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

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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 *H. pylori* strains from all the other established strains.
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Key words: Accessions HQ877021, HQ877022 and HQ877023, *Helicobacter pylori*, isolates, 16S rRNA gene, variations.

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**.** However, by the emergence of the new technology of polymerase chain reaction (PCR), researchers started to detect *H. pylori* in gastric biopsy specimens using PCR, 16S rRNA gene, ureA and cagA genes **(Lee and Megraud, 1996, 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.

Materials And Methods

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.

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)**.

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).

Design of 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.

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.

The Molecular studies were carried out at the Department of Genetics and Cytology, National Research Centre, Cairo, Egypt.

Results

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

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**).

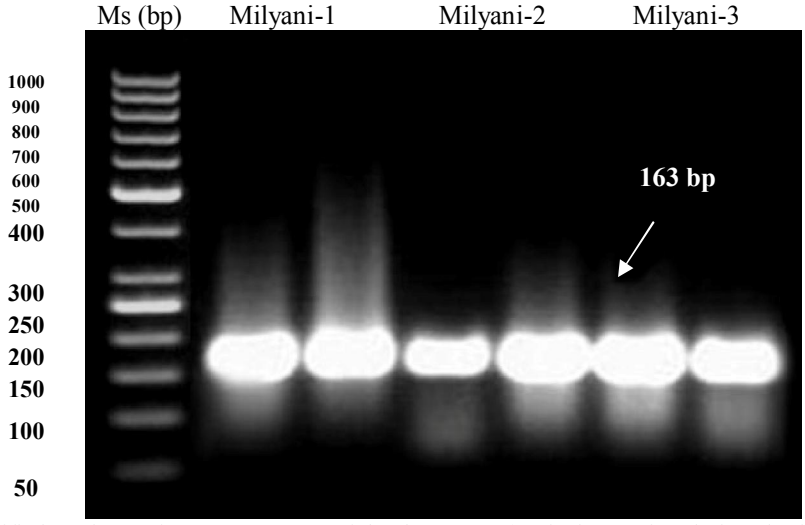


Fig. 1. PCR amplified products of 16S rRNA gene of the three *H. pylori* isolates using designed primer with 163 bp. M= DNA ladder with 100 bp

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 found to be cytosine (C) 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 (…).

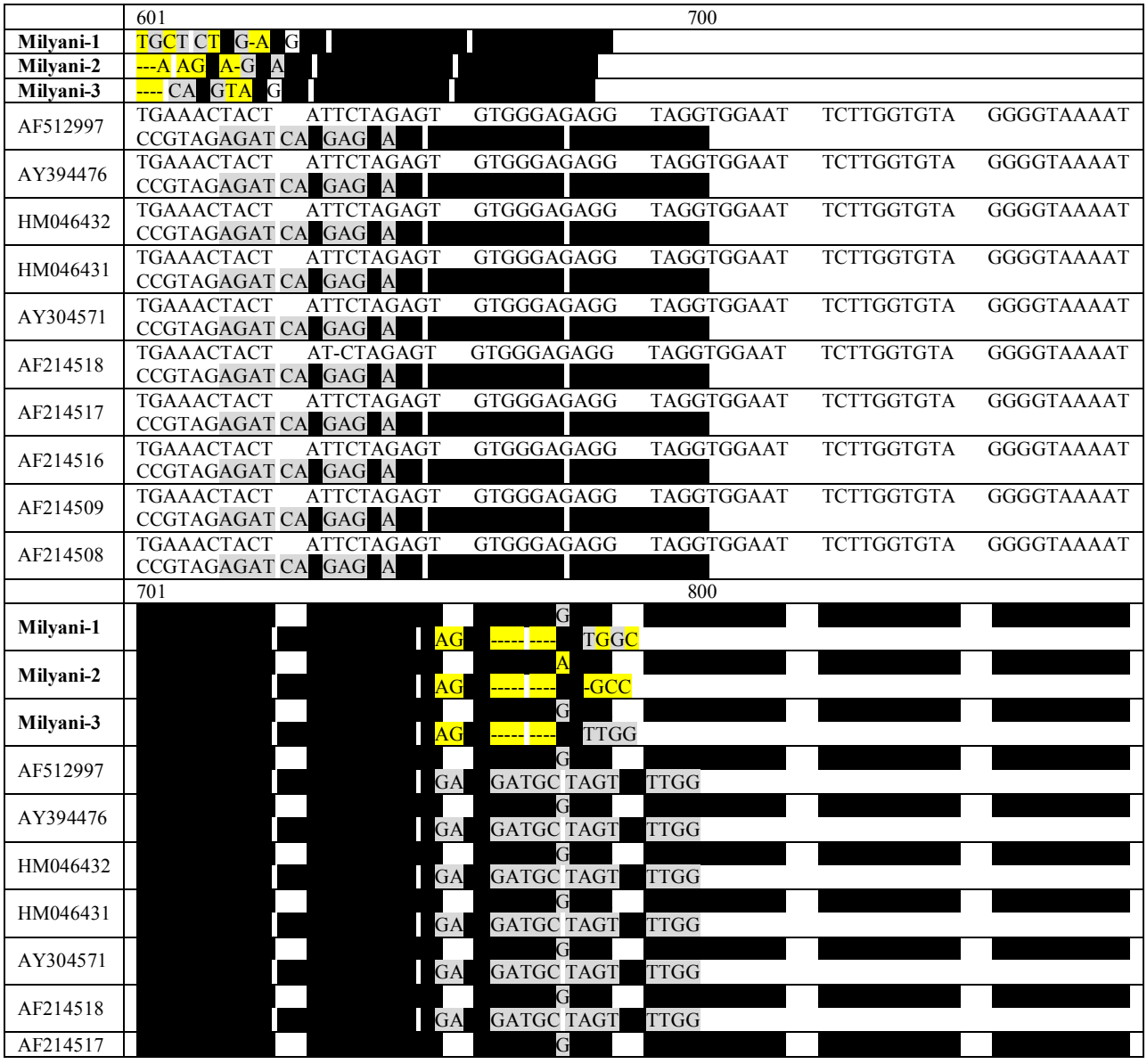
The accession HQ877023 with a fragment size 163 bp (isolate Milyani-3) showed a total number of 40 nucleotide positional differences with base-pair substitutions, whereas the highest number (4) of positional differences was adenine (A) found in HQ877023 that changed to thymine (T) in all other GenBank 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**).

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
Accession	**Isolates**	**Clone**		**Accession**	**Strains**		**Accession**	**Strains**



	GA GATGC TAGT TTGG
AF214516	G GA GATGC TAGT TTGG
AF214509	G GA GATGC TAGT TTGG
AF214508	G GA GATGC TAGT TTGG
	801 843
Milyani-1	GACTCGTGAG AACCC CCCT CACCCAC AG TC AGTGTTC CAT
Milyani-2	ACAACACTTA CCCCC CCAA CCCCCCC AC AC TGTGGGT TGA
Milyani-3	TG----TTGC CACCA ACAT A-----G CA AA CGAAAAC CAACCACCAA C
AF512997	AGGGCTTAGT CTCTC AG-T AATGCAG TA AC CATTAAG CATCCCGCCT GGGGAGTACG GTCGCAAGAT TAAAACTCAA AGGAATAGAC GGGGACCCGC
AY394476	AGGGCTTAGT CTCTC AG-T AATGCAG TA AC CATTAAG CATCCCGCCT GGGGAGTACG GTCGCAAGAT TAAAACTCAA AGGAATAGAC GGGGACCCGC
HM046432	AGGGCTTAGT CTCTC AG-T AATGCAG TA AC CATTAAG CATCCCGCCT GGGGAGTACG GTCGCAAGAT TAAAACTCAA AGGAATAGAC GGGGACCCGC
HM046431	AGGGCTTAGT CTCTC AG-T AATGCAG TA AC CATTAAG CATCCCGCCT GGGGAGTACG GTCGCAAGAT TAAAACTCAA AGGAATAGAC GGGGACCCGC
AY304571	AGGGCTTAGT CTCTC AG-T AATGCAG TA AC CATTAAG CATCCCGCCT GGGGAGTACG GTCGCAAGAT TAAAACTCAA AGGAATAGAC GGGGACCCGC
AF214518	AGGGCTTAGT CTTTC AG-T AATGCAG TA AC CATTAAG CATCCCGCCT GGGGAGTACG GTCGCAAGAT TAAAACTCAA AGGAATAGAC GGGGACCCGC
AF214517	AGGGCTTAGT CTCTC AG-T AATGCAG TA AC CATTAAG CATCCCGCCT GGGGAGTACG GTCGCAAGAT TAAAACTCAA -GGAATAGAC GGGGACCCGC
AF214516	AGGGCTTAGT CTCTC AGAT AATGCAG TA AC CATTAAG CATCCCGCCT GGGGAGTACG GTCGCAAGAT TAAAACTCAA AGGAATAGAC GGGGACCCGC
AF214509	AGGGCTTAGT CTCTC AGAT AATGCAG TA AC CATTAAG CATCCCGCCT GGGGAGTACG GTCGCAAGAT TAAAACTCAA AGGAATAGAC GGGGACCCGC
AF214508	AGGGCTTAGT CTCTC AGAT AATGCAG TA AC CATTAAG 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.

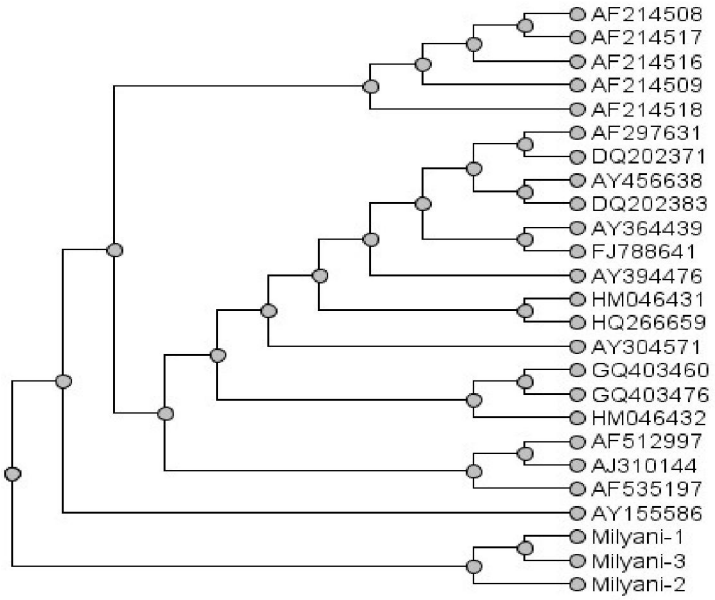


Fig. 3. Phylogenetic relationships between the three *H. pylori* isolates (Milyani-1, -2 and -3) other GenBank related strains, based on 16S ribosomal RNA gene.

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 … (No nucleotide bases)	G …	T …	A …
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	783 801 808 830 835	798 806 810 837		667 672 831 838 839		804	790	793 789 786	794 790 788	675 792 787	819			
Total number of nucleotide positions = (46)			**(8)**			**(12)**			**(9)**			**(6)**			**10**				**1**			
HQ877022	**Milyani-2**	Change at nucleotide positions:	671	674 717 782 803 804	670 806 808 810 820 829 843	815 817 821 822 826 830	800 802 818 824 827	807 812 814 823	672 783 835 838 839 842	798 837			841 834	809 840	790	668 786 789 793	768 790 794 797	669 675 787 792				819
Total number of nucleotide positions = (55)			**(13)**			**(16)**			**(8)**			**(4)**			**13**				**1**			
HQ877023	**Milyani-3**	Change at nucleotide positions:	812 815 832	676 782	808 836 837 843		818 840	810 814 829	783 835			675 801			790 803 805	668 793 789 786 804 824 825	670 794 790 788 806 823	669 787 792 822 826				819
Total number of nucleotide positions = (40)			**(9)**			**(5)**			**(2)**			**(2)**			**21**				**1**			
Overall total number of nucleotide positions = 141			**6** **30**	**11**	**13**	**10** **33**	**14**	**9**	**13** **19**	**6**	**0**	**7** **12**	**2**	**3**	**5** **44**	**14**	**13**	**12**	**1** **3**	**0**	**0**	**2**

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 GenBank based on the 16S ribosomal RNA gene are shown in **Fig. 3**. The dendrogram divided all GenBank 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.

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

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