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Improved PCR-based Methods for Detecting Helical Forms of Helicobacter Pylori

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Abstract The paper would address Helicobacter pylori which is a starting of bacteria in the gastrointestinal tract's human epithelial cells. His disorder is linked with many common illnesses, including recurrent gastritis, peptic ulcers, gastrointestinal lymphoma, and adenocarcinoma. More than 50 percent of the world is found to be infected with Helicobacter pylori. It is found deep inside the stomach of humans, where neutral HP predominates, in epithelial cells that are secreting mucus. This bacteria has been identified as the primary cause of a variety of lymphoma-related gastrointestinal illnesses, including chronic gastritis, excretory ulcer, abdominal cancer, and lymphoma of the gastric mucosa, among others (MALT). In this study, two PCR-based techniques for the detection of Helical types of Helicobacter pylori were improved. Molecular treatment of a strain of Helicobacter Pylori using amplification and direct analysis of 16S rDNA PCR.

Keywords*: molecular marker, PCR, gene, diagnosis, helicobacter pylori King Fahad Hospital*

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1. Introduction

Helicobacter pylori (H. pylori) are small, curved/spiral, highly motile, react negatively on Gram stain. It is a pathogenic bacterium, colonizes the stomach for life [1], it usually induces different human pathological disorders. *H. pylori* infection previously called Campylobacter pylori cause considerable morbidity, and directly imposes on human health, and infects billions of patients worldwide [2]. It inhabits and colonizes the upper digestive system, is considered the main cause of persistent gastritis, peptic ulcers, and gastric cancer. This pathogenic bacterium is transmitted through the contaminated water and food as well as fecal contaminated hands and oral to oral routes [3].

H. Pylori infection is completely asymptomatic or has almost imperceptible symptoms. Only the gastric inflammatory process becomes intense, and symptoms such as abdominal burning that gets worse on an empty stomach, abdominal pain, nausea and vomit, diarrhea, lack of appetite, Frequent belching swelling unexplained weight loss hunger in the morning halitosis [4,5]. The pathogenicity and the colonization of *H. pylori* different strains are virulence factors dependence: 1. Cytotoxin Associated Gene A (cag A) is a predisposing factor for gastric ulcers and cancer. 2. Vacuolating Toxin A (VacA) is a large vacuole inducer and disrupts the gastric epithelial barrier. 3. Both the sialic acid (SabA) and blood group antigen (BabA) binding adhesin are bacterial surface proteins (adhesins) prerequisites for their colonization [6]. Chronic *H. Pyloric* infections were reported in 50% of the world's population. Comparatively, a 10% prevalence among children living in either high or 80% in low-income countries has been estimated [7]. It is particularly common in countries where most of the population is below sixteen years [8]. Recently it is thought that *H. Pyloric* infection is particularly related to low socioeconomic status rather than to race. The prevalence varies over 80% in Japan and South America; about 40% in England and 20% in Scandinavia. The prevalence of infection in the white population of the United States of America, South Africa, and Europe is similar, while the other group's ethnic groups have double prevalence values [9]. Globally, *H. pylori* respectively caused 70-85% and 90-95% gastric and duodenal ulcers. Based on the *H. pylori* published research, it is considered as a group 1 carcinogen and increased the risk for other diseases [10]. In a recent study conducted among Saudi children a high prevalence (49.8%) was reported in Jeddah and Riyadh [11] and 54.9% among Patients with Dyspepsia [12] and 70% with gastric ulcers [13].

2. Materials and Methods

In these 11 years (2008-2018) retrospective, a cross-sectional hospital-based study was carried at the

histopathology laboratory, King Fahad Hospital, Madinah Monawaarrah, KSA. Formalin-fixed paraffin wax embedded blocks (FPEB) made from endoscopic gastric biopsies samples taken from *H. pylori*-infected patients that have previously been detected by light microscopic examination of a histopathological section stained by Hematoxylin and Eosin stain (H &E) and/or Giemsa stains and that kept as archives during the period from 2008 to 2018 were retrieved and were included in the current study.

2.1. Molecular Procedures

To identify the most *H. pyloric* active gene in Madinah isolate, a simultaneously selected sample from peptic ulcer patients obtained by biopsy forceps of endoscope were tested by PCR for the *H. pylori* mutation gene.

2.2. DNA Extraction

To extract DNA from paraffin slices, the researchers employed the following procedure. 100 Fixed Formalin Paraffin-Embedded (FFPE) tissues were dewaxed and deparaffinized, and the tissue pieces were segmented into 50-nanometer (50-nm) tissue pieces (the process comprised 3 washes in xylene for 3 minutes followed by 3 washes in 99.8 percent ethanol for 3 minutes). The DNA was extracted from FFPE tissue sections that were obtained from gastric biopsies of the targeted population as described previously (reference). Briefly, multiple 5-micron thick sections were taken from each block into a DNAse-free RNAse-free 1.5mL microcentrifuge tubes. Then, 700µl of 100% Xylene was added to each 1.5mL tube, the mixture was incubated at 30℃ for 15 min. The tubes were centrifuged at 16,000g for 3 min to remove the supernatant and 700µl of absolute Ethanol was added for washing the sample pellet. The procedure was performed twice more to ensure that all traces of Xylene were eliminated. The tubes were finally Incubated at 37℃ for 15 min to evaporate any Ethanol residue.

The gSYNC DNA Extraction Kit (Geneaid Biotechnology, New Taipei City) was used according to the manufacturer's recommendations to extract genomic DNA from the pelleted cell suspension. After each tube was filled with 200 mL of GST and 20 mL of Proteinase K, it was incubated at 60 degrees Celsius for an overnight period. The supernatant was moved to a fresh 1.5mL microcentrifuge tube after centrifugation at 16000g for 2 minutes. Each tube received 200 mL of GSB, which was gently mixed for 10 seconds before being used. 200µl of absolute Ethanol was added to the mixture before transferring the entire contents to GS columns. Flow-through was removed from the columns by centrifuging them at 16000g for 2 minutes, after which the column was transferred to a fresh 2mL collecting tube. The gDNA was then washed twice using 400µl and 600µl of W1 buffer consecutively. GS columns were loaded with 50 mL of pre-heated elution buffer, which was then allowed to stand for 3 minutes at room temperature to enable the elution buffer to be fully absorbed. DNA was finally eluted from the GS columns by centrifuging the columns at 16000g for 30 seconds. Subsequently, the quantity and quality of DNA from all obtained samples were evaluated using spectrophotometrically measurement of absorbance at 260/280 nm wavelength using NanoDrop 1000 UV-VIS Spectrophotometer (Thermo Fisher Scientific, Massachusetts). The obtained DNA was stored at -20℃ until used.

2.3. Quality Assessment of DNA:

To assess the viability and integrity of extracted DNA from the FFPE sections, we aimed to amplify a housekeeping gene using PCR. 1 l of ATL-1 primers (100pM pH8), 1 l of Template DNA, 8 l of dH2O, and 10 l of GoTaq PCR master mix (Promega, Wisconsin) were used to make the PCR reaction. An initial denaturation at 95℃ for 2 minutes was followed by 35 cycles of denaturation at 95℃ for 15 seconds, annealing at 52℃ for 10 seconds, and elongation at 72℃ for 35 seconds on a Veriti 96-Well Thermal Cycler (Applied Biosystems, Massachusetts). After a final elongation step of 10 minutes at 72℃, the samples were run on a 1.5 percent gel for 40 minutes at 90 volts using the M12 Complete Electrophoresis Package (Edvotek Inc, Washington). ChemiDoc-It2 Imaging was used to observe the amplification bands under UV light.

2.4. PCR Target

Two pieces of isolated DNA were amplified using PCR primers. The primer sequences were based on a published sequence. Primers K1 (5′- CCA CAG CGA TGT GGT CTC AG-3′) and K2 (5′- CTC CAT AAG AGC CAA AGC CC-3′). Primers K3 (5′- GCA CAA GCC AGC CTG ACT G-3′) and K4 (5′- AGC AGT TAT CAC ATC CGT G -3′). The PCR reaction was prepared by adding 1ul of primers (K1, K2) or (K3, K4) (100pM pH8), 1 µl of Template DNA, 8 µl of dH 2O, and 10 µl of GoTaq PCR master mix (Promega, Wisconsin). Applied Biosystems' Veriti 96-Well Thermal Cycler (Applied Biosystems, Massachusetts) was used to perform the thermal cycling, which began with an initial denaturation at 95℃ for 1 minute and continued with 35 cycles of denaturation at 95℃ for 15 seconds, annealing at 55℃ for 15 seconds, and elongation at 72℃ for 30 seconds. The samples were subjected to a final elongation phase at 72℃ for 7 minutes before being run on a 1.5 percent gel for 40 minutes at 90 volts utilizing the M12 Complete Electrophoresis Package (Edvotek Inc., Washington) for 40 minutes. With the help of the ChemiDoc-It2 Imaging System, the amplification bands were seen under ultraviolet light (Analytik Jena, Thuringia).

2.5. DNA Sequence Analysis

Three out of 31 DNA samples that showed acceptable PCR amplification for the ATL1 gene were randomly selected. the Following fragment corresponding to nucleotides to *H. pylori* 23S (GenBank accession number U27270) was amplified using 2 primer sets (K_1 (5′- CCA CAG CGA TGT GGT CTC AG-3′) and K_2 (5′- CTC CAT AAG AGC CAA AGC CC-3′), Primers K_3 (5′- GCA CAA GCC AGC CTG ACT G-3′) and K_4 (5′- AGC AGT TAT CAC ATC CGT G -3′). *H. pylori* 23S ribosomal RNA genes were sequenced and analyzed to perform the DNA alignment.

3. Results

3.1. Molecular Procedures

Total nucleic acid extraction from starting materials showed acceptable results with 31 (31%) samples showing good quality DNA and RNA. Quantification was performed on all extracted DNA samples to assess the required volume when performing our PCR protocols.

3.2. Extracted DNA

DNA was extracted from 100 FFPE tissues sections purified and its concentration was tested using NanoDrop photometer and PCR products were electrophoresed on a 1.2% agarose gel.

3.3. Quality Assessment of DNA

To the ratio of the concentration of DNA's purity was measured, the purity of DNA was quantified by the property of its absorbance at 260 and 280 nm (A260/A280). The result of DNA concentration range from 1.39 to 158.9 and an average of 27.6 ng/µL with a 260/280 ratio range from 0.67 to 3.64 and the average is 1.82. The results of the analyzed DNA purity using full-spectrum spectrophotometer ND-1000 UV-V Spectrophotometer (NanoDrop Technologies) were demonstrated in (Figure 1)

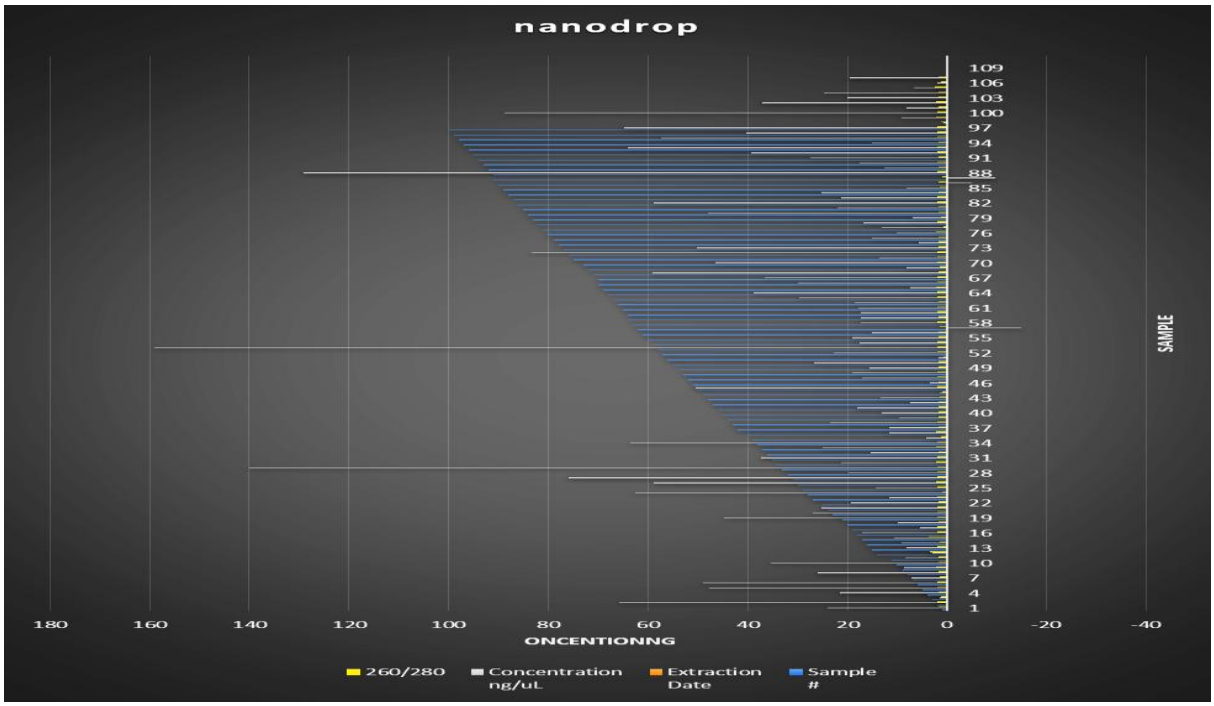

Figure 1. The multiple (100) DNA samples purity using ND-1000 UV-V Spectrophotometer (NanoDrop Technologies)

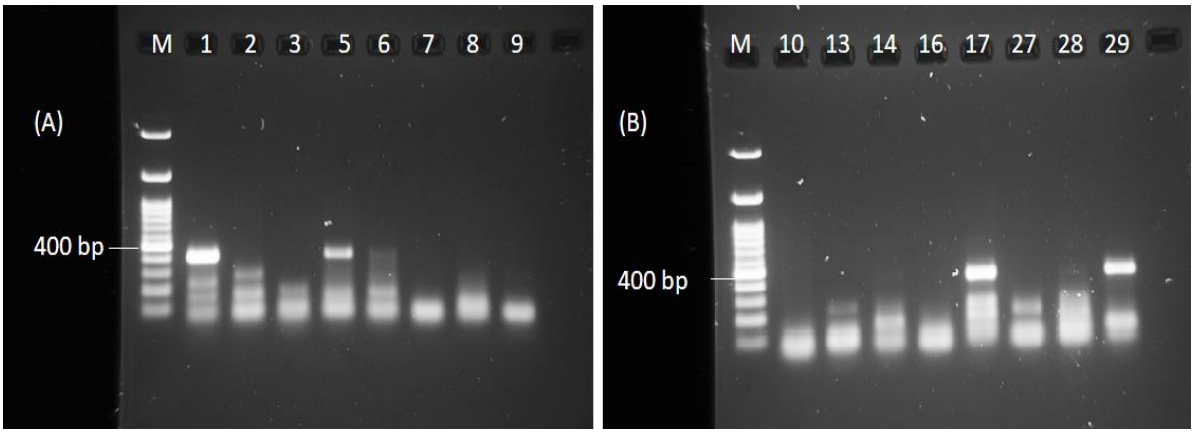

Figure 2. (A, B): PCR first runs (1.5% Agarose gel) used the K_1 and K_2 pairs of oligonucleotides amplified another fragment of the peptidyl transferase region of the 23S rRNA of *H. Pylori*. Lane $_{M,}$ standard size marker, Lane $_{1\text{-}29}$ test samples

3.4. PCR Target

Documentations of *H. pylori* by PCR using the specific primer sets were performed and the results of 1.5% agarose electrophoresis analysis of PCR products were de

To test the integrity of the eluted DNA, PCR was run and the regions of the ATL1 gene from all samples were amplified. ATL1 genes are found in chromosome X of humans and were considered internal control in this study. After that, samples that showed good amplification in the

internal control were used to complete the analysis of this study. In total, 31 samples showed acceptable PCR amplification for the ATL1 gene and were used as starting materials for two suspect PCR runs. The result of the first run used the K1 and K2 pair of oligonucleotides was illustrated in (Figure 4). This pair amplifies a fragment of the peptidyl transferase region of the 23S rRNA of *H. Pylori.* Out of the 100 assessed samples, only 12 samples showed successful amplification of this region. The result of the second run used the K3 and K4 pairs of oligonucleotides were demonstrated in (Figure 5). This pair amplifies another fragment of the peptidyl transferase region of the 23S rRNA of *H. Pylori*. Out of the 100 assessed samples, only 19 samples showed successful amplification of this region.

Monstrated in (Figure 6).

3.5. DNA Sequences

Three random DNA samples out of the 31 samples that showed acceptable PCR amplification for the ATL1 gene were chosen for DNA sequencing from each of the two groups K_1 + K_2 and K_3 + K_4 oligonucleotides. The result of The complete DNA sequencing for *H. pylori* 23S and 5S ribosomal RNA genes sequenced DNA random samples showed point mutation in one of the 3 (33.3%) analyzed samples in both (K_1 + K_2) and (K_3 + K_4) oligonucleotides used. K_1 + K_2 oligonucleotides result in two point's mutation in A2143G and A2203G and were illustrated in (Figure 6) K_3 + K_4 oligonucleotide result in one mutation at the G2727T and demonstrated in (Figure 7).

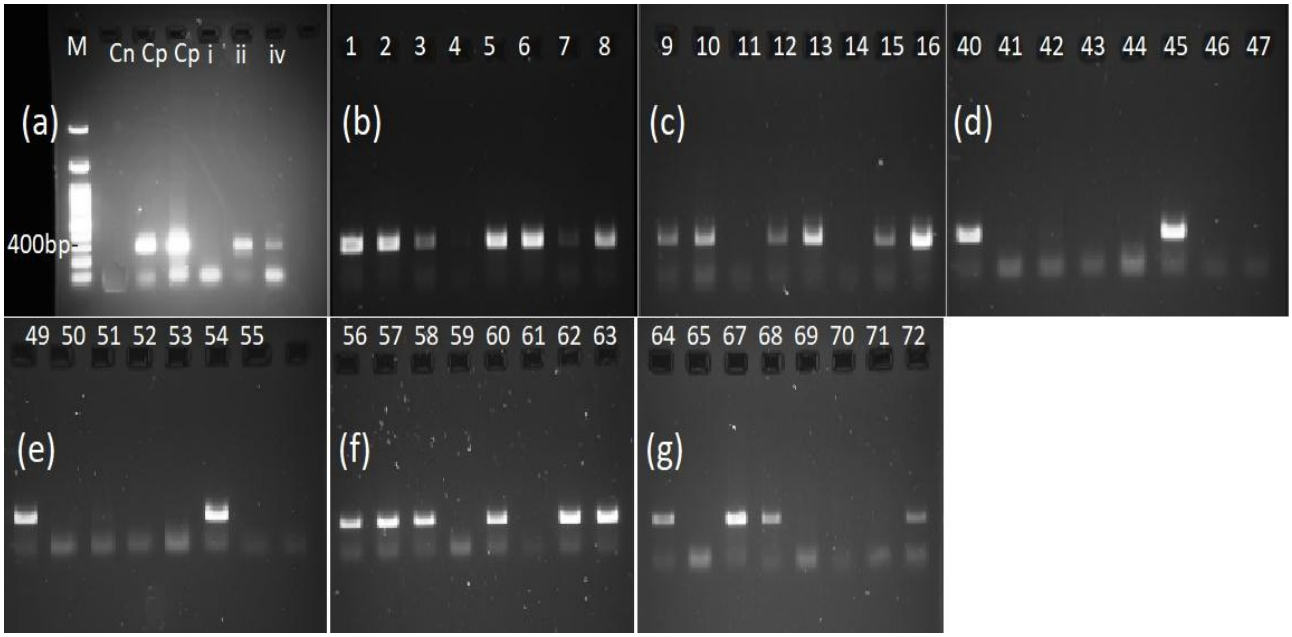

Figure 3. (a-g): Documentations of *H. pylori* by PCR using *H. pylori* specific primer sets. Image of 1.5% Agarose gel Lane $_{M,}$ standard size marker, L_{Cn} negative control, L_{Cp} positive control, $L_{1\text{-}72}$ test samples

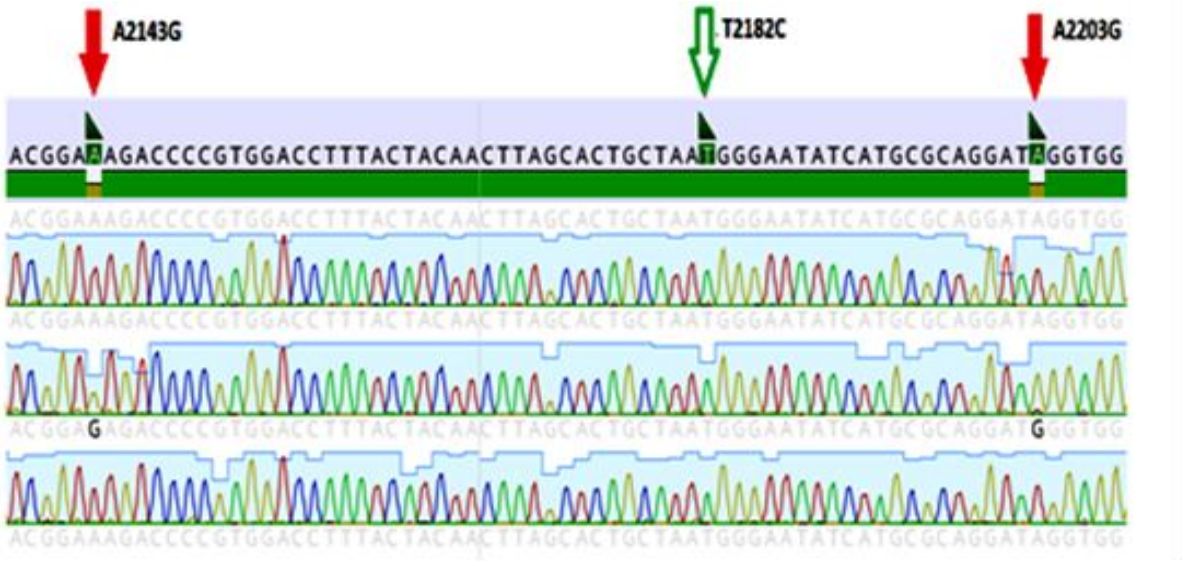

Figure 4. Mutant 23s rRNA gene sequence chromatogram using K_1K_2 oligonucleotides. Sequencing traces with peaks of a fluorescent signal corresponding to peak (A) at A2143G mutation and Peak (A) at A2203G (red arrows) showed point mutations in *H. pylori* 23s rRNA, peak T at T2182C was not detected (green arrow)

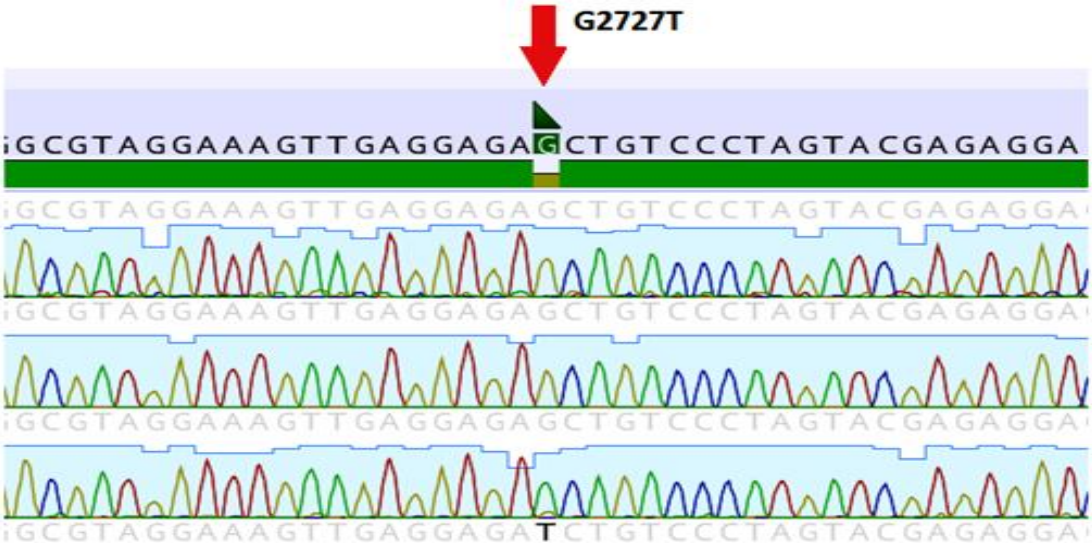

Figure 5. Mutant 23s rRNA gene sequence chromatogram using K3K4 oligonucleotides. Sequencing traces with peaks of a fluorescent signal corresponding to Peak (G) at G2727T mutation (red arrow) showed point mutations in H. pylori 23s rRNA

Helicobacter pylori gene for 23S ribosomal RNA, partial sequence, isolate: Nepal90

Sequence ID: LC184330.1 **Length:** 397 **Number of Matches:** 3

Range 1: 43 to 397 GenBank Graphics ▼ Next Match ▲ Pr

Score	Expect	Identities	Gaps	Strand
645 bits(349)	2e-180	353/355(99%)	0/355(0%)	Plus/Plus

```
Query  851   TAAGAGGAAGTATAATGTGTGACCCCTGCCCGGTGCTCGAAGGTTAAGAGGATGCGTCAG  910
Sbjct  43    TAAGAGGAAGTATAAGGTGTGACGCCTGCCCGGTGCTCGAAGGTTAAGAGGATGCGTCAG  102
Query  911   TCGCAAGATGAAGCGTTGAATTGAAGCCCGAGTAAACGGCGGCCGTAACTATAACGGTCC  970
Sbjct  103   TCGCAAGATGAAGCGTTGAATTGAAGCCCGAGTAAACGGCGGCCGTAACTATAACGGTCC  162
Query  971   TAAGGTAGCGAAATTCCTTGTCGGTTAAATACCGACCTGCATGAATGGCGTAACGAGATG  1030
Sbjct  163   TAAGGTAGCGAAATTCCTTGTCGGTTAAATACCGACCTGCATGAATGGCGTAACGAGATG  222
Query  1031  GGAGCTGTCTCAACCAGAGATTCAGTGAAATTGTAGTGGAGGTGAAAATTCCTCCTACCC  1090
Sbjct  223   GGAGCTGTCTCAACCAGAGATTCAGTGAAATTGTAGTGGAGGTGAAAATTCCTCCTACCC  282
Query  1091  GCGGCAAGACGGAAAGACCCCGTGGACCTTTACTACAACTTAGCACTGCTAATGGGAATA  1150
Sbjct  283   GCGGCAAGACGGAAAGACCCCGTGGACCTTTACTACAACTTAGCACTGCTAATGGGAATA  342
Query  1151  TCATGCGCAGGATAGGTGGGAGGCTTTGAAGTAAGGGCTTTGGCTCTTATGGAGA  1205
Sbjct  343   TCATGCGCAGGATAGGTGGGAGGCTTTGAAGTAAGGGCTTTGGCTCTTATGGAGA  397
```

Figure 6 Alignment of H. pylori Saudi Arabia isolated (K1) strain. Pairwise sequence alignment of isolates showing identity 99.4% to reference strain Sequence ID: LC184330.1 from Genebank (Query)

Helicobacter pylori strain GCT 97 chromosome, complete genome

Sequence ID: CP048599.1 **Length:** 1656646 **Number of Matches:** 5

Range 1: 1601729 to 1602113 GenBank Graphics ▼ Next Match ▲ Previous M

Score	Expect	Identities	Gaps	Strand
693 bits(375)	0.0	382/385(99%)	2/385(0%)	Plus/Plus

```
Query  11       AGCAGAGACG-ACGTCGGTCATAGTGATCCGGTGGTTCTGTGTGGAAGGGCCATCGCTCA  69
Sbjct  1601729  AGCAGAGACGAAAGTCGGTCATAGTGATCCGGTGGTTCTGTGTGGAAGGGCCATCGCTCA  1601788
Query  70       AAGGATAAAAGGTACCCCGGGGATAACAGGCTGATCTCCCCCAAGAGCTCACATCGACGG  129
Sbjct  1601789  AAGGATAAAAGGTACCCCGGGGATAACAGGCTGATCTCCCCCAAGAGCTCACATCGACGG  1601848
Query  130      GGAGGTTTGGCACCTCGATGTCGGCTCATCGCATCCTGGGGCTGGAGCAGGTCCCAAGGG  189
Sbjct  1601849  GGAGGTTTGGCACCTCGATGTCGGCTCATCGCATCCTGGGGCTGGAGCAGGTCCCAAGGG  1601908
Query  190      TATGGCTGTTCGCCATTTAAAGCGGTACGCGAGCTGGGTTCAGAACGTCGTGAGACAGTT  249
Sbjct  1601909  TATGGCTGTTCGCCATTTAAAGCGGTACGCGAGCTGGGTTCAGAACGTCGTGAGACAGTT  1601968
Query  250      CGGTCCCTATCTGCCGTGGGCGTAGGAAAGTTGAGGAGAGCTGTCCCTAGTACGAGAGGA  309
Sbjct  1601969  CGGTCCCTATCTGCCGTGGGCGTAGGAAAGTTGAGGAGAGCTGTCCCTAGTACGAGAGGA  1602028
Query  310      CCGGGATGGACGTGTCACTGGTGCACCAGTTGTTCTGCCAAGAGCATCGCTGGGTAGCTA  369
Sbjct  1602029  CCGGGATGGACGTGTCACTGGTGCACCAGTTGTTCTGCCAAGAGCATCGCTGGGTAGCTA  1602088
Query  370      CACACGGATGTGATAACTGCT-AAA  393
```

Figure 7. Alignment of H. pylori Saudi Arabia isolated (K3) strain. Pairwise sequence alignment of isolates showing identity 99.2% to reference strain Sequence ID: CP048599.1 from Genebank (Query)

Table 1. *H. pylori* isolate (K_1) strain 23s rRNA gene sequence similarity

Strain	E value	Identity	Accession No.
Nepal114	e 2.00-180	99.44%	LC184333.1
Nepal90	e 2.00E-180	99.44%	LC184330.1
GMC173	e 2.00E-180	99.44%	MH445304.1

Table 2. *H. pylori* isolate (K_3) strain 23s rRNA gene sequence similarity

Strain	E value	Identity	Accession No.
GCT 97 chromosome	e 0.0	99.22%	CP048599.1
G-Mx-2003-108 chromosome	e 0.0	99.22%	CP032044.1
G-Mx-2003-93 chromosome	e 0.0	99.22%	CP032043.1
G-Mx-2006-152 chromosome	e 0.0	99.22%	CP032040.1
G-Mx-2006-583 chromosome	e 0.0	99.22%	CP032039.1
G-Mx-2011-124 chromosome	e 0.0	99.22%	CP032038.1
C-Mx-2011-145 chromosome	e 0.0	99.22%	CP032034.1
C-Mx-2011-152 chromosome	e 0.0	99.22%	CP032025.1
C-Mx-2010-12 chromosome	e 0.0	99.22%	CP032024.1
C-Mx-2010-3 chromosome	e 0.0	99.22%	CP032023.1
C-Mx-2011-69 chromosome	e 0.0	99.22%	CP032020.1
B140 chromosome, complete	e 0.0	99.22%	CP024948.1
B136A chromosome, complete	e 0.0	99.22%	CP024949.1
B130A chromosome, complete	e 0.0	99.22%	CP024950.1
B125A chromosome, complete	e 0.0	99.22%	CP024952.1
13-A-EK8 chromosome	e 0.0	99.22%	CP032912.1
19-A-EK3 chromosome,	e 0.0	99.22%	CP032911.1
20-A-EK1 chromosome,	e 0.0	99.22%	CP032910.1
21-A-EK1 chromosome,	e 0.0	99.22%	CP032909.1
25-A-EK1 chromosome,	e 0.0	99.22%	CP032906.1

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