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Evaluation of Four Different DNA Targets in Polymerase Chain Reaction for Detection and Genotyping of *Helicobacter pylori*

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***Abstract*—**Polymerase chain reaction (PCR) assays targeting genomic DNA segments have been established for the detection of *Helicobacter pylori* in clinical specimens. However, the data on comparative evaluations of various targets in detection of *H. pylori* are limited. Furthermore, the frequencies of *vacA* (*s1* and *s2*) and *cagA* genotypes, which are suggested to be involved in the pathogenesis of *H. pylori* in other parts of the world, are not well studied in Kuwait. The aim of this study was to evaluate PCR assays for the detection and genotyping of *H. pylori* by targeting the amplification of DNA targets from four genomic segments. The genomic DNA were isolated from 72 clinical isolates of *H. pylori* and tested in PCR with four pairs of oligonucleotides primers, i.e. ECH-U/ECH-L, ET-5U/ET-5L, CagAF/CagAR and Vac1F/Vac1XR, which were expected to amplify targets of various sizes (471 bp, 230 bp, 183 bp and 176/203 bp, respectively) from the genomic DNA of *H. pylori.* The PCR-amplified DNA were analyzed by agarose gel electrophoresis. PCR products of expected size were obtained with all primer pairs by using genomic DNA isolated from *H. pylori*. DNA dilution experiments showed that the most sensitive PCR target was 471 bp DNA amplified by the primers ECH-U/ECH-L, followed by the targets of Vac1F/Vac1XR (176 bp/203 DNA), CagAF/CagAR (183 bp DNA) and ET-5U/ET-5L (230 bp DNA). However, when tested with undiluted genomic DNA isolated from single colonies of all isolates, the Vac1F/Vac1XR target provided the maximum positive results (71/72 (99% positives)), followed by ECH-U/ECH-L (69/72 (93% positives)), ET-5U/ET-5L (51/72 (71% positives)) and CagAF/CagAR (26/72 (46% positives)). The results of genotyping experiments showed that *vacA s1* (46% positive) and *vacA s2* (54% positive) genotypes were almost equally associated with VaCA+/CagA- isolates ($P > 0.05$), but with VacA+/CagA+ isolates, S1 genotype (92% positive) was more frequently detected than S2 genotype (8% positive) ($P< 0.0001$). In conclusion, among the primer pairs tested, Vac1F/Vac1XR provided the best results for detection of *H. pylori*. The genotyping experiments showed that *vacA s1* and *vacA s2* genotypes were almost equally associated with $vaCA^{+}/cagA^{-}$ isolates, but *vacA s1* genotype had a significantly increased association with $vacA^{+}/cagA^{+}$ isolates.

***Keywords*—**H. pylori; detection; genotyping; Kuwait.

I. Introduction

HELICOBACTER *pylori* is an organism colonizing the gastric mucosa of humans. The research performed by Warren and Marshall in the 1980s showed that this organism was associated with gastritis and peptic ulcer disease [1]. Chronic *H. pylori* infection is also implicated in gastric cancers [2]. *H. pylori* infection is the most common infection in humans with about half of the world's population being chronically infected [3]. The diagnosis of *H. pylori* infection generally includes a combination of non-invasive and invasive procedures that include classical and molecular methods [4], [5]. Invasive tests for *H. pylori* infection include endoscopy followed by classical histological examination and/or culture to demonstrate the presence of the bacterium [5]. Among molecular tests, PCR assays targeting various genomic DNA segments (417 bp DNA, 230 bp DNA and *vacA* and *cagA* genes, etc.) have been established for the detection and/or genotyping of *H. pylori* in clinical specimens [6]. However, the comparative evaluation of these targets in the detection and/or genotyping of *H. pylori* has not been performed. Moreover, the distributions of *vacA* and *cagA* genotypes, which are suggested to be involved in the pathogenesis of *H. pylori* in other parts of the world [7], are not well studied in Kuwait**.**

In this study, PCR assays were established for the detection of *H. pylori* by targeting the amplification of the above stated four different genomic DNA segments by using target-specific primers. These PCR assays were evaluated for detection and/or genotyping of *H. pylori* by using clinical isolates from patients residing in Kuwait and attending the Al-Adan Hospital in Kuwait.

II. Materials and Methods

A. Clinical Isolates of H. pylori

A total of 72 *H. pylori* isolates were obtained after *in vitro* culture of the organism from gastric biopsies of patients suffering from diseases indicative of *H. pylori* infection from Al-Adan Hospital in Kuwait. Single colonies from the blood agar plates were picked up, suspended in 1 ml Tris-EDTA (TE) buffer in Eppendorf tubes, and heated at 95°C for 25 minutes to release DNA. The amount of DNA was quantitated by spectrophotometry and stored at -20°C.

B. Oligonucleotide Primers

Four pairs of oligonucleotides primers were used in this study. The details (primer designation, nucleotide sequence and size of target DNA) and the original references of their use are given in Table I. All the primers were synthesized commercially (Interactiva Biotechnologies GmbH, Ulm, Germany).

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TABLE I
THE DESIGNATION, NUCLEOTIDE SEQUENCE OF OLIGONUCLEOTIDE PRIMERS AND THE SIZE OF TARGET DNA IN THE GENOME OF *H. PYLORI*

Primer designation	Nucleotide sequence	Size of target (bp)	Reference
ECH-U	5'-CCCTCACGCCATCAGTCCCAAAAA-3'	417	[8]
ECH-L	5'-AAGAAGTCAAAAACGCCCCAAAAC-3'		
ET-5U	5'-GGCAAATCATAAGTCCGCAGAA-3'	230	[9]
ET-5L	5'-TGAGACTTTCCTAGAAGCGGTGTT-3'		
CagAF	5' TTGACCAACAACCACAAACCGAAG-3'	183	[10]
CagAR	5'-CTTCCCTTAATTGCGAGATTCC-3'		
Vac1F	5'-ATGGAAATACAACAAACACAC-3'	176/203	[10]
Vac1XR	5'-CCTGAAACCGTTCCTACA GC-3'		

C. Amplification of Target DNA by PCR

The above mentioned primers were used in PCR assays for the amplification of targets from the genomic DNA of clinical isolates of *H. pylori* according to standard procedures [11], [12]. In brief, the reaction mixtures (50 µl) in a PCR tube contained 1x DNA buffer II, 25 mM magnesium chloride, 1 U AmpliTaq DNA Polymerase (ThermoFisher Scientific, Waltham, MA, USA), 25 pmoles of each forward and reverse primer and 5 µl of genomic DNA. A negative control tube had all the ingredients except the genomic DNA. The PCRs were performed in a Perkin Elmer GeneAmp 9600 PCR System Thermal Cycler (Perkin Elmer Inc., Waltham, MA, USA). The cycling parameters included an initial denaturation at 95°C for 3 minutes, followed by 40 cycles of amplification with each cycle consisting of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute and extension at 74°C for 1 minute, followed by a final extension step at 74°C for 5 minutes.

D. Agarose Gel Electrophoresis

The amplified DNA were analyzed by agarose gel electrophoresis according to standard procedures [13]. In brief, aliquots of amplified samples (18 µl) were electrophoresed on 2% agarose in Tris-Acetate-EDTA (TAE) buffer. The gels were stained with ethidium bromide. The bands of amplified DNA were visualized under ultraviolet light and photographed. The sizes of amplified DNA were determined by comparing the band size with the band sizes of 123 bp DNA ladder used as molecular weight marker.

E. Statistical Analysis

Fisher exact test was used to analyze the data and p values <0.05 were considered significant.

III. RESULTS AND DISCUSSION

All of the four pairs of primers were tested in PCR with DNA isolated from representative clinical isolates of *H. pylori*. The results showed that PCR products of expected size were obtained with all the four primer pairs (Fig. 1). These results suggested that the method of genomic DNA isolation, the PCR conditions as well as the primers used in the study were appropriate for detection and typing of clinical isolates of *H. pylori*, as reported previously [8], [9], [11], [12].

To determine the analytical sensitivity of each primer pair for target amplification, PCRs were performed with 10-fold serial dilutions of genomic DNA starting from 1 ng (DNA from approximately 500,000 organisms) to 1 fg (DNA from approximately 0.5 organisms). The results showed that the most sensitive PCR target was 471 bp DNA amplified by the primers ECH-U/ECH-L, which could detect the target from approximately 50 organisms, followed by the primers VacAF/VacAR (detection limit = 500 organisms). The primers CagAF/CagAR and ET-5U/ET-5L showed lower sensitivities (detection limit = 5000 organisms) (Table II). Our results are in agreement with other reports showing the best analytical sensitivity of ECH-U/ECH-L for detection of *H. pylori* by PCR [9], [12].

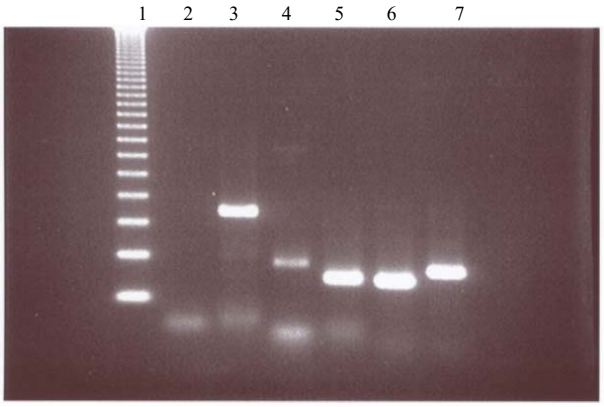

Fig. 1 Agarose gel electrophoresis of *H. pylori* DNA amplified in PCR using different primer pairs. Lane 1= 123 bp DNA ladder (molecular weight marker), Lane 2= Negative control lacking *H. pylori* genomic DNA, Lane 3= PCR product with ECH-U/ECH-L primer (417 bp), Lane 4= PCR product with ET-5U/ET-5L primer (230 bp), Lane 5= PCR product with CagAF/CagAR primer (183 bp), Lane 6= PCR product with Vac1F/Vac1xR primer (176 bp), Lane 7= PCR product with Vac1F/Vac1xR primer (203 bp)

In order to evaluate all of the four primer pairs for molecular identification of *H. pylori*, the genomic DNA from 72 clinical isolates were tested for amplification of their targets by PCR. The concentration of *H. pylori* genomic DNA in all these experiments were adjusted to 1 ng, which was at least 100 times more than the minimum concentrations required, as shown in Table II. This was done to avoid the limitation with respect to the quantity of DNA used. The results showed that the specific targets were amplified from genomic DNA of 67/72 (93%) and 71/72 (99%) with ECH-U/ECH-L and the Vac1F/Vac1xR, respectively, and the differences were statistically non-significant (P >0.05) (Table III). Among the remaining two primer pairs, ET-5U/ET-5L amplified targets from 51/72 (71%), which was significantly

less than the targets amplified by ECH-U/ECH-L (P < 0.0005) and the Vac1F/Vac1xR (P< 0.00001). On the other hand, the primers CagAF/CagAR amplified the specific target from the lowest number of *H. pylori* isolates (positives = 26/72, 36%), which was significantly less than the targets amplified by ECH-U/ECH-L (P< 0.00001), Vac1F/Vac1xR (P< 0.00001) and ET-5U/ET-5L (P < 0.01) (Table III). These results demonstrated that VacA gene is the most sensitive target and CagA gene is the least sensitive target for detection of *H. pylori* due to the presence or the absence of the respective genes. Similar results have been reported from other parts of the world as well [14]-[18].

TABLE II
EFFECT OF *H. PYLORI* GENOMIC DNA CONCENTRATION ON THE AMPLIFICATION OF TARGETS BY USING VARIOUS PRIMER PAIRS IN PCR

Primer pair	PCR results with various genomic DNA concentrations						
	1 ng	100 pg	10 pg	1 pg	100 fg	10 fg	1 fg
ECH-U/ECH-L	+ve	+ve	+ve	+ve	+ve	-ve	-ve
ET-5U/ET-5L	+ve	+ve	+ve	-ve	-ve	-ve	-ve
CagAF/CagAR	+ve	+ve	+ve	-ve	-ve	-ve	-ve
Vac1F/Vac1XR	+ve	+ve	+ve	+ve	-ve	-ve	-ve

TABLE III
PCR AMPLIFICATION OF DNA TARGETS USING DIFFERENT PRIMER PAIRS WITH 72 CLINICAL ISOLATES OF *H. PYLORI*

Primer pair	No. positive/tested	% positive
ECHU/ECHL	67/72	93%
ET5U/ET5L	51/72	71%
CagAF/CagAR	26/72	36%
Vac1F/Vac1XR	71/72	99%

The *vacA* and *cagA* genes encode for cytotoxins that have been suggested to have roles in the virulence of *H. pylori* [19], [20]. Among these genes, *vacA* is polymorphic and has variants, e.g. *s1* and *s2* [19], [20]. The annealing sites for the primers Vac1F/Vac1xR are the same for *vacA s1* and *vacA s2* genotypes but they differ in the internal sequences with *s1* variant having a smaller size (176 bp) than *s2* variant (203 bp), when these targets are amplified by PCR, due to a difference of 27 bases among the two variants [11]. The *vacA s1* variant is associated with virulence but not the *vacA s2* variant [18], [21]. In this study, the results of genotyping experiments showed that the number of *vacA s1* isolates were significantly more than *vacA s2* isolates (P <0.01) (Table IV).

TABLE IV
FREQUENCY OF *S1* AND *S2* GENOTYPES AMONG *VACA*$^+$ ISOLATES

Number of *vacA*$^+$ isolates	*vacA s1*		*vacA s2*		P
	P/T	% positive	P/T	% positive	
71	44/71	62%	27/71	38%	<0.01

P/T = No. positive / No. tested

The *vacA* genotyping results were further analyzed with respect to the association of *vacA s1* and *s2* variants in relation to *cagA* status. This analysis revealed that *vacA s1* (46% positive) and *vacA s2* (54% positive) genotypes were almost equally associated with *vacA*$^+$/*cagA*$^-$ isolates (P >0.05). However, in case of *vacA*$^+$/*cagA*$^+$ isolates, *s1* genotype (92% positive) was more frequently detected than *s2* genotype (8% positive) (P < 0.00001) (Table IV). It has been suggested that the association of *cagA* with pathogenesis could be due to fact that it is significantly more associated with the pathogenic *vacA s1*genotype [21], [22].

TABLE V
VACA S1 AND *VACA S2* GENOTYPE DISTRIBUTION WITH RESPECT TO *CAGA* STATUS AMONG 71 *VACA*$^+$ CLINICAL ISOLATES OF *H. PYLORI*

cagA status	*vacA s1*		*vacA s2*		P
	P/T*	% positive	P/T	% positive	
cagA positive (n = 25)	23/25	92%	2/25	8%	<0.00001
cagA negative (n = 25)	21/46	46%	25/46	4%	>0.05

P/T = No. positive / No. tested

IV. CONCLUSION

Among the four sets of primer pairs tested for the detection of *H. pylori*, Vac1F/Vac1XR provided the best result, and hence, this primer pair may provide optimal sensitivity for the specific detection of *H. pylori* in clinical specimens. The genotyping experiments showed that *vacA s*1 and *vacA s2* genotypes were almost equally associated with *vacA*$^+$/*cagA*$^-$ isolates, but an overwhelming majority of *vacA*$^+$/*cagA*$^+$ isolates had *vacA s1* genotype, suggesting that the association of *cagA* with pathogenesis could be due to its increased association with *vacA s1* genotype.

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