Journal of Genetic and Environmental Resources Conservation, 2016, 4(2):135-140.
www.jgerc.com

Polymorphism of 16srRNA gene of *Helicobacter pylori*

Haider K. Al-Rubai

Forensic DNA Research and Training Center, Al-Nahrain University, Baghdad, Iraq.
hayderkhadum@yahoo.com

Abstract

The aim of this study was to investigate the polymorphisms of *Helicobacter pylori* based on 16srRNA gene sequencing. To achieve this goal, biopsy samples were collected from 30 patients with various gastrointestinal symptoms, were applied for microbiological analysis which include: urease test bacterial culture using selective media DNA was isolated from *H. pylori* and the 16srRNA gene were amplified by using specific primer, then sequencing of nucleic acid of genes was performed by machine is AB13730XL, applied biosystem, macro gen company, the DNA sequencing results of flank sense of 16srRNA gene from 30 strains of *H. pylori* was found to be compatible 99% and score 1517 and expect 0.0 of the 16srRNA gene of *Helicobacter pylori* strain BO418 from the Gene Bank, The expected substitution were found after the analysis of the sequences there were 5 transversion and one transition in the 30 *Helicobacter pylori* with Sequence ID emb|AJ310144.1| location at range of nucleotide from 5128 to 5408, compared with data obtained from gene bank, these finding lead to conclusion, our assay allows rapid detection of polymorphism in *H. pylori*.

Key words: *Helicobacter pylori*, 16srRNA gene, Polymorphism, Sequencing.

Introduction

Helicobacter pylori is a microaerophilic, gram-negative bacteria that colonizes the gastric mucosa of approximately 50% of the world's population (McMulty *et al*., 2002). It is a primary pathogenic factor in benign and malignant gastro duodenal disease (Gisbert and Pajares, 2002; Jean-Philippe *et al.,* 2006; Kobayashi *et al.,* 2004). It is a Gram-negative, microaerophilic bacterium found usually in mucous layer or the epithelial lining of the stomach *Helicobacter pylori* is a spiral-shaped bacterium that it causes more than 90% of duodenal ulcers. Worldwide, chronic infection. decreasing in many parts of the world (McMulty *et al*., 2002), gastric malignanc; dyspeptic symptoms. *H. pylori* is one of the most genetically diverse bacterial species, displaying from 2.7% to 8.0% of DNA sequence polymorphism (Gisbert and Pajares, 2002). *H. pylori* is considered a risk factor for developing gastric carcinoma (Jean-Philippe *et al.,* 2006). *H. pylori* have two major protein first CagA proteins: the Western and the second in East Asian CagA. The East Asian CagA-positive H. pylori infection is more closely associated with gastric cancer (Kobayashi *et al.,* 2004). Methods that accurately detect *H. pylori* infection by direct demonstration of *H. pylori* in gastric biopsy specimens is possible through the use of culture, histological examination with several stains, and assays for rapid urease activity (Boor *et al.,* 2011). Recently, assays based on PCR technology were employed to detect the presence of H. pylori DNA by using several gene for example the cytotoxin-associated antigen (cagA) gene in H. pylori (Chong *et al.,* 2015). Other indicator for *H. pylori* is the Rapid Urease testing identifies active *H. pylori* infection through the organism's urease activity. Gastric biopsies are obtained and placed into an agar gel or on a reaction strip containing urea, a buffer, and a pH-sensitive indicator (De Reuse *et al.,* 2014). The 16S rRNA gene sequence is about 1,550 bp long and is composed of both variable and conserved regions. The gene is large enough, with sufficient interspecific polymorphisms of 16S rRNA gene, to provide distinguishing and statistically valid measurements (Brodersen *et al.,* 2009). Universal primers are usually chosen as complementary to the conserved regions at the beginning of the gene and at either the 540-bp region or at the end of the whole sequence about the 1,550-bp region, and the sequence of the variable region in between is used for the comparative taxonomy (Chopra and Roberts 2001).

Materials and Methods

A total of 30 patients with various gastrointestinal symptoms representing different age groups from both sexes were under want samples were obtained from endoscopy

department in Al-Kadhimya hospital in Baghdad, Iraq. Patients were advised to fast for overnight before endoscopy. Endoscopies performed under local anesthesia (xylocaine). The endoscopy was disinfected with (2%) glutaraldehyde (cidex) before and after each procedure. Biopsy forceps were washed with water and disinfected with glutaraldehyde (cidex) for (10min), then washed with distilled water before each procedure. During upper gastrointestinal endoscopy, four gastric biopsy specimens were taken (3-4) cm, two from each of corpus (body) and antrum region of the stomach. One biopsy from each region was fixed in 10% formal buffer saline for histological investigation and the other was used for bacteriological investigation. Biopsy specimens were transported to the laboratory in 0.5ml Brain - heart infusion broth with ice and kept at 4C for no longer than 4hrs before processing. The biopsy samples were minced and homogenized between the frosted ends of sterile microscope sides in a sterile petridishes near benzene burner, then subjected for the following tests: phenol red (as an indicator) and incubated at (37^{o}C). Slants were examined for color change from yellow to pink before and after (1hr) and after (24hrs). The test was not finally declared as negative till 24hrs. The second minced biopsy was inoculated in Brain-heart infusion broth and on each of selective media (Columbia, Brain-heart infusion) agar media plates and non-selective media (Blood) agar media plates that was used for primary isolation of *H. pylori*. The cultures were incubated at 37^{o}C under microaerophilic conditions in an anaerobic jar with a gas generating kit. Plates were examined for positive growth for intervals of 3-5 days for the selective media and 7 days for the nonselective media before discarding as negative. For positive growth, the colonies must be tiny, glistering, translucent or gray and covered with entire edges.

Urease test of colonies: Grown colonies were picked from the agar plate with a sterile loop and then inoculated on urea agar slant. Positive result was detected by changing color from yellow to pink within few minutes.

DNA extraction and polymerase chain reaction: A single colony of cultivated bacteria, which had been incubated overnight, suspended into 1ml of distilled water, centrifuged at 14000xg for 2min., then the supernatant discarded, after that 120µL of lysostaphin (10mg/l; Sigma) was added. DNA extracted using mini DNA extraction kit (Promega) according to manufacture instructions. Specific primers were designed for amplification by using a forward primer (*16s RNA* F: 5′- AGA GTT TGA TCC TGG CTC AG -3′) and a reverse primer (*16s RNA* R:5′ GGT TAC CTT GTT ACG ACT T -3′) (Primers set supplied by IDT (Integrated DNA Technologies company, Canada). PCR reaction was conducted in 25µl of a reaction mixture containing 2µl of DNA, 12.5µl *GoTaq0T® Green* Master (Promega, CA), (0.5µl) 25mM MgCl2, 2µl of (10 Pmol\ µl) of each primer, 2µl of distilled water. Amplification program was 1 cycle at 94°C for 1min; 35 cycles of 94°C for 1min, 63°C for 1min, 72°C for 1min; 72°C for 10min, using the Mastercycler (Eppendorf) (Dapeng *et al.,*2010). The amplified product was subjected to 1.5% agarose gel electrophoresis, and visualized under UV (Imagemaster VDS, Pharmacia Biotech, USA) after Red safe staining. Positive PCR product samples were sent for sequence analysis; nd 25µl (10 pmol) from the forward primer. The samples were treated with AB13730XL APPLIED BIOSYSTEMS machine in national instrumentation centre for environmental management NICM/USA company online at (http://nicem.snu.ac.kr/main/?en_skin=index.html). The result of the sequence analysis was analysed by blast in the National Centre Biotechnology Information (NCBI) online at (http:// www.ncbi.nlm.nih.gov) and Bio Edit program.

Results and Discussion

Patients with dyspepsia included 15 female and 15 male, aging between 19-70 years and mean age 45. They were underwent for diagnostic upper gastrointestinal endoscopy at endoscopy department in Al-Kadhmya Hospital in Baghdad, Iraq. Several gastric biopsy specimens were taken from antrum and body. Those isolates were collected had been identified at the species level using morphological characteristics and biochemical tests like urease production 100% of the isolates gave positive results for urease test. All the processes of DNA amplification were performed with the use of 16srRNA gene for the confirmation of *Helicobacter pylori* stains following the procedure published by Dapeng *et al.* (2010). 16srRNA gene was successfully amplified using specific PCR primer amplification of 16srRNA gene of 30 strains of *Helicobacter pylori* collected in the present study to confirm the presence of 16srRNA gene in the strains. The DNA extracts was subjected to PCR analysis to confirm the possible presence of 16srRNA gene. As expected DNA from all *Helicobacter pylori* produced clean bands upon amplification with 16srRNA gene set of specific primer. Figure (1) appeared that molecular weight of 16srRNA gene was 1500bp in the PCR product of *Helicobacter pylori* strains was exclusively used to proceed for the sequencing analysis assay to detect the polymorphism in gene content.

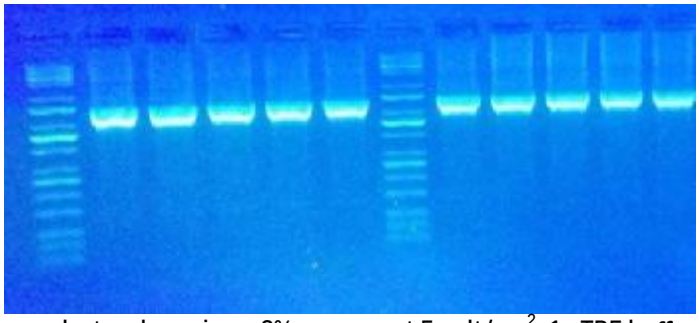

Figure (1) The product was electrophoresis on 2% agarose at 5 volt/cm^2. 1x TBE buffer for 1:30 hours. N: DNA ladder (100), lane (1-10) PCR product of band size 1500bp, visualized under U.V light.

Sequencing of this gene was performed to detect mutation related to development of polymorphism. Sequences alignment using BLAST and BioEdit showed that the 99% similarity with NCBI, score 1517 and expect 0.0 of the 16srRNA gene of *Helicobacter pylori* strain BO418 from the gene bank (Figure 2).

Helicobacter pylori partial 16S rRNA gene, strain BO418 Sequence ID: emb|AJ310144.1|

Score	Expect	Identities	Gaps	Strand
1517 bits(821)	0.0	833/839(99%)	0/839(0%)	Plus/Plus

```
Query  1    CTGGCGGCGTGCCTAATACATGCAAGTCGAACGATGAAGCTTCTAGCTTGCTAGAATGCT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    CTGGCGGCGTGCCTAATACATGCAAGTCGAACGATGAAGCTTCTAGCTTGCTAGAATGCT  60

Query  61   GATTAGTGGCGCACGGGTGAGTAACGCATAGGTCATGTGCCTCTTAGTTTGGGATAGCCA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GATTAGTGGCGCACGGGTGAGTAACGCATAGGTCATGTGCCTCTTAGTTTGGGATAGCCA  120

Query  121  TTGGAAACGTTGATTAATACCAGATACTCCCTACGGGGGAAAGATTTATCGCTAAGAGAT  180
            ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TTGGAAACGATGATTAATACCAGATACTCCCTACGGGGGAAAGATTTATCGCTAAGAGAT  180

Query  181  CAGCCTATGTCCTATCAGCTTGTTGGTAAGGTAATGGCTTCCCAAGGCTATGACGGGTAT  240
            |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct  181  CAGCCTATGTCCTATCAGCTTGTTGGTAAGGTAATGGCTTACCAAGGCTATGACGGGTAT  240

Query  241  CCGGCCTGAGAGGGTGAACGGACACACTGGAACTGAGACACGGTCCAGACTCCTACGGGA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  CCGGCCTGAGAGGGTGAACGGACACACTGGAACTGAGACACGGTCCAGACTCCTACGGGA  300

Query  301  GGCAGCAATAGGGAATATTGCTCAATGGGGGAAACCCTGAAGCAGCAACGCCGCGTGGAG  360
            ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  GGCAGCAGTAGGGAATATTGCTCAATGGGGGAAACCCTGAAGCAGCAACGCCGCGTGGAG  360

Query  361  GATGAAGGTTTTAGGATTGTAAACTCCTTTTGTTAGAGAAGATAATGACGGTATCTAACG  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  GATGAAGGTTTTAGGATTGTAAACTCCTTTTGTTAGAGAAGATAATGACGGTATCTAACG  420

Query  421  AATAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAC  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  AATAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAC  480

Query  481  TCGGAATCACTGGGCGTAAAGAGCGCGTAGGCGGGATAGTCAGTCAGGTGGGAAATCCTA  540
            |||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
```

```
Sbjct 481 TCGGAATCACTGGGCGTAAAGAGCGCGTAGGCGGGATAGTCAGTCAGGTGTGAAATCCTA 540

Query 541 TGGCTTAACCATAGAACTGCATTTGAAACTACTATTCTAGAGTGTGGGAGAGGTAGGTGG 600
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 541 TGGCTTAACCATAGAACTGCATTTGAAACTACTATTCTAGAGTGTGGGAGAGGTAGGTGG 600

Query 601 AATTCTTGGTGTAGGGGTAAAATCCGTAGAGATCAAGAGGAATACTCATTGCGAAGGCGA 660
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 601 AATTCTTGGTGTAGGGGTAAAATCCGTAGAGATCAAGAGGAATACTCATTGCGAAGGCGA 660

Query 661 CCTGCTGGAACATTACTGACGCTGATTCCGCGAAAGCGTGGGGAGCAAACAGGATGAGAT 720
      |||||||||||||||||||||||||| |||||||||||||||||||||||||| ||||
Sbjct 661 CCTGCTGGAACATTACTGACGCTGATTGCGCGAAAGCGTGGGGAGCAAACAGGATTAGAT 720

Query 721 ACCCTGGTAGTCCACGCCCTAAACGATGGATGCTAGTTGTTGGAGGGCTTAGTCTCTCCA 780
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 721 ACCCTGGTAGTCCACGCCCTAAACGATGGATGCTAGTTGTTGGAGGGCTTAGTCTCTCCA 780

Query 781 GTAATGCCACTAACGCATTAAGCATCCCGCCTGGGGAGTACGGTCGCAAGATTAAAACT 839
      |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 781 GTAATGCCACTAACGCATTAAGCATCCCGCCTGGGGAGTACGGTCGCAAGATTAAAACT 839
```

Figure (2): Sequencing of sense flanking the partial 16srRNA gene in *Helicobacter pylori* compared with standard 16srRNA obtained from Gene Bank. Query represents of sample; Sbject represent of database of National Center Biotechnology Information (NCBI).

The expected polymorphism were found after the analysis of the sequences as listed in table (1). There are 5 transversion substitution in the 30 *Helicobacter pylori* with sequence ID emb|AJ310144.1| location at range of nucleotide from 5128 to 5408 that caused change adenine to thiamin, adenine to cytocine, thiamin to guanine, guanine to cytosine and thiamin to guanine, and transition, resulted in a change of guanine to adenine compared with data obtained from gene bank as show in Figure (2).

Table (1): Types of mutations detected in partial 16srRNA gene in *Helicobacter pylori*

Type of mutation	Nucleotide	Range of nucleotide	Sequence ID
Transversion	A>T	5128 to 5408	emb|AJ310144.1|
Tansversion	A>C	5128 to 5408	emb|AJ310144.1|
Transition	G>A		emb|AJ310144.1|
Transversion	T>G		emb|AJ310144.1|
Transversion	G>C		emb|AJ310144.1|
Transversion	T>G		

The 16S rRNA gene is used as the standard for classification and identification of microbes, because it is present in most microbes and shows proper changes. Type strains of 16S rRNA gene sequences for most bacteria and archaea are available on public databases such as NCBI. However, the quality of the sequences found on these databases is often not validated. Therefore, secondary databases that collect only 16S rRNA sequences are widely used (Brodersen *et al.,* 2009). Conventional methods to assess levels of Polymorphism of *H. pylori* are based on culture in combination with agar dilution (Wu *et al.,* 2002 and Realdi *et al.,* 2009). Since sequencing analysis

seems to be restricted to the occurrence of specific mutations in a small region of the 16S rRNA molecule (Gerrits *et al.,* 2002; Dailidiene *et al.,* 2002 and Trieber, and Taylor, 2012), molecular methods an attractive and alternative. In the present study a PCR-based on sequencing analysis was used to detect the presence of the substitution in the 16S rRNA genes. This assay distinguishes the high-level of polymorphism in isolates from the data from equencing analysis of 16S rRNA genes in *H. pylori* strains. Since all the isolates show high-level of polymorphism that's may be linked to therapy failure (Megraud *et al.,* 1999; Silva *et al.,* 2002 and Godoy *et al.,* 2003), this sequencing analysis approach is useful for the detection of clinically relevant levels of polymorphism in *H. pylori*. It is striking that all characterized *H. pylori* isolates contain mutations in the exact same 16S rRNA region, especially because these isolates were obtained from dyspeptic patients living in same geographic regions (Gerrits *et al.,* 2010). This observation suggests that *H. pylori* require mutations within the 16S rRNA primary binding site for antibiotic resistance. Probably this resistance arises by mutations, although the acquisition of mutant 16S rRNA alleles through horizontal gene transfer cannot be excluded (Tomb *et al.,* 2007 and Alm *et al.,* 2009), these finding lead to conclusion, our assay allows rapid detection of polymorphism in *H. pylori*.

References

Alm, R.A.; Ling, L.S.; Moir, D.T.; King, B.L.; Brown; E.D.; Doig, P.C.; Smith, D.R.; Noonan; B.; Guild, B.C.; deJonge, B.L. 2009. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. Nature, 397: 176-180.

Boor, K. J.; M.L. Duncan and Price,C.W. 2011. Genetic and transcriptional organization of the region encoding the-subunit of *Bacillus subtilis* RNA polymerase. J. Biol. Chem., 270: 20329–20336.

Brodersen, D.E.; Clemons Jr.; W.M., Carter.; A.P. Morgan-Warren.; R.J., Wimberly, B.T.; and Ramakrishnan, V. 2009 The structural basis for the action of the antibiotics tetracycline, pactamycin, and hygromycin Bon the 30S ribosomal subunit. Cell, 103: 1143-1154.

Chong, S.K.F.; Lou, Q.; Fitzgerald, J.F. and Lee, C.H. 2015. Evaluation of 16S rRNA gene PCR with primers Hp1 and Hp_2 for detection of *Helicobacter pylori*. J. Clin. Microbiol., 34: 2728–2730.

Chopra, I. and Roberts, M. 2001. Tetracycline antibiotics : mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol. Mol. Biol. Rev., 65: 232-260.

De Reuse, H.; Labigne, A. and Mengin-Lecreulx, D. 2014. The *Helicobacter pylori ureC* gene codes for a phosphoglucosamine mutase. J. Bacteriol., 179: 3488–3493.

Dailidiene, D.; Bertoli, M.T.; Miciuleviciene, J.; Mukhopadhyay, A.K.; Dailide, G., Pascasio; M.A., Kupcinskas; L. and Berg, D.E. 2002 Emergence of tetracycline resistance in Helicobacter pylori: multiple mutational changes in 16S ribosomal DNA and other genetic loci. Antimicrob. Agents Chemother., 46: 3940-3946.

Dapeng, Z.; Zhifang L.; Jian D.; Wenjuan Li and Yundong S. 2010. A standardized mouse model of Helicobacter pylori infection. J. Med. Microbiol., 59: 259–265.

Gerrits, M.M.; Berning, M.; van Vliet; A.H.M.; Kuipers, E.J. and Kusters, J.G. 2010 Effects of 16S rRNA gene mutations on tetracycline resistance in *Helicobacter pylori*. Antimicrob. Agents Chemother., 47: 2984-2986.

Gerrits, M.M.; de Zoete, M.R.; Arents, N.L.; Kuipers, E.J. and Kusters, J.G. 2002. 16S rRNA mutation-mediated tetracycline resistance in Helicobacter pylori. Antimicrob. Agents Chemother., 46: 2996-3000.

Gisbert, J.P. and Pajares, J.M. 2002. Review article : *Helicobacter pylori* 'rescue' regimen when proton pump inhibitor-based triple therapies fail. Aliment. Pharmacol. Ther., 16: 1047-1057.

Godoy, A.P.; Ribeiro, M.L.; Benvengo, Y.H.B.; Vitiello, L.; Miranda, M.C.B.; Mendonca, S. and Pedrazzoli Jr., J. 2003. Analysis of antimicrobial susceptibility and virulence factors in *Helicobacter pylori* clinical isolates. BMC Gastroenterol., 320.

Jean-Philippe V.; Nicolas, F. and Yves, V. 2006. How to treat *Helicobacter pylori*. First-line, second-line, and future therapies. BMC Bioinformatics, 7(520): 1471-2105.

Kobayashi, I.; Hiroe, M.; Saika, T.; Nishida, M.; Fujioka, T. and Nasu, M. 2004. Microdilution method with air-dried microplate for determining MICs of clarithromycin and amoxicillin for *H. pylori* species. J. Med. Microbiol., 53: 403-406.

McMulty, C.; Owen, R.; Tompkins, D.; Hawtin, P.; McColl, K.; Price, A.; Smith, G.; Teare, L. 2002. *Helicobacter pylori* distribution, and response to triple therapy. Hum. Pathol., 24: 577-583.

Megraud, F.; Lehn, N.; Lind, T.; Bayerdor!er, E.;

O'Morain, C.; Spiller, R.; Unge, P.; Veldhuyzen van Zanten, S.J.; Wrangstadh, M. and Burman, C.F. 1999. Antimicrobial susceptibility testing of Helicobacter pylori in a large multicenter trial : the MACH 2 study. Antimicrob. Agents Chemother., 43: 2747-2752.

Realdi, G.; Dore, M.P.; Piana, A.; Atzei, A.; Carta, M.; Cugia, L.; Manca, A.; Are, B.M.; Massarelli, G.; Mura, I.; Maida, A. and Graham, D.Y. 2009. Pretreatment antibiotic resistance in *Helicobacter pylori* infection: results of three randomized controlled studies. Helicobacter, 4: 106- 112.

Silva, F.M.; Eisig, J.N.; Chehter, E.Z.; Silva, J.J. and Laudanna, A.A. 2002. Omeprazole, furazolidone, and tetracycline : an eradication treatment for resistant Helicobacter pylori in Brazilian patients with peptic ulcer disease. Rev. Hosp. Clin. Fac. Med. Sao Paulo, 57: 205-208.

Tomb, J.F.; White, O.; Kerlavage, A.R.; Clayton, R.A.; Sutton, G.G.; Fleischmann, R.D.; Ketchum, K.A.; Klenk, H.P.; Gill, S.; Dougherty, B.A.; Nelson, K. 2007. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature, 388: 539-547.

Trieber, C.A. and Taylor, D.E. 2012. Mutations in the 16S rRNA genes of Helicobacter pylori mediate resistance to tetracycline. J. Bacteriol., 184: 2131-2140.

Wu, H.; Shi, X.D.; Wang, H.T. and Liu, J.X. 2000. Resistance of Helicobacter pylori to metronidazole, tetracycline and amoxycillin. J. Antimicrob. Chemother., 46: 121-123.