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Construction of a prokaryotic expression system of *vacA* gene and detection of *vacA* gene, VacA protein in *Helicobacter pylori* isolates and ant-VacA antibody in patients' sera

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Abstract

AIM: To construct a recombinant prokaryotic expression vector inserted with *Helicobacter pylori vacA* gene and identify the immunity of the expressed recombinant protein, and to determine prevalence of *vacA*-carrying/VacA expressing *H pylori* isolates and seroprevalence of specific ant-VacA antibody in *H pylori* infected patients.

METHODS: Polymerase chain reaction technique was used to amplify complete *vacA* gene of *H pylori* strain NCTC11637 and to detect *vacA* gene in 109 *H pylori* isolates. The amplification product of the complete *vacA* gene was sequenced after T-A cloning. A recombinant expression vector inserted with a complete *vacA* gene fragment, named as *pET32a-vacA*, was constructed. Expression of the target recombinant protein VacA (rVacA) was examined by SDS-PAGE. Western blot using commercial antibodies against whole cell of *H pylori* and an immunodiffusion assay using self-prepared rabbit anti-rVacA antibody were applied to determine immunoreaction and antigenicity of rVacA. Two ELISA methods were established to detect VacA expression in *H pylori* isolates and the specific anti-VacA antibody in sera from 125 patients infected with *H pylori*.

RESULTS: In comparison with the reported corresponding sequences, homologies of nucleotide and putative amino acid sequences of the cloned *vacA* gene were 99.82% and 100%, respectively. The constructed recombinant prokaryotic expression system efficiently produced rVacA. rVacA was able to combine with the commercial antibodies against whole cell of *H pylori* and to induce the immunized rabbit to produce specific antibody with an immunodiffusion titer of 1:4. All tested *H pylori* isolates carried *vacA* gene, but only 66.1% expressed VacA protein. Of the serum samples tested, 42.4% were positive for specific anti-VacA antibody.

CONCLUSION: A prokaryotic expression system of *H pylori vacA* gene was successfully constructed. The expressed rVacA can be used to detect specific anti-VacA antibody in human and to prepare antiserum in animals. The high frequency of *vacA* gene in *H pylori* isolates, but with a low frequency of VacA expression and specific anti-VacA antibody in *H pylori* infected patients implies that VacA is not an ideal antigen for *H pylori* vaccine.

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INTRODUCTION

In China, gastritis and peptic ulcer are two most prevalent gastric diseases, and gastric cancer is one of the malignant tumors with high morbidities[1-34]. *Helicobacter pylori* is recognized as a human-specific gastric pathogen that colonizes the stomachs of at least half of the world's populations[35]. Most infected individuals are asymptomatic. However, in some subjects, the infection causes acute/chronic gastritis or peptic ulceration, and plays an important role in the development of gastric adenocarcinoma, mucosa-associated lymphoid tissue (MALT) lymphoma and primary gastric non-Hodgkin's lymphoma[36-43].

Vacuolating cytotoxin, an important pathogenic factor of *H pylori*, is able to cause vacuolar degeneration in epithelial cells such as HeLa and RK-13 cell lines[44-46]. The gene of vacuolating cytotoxin has 3 864-3 888 bp in size containing a region for a signal peptide at the amino-end, a fragment with approximate 2 241 bp (*vacA*) and a region for a peptide at the cariboxyl-end[47]. The signal and C-end peptides are left in inner and outer membranes of *H pylori* during secreting the cytotoxin[48]. VacA, responsible for the toxicity of vacuolating cytotoxin, is excreted out of the bacterium[49]. In some of the previous studies, VacA was demonstrated as a fine antigen for *H pylori* vaccine[50,51]. However, it has been reported that almost all *H pylori* strains carry *vacA* gene but VacA is detectable only in 50-60% of the strains[52]. For a fine practical strategy of genetic engineering vaccine development, an antigen candidate must satisfy the requirements including exposure on the surface of bacterial body, universal distribution in different strains and strong antigenicity to induce a specific antibody. So it is a critical subject to determine VacA expression in different *H pylori* strains and the prevalence of specific anti-VacA antibody in infected individuals from various geographical areas. In this study, a recombinant expression plasmid containing complete *vacA* gene was constructed. By using ELISA, VacA expression in different *H pylori* isolates and seroprevalence of anti-VacA specific antibody from *H pylori* infected patients were determined.

MATERIALS AND METHODS

Materials

H pylori strain NCTC11637 was kept in our laboratory. Primers for polymerase chain reaction (PCR) amplification were synthezed by BioAsia (Shanghai, China). Taq-plus high fidelity

PCR kit and restriction endonucleases were purchased from TaKaRa (Dalian, China). The T-A Cloning kit and sequencing service were provided by BBST (Shanghai, China). A plasmid *pET32a* as an expression vector and *E. coli* BL21 DE3 as a host cell were provided by Novagen (Novagen, Madison, USA). Rabbit antiserum against whole cell of *H pylori*, HRP-labeling sheep antisera against rabbit IgG and against human IgG were purchased from DAKO (Glostrup, Denmark) and Jackson ImmunoResearch (West Grove, USA), respectively. Overall, 156 patients who were referred for upper endoscopy at four hospitals in Hangzhou during November 2001 to February 2003 due to various gastroduodenal diseases and who had a positive urease test on gastric biopsy specimens and serum samples collected, were included in the study. Reagents used in isolation and identification of *H pylori* were purchased from bioMérieux (Marcy I' Etoile, France).

Isolation and identification of H pylori

Each biopsy specimen was homogenized with a tissue grinder and then inoculated on Columbia agar plates supplemented with 80 mL/L sheep blood, 5 g/L cyclodextrin, 5 mg/L trimethoprim, 10 mg/L vancomycin, 2.5 mg/L amphotericin B and 2 500 U/L cefsoludin. The plates were incubated at 37 ℃ under microaerobic conditions (50 mL/L O_2, 100 mL/L CO_2 and 850 mL/L N_2) for 3 to 5 d. A bacterial isolate was identified as *H pylori* according to typical Gram staining morphology, biochemical tests positive for urease and oxidase, and slide agglutination with the commercial rabbit antibodies against whole cell of *H pylori*. A total of 126(80.1%) *H pylori* strains isolated from the 156 specimens were well-characterized.

Preparation of DNA template

Genomic DNA from each *H pylori* strain was extracted by the conventional phenol-chloroform method and DNase-free RNase treatment. The obtained DNA was dissolved in TE buffer. Concentration and purity of the DNA preparations were determined by ultraviolet spectrophotometry[53].

Polymerase chain reaction

Primers were designed to amplify complete *vacA* gene from *H pylori* strain NCTC11637 based on the published data (GenBank accession No.: AF049653)[54]. The sequences of sense primer with an endonuclease site of *Eco*R I and antisense primer with an endonuclease site of *Xho* I were 5'-GAGGAATTCATGGAAATACAACAAACACACCGC-3' and 5'-GGACTCGAGTTAATTGGTACCTGTAGAA ACATTACC-3', respectively. The total volume per PCR was 100 μL containing 2.5 mol/L each dNTP, 250 nmol/L each of the 2 primers, 15 mol/L $MgCl_2$, 3.0 U Taq-plus polymerase, 100 ng DNA template and 1×PCR buffer (pH8.3). The parameters for PCR were 94 ℃ for 5 min, ×1; 94 ℃ for 30 s, 58 ℃ for 30 s, 72 ℃ for 120 s, ×10; 94 ℃ for 30 s, 58 ℃ for 30 s, 72 ℃ for 135 s (15 s addition for the each of the following cycles), ×15; and then 72 ℃ for 15 min, ×1.

The sequences of primers for detecting *vacA* gene in *H pylori* isolates were 5'-TCAATATCAACAAGCTC-3' (sense), and 5'-CCGCATGCTTTAATGTC-3' (antisense)[55]. The reagents and the concentrations, reaction volume and PCR parameters were the same as those for amplifying complete *vacA* gene, except primer difference, annealing temperature at 52 ℃, extensive time for 60 s and 35 cycles.

The results of PCR were visualized under UV light after electrophoresis in 10 g/L agarose pre-stained with ethidium bromide. The expected sizes of the target amplification fragments with whole length of *vacA* gene for cloning and partial length of *vacA* gene for detection were 2 241 bp and 787 bp, respectively.

Cloning and sequencing

The target amplification fragment of complete *vacA* gene from *H pylori* strain NCTC11637 was cloned into plasmid vector *pUCm*-T (*pUCm*-T-*vacA*) by using the T-A Cloning kit according to the manufacturer's instruction. The recombinant plasmid was amplified in *E. coli* DH5α and then extracted by Sambrook's method[53]. A professional company (BBST) was responsible for nucleotide sequence analysis of the inserted fragment. Two plasmids, *pUCm*-T-*vacA* and *pET32a*, in 2 different strains of *E. coli* DH5α after amplified in LB medium were extracted and then digested with *Eco*RI and *Xho* I, respectively[53]. The target fragments of *vacA* gene and *pET32a* were recovered and then ligased. The recombinant expression vector *pET32a-vacA* was transformed into *E. coli* BL21DE3, and the expression system was named as *pET32a-vacA-E. coliBL21DE3*. *vacA* gene inserted in *pET32a* plasmid was sequenced again.

Expression and identification of the target recombinant protein

pET32a-vacA-E. coliBL21DE3 was rotatively cultured in LB medium at 37 ℃ and induced by isopropylthio-β-D-galactoside (IPTG) at different concentrations of 1.0, 0.5 and 0.1 mmol/L Supernatant and precipitate of the culture after incubation were separated by centrifugation and then the bacterial pallet was ultrasonically broken (300 V, 5 s×3). SDS-PAGE was used to measure the molecular weight and output of rVacA, and Ni-NTA affinity chromatography was applied to collect the recombinant protein. The commercial rabbit antiserum against whole cell of *H pylori* and HRP-labeling sheep antiserum against rabbit IgG were respectively used as the first and second antibodies to determine the immunoreactivity of rVacA by Western blot. Rabbits were immunized with rVacA to prepare the antiserum. An immunodiffusion assay was performed to determine antigenicity of rVacA.

ELISA

By using rVacA as the coated antigen at concentration of 20 μg/mL, a patient serum sample (1:200 dilution) as the first antibody and HRP-labeling sheep antibody against human IgG (1:4 000 dilution) as the second antibody, antibody against VacA in sera of the 126 *H pylori* infected patients were detected. The result of ELISA for a patient's serum sample was considered as positive if the value of optical density at 490 nm (A_{490}) was over the mean plus 3 SD of five different negative serum samples[56]. VacA expression in clinical isolates of *H pylori* was examined by using the ultrasonic supernatant of each isolate (50 μg/mL) as the coated antigen, the self-prepared rabbit anti-rVacA serum (1:2 000 dilution) as the first antibody and HRP-labeling sheep antibody against rabbit IgG (1:4 000 dilution) as the second antibody. The result of ELISA for a *H pylori* ultrasonic supernatant sample was considered as positive if its OD_{490} value was over the mean plus 3 SD of five separated *E. coli* ATCC 25922 ultrasonic supernatant samples at the same protein concentration[56].

Data analysis

The nucleotide and putative amino acid sequences of the cloned *vacA* gene were compared for homology with the published corresponding sequences (GenBank accession No. AF049653)[54].

RESULTS

PCR

All tested *H pylori* isolates (109/109) were positive for *vacA* gene. The target amplification products with whole length of *vacA* gene from *H pylori* strain NCTC11637 and with partial

fragment of *vacA* gene from *H pylori* isolate were shown in Figure 1, respectively.

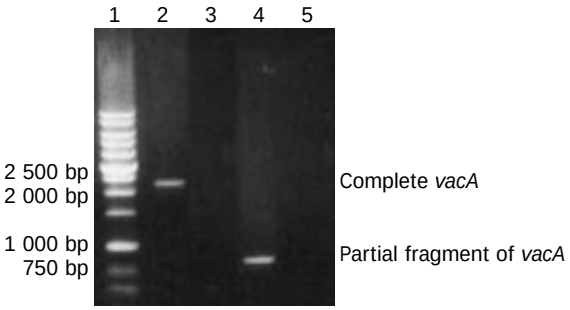

Figure 1 The target amplification products with whole length of *vacA* gene from *H pylori* strain NCTC11637 and partial fragment of *vacA* gene from a *H pylori* isolate. Lane 1, DNA marker; lane 2, an amplification fragment of complete *vacA* gene from *H pylori* strain NCTC11637; lane 4, an amplification fragment of partial *vacA* gene from a *H pylori* isolate; and lanes 3 and 5, blank controls.

Nucleotide sequence analysis

The nucleotide sequences of *vacA* gene in *pUCm*-T-*vacA* and *pET32a-vacA* were completely same. The nucleotide and putative amino acid sequences of the cloned *vacA* gene (Figure 2) showed 99.82% and 100% homologies, respectively, compared with the published sequence from *H pylori* strain NCTC11637 *vacA* gene (GenBank accession No.: AF049653)[54].

Expression of the target recombinant protein

IPTG at concentrations of 1.0, 0.5 and 0.1 mmol/L efficiently induced the expression of rVacA in the *pET32a-vacA-E. coli BL21DE3* system. The expressed rVacA was mainly presented in ultrasonic precipitates, and the output was approximate 15% of the total bacterial proteins (Figure 3).

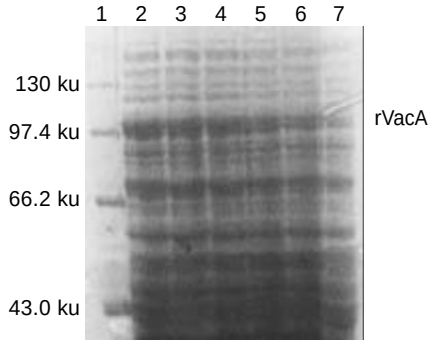

Figure 3 Expression of rVacA induced by IPTG at different concentrations. Lane 1, the protein marker; lanes 2-4, IPTG at 1.0, 0.5, 0.1 mmol/L respectively; lanes 5 and 6, bacterial precipitate and supernatant with IPTG at 0.5 mmol/L, respectively; and lane 7, the negative control.

Immunoreactivity and antigenicity of rVacA

The commercial rabbit antibodies against whole cell of *H pylori* combined with rVacA as confirmed by Western blot (Figure 4). The titer demonstrated by immunodiffusion assay between rVacA and rabbit anti-rVacA serum was 1:4.

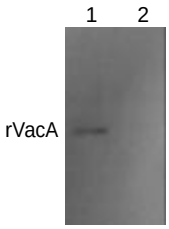

Figure 4 Western blot result of rabbit antibody against whole cell of *H pylori* and rVacA. Lane 1, rVacA expressed by *pET32a-vacA-E.coliBL21DE3*; and lane 2: negative control of *E.coliBL21DE3*.

```
1    atggaaatacaacaaacacaccgcaaaatcaatcgccctttggtttctcttgctttagta
1    M  E  I  Q  Q  T  H  R  K  I  N  R  P  L  V  S  L  A  L  V

61   ggagcgttagtcagcatcacaccgcaacaaagtcatgccgccttttcacaaccgtgatc
21   G  A  L  V  S  I  T  P  Q  Q  S  H  A  A  F  F  T  T  V  I

121  attccagccattgttgggggatCgctacaggcgctgctgtaggaacggtctcagggctt
41   I  P  A  I  V  G  G  I  A  T  G  A  A  V  G  T  V  S  G  L

181  cttAgctgggggctcaacaagccgaagaagccaataaaaccccagataaacccgataaa
61   L  S  W  G  L  K  Q  A  E  E  A  N  K  T  P  D  K  P  D  K

241  gtttggcgcattcaagcaggaagaggcttcaataatttcctcacaaggaatacgactta
81   V  W  R  I  Q  A  G  K  G  F  N  N  F  P  H  K  E  Y  D  L

301  tacaaatccctttatccagtaagattgatggaggctgggattgggggaatgccgctagg
101  Y  K  S  L  L  S  S  K  I  D  G  G  W  D  W  G  N  A  A  R

361  cattattgggtcaaaggcgggcaatggaacaagcttgaagtggatatgaaagacgctgta
121  H  Y  W  V  K  G  G  Q  W  N  K  L  E  V  D  M  K  D  A  V

421  gggacttataaactttcaggcctaatcaactttactggtggggatttagatgtcaatatg
141  G  T  Y  K  L  S  G  L  I  N  F  T  G  G  D  L  D  V  N  M

481  caaaaagccactttgcgcttgggccaattcaatggcaattctttcacaagctataaggat
161  Q  K  A  T  L  R  L  G  Q  F  N  G  N  S  F  T  S  Y  K  D

541  agTgctgatcgcaccacgagagtggatttcaacgctaaaaatatcttaattgataatttt
181  S  A  D  R  T  T  R  V  D  F  N  A  K  N  I  L  I  D  N  F

601  ttagaaatcaataatcgtgtgggttctggagccgggaggaaagccagctctacggtttta
201  L  E  I  N  N  R  V  G  S  G  A  G  R  K  A  S  S  T  V  L

661  actttgcaagcttcagaagggatcactagcagtaaaaacgctgaaatttctctttatgat
221  T  L  Q  A  S  E  G  I  T  S  S  K  N  A  E  I  S  L  Y  D
```

```
721  ggtgccacgctcaatttggcttcaagcagtgttaaattaatgggtaatgtgtggatgggc
241  G  A  T  L  N  L  A  S  S  S  V  K  L  M  G  N  V  W  M  G

781  cgtttgcaatacgtgggagcgtatctggccccttcatacagcacgataaacacttcaaaa
261  R  L  Q  Y  V  G  A  Y  L  A  P  S  Y  S  T  I  N  T  S  K

841  gtgacaggggaagtgaattttaaccatctcactgtgggcgatcacaacgctgctcaagca
281  V  T  G  E  V  N  F  N  H  L  T  V  G  D  H  N  A  A  Q  A

901  ggcattatcgctagtaacaagactcatattggcacattggatttgtggcaaagcgcgggG
301  G  I  I  A  S  N  K  T  H  I  G  T  L  D  L  W  Q  S  A  G

961  ctaaacattatcgcccctccagaaggcggttataaggataaacctaaggataaacctagt
321  L  N  I  I  A  P  P  E  G  G  Y  K  D  K  P  K  D  K  P  S

1021 aacaccacgcaaaataatgctaacaacaaccaacaaaacagcgctcaaaacaataataac
341  N  T  T  Q  N  N  A  N  N  N  Q  Q  N  S  A  Q  N  N  N  N

1081 actcaggttattaacccacccaatagcgCgcaaaaaacagaaattcaacccacgcaagtc
361  T  Q  V  I  N  P  P  N  S  A  Q  K  T  E  I  Q  P  T  Q  V

1141 attaatgggccttttgctggtggcaaagacacggtggtcaatatcaaccgcatcaacact
381  I  N  G  P  F  A  G  G  K  D  T  V  V  N  I  N  R  I  N  T

1201 aacgctgatggcacgattagagtgggagggtataaagcttctcttaccaccaatgcggct
401  N  A  D  G  T  I  R  V  G  G  Y  K  A  S  L  T  T  N  A  A

1261 catttgcatatcggcaaaggcggtatcaatctgtccaatcaagcgagcgggcgttcttta
421  H  L  H  I  G  K  G  G  I  N  L  S  N  Q  A  S  G  R  S  L

1321 ttggtggaaaatctaaccgggaatatcaccgttgatgggcctttaagagtgaataaccaa
441  L  V  E  N  L  T  G  N  I  T  V  D  G  P  L  R  V  N  N  Q

1381 gtgggtggttatgctcttgcaggatcaaacgcgaattttgagtttaaggctggcacggat
461  V  G  G  Y  A  L  A  G  S  N  A  N  F  E  F  K  A  G  T  D

1441 accaaaaacggcacagccacttttaataacgatattagtttgggaagatttgtgaattta
481  T  K  N  G  T  A  T  F  N  N  D  I  S  L  G  R  F  V  N  L

1501 aaagtggatgctcatacagctaattttaaaggtattgatacgggtaatggtggtttcaac
501  K  V  D  A  H  T  A  N  F  K  G  I  D  T  G  N  G  G  F  N

1561 accttggattttagtggcgttacagacaaagtcaatatcaacaagctcatcacagcttcc
521  T  L  D  F  S  G  V  T  D  K  V  N  I  N  K  L  I  T  A  S

1621 actaatgtggccattaaaaacttcaacattaatgaattgttggttaagaccaatggggtg
541  T  N  V  A  I  K  N  F  N  I  N  E  L  L  V  K  T  N  G  V

1681 agtgtgggggaatacactcattttagcgaagatataggcagtcaatcgcgcatcaacacc
561  S  V  G  E  Y  T  H  F  S  E  D  I  G  S  Q  S  R  I  N  T

1741 gtgcgtttagaaactggcactaggtcaatcttttctgggggtgtcaaatttaaaagcggc
581  V  R  L  E  T  G  T  R  S  I  F  S  G  G  V  K  F  K  S  G

1801 gaaaaattggttatagatgagttttactatagcccttggaattattttgacgctaggaat
601  E  K  L  V  I  D  E  F  Y  Y  S  P  W  N  Y  F  D  A  R  N

1861 attaaaaatgttgaaatcaccagaaaattcgcttcttcaaccccagaaaaccccttggggc
621  I  K  N  V  E  I  T  R  K  F  A  S  S  T  P  E  N  P  W  G

1921 acatcaaaActcatgtttaataatctaaccctgggtcaaaatgcggtcatggactatagt
641  T  S  K  L  M  F  N  N  L  T  L  G  Q  N  A  V  M  D  Y  S

1981 caattttcaaatttaaccattcaggggattttatcaacaatcaaggcactatcaactat
661  Q  F  S  N  L  T  I  Q  G  D  F  I  N  N  Q  G  T  I  N  Y

2041 ctggtccgaggcgggaaagtggcaaccttaaatgtaggcaatgcagcagctatgatgttt
681  L  V  R  G  G  K  V  A  T  L  N  V  G  N  A  A  A  M  M  F

2101 aataatgatatagacagcgcgaccggattttacaaaccgctcatcaagattaacagcgct
701  N  N  D  I  D  S  A  T  G  F  Y  K  P  L  I  K  I  N  S  A

2161 caagatctcattaaaaatacagagcatgttttattgaaagcgaaaatcattggttatggt
721  Q  D  L  I  K  N  T  E  H  V  L  L  K  A  K  I  I  G  Y  G

2221 aatgtttctacaggtaccaattaa
741  N  V  S  T  G  T  N  *
```

Figure 2 Nucleotide and putative amino acid sequences of *vacA* gene from *H pylori* strain NCTC 11637. Note: Underlined areas indicate the position of primers; C, T, C and A are replaced by t, c, g and g in the nucleotide sequence from strain NCTC 11637, respectively, but the encoded amino acid residuals are not altered (are the changes correct?). "*" means stop codon.

ELISA

Since the mean±SD of A_{490} values of the five negative serum samples was 0.275±0.111 in the detection of specific antibodies in patients' sera, the positive reference value was 0.625. According to the reference value, 42.4%(53/125, one serum sample was contaminated) of the tested patients' serum samples were positive for antibody against rVacA with an A_{490} value range from 0.63-1.21. Since the mean±SD of A_{490} values of the five negative bacterial controls was 0.098±0.036 in the detection of VacA expression in *H pylori* isolates, the positive reference value was 0.205. According to the reference value, 66.1%(72/109, other 17 isolates could not be revived from -70 ℃) of the tested *H pylori* isolates were positive for the epitope of rVacA with an A_{490} value range from 0.27-1.73.

DISCUSSION

Amoxciilin and metronidazole, used as routine therapeutic antibiotics in triple therapies, efficiently eradicate *H pylori* infection *in vivo*[57-59]. However, there are problems such as side effects, emergence of drug resistance and re-infection after withdrawal of the antibiotics, *etc*. It is generally considered that *H pylori* vaccination is the optimal strategy for the prevention and control of *H pylori* infection.

VacA, confirmed as a unique *H pylori* exotoxin with strong antigenicity[47,48], has been considered to be an antigen candidate in *H pylori* vaccine[50,51]. However, the low prevalence (50-60%) of VacA expression in *H pylori* strains and rare data on specific antibody inducement after *H pylori* infection in human make this consideration difficult to test. Therefore, we constructed a prokaryotic expression system of *H pylori vacA* gene, used rVacA to detect anti-VacA antibody in *H pylori* infected patients and prepared rabbit anti-rVacA serum to determine VacA expression in *H pylori* isolates, in order to determine the potential of VacA as an antigen in *H pylori* vaccine development.

The present study demonstrated high homologies (99.82% and 100 %) of the nucleotide and putative amino acid sequences of the cloned *vacA* gene compared with the reported (GenBank accession No.: AF049653)[54] and efficient expression of *pET32a-vacA-E. coliBL21DE3*, indicating the successful establishment of a prokaryotic expression system of this target gene. The high specificity, immunoreactivity and strong antigenicity of rVacA exhibited in this study are beneficial to establish ELISAs to detect VacA-specific antibody in *H pylori* infected patients and VacA expression in *H pylori* isolates.

All tested *H pylori* isolates were positive for *vacA* gene by PCR. However, expression of VacA, not like those of UreB and HpaA in our previous studies[60], was detectable only in 66.1% of the tested *H pylori* isolates. The lower positive detection rate (42.4%) for VacA-specific antibody in sera of *H pylori* infected patients was also found. The fact that the prevalence of VacA expression is lower than that of *vacA* gene in *H pylori* strains suggests possible mutation of the gene[44], and the even lower seroprevalence of specific anti-VacA antibody in infected patients is probably due to the defect of signal peptide in the protein[61].

In conclusion, a prokaryotic expression system of *H pylori vacA* gene was successfully constructed. The expressed rVacA can be used to detect specific anti-VacA antibody in human and to prepare antiserum in animals. However, the evidence obtained from this study indicates a poor potential for VacA as an antigen in the development of *H pylori* vaccine. In addition, detection of specific anti-VacA antibody is not a reliable diagnostic indicator as to whether an individual is infected by *H pylori*.

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