for 5 minutes, followed by 33 repeated steps including denaturation at 94°C for one minute, annealing at 58°C for one minute, and extension at 72°C for 30 seconds, concluding with a final elongation step at 72°C for 10 minutes.

PCR product Electrophoresis

The PCR product was electrophoresed to detect the HP-NAP gene using horizontal electrophoresis with one percent agarose gel. The structure of these gels is porous, with pore diameters of about 100 to 500 nm, allowing molecules to be separated by size. First, a one percent agarose gel was prepared by dissolving one gram of agarose powder in 100 ml of TBE (Tris/Borate/EDTA) buffer, which turned into a clear and uniform liquid after heating. The electrophoresis tank was filled with the same buffer used to prepare the gel. After preparing the gel and creating suitable wells with a special comb, 2 microliters of the PCR product were added to the gel. After connecting the electrodes, the device was turned on. Since DNA molecules have a negative charge, they move towards the positive electrode when placed on the gel and under an electric current. The separation of DNA molecules is based solely on their size. Short DNA strands move faster than longer molecules, facilitating the separation of DNA molecules of different sizes. Tracking of samples was done by adding

fluorescent dyes in the gel well. Safe stain (Pishgam, Tehran, Iran) was used for this purpose. After electrophoresis and cutting off the electric current, a DNA marker or ladder was used as a standard to measure the DNA band resulting from PCR. The band at 260 bp was related to HP-NAP. The size of the band obtained from the PCR product on the gel was determined using the 100-2000 bp ladder (Pishgam, Tehran, Iran), and the result was observed using a gel documentation machine. A picture was taken to search for the desired size.

RESULTS

Initially, a small amount from the plate containing the desired bacteria was transferred to a microscope slide for staining and observation of gram-negative bacteria, as seen in Figure 1. After staining and confirming the absence of contamination with other bacteria, a specific urease test was conducted inside the test tube. These bacteria convert urea in the culture medium into ammonia, carbon dioxide, and water. Ammonia resulting from the decomposition of urea causes alkalinity, indicated by the color change of the urea reagent (phenol red) from yellow to light pink, as shown in Figure 2.

Molecular test

The main goal of this research is to determine the HP-NAP gene using the PCR technique. After performing this technique and electrophoresis of the PCR product, the image obtained from the gel documentation machine was analyzed. Considering that the primer design indicated a 260 bp band related to the HP-NAP gene, an appropriate laser was used to detect DNA bands. Consequently, a 100 to 2000 bp ladder, capable of detecting 100 bp DNA fragments, was used. In the figure below, the 260 bp band related to the HP-NAP gene is clearly visible compared to the 200 to 300 bp ladder band. As a result, this protein was also detected in the native bacteria of Iran for further studies (Figure 3).

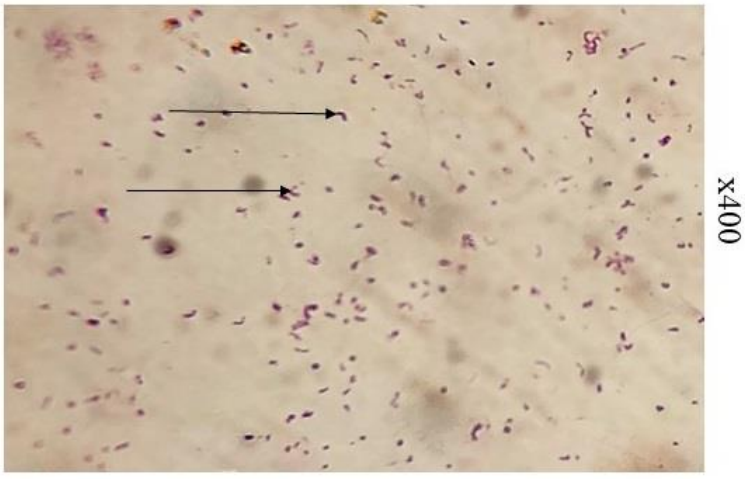

Figure 1: Gram staining of bacteria and observation under the microscope with x400 magnification.

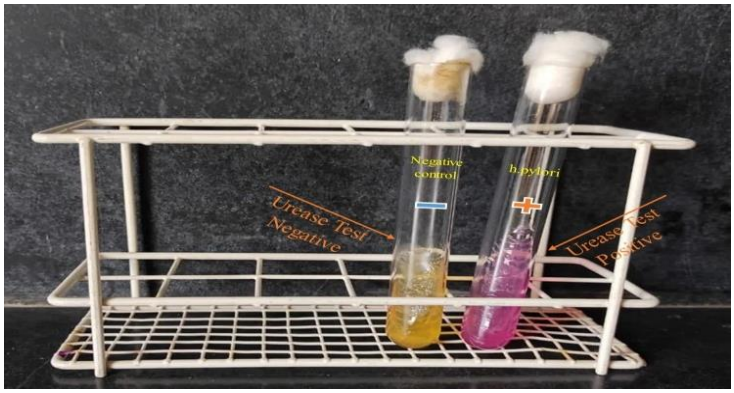

Figure 2: Urease test: the left tube of the negative sample and the right tube of the positive sample (*H.pylori*).

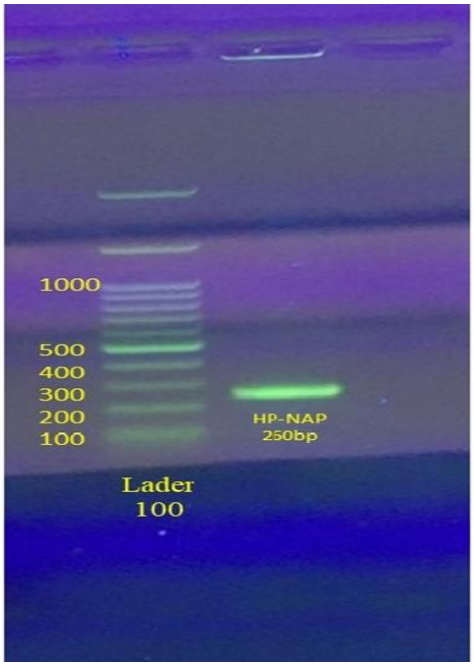

Figure 3: The result of HP-NAP electrophoresis on one percent agarose gel.

DISCUSSION

Helicobacter pylori, an ancient bacterium, often settles in the human stomach and adheres to its cells. *Helicobacter pylori* is likely part of the human stomach's microflora. With improvements in lifestyle and health conditions, and the eradication of *Helicobacter pylori*, its prevalence has decreased drastically in developed countries, while the incidence of asthma and allergic diseases has increased significantly [31, 32]. To confirm the "disappearing microbiota" theory, Blaser states that the loss of certain bacterial species from the old microbiota can change the immune system and its cognitive functions, which should develop early in life. This change can lead to increased sensitivity to some diseases. The loss of these microbiota, observed worldwide, can lead to the emergence of modern diseases. These findings encourage us to understand the reasons for the loss of microbiota [5, 33]. Although eradicating this bacterium reduces the incidence of indigestion, stomach ulcers, and stomach malignancies, there are concerns regarding its complete eradication, including resistance to foreign microbes and increased prevalence of disorders inversely related to *H. pylori* infection, such as obesity, asthma, GERD, and Barrett's esophagus [34, 35]. Completely eradicating this bacterium has

advantages and disadvantages. Therefore, identifying and removing only malignant strains of Helicobacter pylori is crucial for treatment and eradication [36]. Epidemiological studies and experiments show that exposure to *Helicobacter pylori*, especially in childhood, can prevent allergic asthma [37]. However, several recent in vivo studies have shown that live bacteria do not play a role in inducing this protective role. Administration of *Helicobacter pylori* extract in newborns was able to prevent airway inflammation and goblet cell metaplasia. Injection of *Helicobacter pylori* extract can inhibit DCs involved in allergen processing in the mediastinal lymph nodes of the lungs. These results show that *Helicobacter pylori* extract can effectively prevent allergic airway disorders after sensitization [38]. *H. pylori* targets DCs and relies on highly suppressed T-regs. Since HP-NAP is considered a possible regulator of T-regs and can inhibit allergic inflammation of asthma, HP-NAP could be developed as an efficient *H. pylori*-specific vaccine for the treatment of allergic asthma [39-41]. Van Wyk et al. have shown that *Helicobacter pylori* extract can effectively reduce mucus production and multiple features of inflammation in mice re-challenged by house dust mites [42]. Maternal exposure to *H. pylori* can reduce allergic airway inflammation in offspring via T-regs and provide new insights for interventional asthma treatment [43]. *Helicobacter pylori* and humans have evolved together for about 50,000 years. This bacterium is the main cause of gastric ulcer, gastric lymphoma, and gastric adenocarcinoma. The complete disappearance of this bacterium seems to have consequences for humans, as there is now much information about the advantages and disadvantages of this change on digestive system health or disease. However, there is substantial evidence about its extra-intestinal effects, including on allergic asthma. Similar to the inverse association between H. pylori and childhood asthma, *H. pylori* neutrophil-activating protein (HP-NAP) has the ability to shift allergen-specific T cell responses in vitro from Th-2 responses to Th-1 responses. Additionally, HP-NAP in mice led to the inhibition of bronchial inflammation in line with Th-2 responses of allergic asthma [37].

CONCLUSION

Overall theories support the view that the increased prevalence and severity of asthma and allergy in Western countries may be related, at least in part, to the reduction of *H. pylori* infection, which can cause long-term induction of a Th1 background and lead to the prevention and treatment of asthma and allergic diseases. In this research, we identified the gene of this protein in native bacteria by molecular PCR method, and there is hope that by extracting and purifying this protein, it will be used in clinical cases of allergic asthma. If using this peptide has positive effects in controlling and preventing allergic asthma, the possibility of using it as medicine or a vaccine for target patients will be initiated.

ETHICS

Approved.

CONFLICT OF INTEREST

There is no conflict of interest.

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