ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES Knowledge to Innovation

Vol 7, Issue 3, 2014

ISSN - 0974-2441

Original Article

CLONINIG AND EXPRESSION OF BAB-PATHOGENICITY ISLAND ANTIGENS FOR THE PRODUCTION OF VACCINE AGAINST HELICOBACTER PYLORI, THE RISK FACTOR FOR GASTRIC CANCER

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Received: 25 February 2014, Revised and Accepted: 31 March 2014

ABSTRACT

BabA2, one of the two allele of BabA gene and a member protein of Helicobacter pylori, plays a vital role in assisting bacterial colonization in the stomach. However, its association with H pylori -related gastroduodenal diseases still remains unclear. In the present study, babA2 gene from Helicobacter pylori was amplified using specific primers and then cloned in pTZ57R/T and transformed into DH5-α cells successfully. Transformation was confirmed with plasmid extraction and followed by restriction digestion. IPTG was used as an inducer for the expression of babA2 protein and the protein was successfully isolated and quantified. The quantified protein was subjected to SDS PAGE to evaluate the expression of that protein. The sequence analysis have shown 99% "perfect" match with sequences of their corresponding gene (babA2) from GenBank as determined by using BLAST (version 2.7). Inserted babA2 gene was expressed significantly in the prokaryotic expression system, and specific strip at ~ 75 KDa was demonstrated in SDS-PAGE. Further study is needed to substantiate that the expressed protein will act as an antigen for the humoral immunity against the Helicobacter pylori.

Keywords: Helicobacter pylori, Genomic DNA, PCR, Cloning, Bab-A gene

INTRODUCTION

It has been well known that Helicobacter pyloris infection which causes active gastritis and potentially advances to chronic gastritis with atrophy, duodenal ulcer, gastric adenocarcinoma, or mucosa-associated lymphoid tissue (MALT) lymphoma depends on environmental conditions, host and bacterial virulence factors [1]. Many epidemiological studies have observed a strong association between the presence of duodenal ulcer and the bacterial virulence factors including vacuolating cytotoxin (VacA) and cytotoxin-associated antigen (CagA) that were identified [2]. Besides these VacA and CagA, bacterial adhesion factor has been considered to play a key role in the pathogenesis of gastritis caused by H. pylori in humans [3]. The blood-group antigen-binding adhesin, BabA, has been demonstrated to aid in the adherence of H. pylori to human Lewisb (a-1,3y4- difucosylated) blood-group antigens on gastric epithelial cells [4]. Using PCR, BabA which has been isolated and cloned led to the identification of babA2 genotype encoded by H. pylori strains [5].

Although, H pylori infection could be treated and eliminated by using antibiotics, owing to the high cost of combination therapy and the crisis of antibiotic resistance, this has resulted in the development of vaccines [6, 7]. At present, the antigens that have been approved for the development of H pylori vaccine, which mainly involves in blocking the toxicity factors of H pylori, are urease, vacuolating cytotoxin, catalase, etc. Few studies have suggested that vaccine antigens that focus on adhesions, which aids in the successful colonization of H pylori in the human gastric mucosa, could help in developing the vaccine against H pylori infection effectively [8].

In an attempt to understand the clinical relevance of the H. pylori adherence factor BabA2 in India as well as to support its use in the development of H pylori vaccine, the aims of the present study was to find if BabA2 isolated from H pylori could be produced in Escherichia coli strain as well as to study the sequence of the protein expressed by the recombinant plasmid of H pylori BabA2 gene with reference to the GENBANK [5].

MATERIALS AND METHODS

DNA Isolation and PCR

The bacterial isolate was taken from the bacterial repository of Credora Life sciences, Bangalore, India. Genomic DNA was isolated from *Helicobacter pylori* using the chloroform, and isoamyl alcohol (24:1) extraction method. In order to amplify the specific gene of our interest babA2 product, PCR was performed using the following conditions: complete denaturation: 95°C for 2 min; Annealing: 52°C for 30 sec; Extension: 72°C for 1 min, followed by 30 cycles of amplification and the final elongation step (72°C for 2 min) using Forward Primer 5'GAA GCC TTA GCT GGT GAA GGT 3'and Reverse primer 5' CGG TTG TGG GGT AGT GAT AGA 3'. PCR products were separated and analyzed on 1 % agarose gel electrophoresis.

Cloning of babA2 gene

The PCR product of babA2 gene was eluted from the gel and cloned in T vector pTZ57R/T (Fermentas, Germany) as per manufacturer's instructions. The ligated mix was then transformed into competent E. coli DH5α cells, by CaCl2 method. The transformants were plated on Luria broth (LB) agar supplemented with Ampicillin (50mg/mL), in addition with IPTG (40mg/mL), and X-gal (20mg/mL). Cells were incubated at 37º C for overnight. Blue–White screening colony selection method was performed to choose the white colored recombinant clone and followed by colony PCR amplification was performed for confirmation of cloning of our gene of interest (babA2 gene).

Plasmid Isolation

The plasmid was isolated from positive clones by alkali-lysis method described by Sambrook *et a l.* Briefly, 2ml of Overnight culture was centrifuged. The cell pellet was resuspended in 200µl ice cold lysis solution 1 (which consist of 15% glucose, 25mM Tris, 10mM EDTA) and followed by vortexed gently. Then added 400 µl of freshly prepared solution 2 (which consist of 0.2N NaOH, 1% SDS) and 50 µl solution 3 (3M Sodium acetate). Centrifugation was done at 10,000 rpm for 10 minutes and to the supernatant equal volume of iso-propanol was added and incubated at RT for 15 min. Then

centrifuged at 10000 rpm for 10 min, removed the supernatant and pellet was dissolved in 50µl TE buffer for further use.

Confirmation of clone by plasmid isolation and restriction digestion

The recombinant white colonies were isolated from the LB-Ampicillin agar plates and inoculated in LB Ampicillin containing broth and incubated at 37°C for overnight. Plasmid DNA was isolated by Alkaline-lysis method. The purified plasmid was subjected to restriction digestion using *Bam*H1and EcoR1. The release of the gene product was checked on 1% agarose gel electrophoresis.

Confirmation of clone by plasmid isolation and restriction digestion

Ligated plasmid was transformed in to the bacterium DH5α as described earlier and plated on LB agar- Ampicillin plates. The white colonies from the LB- Ampicillin agar plates were inoculated in LB-Ampicillin broth and incubated at 37°C for overnight and plasmid was isolated by Alkaline lysis method. The transformed bacteria were inoculated in 50 ml of LB broth containing IPTG (0.5mM) as inducer for the expression of the gene. *E.coli* strain without plasmid was used as control. The inoculated culture flask was incubated at 37°C at 150 rpm for 24 hours in an orbital incubator shaker.

Protein extraction and estimation

The bacterial cells from 50 ml culture were harvested by centrifugation (7,000 x g) at 4°C, washed with sterile distilled water. The cells were then suspended in 10 ml of ice-cold acetone (analytical grade), allowed to stand on ice for 5 min, and collected by centrifugation (7,000 x g) at 4°C. Residual acetone was removed by air drying, and the proteins were then extracted by incubating with 1.0 ml of 1% sodium dodecyl sulfate (SDS) for 2 min. The protein concentration was estimated at 280nm using UV-VIS spectrophotomer (Vivaspec Biophotometer, Germany). From the stock 1µl Protein was mixed with 99-µl sterile distilled water to get 100 times dilution. The diluted protein concentration was directly estimated by Vivaspec Biophotometer. SDS PAGE is done for further seperation.

RESULT

Culturing and Morphological studies

The bacteria were cultured in brain heart infusion media. The cells were stained by Gram staining. The Gram negative spiral shaped rods are visible at 100x oil immersion (Olympus, Japan).

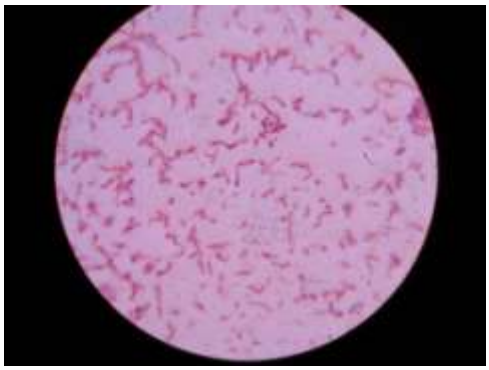

Fig.1: Slide showing the Gram staining of *Helicobacter pylori*

Genomic DNA isolation and quantification

The Gram negative bacteria were cultured in the Brain heart infusion broth media and genomic DNA was isolated by modified CTAB method. The isolated DNA was electrophorized in 1% Agarose gel (Fig.4). The quantity and quality of DNA was analyzed by UV visible spectrophotometer and the data was shown in the table 14. The purity of DNA was indicated by A260/A280 ratio, where the value from 1.8 to 2.0 was considered as high purity. Nevertheless, the extracted DNA is considered of adequate purity if A260/A280 is > 1.5.

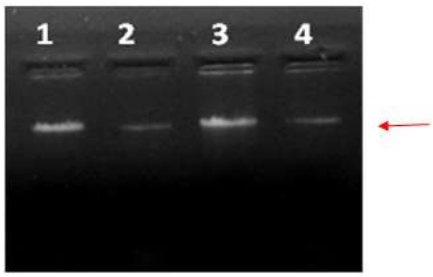

Fig. 2: Genomic DNA isolated from *Helicobacter pylori* (Lane 1, 2, 3, 4 - Genomic DNA).

PCR amplification of the *bab*A2 gene

Species specific primers were designed for the *Helicobacter pylori* using the sequences of *babA2* gene available in NCBI GenBank using Primer 3 Software. The predicted primers were validated initially *in silico* and subsequently in wet lab. The primers could yield an amplicon of the expected size specific to *babA2* gene. The primers were found to produce ~2112 bp amplicon which shown in the electrophoresis experiment.

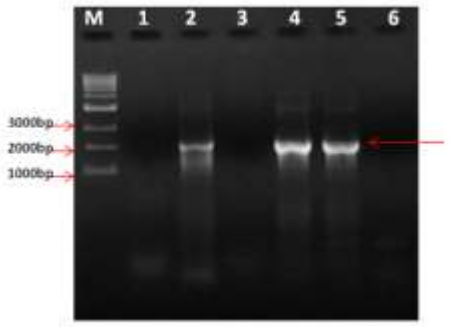

Fig. 3: PCR amplification of *bab*A2 partial gene by specific primers (M- 1kb ladder, Lane 1, 2, 3, 4, 5- *bab*A gene product, 6-Negative control)

The recently described blood group antigen-binding adhesin *bab*A has been shown to mediate adherence of *Helicobacter pylori* to Lewis b (α-1,3/4-difucosylated) receptors on gastric epithelium. Although three *bab* alleles have been identified (*babA1*, *babA2*, and *babB*), only the *babA2* gene product is necessary for Lewis b binding activity (Dixon *et al.,* 1996). Studies in Western countries have demonstrated associations between *babA2*-positive status and duodenal ulcer as well as gastric carcinoma (Gerhard *et al.,* 1999). However, in Asian countries, most of the circulating *Helicobacter pylori* strains are *babA2* positive, whether or not they were isolated from symptomatic or diseased patients (Kim et al., 2001).

Cloning of PCR product in to T vector

PCR yielded a specific amplicon of 1060-bp in *Helicobacter pylori* strain. The fragment was separated in gel and purified the product using gel extraction kit.

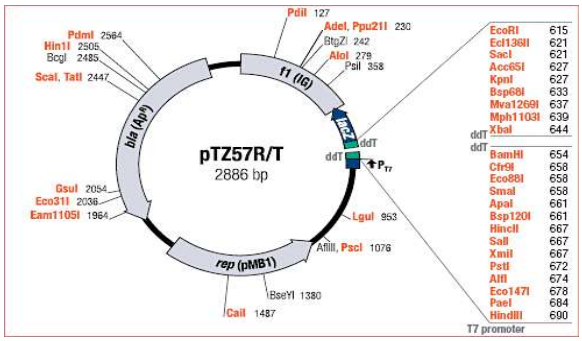

Fig.4: Map of T vector pTZ57R/T (Fermentas, Germany)

The purified PCR product was quantified and ligated with cloning vector using T4 DNA ligase enzyme. The ligated plasmid was transformed in to *E.coli* bacterial strain DH5-α. The transformation was done by heat shock method and transformed cell was cultured in the Xgal-IPTG-Ampicillin-LB Agar plate at 37°C for overnight. The white colonies were picked up from the plates and cultured in Ampicillin containing LB broth.

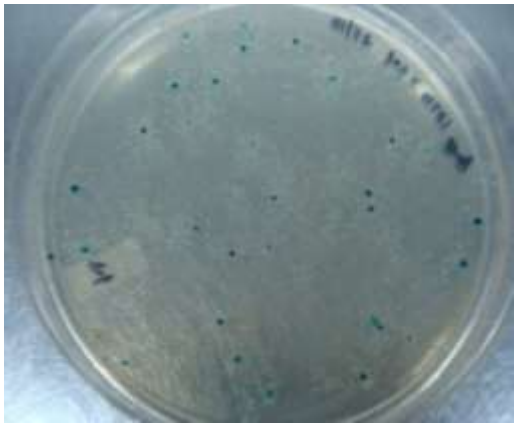

Fig. 5: Blue white selection of the transformed bacterial cells in the X gal-IPTG-Ampicillin-LB Agar

Plasmid Isolation and Confirmation of clone by restriction digestion

Plasmid was isolated from the transformed cells by using alkaline lysis method. The isolated plasmid was electrophorized on 1% Agarose gel. The purified plasmid was subjected to restriction digestion using *Bam* H1 and *Eco*R 1 (Merck, India). After incubation at 37°C for 4 hours the restricted product was electrophorized on 1% Agarose gel. The release of the gene product was visualized in the gel.

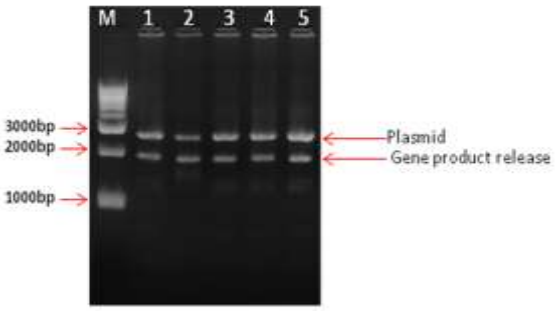

Fig.6: Restriction digestion of ligated plasmid using *Bam* H1 and *Eco*R 1. M: marker, Lane 1, 2, 3, 4, 5 showing both the plasmid DNA and Gene product release.

Sequence data:

The gene was identified by sequencing of plasmid. An approximately 2112-bp region of the *bab*A2 gene was sequenced at Eurofins, Bangalore. The sequence data was shown bellow**.** Nucleotide sequence analysis of gene was used to investigate the identity of bacterial babA2 gene of *Helicobacter pylori*. To demonstrate the quality and accuracy of results provided from a public database, we compared sequences to their corresponding GenBank sequences. The sequence had "perfect" match (similarity, 99%) with sequences of their corresponding gene (*bab*A2) from GenBank as determined by using BLAST (version 2.7). An association between the severity of the antral and oxyntic gastritis with *babA2* or *cagA* status was evaluated in the patients without duodenal ulcer and gastric carcinoma by the Mann-Whitney two-tailed test. Models of logistic regression adjusting for potential confounding factors, such as age and gender, were constructed, as it has previously been demonstrated that the presence of some virulence markers increase with age. Since *babA2* and *cagA* were closely linked, *cagA* status was included as an independent variable when *babA*2 was the reference, and vice versa.

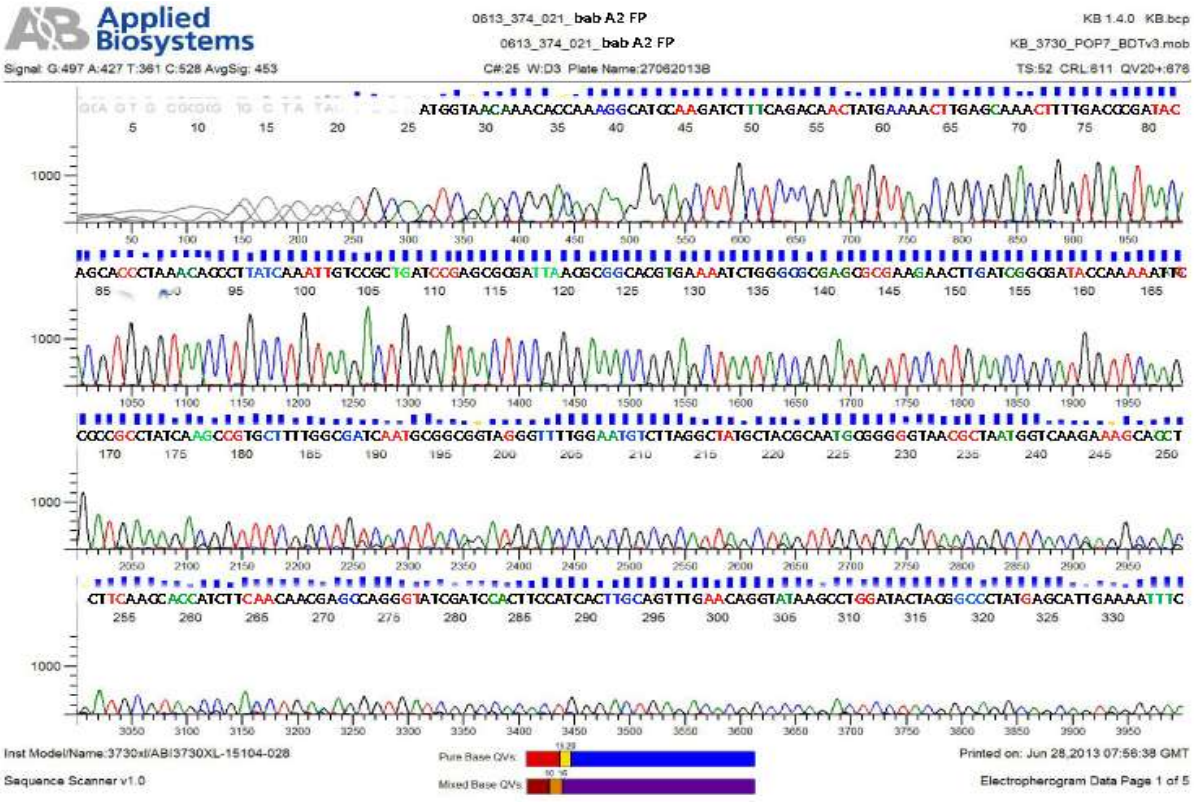

Fig.7: Showing the sequencing dendrogram of *bab*A2 gene

Sequence alignment by clustal W

The *bab*A2 gene sequence of *Helicobacter pylori* is also available in GenBank database, which is more similar to this sequence (99% similarity). The N-J tree with branch length was plotted using ClustalW sequence alignment (http://align.genome.jp/), showing the relationship of *bab*A2 gene among the closest *Helicobacter pylori strains* in the NCBI database. This regional difference may be due to an actual low frequency of the gene in the *Helicobacter pylori* strains that circulate in Portugal and Brazil or to variability within the gene that impairs PCR amplification. Although we cannot rule out the last possibility, it seems less probable, since the annealing sites of the primers we used were not from the region with the greatest diversity observed in *babA2.*

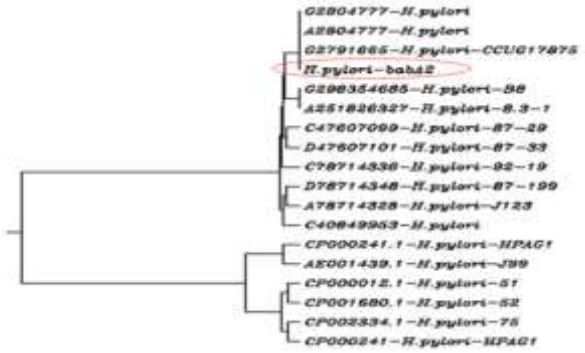

Fig.8: A tree plot was constructed with the NJ method using ~2200bp fragment of the *babA2* gene showing the relationship of *Helicobacter pylori*

However, in Asia, most of the *Helicobacter pylori* strains are *babA2*-positive, irrespective of clinical outcome. Thus, conclusions about the relationship between *Helicobacter pylori* genotypes and clinical outcome derived from one geographic region may not be true for other geographic regions. The relationship between *babA2*-positive *Helicobacter pylori* and an increased risk of developing clinical outcomes is controversial [9], because the presence of *babA2* is not always to reflect the *bab*A binding activity due to regulation by the number of transcriptional start adenine [poly (A)] residues in the promoter region and the presence of chimeric *babA/B* or *babB/A* genes [5]. Moreover, it is relatively difficult to detect the *babA2* gene by PCR with a single primer pair due to high homology between the sequences of *babA1* and *babA2.*

***bab*A2 gene expression analysis**

The T vector clone was restricted with restriction enzyme (*Eco*RI and *Bam*H1) and the released gene product was gel purified using gel extraction kit. The purified gene fragment was quantified and ligated with linearized pET20b expression vector (Novagen, Germany) using T4 DNA ligase. Inserted *bab*A2 gene was expressed significantly in the prokaryotic expression system, and specific strip at ~ 75 KDa was demonstrated in SDS-PAGE (Fig.9) [10], analyzed the expression of *ure*A, *cag*A, *vac*A genes after prolonged incubation in a liquid medium [11], explored the virulence and the potential pathogenicity of *H. pylori* transformed from spiral form by exposure to antibiotic in, and found that the content of the protein with the molecular weight over *Mw* 74 000 decreased, but *vac*A, *cag*A, *ure*A, *ure*B, *bab*A gene remained to be preserved, so they concluded that the virulence and the proteins with molecular weight over *Mw* 74 000 in coccoid *H pylori* decrease, but no deletion exists in amplification fragments from *ure*A, *ure*B, *hpa*A, *vac*A and *cag*A genes, and suggested that *H. pylori* may have potential pathogenicity. Furthermore, confirmed that the transcription and translation of cagA and vacA gene might actively take place in *H. pylori* cell [12].

DISCUSSION

The gene was identified by sequencing of plasmid. An approximately 2112-bp region of the *bab*A2 gene was sequenced at Eurofins, Bangalore. The sequence data was shown bellow**.** Nucleotide sequence analysis of gene was used to investigate the identity of bacterial babA2 gene of *Helicobacter pylori*. To demonstrate the quality and accuracy of results provided from a public database, we compared sequences to their corresponding GenBank sequences. The sequence had "perfect" match (similarity, 99%) with sequences of their corresponding gene (*bab*A2) from GenBank as determined by using BLAST (version 2.7). An association between the severity of the antral and oxyntic gastritis with *babA2* or *cagA* status was evaluated in the patients without duodenal ulcer and gastric carcinoma by the Mann-Whitney two-tailed test. Models of logistic regression adjusting for potential confounding factors, such as age and gender, were constructed, as it has previously been demonstrated that the presence of some virulence markers increase with age. Since *babA2* and *cagA* were closely linked, *cagA* status was included as an independent variable when *babA*2 was the reference, and vice versa [5].

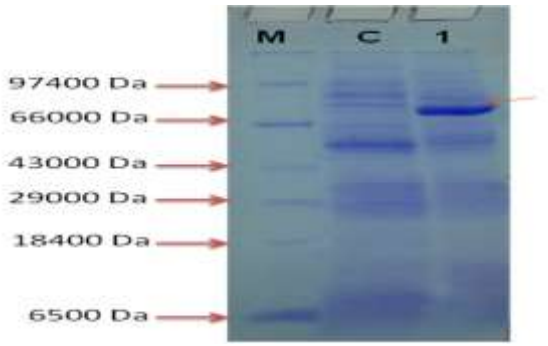

Fig.9: Analysis of *bab*A2 gene expression (M- Marker; C- Control; 1- *bab*A2 Protein product)

CONCLUTION

Increased evidence suggest that bacterial adherence factors could contribute further to the specific tropism and pathogenicity of H. pylori in the human gastric epithelium. Consequently, in the present study, the clinical relevance of the H. pylori adherence factor BabA2 has been investigated by isolating the clinical isolates of H. pylori and then successfully transforming the recombinant plasmid DNA encoding BabA2 gene into E.coli strains. The findings of the present study suggest that BabA2 of H pylori potentially a better entrant as a vaccine constituent.

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