Variations of VacA nucleotide and domain sequences of *Helicobacter pylori* coccoid forms isolated from a patient in Makkah City, Saudi Arabia

***[1]Rajaa M. Milyani and [2]Osama E. El-Sayed**

[1]Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia.
[2]National Research Centre, Genetics and Cytology Department Cairo, Egypt.

*Corresponding author:helicobacter2011@hotmail.com

ABSTRACT

Helicobacter pylori strain Makkah 7 was originally isolated from gastric biopsy of a patient complaining of chronic gastritis in Makkah City, Saudi Arabia. The resulted identified colonies were transferred to Cryovile containing thioglycolate broth with 15% glycerol and stored at -40°C for ten years. Storage under such conditions induced the transformation of the strain to coccoid forms. PCR amplification of Makkah 7 (accession no. HQ622108) revealed the 750 bp fragment that represented the VacA gene. Sequence alignment of VacA gene was performed and total number of 235 nucleotide positional differences with base-pair substitutions was identified compared with GenBank strains of *H. pylori*. Translation of the VacA sequence induced a putative signal polypeptide of 199 amino acids with a molecular weight of 140 kDa and 8.79 isoelectric point. The amino acid sequence comparison revealed that the predicted protein is a vacuolating cytotoxin A autotransporter domain (accession no. ADU05468). Amino acids alignment of Makkah 7 compared with 100 *H. pylori* strains obtained from GenBank revealed mutations in different positions. Phylogenetic analyses based on both VacA gene and its peptide domain sequences showed that Makkah 7 formed a phylogenetically distinct and a unique group. These findings emphasize the large variations in coccoid forms of *H. pylori* Makkah 7 strain which may influence its pathogenicity and characteristics.[Rajaa M. Milyani. Variations in Vac A nucleotides and peptides domain of *Helicobacter pylori* coccoid forms isolated from a patient suffering from gastritis in Makkah City, Saudi Arabia. Nature and Science 2011;9(8):122-137]. (ISSN: 1545-0740). http://www.sciencepub.net.

Key words: Accession HQ877021, *Helicobacter pylori*, coccoid forms, amino acids, VacA gene, sequence variations.

1.Introduction

Helicobacter pylori (*H. Pylori*) is spiral-shaped Gram-negative bacteria that are highly motile, micro-aerophylic and liable to transform into coccoid forms that are metabolically active but uncultivable when exposed to unfavorable conditions, **Bode *et al.* (1993);Azevedo, *et al*. (2007**).

Since the discovery of *H. Pylori* by **Warren and Marshall (1983)** as the aetiological agent of type B gastritis, a large accumulated data have been established –yet- with many unresolved problems. Although *H. Pylori* is an important human pathogen that causes peptic ulcer, duodenal ulcer, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma in addition to their possible role in cardiovascular disease (**Aceti, *et al.* (2004); Zhao, *et al.* (2007**) the mode of transmission, successful therapy and the efficacy of vaccines to prevent *H. Pylori* infection is still not clear and far away from our understanding. Coccoid forms of *H. Pylori* are still a mystery to many scholars, though their role in pathogenisity has been documented **Chan *et al.*,(1994); Saito *et al.*,(2003);Azevedo *et al.,*(2007); Milyani(2011**). Many researchers were attracted to the coccoid phase of the bacterium, started serious and important investigations - mostly up to the molecular levels – hoping to reveal the unknown so they can resolve the different troublesome problems that patients, physicians, pharmacologists and scientists face.

Several *H. Pylori* virulence factors in both spiral and coccoid forms have been identified and established in playing a role in its pathogenicity and the most important determinants are VacA and cagA genes,**Xiang *et al*., (1995);She(2001);Falush *et al.* (2003**). VacA encoded by the VacA gene, is known to be a key pathogenicity factor of *H. pylori* which infects the gastric mucosa of more than half of the human population worldwide (**Blaser and Atherton 2004**). VacA is a cytotoxin of the gastric epithelial cell layer and a potent immune-regulatory toxin inducing apoptosis in epithelial cells (**Gebert *et al.* (2004**). Furthermore, *H. Pylori* VacA sequences are well-characterized markers of *H. pylori* virulence, and have been identified as a strong marker of *H. pylori*-associated disease **Chung *et al.* (2010**). In addition, **Blaser and Atherton (2004)** recorded that the VacA gene, present as a single copy in the genomes of many *H. pylori* tested, and is highly polymorphic. On the other hand, **Yamaoka (2009)** stated that there are two types of cagA: the East Asian type and the Western type. Moreover, **Gangwer *et al*. (2010)**

reported that phylogenetic reconstructions of VacA allowed the subdivision of VacA sequences into three main groups with distinct geographic distributions. They postulated that the divergence of the three groups is principally due to positively-selected peptide domain sequence changes.

The aim of the present study was to determine and analyze the sequence of VacA gene and its polypeptide domain in a new *H. pylori* coccoid form isolated from a male patient suffering from chronic gastritis in Makkah Almokarama City, Saudi Arabia

2.MATERIALS AND METHODS

2.1.Materials:

2.1.1.Clinical specimen and bacterial isolate

Helicobacter pylori was isolated from a gastric biopsy of a male patient complaining of chronic gastritis, the clinical specimen was provided by the Gastroenterology department at Al-Noor Specialist Hospital at Makah almokarrama City, Saudi Arabia.

2.1.2. **Design specific primer for VacA gene**

The VacA gene was detected using two primers: VacA -F (5′ GCCAATTCAATGGCAATTCT 3′) and VacA -R: (5′ CGCTTGATTGGACAGATTGA 3′) procured from Bioron GmbH (Germany). The two primers were designed from the very similar sequences within the consensus gene regions of NCBI GenBank.

2.2.Methods:

2.2.1.Culture and Identification

Gastric biopsy was cultured on Blood and Chocolate agar using the rubbing technique and the plates were incubated under micro-aerophylic conditions at 37°C for five days ,**Milyani and Barhameen, (2004**). Identification was by morphological studies, urease, catalase and oxidase tests in addition to motility, **Lee and Megraud, (1996**). The colonies were sub-cultured on three blood agar plates and after five days incubation, the harvested colonies were transferred to a Cryovile filled with 0.5 ml Thioglycolate broth with 15% glycerol and stored at - 40°C as stock culture for further studies. Ten years later, a 10 µl diameter sterile disposable loop (Sara Med. Saudi Arabia) was dipped in the stock culture and a loopfull was streaked on both Blood and Chocolate agar and incubated under the appropriate conditions as mentioned above ,**Milyani (2011**).

2.2.2.DNA extraction and PCR amplification of VacA gene *H. pylori* coccoid forms

Total DNA was extracted using the Wizard® SV kit (Promega, Madison, USA). PCR-amplification reaction was used 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 (de-naturation), 40 cycles of 94°C for 30 sec, 49°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 0.8 µg/µl ethidium bromide and visualized under UV light. The size of the VacA fragment of 750 bp was estimated based on a 50 bp DNA ladder (Bioron, Germany).

2.2.3.VacA gene purification, sequencing and analysis

PCR product of 750 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 VacA fragment was sequenced on an Applied Biosystems automatic sequencer (ABI PRISM® 1200 DNA Sequencer, Bioron GmbH, Germany).

2.2.4.Nucleotide sequence accession number

The GenBank accession number for the partial nucleotide sequences of Vac A gene from *H. pylori* isolate; Makkah 7 is HQ622108.

2.2.5.Vacuolating cytotoxin polypeptide domain accession number

The GenBank accession number for the putative conserved domain of vacuolating cytotoxin (Vac A) is ADU05468.

2.2.6.Sequence alignment of *H. pylori* VacA gene

Sequence was 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). Highly conserved residues have a black background, whereas partially conserved residues are shown with a gray shaded background. Numbering at the end of each line refers to the position in the alignment.

2.2.7.Phylogenetic data analysis

Genetic distances were obtained using Kimura's two-parameter model (**Kimura 1980**). A phylogenetic tree and dendrogram were constructed using multiple alignment of the VacA from *H. pylori* isolates and strains by the neighbour-joining method (**Saitou and Nei, 1987**) with the Geneious Pro 4.5.4 program.

3.RESULTS

3.1.Culture and identification

Culture of gastric biopsy revealed typical morphology of *H. pylori* colonies, proven by positive urease, catalase and oxidase tests. Gram culture - using phase contrast microscope showed coccoid forms indicating complete transformation of the culture to coccoid.

stain also showed Gram negative S-shaped bacteria, in addition, to the well recognized motility of *H. pylori*. However, culturing from the stock culture after ten years gave undetectable viable counts, and examining a drop from the stock

3.2.PCR amplification and sequence analysis of Vac A gene of *H. pylori* coccoid forms isolate

PCR amplifications of *H. pylori* isolate (Makkah-7) revealed the fragment with expected size of 750 bp that represented the VacA gene (Fig. 1).

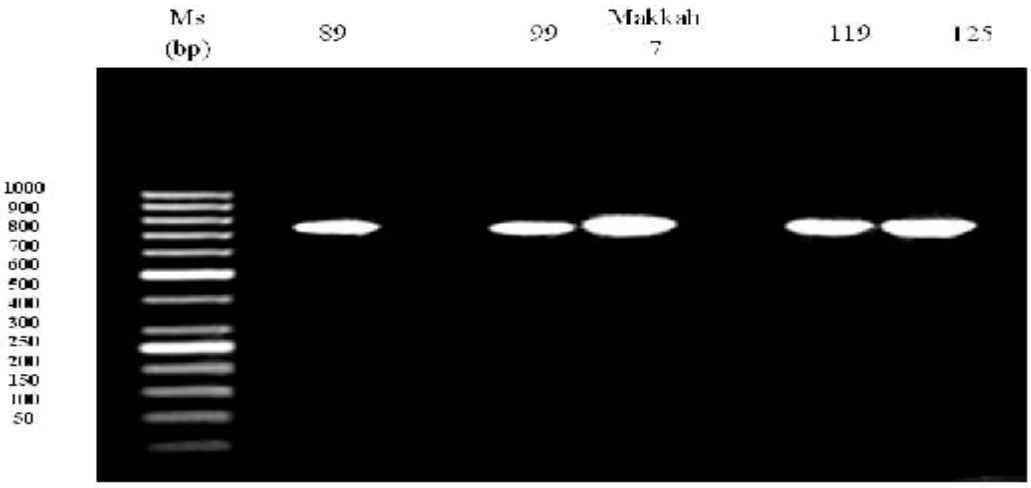


Fig. 1. PCR amplified products of Vac A gene of *H. pylori* isolate (Makkah 7) and other coccoid form isolates using designed primer with 750 bp. M– DNA ladder with 50 bp.

The 750 bp fragment of Vac A gene of *H. pylori* strain Makkah 7 was delivered to GenBank/NCBI database according to the expected size of the designed primer pair and to the appearance on the agarose gel electrophoresis (Fig. 1). However, the size of the fragment was finally reduced by the GenBank and submitted as 599 bp. This reduction in fragment size normally occurs when extraction of the fragments is from genomic DNA and not from sub-cloning experiment.

A 599 bp nucleotide sequence of the partial Vac A gene from isolate Makkah 7 (HQ622108) was aligned and compared in the GenBank using the BLAST search. A total of 87 Vac A gene partial sequences from different accessions of *H. pylori* included different isolates and strains were identified (Table 1). Sequence Blast alignment showed that the homology of Vac A coccoid isolate (HQ622108) and the reported accession sequences was 85 to 95% maximum.

Sequence alignments of the Vac A gene of isolate Makkah-7 (HQ622108) compared with *H. pylori* GenBank strains and isolates revealed positional differences in nucleotide sequences and base-pair substitutions between the isolate and several isolates and strains (Fig. 2).

The accession HQ622108 with a fragment size 599 bp (isolate Makkah-7) revealed a total number of 235 nucleotide positional differences with base-pair substitutions. The highest number (29) with positional differences with base-pair substitutions was obtained from Guanine (G) that changed to (A) and from Adenine (A) to Thymine (T), followed by Thymine (T) to Adenine (A) with 27 positional differences. In contrary, the lowest number with 7 positional differences was detected in Guanine (G) that changed to cytosine (C), followed by 11 from (C) to (G) and (T) to (G).

The alignment sequence of 599 bp was divided into parts according to the starting number of nucleotides and each part included 100 bp. The highest number of positional differences (45) was from 3200 to 3300, followed by the range from 3001 to 3100 with 43, then by the range from 2900 to 3000 with 42 positional differences. In the first part with a range of 2800 to 2900, Guanine (G) was changed in adenine (A) in 7 positional differences, followed by (A) changed to (G) and (T) to (A) in 6 positional differences. Adenine (A) was changed to cytosine (C) and (G) changed to (A) in 6 positional differences as shown in Table (2).

Table 1. Blast search of VacA gene sequence identity between the *H. pylori* isolate (Makkah-7) and GenBank sequences.

Accession	Strain	%	Accession	Strain	%	Accession	Strain	%	Accession	Strain	%
AB190966	F92	93	AF049649	F69	89	AF195018	1939	85	AY663830	249a3	93
AB190965	F80	93	AF049629	F47	89	AF195017	184	93			
AJ418360	NQ1701	93	AF049626	F42	89	AF195010	55	89	CP002076	Cuz20	91
AB190973	OK130	90	AF049647	F64	90				CP002073	SJM180	93
AB190964	F32	89	AF049624	F34	90	AF361702	CHN5038c	90	CP002071	Sat464	92
AB190958	F13	89	AF049631	F52	90	AF361701	CHN4611a	89			
AB190961	F17	89	AF049648	F68	92	AF361700	CHN3554a	89	GQ331981	DL2	89
AB190969	OK109	89	AF049641	F33	92				GQ331982	L7	90
AB190968	OK107	90	AF049646	F62	92	AJ418362	LSU1014-6	86	GQ331976	PG227	89
AB190986	OK204	90	AF049619	F20	92	AJ418361	LSU1014-1	86	GQ331983	L8	90
AB190975	OK144	90	AF049620	F21	90	AJ418358	NQ1624	94	GQ331977	MZ1	89
AB190983	OK185	90	AF049621	F28	89	AJ438933	HP-HJM 24	93	GQ331984	L1	90
AB190980	OK179	91	AF049634	F57	90	AJ438923	HP-HJM 14	93	GQ331974	PG218	90
AB190979	OK160	90	AF049639	F87	89				GQ331980	DL1	85
AB190974	OK139	90	AF049627	F44	89	AJ438930	HP-HJM 21	92			
AB190981	OK180	88	AF049632	F55	89	AJ438921	HP-HJM 12	92	FM991728	B38	93
AB190982	OK181	88	AF049637	F67	95	AJ438926	HP-HJM 17	86	FN598874	B8	95
AB190962	F18	90	AF049645	F61	89						
AB190960	F16	90				AE001439	J99	91	HM047646	K47	92
AB190977	OK158	90	AF071095	F37	93						
AB190972	OK129	90	AF071096	F71	90	AM997176	G27	85	U95971	95-54 (J128)	93
AB190988	OK210	90							U07145	NCTC 11638	93
AB190987	OK205	91	AF191641	AFN1156	92	AY232454	Iran Hel-155	90			
AB190984	OK187	93	AF191642	AFN4124	92						
AB190976	OK155	93				AY848858	MDC1	94			
			AF191645	AFNG114	92	AY663831	249a5	93			
AF049622	F29	85	AF191643	AFN4769	92	AY765346	MV008-P				

3.3.Phylogenetic analysis of VacA gene of Makkah-7

The phylogenetic tree represented the relationship between coccoid form *H. pylori* isolate (HQ622108) and all described *H. pylori* related strains obtained from GenBank based on the VacA gene as shown in Fig. (3). The dendrogram divided all GenBank strains into two clusters; one contained the *H. pylori* isolate (HQ622108) and the second cluster comprised all GenBank strains. The Makkah-7 isolate formed a phylogenetically distinct cluster separate from all other species.

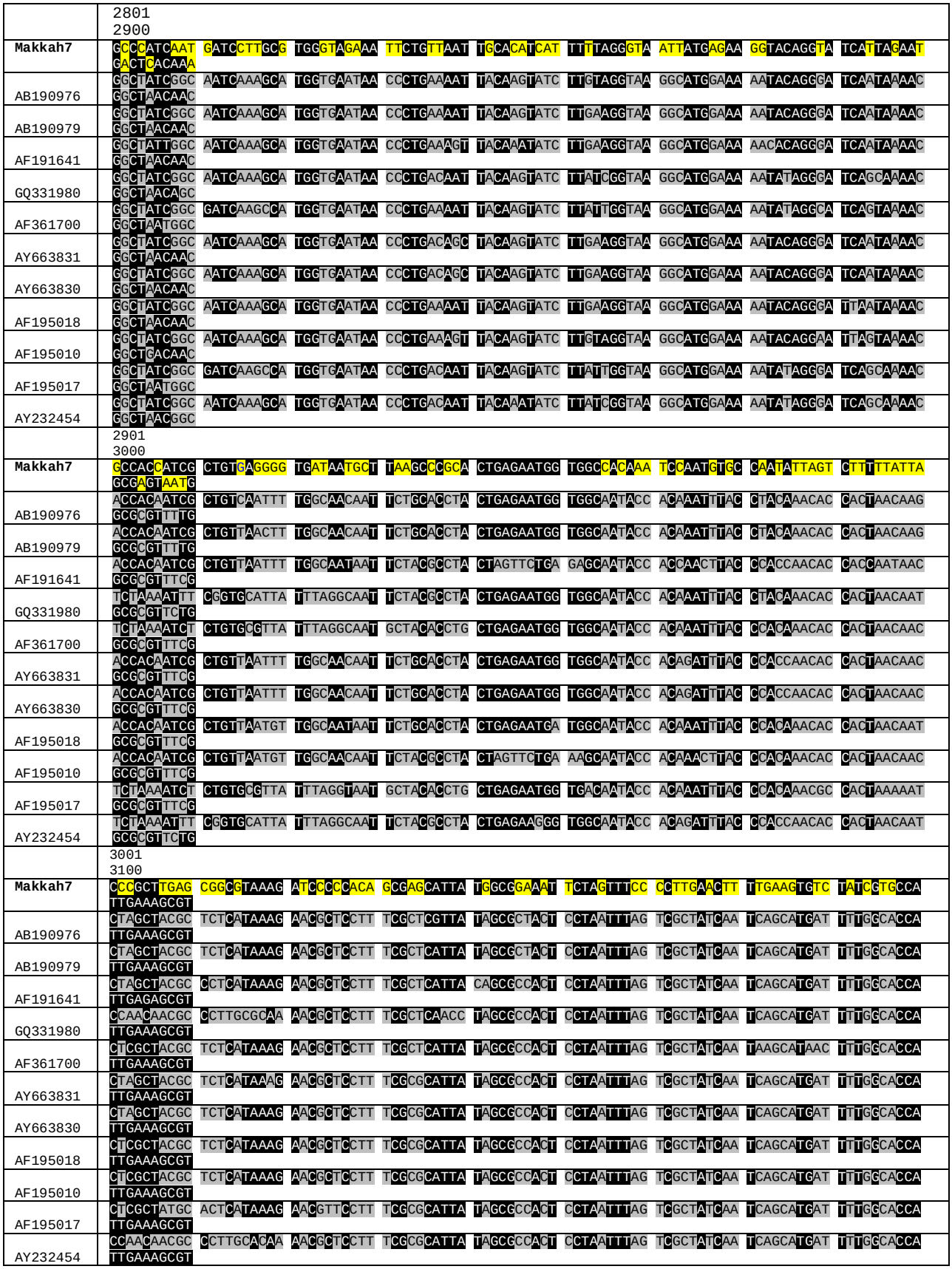
3.4.Polymorphisms in vacuolating cytotoxin A domain

Translation of the 599-nucleotide long of the VacA sequence induced a putative signal polypeptide of 199 amino acids with a molecular weight of about 140 kDa and 8.79. iso-electric point. of An amino acid sequence comparison using BLASTP (http://www.ncbi.nlm.nih.gov/BLAST) revealed that the predicted protein is a vacuolating cytotoxin A (VacA) putative conserved domain as shown in Table (3). Moreover, amino acids alignment of vacuolating cytotoxin A of isolate Makkah 7 (ADU05468) compared with 100 obtained GenBank *H. pylori* strains and isolates revealed mutations in different positions (Table 4 and Fig. 4).

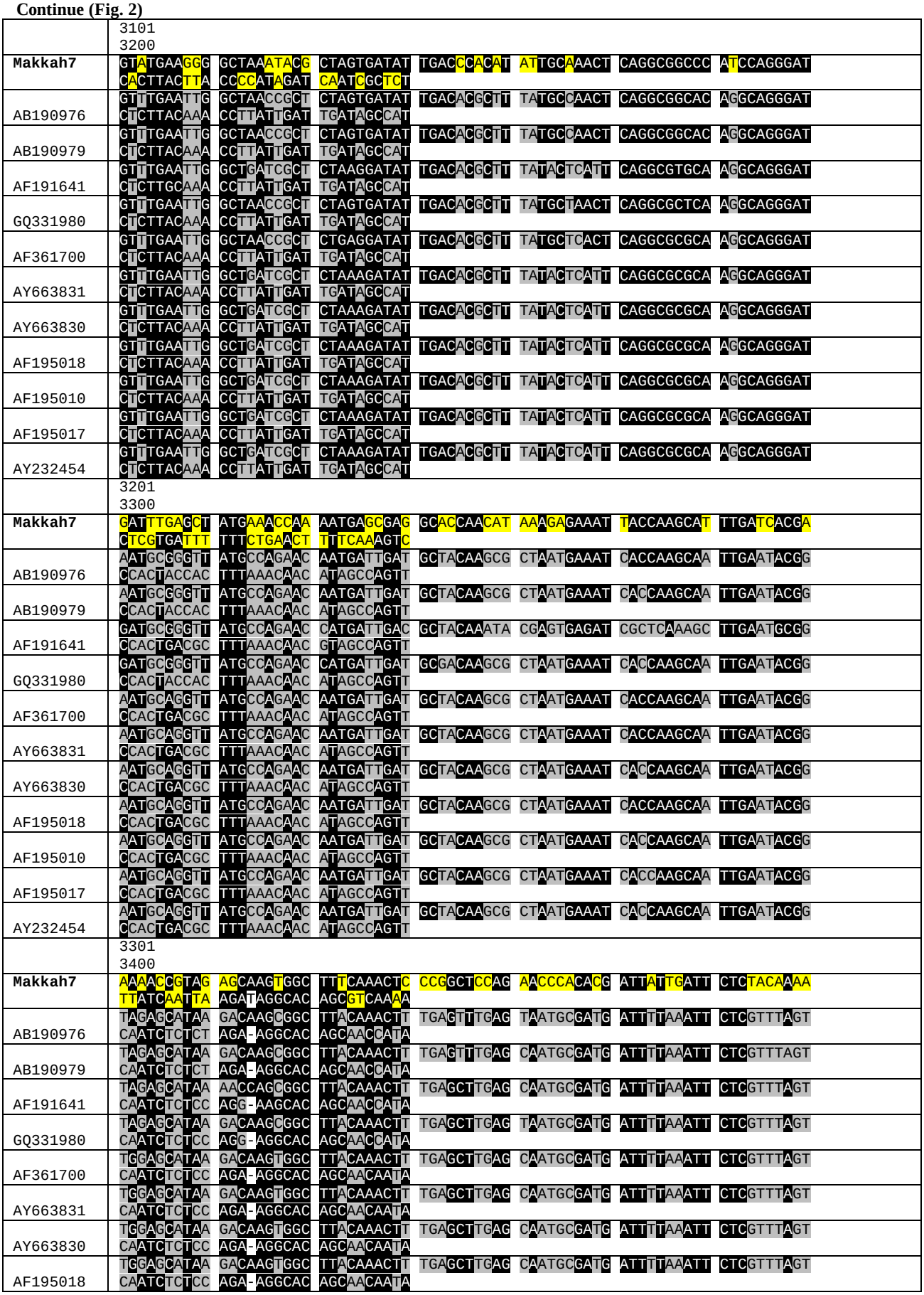
Table 2. Positional differences and base pair substitutions in nucleotide sequences between Makkah-7 *H. pylori* isolate and numerous strains based on VacA gene similarity.

Exist in Makkah-7 as:	Nucleotide sequence range	A	A	A	C	C	C	G	G	G	T	T	T
Changed in NCBI to:		C	G	T	A	G	T	A	T	C	A	C	G
Changes in nucleotide positions:	2800-2900	2863	2808	2828	2815	2802	2804	2811	2829		2816	2810	2825
		2900	2809	2849	2845			2820	2858		2817	2831	2853
			2846		2848			2842			2836	2832	2862
			2861		2895			2868			2837	2850	2879
			2867					2871			2859	2890	2891
			2892					2872			2874		
								2887					
	T=41	2	6	2	4	1	1	7	2	0	6	5	5
	2900-3000	2932	2923	2933	2906		2939	2901	2918	2915	2982	2924	2952
		2959	2990	2972	2929		2957	2917	2919	2938	2985	2927	
		2960		2997	2936			2928	2920		2986	2974	
		2978		2998	2955			2965	2967		2988	2980	
		2982			2963			2969			2989	2983	
		2994						2979					
	T=42	6	2	4	5	0	2	6	4	2	5	5	1
	3000-3100	3028		3030	3003	3024	3002	3015	3013	3008	3007	3051	3063
		3034		3047	3059	3060	3011	3042	3031	3010	3022	3072	3064
		3039		3067		3084	3026	3055	3035	3012	3069	3086	
		3075		3082			3029	3073	3065	3046	3070		
							3061	3076			3079		
							3080	3087					
	T=43	4	0	4	2	3	6	6	4	4	5	3	2
	3100-3200	3116	3118	3103	3145		3183		3108			3117	
		3156	3147	3149	3195		3184		3109			3198	
			3162	3151			3191		3120				
			3192	3152			3199						
				3172									
				3178									
				3179									
				3187									
	T=25	2	4	8	2	0	4	0	3	0	0	2	0
	3200-3300	3214	3270	3233	3218	3217	3209	3201	3226	3274	3252	3205	3204
		3215		3245	3273	3238	3227	3206	3230		3260	3251	3240
		3220			3284	3294	3234	3244			3265	3272	
		3239			3289		3266	3286			3279	3278	
		3241					3300				3285	3280	
		3287									3291	3290	
		3295									3293		
		3296											
	T=45	8	1	2	4	3	5	4	2	1	7	6	2
	3300-3400	3346	3303	3301	3343	3305	3337	3307			3323	3317	3364
		3377	3311	3341		3332	3330	3310			3356	3371	
			3369	3354		3338	3331	3312			3372	3379	
				3365		3345	3344	3333			3395		
				3367			3349	3357					
				3370			3366	3394					
				3376									
				3380									
				3399									
	T=39	2	3	9	1	4	6	6	0	0	4	3	1
	Total=235	24	16	29	18	11	24	29	15	7	27	24	11

Fig. 2. Sequence alignment of 599 bp of VacA gene in *H. pylori* isolate (Makkah-7) compared with other isolates and strains existed in NCBI GenBank. Conserved nucleotides between my 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 my isolates. Dashes correspond to gaps introduced to optimize the alignments



Continue (Fig. 2)



AF195010	TGGAGCATAA GACAAGTGGC TTACAAACTT TGAGCTTGAG CAATGCGATG ATTTTAAATT CTCGTTTAGT CAATCTCTCC AGA-AGGCAC AGCAACAATA
AF195017	TGGAGCATAA GACAAGTGGC TTACAAACTT TGAGCTTGAG CAATGCGATG ATTTTAAATT CTCGTTTAGT CAATCTCTCC AGA-AGGCAC AGCAACAATA
AY232454	TGGAGCATAA GACAAGTGGC TTACAAACTT TGAGCTTGAG CAATGCGATG ATTTTAAATT CTCGTTTAGT CAATCTCTCC AGA-AGGCAC AGCAACAATA

Continue (Fig. 2)

	3401 3500
Makkah7	TTCACTCGTT CGTGGTGCGG TTAGCCACCC TA
AB190976	TTGACTCGTT CGCTCAACGC TTA-CAAGCT TT
AB190979	TTGACTCGTT CGCTCAACGC TTA-CAAGCT TT
AF191641	TTAACTCGTT CGCTCAACGC TTA-CAAGCT TT
GQ331980	TTGACTCGTT CGCTAAGCGC TTA-CAAGCT TT
AF361700	TTGACTCATT CGCCAGACGC TTG-CAAGCT TT
AY663831	TTGACTCATT CGCCAGACGC TTG-CAAGCT TT
AY663830	TTGACTCATT CGCCAGACGC TTG-CAAGCT TT
AF195018	TTGACTCATT CGCCAGACGC TTG-CAAGCT TT
AF195010	TTGACTCATT CGCCAGACGC TTG-CAAGCT TT
AF195017	TTGACTCATT CGCCAGACGC TTG-CAAGCT TT
AY232454	TTGACTCATT CGCCAGACGC TTG-CAAGCT TT

Table 3. Vaculating cytotoxin A (VacA) putative conserved domains of Makkah-7 *H. pylori* isolate.

RWANRTTRVNFDAKNILIDNFVEINNRVGSGAGRKASSTVLTLKSSE
KITSRENAEISLYDGATLNLVSSSNQSVDLWGKVWMGRLQYVGAYL
APSYSTIDTSKVQGETNFRHLAVGDQNAAQAGIIANKKTNIGTLDLW
QSAGLSIITPPEGGYESKTKDTPSQNNPKNDVQKTEIQPTQVIDGPFAG
GKDTAVNIFH

Fig. 3. Phylogenetic relationships between coccoid form *H. pylori* isolate (Makkah-7) and other GenBank related strains based on VacA gene.

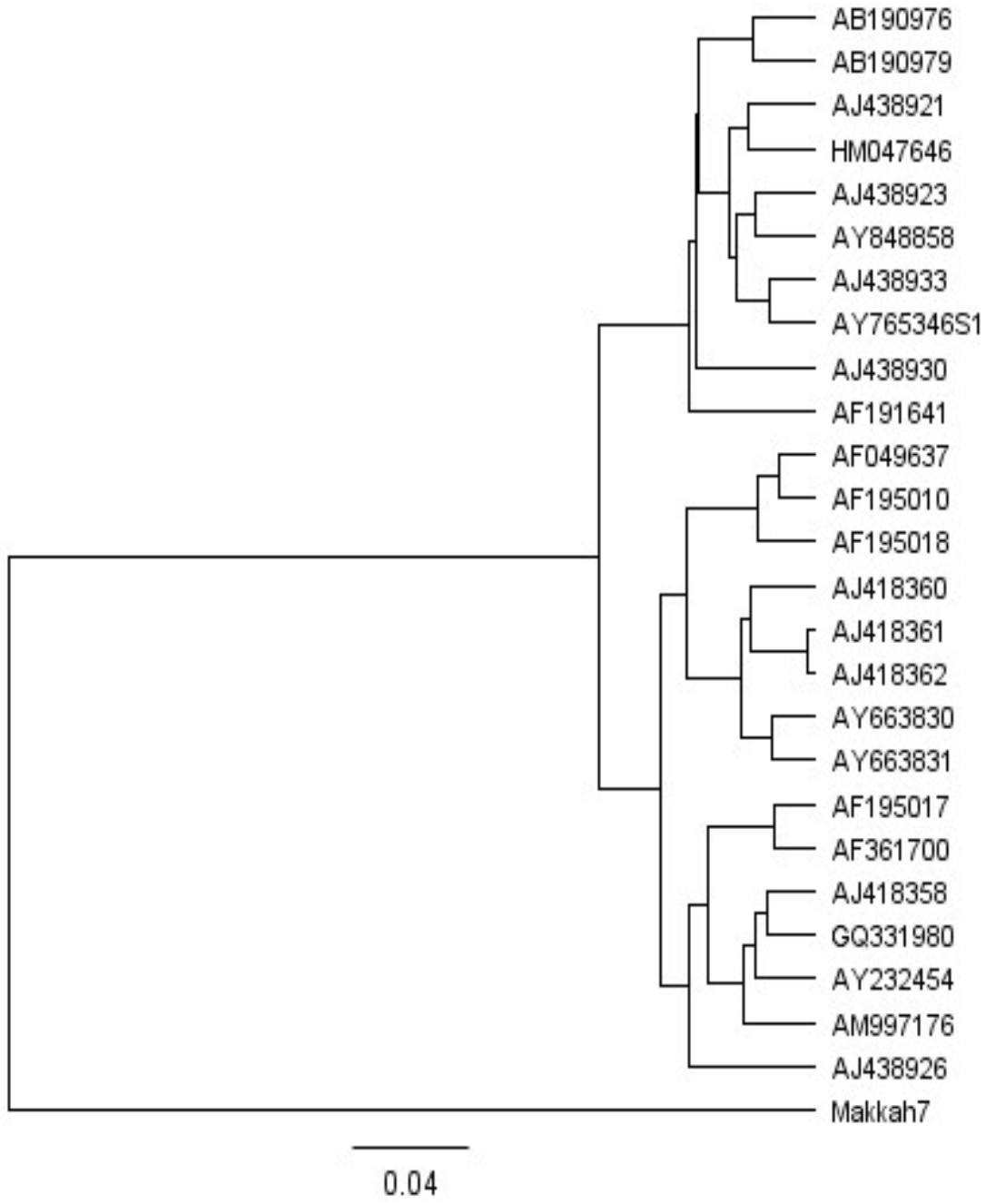


Table 4. BLASTP search of vacuolating cytotoxin A putative domains of Makkah-7 *H. pylori* isolate (ADU05468) and GenBank protein sequences.

AAC77451	AAK56858	ADH10184	BAD51795	CAD27667
	AAK56860	ADH10179	BAD51773	CAD27674
AAG28443	AAK56857	ADH10176	BAD51781	CAD27690
	AAK56856	ADH10182	BAD51774	CAD27669
AAD04264		ADH10178	BAD51770	CAD27678
AAD04268	AAF26509	ADH10183	BAD51786	CAD27688
AAD04270	AAF26502		BAD51784	CAD27685
AAD04269	AAF26510	ADO04098	BAD51780	CAD27676
AAD04277	AAF26501	ADO05607	BAD51772	
AAD04280	AAF26508		BAD51789	NP_223537
AAD04285	AAF86763	ADP02391	BAD51778	
AAD04275		ADP02390	BAD51785	Q48247
AAD04266	AAL83504		BAD51797	Q48253
AAD04283	AAL83503	ADU80120	BAJ56545	
AAD04262		ADU81729	BAD51796	ZP_03438412
AAD04265	AAX49347	ADU41215	BAD51793	ZP_03239901
AAD04272		ADU83300	BAD51792	ZP_03440112
AAD04288	ACX98036	ADU05468	BAD51794	
AAD04279			BAD51790	YP_003057599
AAD04289	ADK63280	BAJ59976	BAD51791	YP_001910400
AAD04281	ADK63300		BAD51783	YP_002266461
AAD04276	ADK63323	BAD51798	BAD51777	
AAD04261		BAD51768	BAD51771	
AAD04267	ADI34974	BAD51782	BAD51779	

Fig. 4. Sequence alignment of vacuolating cytotoxin A putative domain in Makkah-7 isolate (ADU05468) compared with other isolates and strains in NCBI GenBank.

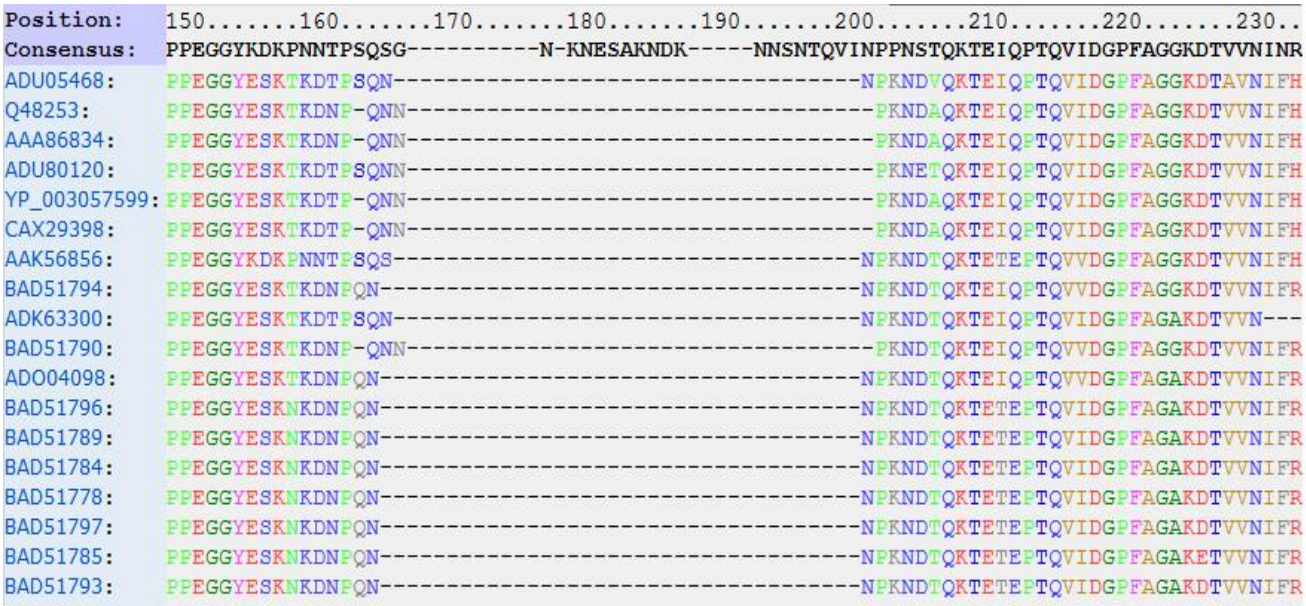
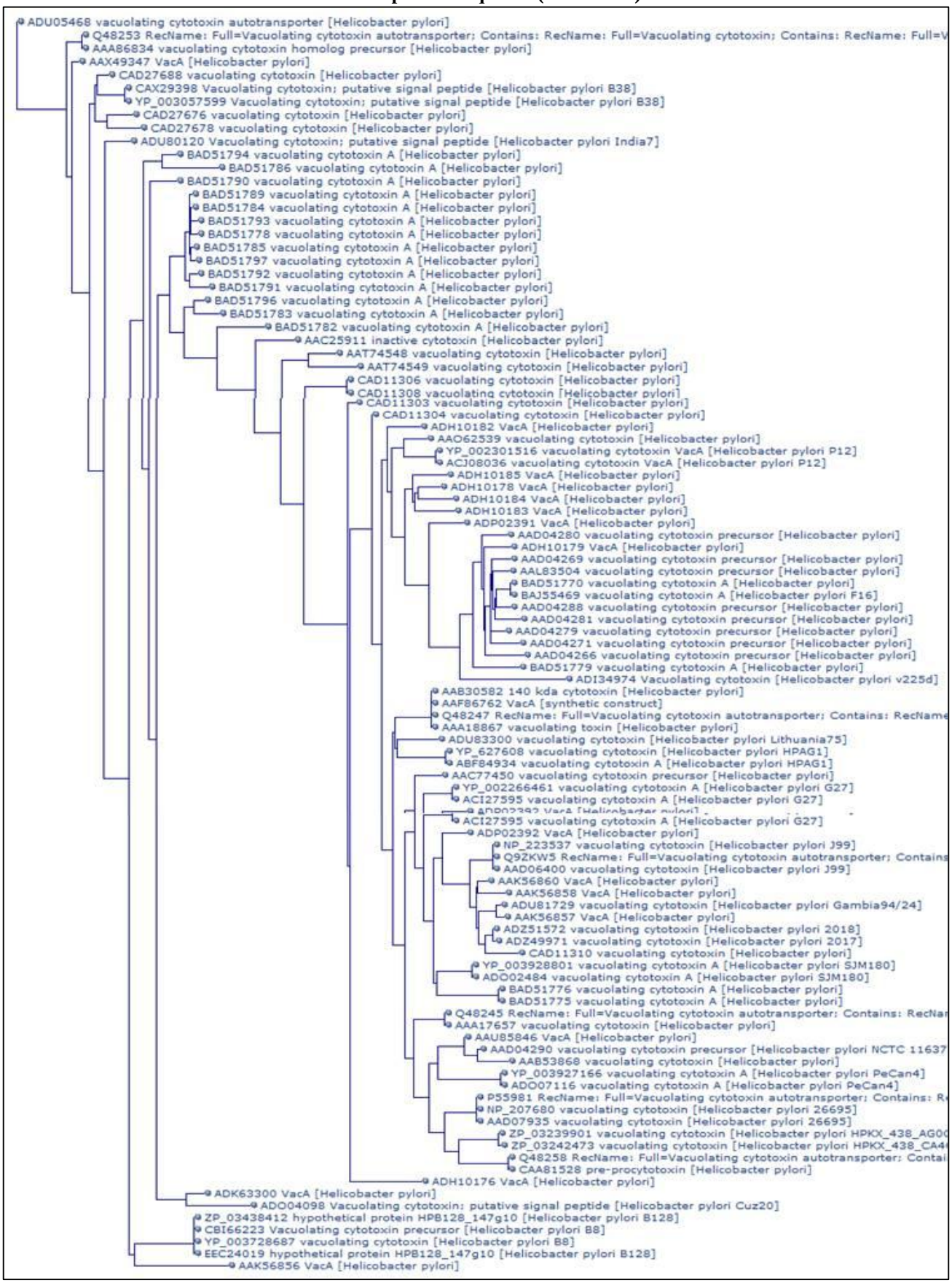


Fig. 5. Phylogenetic relationships between coccoid form *H. pylori* isolate (Makkah-7) and other GenBank related strains based on VacA protein sequence (ADU05468).



Total of 33 amino acids of Makkah-7 have changed to other GenBank *H. pylori* amino acids at different positions (Table 5). On the other hand, the highest numbers of amino acids (9) that changed to Asparagine (Asn) were as follows: Alanine (Ala) changed to Asn at positions 12, 101 and 161; Lysine (Lys) to Asn at 80 and 160; Serine (Ser) to Asn at 70 and 146; Phenylalanine (Phe) to Asn at 233 and Arginine (Arg) to Asn at 112. Moreover, Asn changed to Aspartic acid (Asp) at positions 4 and 10; (Arg to Asp) at 1; (Ser to Asp) at 157. Other amino acids were uniquely changed to individual GenBank amino acids, such as Lys to Glycine (Gly) at 48, Asn to Histidine (His) at 133, Tryptophan (Trp) to Methionine (Met) at 78. The VacA protein (ADU05468) and VacA nucleotide trees of Makka-7 revealed the same distinctive group that is distant from other GenBank strains (Fig. 5).

Table 5. Positional differences in translated protein sequences of vacuolating cytotoxin A putative peptide domain between Makkah-7 *H. pylori* isolate and numerous strains.

		Signal	Present in the GenBank accessions as:														
			A	D	E	G	H	I	K	M	N	P	Q	S	T	V	
Present in Makka-7 as:	Alanine (Ala)	A														229	1
	Aspartic acid (Asp)	D							76		12			206			3
											101						1
											161						1
	Glutamic acid (Glu)	E							53								1
									156								1
	Phenylalanine (Phe)	F									233						1
	Lysine (Lys)	K				48					80	204	44				4
											160						1
	Asparagine (Asn)	N		4			133							129			3
				10										166			2
	Glutamine (Gln)	Q													106		1
	Arginine (Arg)	R		1							112						2
	Serine (Ser)	S	45	157							70						3
											146						1
	Threonine (Thr)	T	149									159				109	3
	Tryptophan (Trp)	W								78				2			2
	Valine (Val)	V	68												207		2
	Total		**3**	**4**	**0**	**1**	**1**	**0**	**3**	**1**	**9**	**2**	**1**	**4**	**2**	**2**	**33**

G=Glycine (Gly), M=Methionine (Met), H =Histidine (His)

4.DISCUSSION

Preservation of *H. pylori* in gastric biopsies or culture is usually carried out by keeping the harvested colonies or biopsy in Brain heart Infusion glycerol and freeze at -70 to -80°C, or in liquid nitrogen at -196°C. The recovery rate using these methods is 80-90% after six years, while routine freezing is not successful (**Lee and Megraud 1996).** However, unexpectedly **Milyani and Barhameen (2004) f**ound that 13 gastric biopsies gave positive growth of *H. pylori* after freezing at -20°C for 15 days. At the present study, the prolonged storage (ten years) of *H. pylori* under -40°C in thioglycolate broth + 15% glycerol rendered the spiral forms to transform into coccoid forms, this is in accordance with **Catrenich and Maki (1991); Reynolds and Penn (1994); Milyani (2011)** who managed to induce coccoid forms under extended incubation. Although, others also induced coccoid forms by subjecting fresh cultures to antibiotics, harsh environment or keeping *H. pylori* in sterile tap water outdoor at 35-45°C for 24 hours and weeks **(Bode, *et al*. 1993; Andersen and Wadstrom, 2001; Nilsson, *et al*., 2002; Milyani unpublished data).**

The results obtained at the present work confirmed that the coccoid forms of *H. pylori* strain under study (Makkah 7 accession no.HQ622108) is VacA positive with variations in VacA gene and peptide domain sequences. Our findings agree with **She *et al*., (2001); Wang and Wang (2004)** who reported that their data indicate that coccoid *H. pylori* contains UreA, UreB, hpaA, VacA, and Cage genes and contains complete VacA gene, and could synthesize its protein, which may be a potential pathogen. On the other hand, **Argent, *et al*., (2008)** stated that most strains of *H. pylori* possess both CagA and VacA virulence factors which down regulate each other's effects on epithelial cells. Interestingly, many researchers recorded that their patients were colonized by multiple strains of *H. pylori*. **(Kim, *et al*,. (2009);Ben Mansour, *et al*., (2010)** and surprisingly, the results from biopsy samples produced different results when compared with those obtained from *H. pylori* isolates, especially for VacA s1, and IceA. Nevertheless, the present results show that VacA *H. pylori* Makkah 7 strain is distinct from other isolates based on VacA gene and peptide domain and according to the phylogenetic studies (Fig. 3 and 5). This distinction has been also recorded when other *H. pylori* were isolated from the same city (Makkah) based on 16S rRNA analysis namely Milyani 1, Milyani 2 and Milyani 3 with accession numbers HQ877021, HQ877022 and HQ877023 respectively **(Milyani 2011)**. In contrast, **Kumar *et al*., (2010)** according to their phylogenetic analysis postulated that the Indian strains of *H. pylori* show close homology to those from Taiwan and/or Brazil and that genetic diversity among *H. pylori* isolates is widely prevalent regardless of the region from which they are isolated.

The present study revealed positional alternations in 33 amino acids among 199 amino acids of vaculating cytotoxin domain. However, such mutations may not actually influence the production of vaculating cytotoxin by Makkka 7 strain (ADU05468). This has been evidently obtained from Table (4) that represented the alignment of 100 accession of vacA domain with 100% similarity. On the other hand, **Ivie, *et al*., (2008) and Genisset, *et al*., (2006)** managed to introduce a small deletion mutation in both aspartic acids 346 and glycine 347 into the *H. pylori* chromosomal VacA gene. Similar to wild-type VacA, the VacA mutant was proteolytically processed, secreted, and bound to eukaryotic cells, but VacA 346-347 did not cause cell vacuolation or membrane depolarization.

The present findings provide additional information on VacA nucleotide and peptide domain isolated from Makkah City that is entirely different from other isolated *H. pylori*. These results also, emphasize the paramount importance of studying *H. pylori* isolates from Saudi population to develop a global understanding of gastric diseases. The factors influencing variations of Makkah 7 *H. pylori* strain are still to be elucidated and indeed these variations may be incriminated in pathogenicity and other characteristics **(Milyani 2011).** Coccoid forms of *H. pylori* and their virulence genes should not be underestimated and vigorous research and further studies should be carried out since many established date have shown their pathogenicity and their possible role in transmission and in therapeutic failure.

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