Nucleotide variations of 16S rRNA gene of VacA positive *Helicobacter pylori* strains isolated from human Gastric Biopsies in Saudi Arabia

Milyani MR

[1]Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia
*Corresponding author: rajaamilyani@hotmail.com

ABSTRACT: Three isolates of *Helicobacter pylori* (*H. pylori*) were originally isolated from gastric biopsies taken from patients complaining of gastric disorders in Makkah City, Saudi Arabia. The isolates that previously revealed to be vaculating cytotoxin A positive were identified by 16S rRNA gene as *H. pylori* using a primer pair designed from the similar sequences within consensus regions of GenBank *H. pylori* to amplify the 163 bp fragment. Sequence alignments of 16S rRNA gene were performed and total numbers of 46, 55 and 40 nucleotide positional differences with base-pair substitutions were identified for these isolates compared to GenBank strains of *H. pylori*. Phylogenetic analyses based on 16S rRNA gene sequences showed that the three *H. pylori* strains formed a phylogenetically distinct group, separate from all other species of *H. pylori*. The three isolates were hence coined as *H. pylori* Milyani-1, -2 and -3 at GenBank database under the accession numbers HQ877021, HQ877022 and HQ877023, respectively. The obtained results evidently indicated a large diversity with unique characteristics of the three Saudi Arabian *H. pylori* strains from all the other established strains.

1.INTRODUCTION

Helicobacter pylori is a Gram-negative, microaerophilic, motile bacterium, that has been implicated in the aetiology of most gastritis, duodenal ulcers and is associated with lymphoproliferative disorders as well as gastric carcinoma. Some evidence also suggests that it is a possible factor in the development of cardiovascular disease (**Cover and Blaser 1992;Aceti *et al*. 2004**).

At the discovery of *H. pylori*, identification was carried out by morphological, biochemical and physiological studies, in addition to histological and serological investigations **(Lee and Megraud, 1996).** However, by the emergence of the new technology of polymerase chain reaction (PCR), researchers started to detect *H. pylori* using PCR, 16S rRNA gene, ureA and cagA genes (**Clayton *et al*., 1992, Twing *et al*. 2011).** The sequence of a fragment of the 16S rRNA gene was determined by **Khan *et al*. (2000)** for ten strains of *H. pylori* to examine the contribution of point mutation within a conserved gene. There were few differences between the sequences from the various strains and it was concluded that such differences were not the most important source of diversity**. Monstein *et al*. (2001)** used real-time DNA sequence analysis of *H. pylori* 16S rRNA gene fragments by pyro-sequencing for rapid molecular identification and

sub-typing of clinical isolates. The latter experiment showed that subtle DNA sequence variation occurs sufficiently often in the 16S rRNA variable V1 and V3 regions of *H. pylori* and the authors concluded that their findings can provide a consistent system for sub-typing. On the other hand, **Trieber and Taylor** (**2002**) identified several unique sequence variations in the 16S rRNA genes of *H. pylori* strain 26695, and these have been placed on a secondary structure model of the *H. pylori* 16S rRNA. Moreover, natural transformation with the 16S rRNA genes from *H. pylori* resistant strains conferred tetracycline resistance on susceptible strains. Furthermore, it has now been established that using PCR of 16S rRNA gene is considered as a powerful tool for identification of different types of

micro-organisms. It has also become the primary method for determining prokaryotic phylogeny and is currently the basis for prokaryotic systematic (**Monsttein *et al.* 2000, Dewhirst *et al.* 2005**).

The aim of the present study was to identify three new *H. pylori* isolates obtained from three male patients complaining of gastric disorders at Makkah Almokarama City, Saudi Arabia, based on the variation analysis of 16S rRNA gene sequences.

2.MATERIALS AND METHODS
2.1.Materials:
The present molecular study was performed in Genetics and Cytology Dept., National Research Centre (NRC), Cairo, Egypt.

2.1.1.Clinical specimens:
Three gastric biopsies obtained from three male patients complaining of chronic active gastritis were provided by Gastroenterology-endoscopy Consultant at Al-Noor Specialist Hospital at Makkah Almokarama City, Saudi Arabia.

2.1.2. Specific primers for 16S rRNA gene
Ten NCBI-different accessions (DQ059083, DQ059082, U01332, U01331, U01330, U01329, U01328, AJ310144, AJ310143 and HPU00679) of *H. pylori* 16S rRNA gene were multiple alignments to design a pair of primers; forward 5 - GTGTGGGAGAGGTAGGTGGA-3 and reverse 5 - GTTTAGGGCGTGGACTACCA-3 with a product size of 163 bp.

2.2.Methods:
2.2.1.Isolation and identification:
The gastric biopsies were cultured and incubated under microaerophylic conditions on Blood and Chocolate agar at 37° C for five days. Culture and Identification was carried out according to **Milyani and Barhameen (2004)**.

2.2.2.DNA extraction and PCR amplification of 16S rRNA gene
DNA extraction was performed using the Wizard® SV kit (Promega, Madison, USA). PCR-amplification reaction was used according to **Williams *et al.* (1990)** in a final volume of 25 μl containing 10X PCR buffer (10 mM Tris-HCl, 50

mM $MgCl_2$, 2 mM dNTPs, 10 mM of each forward and reverse primers, 50 ng of template DNA and 5 U of *Taq* polymerase (Promega, USA). Reactions were performed in a thermocycler (Biometra, GmbH, Germany) and PCR was performed as one cycle of 94°C for 3 min (denaturation), 40 cycles of 94°C for 30 sec, 36°C for 1 min and 72°C for 1 min (annealing) and with a final extension of 5 min at 72°C. PCR amplified product was analyzed using 1.2% agarose gel electrophoresis in 1X TBE buffer by staining with 5 μg/μl ethidium bromide and visualized under UV light. The size of the 16S rRNA fragment of 163 bp was estimated based on a 100 bp DNA ladder (Bioron, Germany).

2.2.3. 16S rRNA gene purification, sequencing and analysis
PCR product of 163 bp was purified with the QIA quick PCR Purification Kit (Qiagen GmbH, Germany) according to the manufacturer's instructions. DNA was eluted in 20 μl of sterile water. The 16S rRNA fragment was sequenced on an Applied Biosystems automatic sequencer (ABI PRISM® 1200 DNA Sequencer, Bioron GmbH, Germany).

Sequences were compared with sequences of representatives of the most related *H. pylori* strains deposited in GenBank and sequencing-genome databases by using the BLAST search (http://www.ncbi.nlm.nih.gov/blast). Analysis was performed using Geneious Pro 4.5.4 program. A phylogenetic tree was supported from 500 bootstrap replicates and a dendrogram was constructed using multiple alignment of the 16S rRNA from *H. pylori* isolates and strains

3.RESULTS
The colonies obtained from culturing the three gastric biopsies showed typical cellular morphology of *H. pylo* in addition to motility and to their vigorous positivity to urease, oxidase and catalase.

3.1.PCR amplification of 16S rRNA in *Helicobacter pylori* isolates
PCR amplifications of the three *H. pylori* isolates revealed the fragments with expected sizes of 163 bp that represented the 16S rRNA gene (**Fig. 1**).
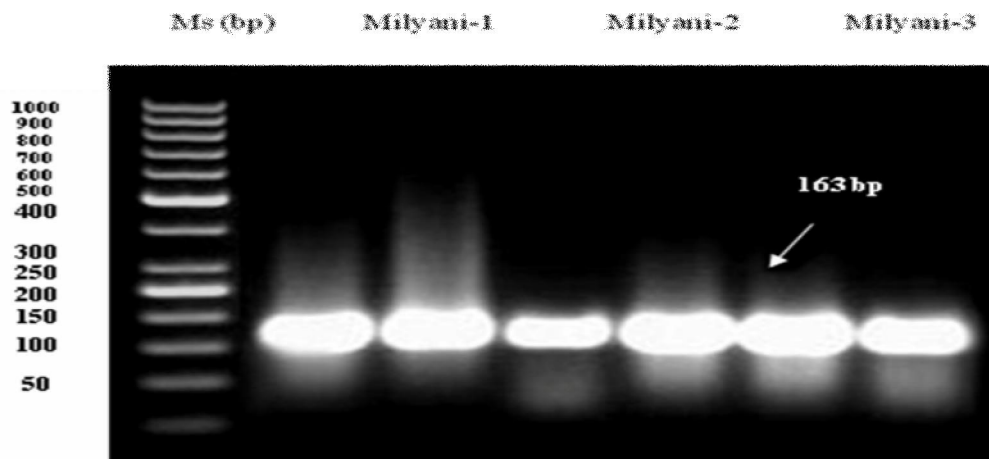
137

Fig. 1. PCR amplified products of 16S rRNA gene of the three *H. pylori* isolates using designed primer with 163 bp. M= DNA ladder with 100 bp

3.2. Sequence analysis of PCR-amplified 16S rRNA of the *H. pylori* isolates

A 163 bp nucleotide sequence of the partial 16S rRNA gene from the three *H. pylori* isolates were aligned and compared in the GenBank using the BLAST search. A total of 121 to 129 16S ribosomal RNA gene partial sequences from different accessions of *H. pylori* included different strains, 9 isolates and one clone were identified **Table (1)**. Blast alignment revealed several accessions of *H. pylori* scored 99% maximum identity for Milyani-1 and 98% maximum identity for Milyani-2 and Milyani-3, except strain DA (AY366422) with 96%.

Sequence alignments of the 16S rRNA gene of the *H. pylori* isolates (HQ877021, HQ877021 and HQ877021, respectively) compared with *H. pylori* GenBank strains and isolates revealed positional differences in nucleotide sequences and base-pair substitutions between the three isolates and the numerous isolates and strains **(Fig. 2)**.

The accession HQ877021 with a fragment size 163 bp (isolate Milyani-1) showed a total number of 46 nucleotide positional differences with base-pair substitutions, whereas the highest number (6) of positional differences was cytosine (C) found that changed to Guanine (G), followed by (G) to Adenine (A) in 5 positional differences and five from Thymine (T) to (A) in all other GenBank isolates and strains as shown in **Table 2**. A single base change or

a mixed base (more than one nucleotide determined at a single position) is considered as a new 16S type. Four other nucleotide positional differences were obtained from A' G, C' A and G' T. Moreover, three of four nucleotide positional differences were obtained from (…) with no nucleotide base to G, T, A and one from (…) to C. One positional difference at nucleotide number 819 was detected from C to (…).

The accession HQ877022 with a fragment size 163 bp (isolate Milyani-2) showed a total number of 55 nucleotide positional differences with base-pair substitutions, whereas the highest number (7) of positional differences was adenine (A) found in HQ877023 that changed to thymine (T) in all other GenBank isolates and strains as shown in **Table (2)**. Six nucleotide positional differences were obtained from C' A, C' G and G' A and one with five positional differences from A' G. Moreover, three of four nucleotide positional differences were obtained from (() with no nucleotide base to G, T, A and one from (() to C. One positional difference at nucleotide number 819 was detected from A to ((.

The accession HQ877023 with a fragment size 163 bp (isolate Milyani-3) showed a total number of 40 nucleotide positional differences

138

with base-pair substitutions, whereas the highest number (4) of positional differences was adenine (A) found in HQ877023 that changed to thymine (T) in all other Gene Bank isolates and strains as shown in Table (2). Three nucleotide positional differences were obtained from A(C and C(T and four with two positional differences from A(G, C(G, G(A and T(A. Moreover, 7, 6, 5 and 3 nucleotide positional differences were obtained from (() with no nucleotide base and changed to G, T, A and C, respectively. One positional difference at nucleotide number 819 was detected from A to (().

Consequently, the overall total number of nucleotide positions of the three isolates was 141 and the highest changes in nucleotide positions were 14 from C'G, followed by 13 from A'T and G'A. However, the lowest changes were two from T'C and three from T'G with no change was obtained from G'C (**Table 2).**

139

Table 1. **Blast search of 16S rRNA gene sequence identity between the three *H. pylori* isolates (Milyani-1, -2 and -3) and GenBank sequences.**

Accession	Isolates	Clone	Accession	Strains	Accession	Strains
GU165836		ISMA-03-DP	GQ403476	C114	GQ403465	M57
AF512997	GB-38		GQ403474	C74	GQ403464	C56
AF214518	GB-16		GQ403473	M74	GQ403463	M56
AF214517	GB-9		GQ403472	C64	GQ403460	C48
AF214516	IHD-32		GQ403471	M64	GQ403459	M48
AF214509	WE64a		GQ403470	C59	GQ403454	C38
HM046432	WG56		GQ403469	M59	GQ403453	M38
HM046431	C51		GQ403468	C58	GQ403452	C35
AY394476	IHD-11		GQ403467	M58	GQ403451	M35
AF214508	MDA-1397		GQ403466	C57	GQ403450	C34
			GQ403449	M34	AY364437	LPB582-99
			GQ403444	C26	AY304571	LPB-64B
			GQ403443	M26	AY304570	LPB-3B
			GQ403438	C23	AY304569	LPB-1B
			GQ403437	M23	AY304551	LPB-5V
			GQ403436	C22	AY456638	SS1
			GQ403435	M22	AF297631	SA-4
			GQ403434	C20	AF297630	SA-3
			GQ403433	M20	AY062898	108
			GQ403432	C19	AY155586	1200
			GQ403431	M19	AF535197	Lit76
			HM243135	CD4	AF535196	Lit69
			HM099656	407D5	AF535195	Sal10
			FJ788641	08561-160209	AF535194	Sal05
			FJ788640	311A-160209	DQ059083	B
			AY593991	LPB36-03	DQ059082	A
			AY593990	LPB05-02	AY366424	PA
			AY593988	LPB258-01	AY366423	MB
			AY593987	LPB638-99	AY366421	SS1
			AY593986	LPB581-99	AJ310144	BO418
			AY364439	LPB427-01	AJ310143	BO417
			AY364438	LPB473-00	AY366422	DA

140

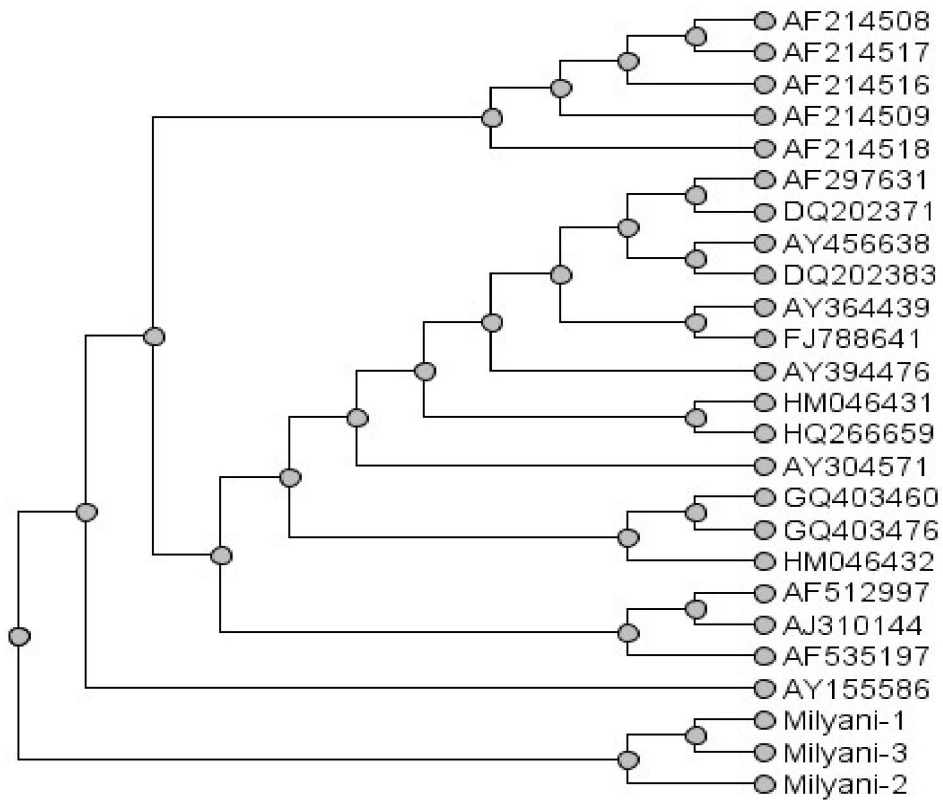|	601 700
Milyani-1	TGCT CTAG-AGGAT ACTCATTGCG AAGGCGACCT
Milyani-2	---A AGAA-GGAAT ACTCATTGCG AAGGCGACCT
Milyani-3	---- CAAGTAGGAT ACTCATTGCG AAGGCGACCT
AF512997	TGAAACTACT ATTCTAGAGT GTGGGAGAGG TAGGTGGAAT TCTTGGTGTA GGGGTAAAAT CCGTAGAGAT CAAGAGGAAT ACTCATTGCG AAGGCGACCT
AY394476	TGAAACTACT ATTCTAGAGT GTGGGAGAGG TAGGTGGAAT TCTTGGTGTA GGGGTAAAAT CCGTAGAGAT CAAGAGGAAT ACTCATTGCG AAGGCGACCT
HM046432	TGAAACTACT ATTCTAGAGT GTGGGAGAGG TAGGTGGAAT TCTTGGTGTA GGGGTAAAAT CCGTAGAGAT CAAGAGGAAT ACTCATTGCG AAGGCGACCT
HM046431	TGAAACTACT ATTCTAGAGT GTGGGAGAGG TAGGTGGAAT TCTTGGTGTA GGGGTAAAAT CCGTAGAGAT CAAGAGGAAT ACTCATTGCG AAGGCGACCT
AY304571	TGAAACTACT ATTCTAGAGT GTGGGAGAGG TAGGTGGAAT TCTTGGTGTA GGGGTAAAAT CCGTAGAGAT CAAGAGGAAT ACTCATTGCG AAGGCGACCT
AF214518	TGAAACTACT AT-CTAGAGT GTGGGAGAGG TAGGTGGAAT TCTTGGTGTA GGGGTAAAAT CCGTAGAGAT CAAGAGGAAT ACTCATTGCG AAGGCGACCT
AF214517	TGAAACTACT ATTCTAGAGT GTGGGAGAGG TAGGTGGAAT TCTTGGTGTA GGGGTAAAAT CCGTAGAGAT CAAGAGGAAT ACTCATTGCG AAGGCGACCT
AF214516	TGAAACTACT ATTCTAGAGT GTGGGAGAGG TAGGTGGAAT TCTTGGTGTA GGGGTAAAAT CCGTAGAGAT CAAGAGGAAT ACTCATTGCG AAGGCGACCT
AF214509	TGAAACTACT ATTCTAGAGT GTGGGAGAGG TAGGTGGAAT TCTTGGTGTA GGGGTAAAAT CCGTAGAGAT CAAGAGGAAT ACTCATTGCG AAGGCGACCT
AF214508	TGAAACTACT ATTCTAGAGT GTGGGAGAGG TAGGTGGAAT TCTTGGTGTA GGGGTAAAAT CCGTAGAGAT CAAGAGGAAT ACTCATTGCG AAGGCGACCT
	701 800
Milyani-1	GCTGGAACAT TACTGACGCT GATTGCGCGA AAGCGTGGGG AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCCCTAAA CAGTG----- ----TGTGGC
Milyani-2	GCTGGAACAT TACTGACGCT GATTGCACGA AAGCGTGGGG AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCCCTAAA CAGTG----- ----TG-GCC
Milyani-3	GCTGGAACAT TACTGACGCT GATTGCGCGA AAGCGTGGGG AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCCCTAAA CAGTG----- ----TGTTGG
AF512997	GCTGGAACAT TACTGACGCT GATTGCGCGA AAGCGTGGGG AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCCCTAAA CGATGGATGC TAGTTGTTGG
AY394476	GCTGGAACAT TACTGACGCT GATTGCGCGA AAGCGTGGGG AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCCCTAAA CGATGGATGC TAGTTGTTGG
HM046432	GCTGGAACAT TACTGACGCT GATTGCGCGA AAGCGTGGGG AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCCCTAAA CGATGGATGC TAGTTGTTGG
HM046431	GCTGGAACAT TACTGACGCT GATTGCGCGA AAGCGTGGGG AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCCCTAAA CGATGGATGC TAGTTGTTGG
AY304571	GCTGGAACAT TACTGACGCT GATTGCGCGA AAGCGTGGGG AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCCCTAAA CGATGGATGC TAGTTGTTGG
AF214518	GCTGGAACAT TACTGACGCT GATTGCGCGA AAGCGTGGGG AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCCCTAAA CGATGGATGC TAGTTGTTGG
AF214517	GCTGGAACAT TACTGACGCT GATTGCGCGA AAGCGTGGGG AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCCCTAAA CGATGGATGC TAGTTGTTGG
AF214516	GCTGGAACAT TACTGACGCT GATTGCGCGA AAGCGTGGGG AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCCCTAAA CGATGGATGC TAGTTGTTGG
AF214509	GCTGGAACAT TACTGACGCT GATTGCGCGA AAGCGTGGGG AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCCCTAAA CGATGGATGC TAGTTGTTGG
AF214508	GCTGGAACAT TACTGACGCT GATTGCGCGA AAGCGTGGGG AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCCCTAAA CGATGGATGC TAGTTGTTGG
	801 843
Milyani-1	GACTCGTGAG AACCCCCCCT CACCCACCAG TCGAGTGTTC CAT
Milyani-2	ACAACACTTA CCCCCCCCAA CCCCCCCCAC ACGTGTGGGT TGA
Milyani-3	TG----TTGC CACCACACAT A-----GCCA AAGCGAAAAC CAACCACCAA C
AF512997	AGGGCTTAGT CTCTCCAG-T AATGCAGCTA ACGCATTAAG CATCCCGCCT GGGGAGTACG GTCGCAAGAT TAAAACTCAA AGGAATAGAC GGGGACCCGC

AY394476	AGGGCTTAGT CTCTCCAG-T AATGCAGCTA ACGCATTAAG CATCCCGCCT GGGGAGTACG GTCGCAAGAT TAAAACTCAA AGGAATAGAC GGGGACCCGC
HM046432	AGGGCTTAGT CTCTCCAG-T AATGCAGCTA ACGCATTAAG CATCCCGCCT GGGGAGTACG GTCGCAAGAT TAAAACTCAA AGGAATAGAC GGGGACCCGC
HM046431	AGGGCTTAGT CTCTCCAG-T AATGCAGCTA ACGCATTAAG CATCCCGCCT GGGGAGTACG GTCGCAAGAT TAAAACTCAA AGGAATAGAC GGGGACCCGC
AY304571	AGGGCTTAGT CTCTCCAG-T AATGCAGCTA ACGCATTAAG CATCCCGCCT GGGGAGTACG GTCGCAAGAT TAAAACTCAA AGGAATAGAC GGGGACCCGC
AF214518	AGGGCTTAGT CTTTCCAG-T AATGCAGCTA ACGCATTAAG CATCCCGCCT GGGGAGTACG GTCGCAAGAT TAAAACTCAA AGGAATAGAC GGGGACCCGC
AF214517	AGGGCTTAGT CTCTCCAG-T AATGCAGCTA ACGCATTAAG CATCCCGCCT GGGGAGTACG GTCGCAAGAT TAAAACTCAA - GGAATAGAC GGGGACCCGC
AF214516	AGGGCTTAGT CTCTCCAGAT AATGCAGCTA ACGCATTAAG CATCCCGCCT GGGGAGTACG GTCGCAAGAT TAAAACTCAA AGGAATAGAC GGGGACCCGC
AF214509	AGGGCTTAGT CTCTCCAGAT AATGCAGCTA ACGCATTAAG CATCCCGCCT GGGGAGTACG GTCGCAAGAT TAAAACTCAA AGGAATAGAC GGGGACCCGC
AF214508	AGGGCTTAGT CTCTCCAGAT AATGCAGCTA ACGCATTAAG CATCCCGCCT GGGGAGTACG GTCGCAAGAT TAAAACTCAA AGGAATAGAC GGGGACCCGC

Fig. 2.   Sequence alignment of 163 bp of 16S rRNA gene in three *H. pylori* isolates (Milyani-1, -2 and -3) compared with other isolates and strains existed in NCBI GenBank. Conserved nucleotides between the studied isolates and other sequences are boxed in black. Putative conserved between the different isolates with no identity with isolates are boxed in grey. The yellow box referred to the identity of all accessions except the studied isolates. Dashes correspond to gaps introduced to optimize the alignments.

Table 2.   Positional differences and base pair substitutions in nucleotide sequences between the three *H. pylori* isolates (Milyani-1, -2 and -3) and numerous isolates and strains based on 16S rRNA gene similarity.

Accession	Isolate	Exist in our accessions as: / Changed in NCBI accessions to:	A / C	A / G	A / T	C / A	C / G	C / T	G / A	G / T	G / C	T / A	T / C	T / G	(No nucleotide bases) … / C	… / G	… / T	… / A	C / …	G / …	T / …	A / … (No nucleotide bases)
HQ877021	**Milyani-1**	Change at nucleotide positions:	811 834	676 782 802 809	812 829	669 815 817 821	800 803 818 824 827 840	814 823	801 808 830 835	783 806 810 837	798	667 672 831 838 839		804	790 789 786	793 790 788	794 787 675	792				819
Total number of nucleotide positions = (46)			(8)			(12)			(9)			(6)			10				1			
HQ877022	**Milyani-2**	Change at nucleotide positions:	671 717 782	674	670		799		672 783	798					790 789 793	668 786 790 794	768	669 675 787 792 797				

			G1	G2	G3	G4	G5	G6
			803 806 804 808 810 820 829 843	815 800 807 817 802 812 821 818 814 822 824 823 826 827 830	835 837 838 839 842	841 809 834 840		819
Total number of nucleotide positions = (55)			**(13)**	**(16)**	**(8)**	**(4)**	**13**	**1**
HQ877023	**Milyani-3**	Change at nucleotide positions:	676 782 812 808 815 836 832 837 843	818 810 840 814 829	783 835	675 801	668 670 669 790 793 794 787 789 790 792 786 788 803 804 806 822 805 824 823 826 825	819
Total number of nucleotide positions = (40)			**(9)**	**(5)**	**(2)**	**(2)**	**21**	**1**
Overall total number of nucleotide positions = 141			6 11 13 **30**	10 14 9 **33**	13 6 0 **19**	7 2 3 **12**	5 14 13 12 **44**	1 0 0 2 **3**

Fig. 3. Phylogenetic relationships between the three _H. pylori_ isolates (Milyani-1, -2 and -3) other GenBank related strains, based on 16S ribosomal RNA gene.

The phylogenetic tree represented the relationship between the three *H. pylori* strains (Milyani-1, Milyani-2 and Milyani-3) with their accession numbers (HQ877021, HQ877022 and HQ877023, respectively) and all described *H. pylori* related strains obtained from Gene Bank based on the 16S ribosomal RNA gene are shown in **Fig. 3**. The dendrogram divided all Gene Bank strains into two clusters; one contained the three *H. pylori* strains and the second cluster comprised all GenBank strains. The three strains were divided into two sub-clusters, whereas Milyani-1 and Milyani-3 were linked together and separate from Milyani-2. In general, each of the three local strains formed a phylogenetically distinct cluster, separate from all other species.

4.Discussion

The traditional identification of bacteria in the clinical setting is based on phenotypic characteristics and biochemical tests which is generally not as accurate as identification based on molecular methods. Comparison of the bacterial 16S rRNA gene sequence has emerged as a preferred genetic technique since 16S rRNA gene sequence analysis can identify poorly described, rarely isolated and unculturable bacteria (**Clarridge 2004; Smuts and Lastovica, 2011**). In the present study, the three strains of *H. pylori*; Milyani-1, -2 and -3 isolated from gastric biopsies of patients in Makkah City, Saudi Arabia revealed variations in 16S rRNA sequence in positional differences with base-pair substitutions compared to Gene Bank *H. pylori* strains. On the other hand, each of the three strains displayed a unique phylogenetic cluster and evidently demonstrated a large diversity from all the other isolates and strains established around the world. This is in accordance with **Taylor *et al.* (1992)** who demonstrated that the genome patterns of *H. pylori* are so diverse that almost no two strains from different patients appear related. In addition, **Trieber and Taylor (2002)** identified several unique sequence variations in the 16S rRNA genes of *H. pylori* strain 26695. However, the obtained variations among the three strains under study and the enormous genomic diversity among other *H. pylori* strains is so far not understood. Nevertheless, it might be related to restriction and modification which could change restriction sites, point mutations in certain genes and different positions of genes on *H. pylori*

maps within the approximate 1.7-kb genome (**Taylor 1996**). Furthermore, allelic diversity is so high that almost every unrelated isolate of *H. pylori* has a unique sequence when a fragment of several hundred base pairs is sequenced from housekeeping or virulence genes (**Falush *et al.* 2003**). Others also believe that this allelic diversity is the result of the combination of a high (mutator-type) mutation rate, a high frequency of recombination between strains during mixed colonization and the ability of *H. pylori* to integrate unusually small pieces of exogenous DNA into its chromosome (**Kraft *et al.* 2006**). Consequently, the 16S rRNA of the three *H. pylori* strains are very polymorphic and confirm the conclusion that the three *H. pylori* are unique strains and each of them displayed a unique phylogenetic cluster and evidently indicated a large diversity from all the other isolates and from the established strains in different geographical parts of the world. Moreover, it is well known now, that 16S rRNA PCR test gives 100% specificity and sensitivity (**Moyaert et al. 2008**), thus sequencing and the phylogenetic analysis of the 16S rRNA are often utilized to identify new isolates (**Smuts and Lastovica 2011**).

It should be noticed that at the present study, the 163 bp fragment of 16S rRNA gene of the three *H. pylori* strains were delivered to Gene Bank/NCBI database according to the expected size of the designed primer pair and to their appearance on the agarose gel electrophoresis (Fig. 1). However, the size of the three fragments were finally reduced by the Gene Bank and submitted as 111 bp. This reduction in fragment size normally occurs when extraction of the fragments is from genomic DNA and not from sub-cloning experiment.

Finally, a future challenge is to translate information from 16S rRNA gene sequencing into convenient biochemical testing schemes, making the accuracy of the genotypic identification available to the smaller and routine clinical microbiology laboratories (**Clarridge 2004**). Above all, the obvious variations seen in the new three *H. pylori* strains may indicate differences in virulence factors, biochemical characteristics, antibiotic different patterns and the outcome of the clinical picture. In addition, these variations could be used in the future as new markers for virulence factors, diagnostic, therapeutic purposes for *H. pylori* different diseases and for further studies that would give answers to many question marks about the source, real route of

144

transmission, recurrence of *H. pylori* infection and the role of coccoid forms of *H. pylori* in pathogenesis.

5. References

1. **Aceti A, Are R, Sabino G, Fenu L,Pasquazzi C, Quaranta G, Zechini B and Terrosu P. (2004).** *Helicobacter pylori* active infection in patients with acute coronary heart disease. J. Infect. **49, 8-12.**

2. **Clarridge JE** (2004) Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clin Microbiol Rev* **17**, 840-**862**

3. **Clayton L C, Kleanthous H, Coates J P, Morgan D D and Tabaqchali S (1992).** Sensitive detection of Helicobacter pylori by using polymerase chain reaction. J Clin Microbiol. January; 30(1): 192-200.

4. **Cover T and Blaser M** (1992) Helicobacter pylori and gastroduodenal disease. The Annual Review of Medici ne 42, 135-145.

5. **Dewhirst FE, Shen Z, Scimeca MS, Stokes LN, Boumenna T, Chen T, Paster BJ, Fox JG** (2005) Discordant 16S and 23S rRNA gene phylogenies for the genus *Helicobacter*: implications for phylogenetic inference and systematic. *Journal of Bacteriology* **187**, 6106-6118.

6. **Falush, D., T. Wirth, B. Linz, J. K. Pritchard, M. Stephens, M. Kidd, M. J. Blaser, D. Y. Graham, S. Vacher, G. I. Perez-Perez, Y. Yamaoka, F. Megraud, K. Otto, U. Reichard, E. Katzowitsch, X. Wang, M. Achtman, and S. Suerbaum.** 2003. Traces of human migrations in *Helicobacter pylori* populations. Science **299:**1582–1585.

7. **Khan MM, Stoker NG, Drasar BS (2000).** Sequence diversity of a fragment of the 16S RNA gene from Helicobacter pylori. Microbios 103, 139-150.

8. **Kraft C, Stack A, Josenhans C, Niehus E, Dietrich G, Correa P, Fox JG, Falush D, Suerbaum S** (2006). Genomic changes during chronic *Helicobacter pylori* infection. Journal of Bacteriology **188**, 249-254.

9. **Lee A and Megraud F,** (1996) Helicobacter pylori: techniques for clinical diagnosis and basic research.W.B. Saunders Compony Ltd, London.

10. *Milyani M R, Barhameen AA (2004).* Incidence of *Helicobacter pylori* infection assessed by four different methods in Makkah city (Saudi Arabia). Egypt. J. Biomed. Sci. **14,** 113- 127.

11. **Monstein H, Nikpour-Badr S, Jonasson J** (2001) Rapid molecular identification and subtyping of *Helicobacter pylori* by pyrosequencing of the 16S rRNA variable V1 and V3 regions. *FEMS Microbiology Letters* **199**, 103-107.

12. **Moyaert H, Pasmans F, Ducatelle R, Haesebrouck F, Baele M** (2008) Evaluation of 16S rRNA gene-based PCR assays for genus-level identification of *Helicobacter* species. *Journal of Clinical Microbiology* **46**, 1867-1869.

13. **Smuts HEM and Lastovica AJ** (**2011**) Molecular Characterization of the 16S rRNA Gene of *Helicobacter fennelliae* isolated from stools and blood cultures from paediatric patients in South Africa. *Journal of Pathogens*. 2011, 1-9.

14. **Taylor DE** (1996) Diversity among *Helicobacter pylori* isolates. *Journal of Clinical Microbiology* **34**, 1041.

15. **Taylor DE, Eaton M, Chang N and Salama SM** (1992) Construction of a *Helicobacter pylori* genome map and demonstration of diversity at the genome level. J. Bacteriol. **174,** 6800-6806.

16. **Trieber CA and Taylor DE** (2002) Mutations in the 16S rRNA genes of *Helicobacter pylori* mediate resistance to tetracycline. *Journal of Bacteriology* **184**, 2131-2140.

17. **Twing KI, Kirchman DL, Campbell BJ** (2011) Temporal study of *Helicobacter pylori* presence in coastal freshwater, estuary and marine waters. *Water Research* **45**, 1897- 1905.

18. **Williams J G K, Kubelic A R, Livak K J, Rafalski J A and Tingey S V (1990).** DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18, 6531-6535.

5/19/2011