Construction of prokaryotic expression system of 2 148-bp fragment from cagA gene and detection of cagA gene, CagA protein in Helicobacter pylori isolates and its antibody in sera of patients

Jie Yan, Yuan Wang, Shi-He Shao, Ya-Fei Mao, Hua-Wen Li, Yi-Hui Luo

AIM: To construct a prokaryotic expression system of a Helicobacter pylori (H pylori) cagA gene fragment and establish enzyme-linked immunosorbent assays (ELISA) for detecting CagA and its antibody, so as to understand the manner in which the infection of CagA-expressing H pylori (CagA+ H pylori) isolates causes disease.

METHODS: H pylori strains in gastric biopsy specimens from 156 patients with positive results in rapid urease test were isolated. PCR was used to detect the frequency of cagA gene in the 109 H pylori isolates and to amplify a 2 148-bp fragment (cagA1) of cagA gene from a clinical strain Y06. A prokaryotic expression system of cagA1 gene was constructed, and the expression of the target recombinant protein (rCagA1) was examined by SDS-PAGE. Western blotting and immunodiffusion assay were employed to determine the immunoreactivity and antigenicity of rCagA1, respectively. Two ELISAs were established to detect CagA expression in 109 H pylori isolates and the presence of CagA antibody in the corresponding patients’ sera, and the correlations between infection with CagA+ H pylori and gastritis as well as peptic ulcer were analyzed.

RESULTS: Of all the clinical specimens obtained, 80.8% (126/156) were found to have H pylori isolates and 97.2% of the isolates (106/109) were positive for cagA gene. In comparison with the reported data, the cloned cagA1 fragment possessed 94.83% and 93.30% homologies with the nucleotide and putative amino acid sequences, respectively. The output of rCagA1 produced by the constructed recombinant prokaryotic expression system was approximately 30% of the total bacterial protein. rCagA1 was able to bind to the commercial antibody against the whole-cells of H pylori and to induce the immunized rabbits to produce antibody with an immunodiffusion titer of 1:4. A proportion as high as 92.6% of the H pylori isolates (101/109) expressed CagA and 88.1% of the patients’ serum samples (96/109) were CagA antibody-positive. The percentage of CagA+ H pylori strains (97.9%) isolated from the biopsy specimens of peptic ulcer appeared to be higher than that from gastritis (88.5%), but the difference was not statistically significant (χ²=3.48, P>0.05).

CONCLUSION: rCagA1 produced by the prokaryotic expression system constructed in this study possesses good immunoreactivity and antigenicity, and the established ELISAs can be used to detect CagA of H pylori and its antibody. H pylori isolates show high frequencies of cagA gene and CagA expression, but the infections by CagA+ H pylori strains are not the most decisive factors to cause gastric diseases.

INTRODUCTION

In China, gastritis and peptic ulcer are the most prevalent gastric diseases, and gastric cancer remains one of the most devastating malignant tumors with the highest morbidity[1-20]. Helicobacter pylori (H pylori) has been recognized as a human-specific gastric pathogen that colonizes in the stomach of at least half of the world’s populations[21-32]. Most infected individuals are asymptomatic, whereas in some cases, the infection causes acute, chronic gastritis or peptic ulceration, and plays an important role in the development of peptic ulcer and gastric adenocarcinoma, mucosa-associated lymphoid tissue (MALT) lymphoma and primary gastric non-Hodgkin’s lymphoma[21-32].

So far, no evidence for the toxicity of the protein (CagA) expressed by cytotoxin-associated gene A (cagA) of H pylori has been presented[22-30]. However, previous studies demonstrated that CagA was closely associated with the pathogenicity of H pylori and severity of H pylori-related diseases[26-29]. Many epidemiological data indicated that the positive rate of cagA gene was significantly higher in the H pylori strains isolated from patients with peptic ulcer than in those with gastritis[33]. Patients infected with cagA+ H pylori had a higher risk of developing gastric cancers than those infected with cagA- strains[34-35]. Approximately 60% to 70% of H pylori strains isolated from European and North American populations carried cagA gene[36-38], whereas over 90% of the isolates from Asia-Pacific populations were cagA gene-positive[39-42]. Strong antigenicity of CagA usually induces antibody in patients with cagA+ H pylori infection and this antibody has been considered as a possible specific clinical indicator of H pylori infection[43-45]. However, the data are scarce concerning the correlations between the presence of cagA, CagA expression and antibody production, CagA+ H pylori infection and types.
of the resultant gastric diseases.

In the present study, a recombinant expression plasmid containing a relatively conserved H pylori cagA gene fragment 2 148 bp in length (cagA1) was constructed. H pylori strains in gastric biopsy specimens from patients with gastritis or peptic ulcer were isolated. The frequencies of cagA gene and expressions of H pylori isolates and CagA antibody in patients’ sera were investigated. Furthermore, the correlations among CagA- H pylori infection and types of the resulted gastric diseases were also analyzed for the purpose of understanding the pathogenic effect of CagA and the potential of CagA antibody detection in clinical diagnosis of H pylori infection.

MATERIALS AND METHODS

Materials
A typical H pylori strain named Y06 isolated clinically was used to amplify cagA1 fragment. Primers for PCR amplification were synthesized by BioAsia (Shanghai, China). Taq-plus high fidelity PCR kit and restriction endonucleases were purchased from TaKaRa (Dalian, China). T-A cloning kit and sequencing service were provided by BBST (Shanghai, China). Plasmid pET32a as the expression vector and E.coli BL21DE3 as the host cell were purchased from Novagen (Novagen, Madison, USA). Rabbit anti-serum against the whole cell of H pylori, HRP-labeling sheep anti-serum against rabbit IgG and human IgG host cell were purchased from Novagen (Novagen, Madison, USA). Rabbit anti-serum against the whole cell of H pylori were synthesized by BioAsia (Shanghai, China). Taq-plus polymerase, 100 ng DNA template and 1×PCR buffer (pH 8.3). The parameters for PCR were at 94 °C for 5 min, x1; at 94 °C for 30 s, at 50 °C for 30 s, at 72 °C for 120 s, x10; at 94 °C for 30 s, at 50 °C for 30 s, at 72 °C for 130 s (additional 10 s for each of the following cycles), x20; at 72 °C for 10 min, x1.

To increase the positive detection rate of cagA gene, two sets of primers derived from different regions of cagA gene were applied in PCR. The sequences of F1/B1 primers were 5'-GATAACAGGCAAGCTTTGAGG-3' (sense), 5'-CTGCAAAGATTTTGGCCAGA-3' (antisense)[46]. The sequences of D08/R08 primers were 5'-ATAATGGCTAAT TAGACAACCTTGAGCC-3' (sense), 5'-TTAGAATATCACA AAAACATCAGGCA-3' (antisense)[46]. Except for the primers and DNA templates, all the other reagents and reaction volumes used in PCR for cagA detection were the same as cagA1 amplification. The parameters for the two PCRs were at 94 °C for 5 min, x1; at 94 °C for 30 s, at 55 °C for 1 min, at 72 °C for 90 s, x35; at 72 °C for 7 min, x1.

The results of PCR were observed under UV light after electrophoresis on 1.5% agarose gel-pre-stained with ethidium bromide. The expected sizes of cagA1 amplification fragment and the two target amplification fragments for cagA gene detection were 2 172 bp (including ATG, TAA, and a 18-bp sequence containing cagA gene) and protective nucleotide residuals, 349 bp and 298 bp, respectively. Cloning and sequencing The cagA1 amplification fragment was cloned into plasmid vector pUCm-T (pUCm-T-cagA1) by using the T-A cloning kit according to the manufacturer’s instructions. The recombinant plasmid was amplified in E.coli DH5α and then extracted by Sambrook’s method[46]. A professional company (BBST) was responsible for nucleotide sequence analysis of the inserted fragment. Two plasmids pUCm-T-cagA1 and pET32a were extracted from two different strains of E.coli DH5α after amplification in LB medium and then digested with EcoRV and XhoI, respectively[46]. The fragments cagA1 and pET32a were recovered and ligased. The recombinant expression vector pET32a-cagA1 was transformed into E.coli BL21DE3, and the expression system designated as pET32a-cagA1-E.coli BL21DE3. The cagA1 fragment inserted in pET32a was sequenced again.

Expression and identification of target recombinant protein pET32a-cagA1-E.coli BL21DE3 was rotatively cultured in LB medium at 37 °C under induction with isopropylthio-β-D-galactoside (IPTG) at different concentrations of 1.0, 0.5 and 0.1 mmol/L, respectively. The supernatant and precipitate of the culture after incubation were separated by centrifugation and then the bacterial pellets were ultrasonically fragmented (300 V, 5 x3). SDS-PAGE was used to measure the molecular mass and output of the target recombinant protein (rCagA1). Ni-NTA affinity chromatography was applied to collect rCagA1. The commercial rabbit anti-serum against whole-cell H pylori and HRP-labeling sheep anti-serum against rabbit IgG were used as the first and second antibodies to determine the immunoreactivity of rCagA1 by Western blotting, respectively. Rabbits were immunized with rCagA1 to prepare antiserum. Immunodiffusion assay was performed to determine the antigenicity of rCagA1.

Enzyme-linked immunosorbent assay (ELISA) Using rCagA1 as the coating antigen at the concentration of 20 µg/mL, with the serum sample (1:400 dilution) from a patient as the first antibody and HRP-labeling sheep antibody against human IgG (1:4 000 dilution) as the second antibody, CagA antibody in the sera of the 126 H pylori-infected patients was detected. The result of ELISA for a patient’s serum sample was
considered positive if the value of optical density at 490 nm (OD\textsubscript{490}) exceeded the mean plus 3 standard deviations of 6 different negative serum samples\textsuperscript{(47).} CagA expression in \textit{H pylori} isolates was examined using ultrasonic supernatant of each of the \textit{H pylori} isolates (50 µg/mL) as the coating antigen, self-prepared rabbit anti-CagA1 serum (1:800 dilution) as the first antibody and HRP-labeling sheep antibody against rabbit IgG (1:3 000 dilution) as the second antibody. The result of ELISA for a \textit{H pylori} ultrasonic supernatant sample was considered positive if its OD\textsubscript{490} value was over the mean plus 3 standard deviations of 6 separated \textit{E.coli} DH5α ultrasonic supernatant samples at the same protein concentration\textsuperscript{(47).}

**Analysis of correlation among cagA gene, CagA and its antibody and \textit{H pylori}-related diseases** According to the clinical data and the obtained results, the correlations among infection with \textit{H pylori} carrying cagA gene and expressing CagA isolated from the patients’ gastric biopsy specimens, and the type and severity of gastric diseases in the same patient were analyzed.

**Statistical analysis**

The nucleotide and putative amino acid sequences of the cloned cagA1 fragment were compared for homologies with the published sequences from \textit{H pylori} (GenBank accession No.: AB015416). χ\textsuperscript{2} test was applied to analyze the clinical data, PCR results for cagA detection and ELISA results for CagA detection.

**RESULTS**

**Positivity rate of \textit{H pylori} in clinical isolates**

In the 156 gastric biopsy specimens with positive urease, \textit{H pylori} was detectable in 126 specimens, with a positivity rate of 80.8%.

> Using the primer pairs F1/B1 and D008/R008 respectively, 82.6% and 78.9% of the tested \textit{H pylori} isolates (90/109) were positive for cagA gene, and the total cagA gene positivity rate was 97.2% (106/109). The target amplification products of cagA1 from \textit{H pylori} strain Y06 and two fragments for cagA gene detection from the isolates are shown in Figure 1.

**PCR results**

**Figure 1** Target amplification fragments of cagA gene amplified from \textit{H pylori} isolates with different primers. Lane 1: 100 bp DNA marker; Lanes 2, 4 and 6: Target amplification fragments by using cagA1, F1/ B1 and D008/ R008 primers, respectively; Lanes 3, 5 and 7: Blank controls.

**Nucleotide sequence analysis**

The nucleotide sequence of cagA1 fragment in pUCm-T-cagA1 and pET32α-cagA1 were completely the same. The nucleotide and putative amino acid sequences of the cloned cagA1 fragment showed 94.83% (Figure 2) and 93.30% (Figure 3) homologies with the published sequences from \textit{H pylori} strain NCTC11637 (GenBank accession No.: AB015416), respectively.
Comparison of nucleotide sequence homology of cagA fragments between different H pylori strains. (1): Corresponding nucleotide sequence of cagA fragment from H pylori strain NCTC11637. (2): Sequencing result of cagA fragment from H pylori strain Y06. "///" indicates the deletion mutation of the nucleotide residuals. Underlined is the position of the primers.
Expression of target recombinant protein

IPTG at concentrations of 1.0, 0.5 and 0.1 mmol/L could efficiently induce the expression of rCagA1, which was detected mainly in the ultrasonic precipitate with an output of approximately 30% of the total bacterial proteins (Figure 4).

Immunoreactivity and antigenicity of rCagA1

The commercial rabbit antibody against whole-cell H pylori could bind to rCagA1 as confirmed by Western blotting (Figure 5). Immunodiffusion assay demonstrated a titer of 1:4 between rCagA1 and rabbit anti-rCagA1 serum.

ELISA results

The mean \( A_{490} \) values (mean±SD) of the 6 negative serum samples was 0.37±0.03 in the detection of specific antibodies in sera of patients, and the positive reference value of 0.46 was consequently derived. According to the reference value, 88.1% (96/109) of the tested patients’ serum samples were positive for the rCagA1 antibodies with an \( A_{490} \) value ranging from 0.56 to 1.05 (Table 1). From the mean \( A_{490} \) values of the 5 negative bacterial controls (0.27±0.09) in the detection of CagA1 in H pylori isolates, the positive reference value of 0.54 was

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**Figure 4** rCagA1 expression by pET32a-cagA1-E.coli BL21DE3 induced with IPTG. Lane 1: Protein marker; Lane 2: Blank control; Lane 3: Non-induced; Lanes 4-6: Induced with 0.1, 0.5 and 1.0 mmol/ L IPTG, respectively; Lanes 7 and 8: Bacterial supernatant and precipitate induced with 0.5 mmol/ L IPTG, respectively.

**Figure 5** Western blotting of binding between rCagA1 and commercial rabbit antiserum against whole-cell H pylori. Lanes 1 and 2: 20 \( \mu \)L and 40 \( \mu \)L of rCagA1 extract, respectively; Lane 3: Blank control.
derived. According to the reference value, the epitope of rCagA1 with an A00 value ranging from 0.55% to 0.9792.6%(101/109) was detected in the tested H pylori isolates (Table 1).

### Table 1 Detection of CagA expression in H pylori isolates and CagA antibody in infected patients’ sera

| Tested indicator | Tested cases | Positive cases | Negative cases | Positivity rate (%) |
|------------------|--------------|----------------|----------------|--------------------|
| CagA gene        | 109          | 106            | 3              | 97.2               |
| CagA protein     | 109          | 101            | 8              | 92.6               |
| Anti-CagA        | 109          | 96             | 13             | 88.1               |

### DISCUSSION

CagA expressed by H pylori was demonstrated to induce cellular skeleton rearrangement and interleukin (IL)-8 secretion in gastric epithelial cells[13-32], IL-8, recognized as an inflammatory cytokine, could cause inflammation by inducing gathering of neutrophilic cells[48-49]. Infection with cagA+ H pylori may elevate the risks of atrophic gastritis, intestinal metaplasia and gastric adenocarcinoma[34,36,31], and CagA is therefore considered as the most important pathogenic factor of H pylori.

CagA gene had a single copy located at the terminal end of region I in cag pathogenic island (CPI)[31,32], and was prone to mutation, especially in the 3’-end by insertion of different numbers of repeated sequences, resulting in the great variation in its length ranging from 3 444 to 5 925 bp in different isolates[36,37]. According to the analysis of 37 cagA gene sequences from GenBank, a fragment with approximate 65 bp starting from the 5’-end of cagA gene of different H pylori isolates also exhibited frequent mutations such as replacement, insertion and deletion, etc. Therefore, a relatively conserved fragment of 2 148 bp from the 67th to 2 214th bp at 5’-end of cagA gene was selected for cloning, which was provisionally named as cagA1. In this study, homologies of the nucleotide and amino acid sequences of the cloned cagA fragment reached 94.83% and 93.30% respectively in comparison with the reported sequences in GenBank (accession NO. AB015416). High output of rCagA1, approximately 30% of the total bacterial protein, expressed by the constructed recombinant prokaryotic expression system pET32a-cagA1-E.coli BL21DE3 was confirmed by SDS-PAGE. rCagA1 could be recognized by a commercial antibody against whole-cell H pylori and was able to induce rabbit to produce high-titer antibodies, indicating that rCagA1 with good immunoreactivity and antigenicity can be used in ELISA as a qualified antigen for detecting CagA antibody and for preparing animal antisera to detect CagA.

Yang et al reported that all the cagA+ H pylori isolates were capable of expressing CagA[41]. However, we found that 5 strains of cagA+ H pylori (7.3%) failed to exhibit the expression. Considering the highly likely mutation in cagA genes from different H pylori isolates, this non-expression of CagA was probably due to sequence mutation or abnormal transcription and translation[36,37]. In the present study, 88.1% of the serum samples from the H pylori-infected patients (96/109) were positive for CagA antibody, and the positive rate was only slightly lower than those of cagA gene (97.2%) and CagA (92.6%) of the isolates, suggesting that CagA possesses strong antigenicity and usually induces detectable specific antibody in cagA+ H pylori-infected patients. However, we found in this study that 11.9% of the serum samples were negative for CagA antibody (13/109), including 3 cases (2.8%) of cagA+ H pylori infection, 5.4% of cagA+ H pylori infection and 5.4% of CagA+ H pylori infection. In addition, previously published data and our results suggest that over 90% of the H pylori isolates from Asia-Pacific areas were cagA gene-positive[39-42], whereas 60% to 70% of the H pylori isolates from European and North American areas were positive[36,38], indicating that the presence of CagA antibody could be used as a reference indicator with only a small risk of error for detecting H pylori infection in individuals from Asia-Pacific areas, but not for those from European or North American areas. It should be noted that the positive rate of cagA gene in the H pylori isolates in this study was as low as 78.9% to 82.6%, as detected using a single pair of primers F1/B1 or D008/R008, indicating that using multiple pairs of primers in PCR may increase the positive rate for cagA gene detection.

Covacci et al and Figueiredo et al reported that cagA+ H pylori infection could usually cause serious gastric diseases[17,38]. For example, 90% of H pylori isolates from peptic ulcer patients were cagA gene-positive, while only 50% to 60% of the isolates from superficial gastritis were positive. However, the reports from Asia-Pacific areas did not show a definite correlation between cagA+ H pylori infection and severity of the diseases[39-42]. Although cagA+ H pylori was isolated from chronic gastritis patients at a higher rate (97.9%) than from peptic ulcer patients (88.5%), the difference was not statistically significant (χ2=3.48, P>0.05), probably due to the high rate of cagA gene-carrying H pylori (97.2%) and a relative small population tested in this study.

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