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Detection of *Helicobacter pylori* by enzyme-linked immunosorbent assay of thermophilic helicase-dependent isothermal DNA amplification

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Abstract

An enzyme-linked immunosorbent assay (ELISA) of thermophilic helicase-dependent isothermal DNA amplification (tHDA) was developed for detection of *Helicobacter pylori*. The primers targeting *ureC* were used for the amplification of bacterial DNA by the isothermal digoxigenin (DIG)-labeling tHDA process, resulting in the accumulation of DIG-labeled DNA amplicons. The amplicons were denatured using heat and then hybridized with a specific biotinylated DNA probe, which was noncovalently immobilized on streptavidin-coated microtiter plate. The hybrids were colorimetrically detected by the addition of an anti-DIG antibody HRP conjugate and 2,2-azino-di-(3-ethylbenzthiazolinsulfonate) substrate solution. Results obtained from the gastric biopsy samples showed 90% and 95.7% of sensitivity and specificity, respectively, in comparison with culture results, and 96.6% and 96.8% of sensitivity and specificity, respectively, in comparison with those of the histologic studies. This assay significantly reduces the time needed for the identification of *H. pylori* and has the potential to facilitate early detection of this gastrointestinal pathogen.

Keywords: tHDA-ELISA; Digoxigenin (DIG); *Helicobacter pylori*; *ureC*

1. Introduction

Helicobacter pylori is a Gram-negative microaerophilic bacterium that infects human gastric epithelial cell surfaces. This bacterium causes chronic infection in a large proportion of the world's population and is associated with a number of different clinical conditions (Shahamat et al., 2004; Brooks et al., 2004). Accurate diagnosis is essential for the effective treatment and management of infections that are caused by this organism. To overcome this problem, we have tested a variety of culture-independent methods for the detection of *H. pylori* in clinical samples (Brooks et al., 2004; Chanto Grettel et al., 2002; Chanfu et al., 1997). Polymerase chain reaction (PCR) also has been widely used to detect *H. pylori* from clinical specimens. However, it requires thermocycling to separate the two DNA strands (Lu et al., 1999; He et al., 2002). A completely different approach is that of Kong et al. who came up with the idea of using a DNA helicase to separate the DNA strands rather than heat while working at New England Biolabs (Vincent et al., 2004). This new technique has been named thermophilic helicase-dependent isothermal DNA amplification (tHDA) and does not require thermocycling (An et al., 2005). In this study, we describe a relatively simple diagnostic method that detects

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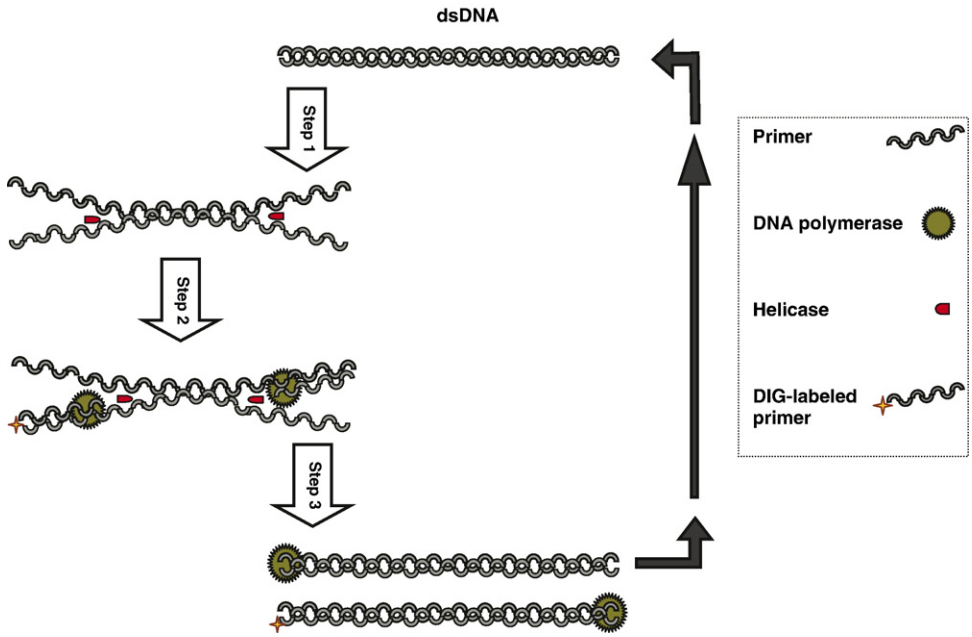

Fig. 1. DIG-labeling tHDA. The figure shows the DIG-labeling tHDA process. Step 1, the helicase unwind DNA duplexes. Step 2, the primers anneal to the ssDNA. Step 3, DNA polymerase extends the primers. One duplex is amplified to 2 duplexes. The dsDNAs are separated by helicase, and this chain reaction repeats itself.

H. pylori in gastric biopsies called *enzyme-linked immunosorbent assay of tHDA (tHDA-ELISA) detection system* (Figs. 1 and 2).

2. Materials and methods

2.1. Bacterial culture and maintenance

The *H. pylori* (ATCC 49503) strain was provided from the Department of Bacteriology, Faculty of Medical Sciences, Iran University of Medical Sciences, Tehran, Iran, preserved in LB broth medium and 20% sterile glycerol. The *Escherichia coli* (ATCC 11775) was used to evaluate the specificity of the tHDA reaction and grown in tryptic soy broth.

2.2. Clinical biopsy samples

One hundred twenty-four human gastric biopsy samples were obtained from patients and analyzed as they were received. Three sets of biopsy specimens from the antrum and the corpus of each patient's stomach were obtained, and each set was used for culture, histology, and tHDA-ELISA assay. The size of specimens was 3.5 to 5 mg in weight. These specimens were collected from patients seen in the Baqiyatallah Hospital, Tehran, Iran, in accordance with the ethical standards of the Helsinki Declaration of 1975 as reflected in the guidelines of the Medical Ethics Committee, Ministry of Health, I. R. Iran. The samples were not collected specifically for the current study. Histologic and microbiologic data were available for all samples tested.

2.3. Nucleic acids and DNA extraction

Purified human tissue DNA and *E. coli* DNA were used to evaluate the impact of nonhomologous nucleic acids on the specificity of the tHDA reaction. The DNA from gastric biopsy specimens, human tissue, and bacterial standard media (*E. coli* ATCC 11775; *H. pylori* ATCC 49503) were extracted using QIAGEN DNeasy tissue extraction kit (QIAGEN, Valencia, CA) according to the manufacturer instructions.

2.4. Determinations of ureC gene copy number in H. pylori

Genomic DNA from *H. pylori* (ATCC 49503) was extracted by the QIAGEN DNeasy tissue extraction kit and dissolved in 1 × TE buffer (10 mmol/L Tris and 1 mmol/L ethylenediaminetetraacetic acid [EDTA], pH 8.0). The absorbance of the DNA solution was measured at 260 nm 3 times, and the mean value was recorded as the actual absorbance. The number of bacteria was calculated as the number of *H. pylori* per microliter = (concentration of DNA solution)/(mean mass of *H. pylori* genome). The mean mass of the *H. pylori* genome was calculated from the mean size of the genome, which was assumed to be 1.6 Mb (He et al., 2002).

2.5. Primers and probe

A primer pair was selected from published *H. pylori ureC* gene nucleic acid sequences (Genbank accession number M60398.1), synthesized by MWG Biotech, Germany. This pair of primers amplifies a 292-base pair (bp) of the target

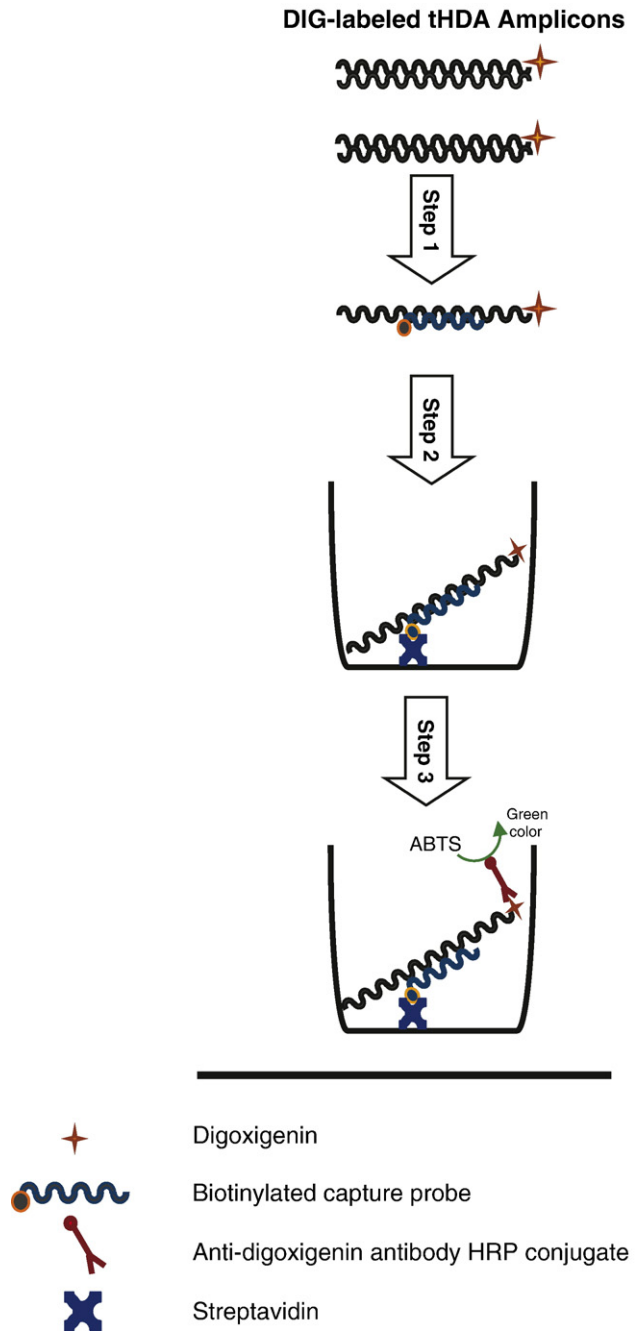

Fig. 2. ELISA of DIG-labeled tHDA amplicons. Step 1, the single-stranded (denatured) DIG-labeled tHDA amplicon is associated with complement biotinylated capture probe. Step 2, the hybrid is transferred to streptavidin-coated well to immobilize. Step 3, the ELISA DIG detection is performed to develop color.

DNA that includes the species-specific region for oligonucleotide probe to identify the *H. pylori* (Table 1). The reverse primer (HPU R) bears the digoxigenin (DIG)-11-dUTP at its 5′ end. Biotin-modified probe (HPU P) corresponding to an internal region defined by the primers were synthesized by MWG Biotech and used for the detection of amplified target DNA.

2.6. tHDA process

The reagents for tHDA were all from New England Biolabs. To set up a 25-μL DIG-labeling tHDA, we prepared 12.5 μL premix of DNA template and primers (2 μmol/L of each primer) in a 0.2-mL microcentrifuge tube in a sterile hood by combining 1.25 μL of 10× annealing buffer, 3.5 mmol/L $MgSO_4$, and ddH_2O to a volume of 25 μL. The premix was incubated at 95 °C for 2 min and then 60 °C for 3 min using an incubator. An equal of 2× tHDA mix (200 μmol/L dNTPS, 3 mmol/L dATP, 10 U of Bst polymerase, 50 ng of Tte-UvrD helicase, ±200 ng Tte-MutL, and ddH_2O)—thawed and kept on ice—was added to the premix and gently mixed by pipetting followed by brief centrifugation. The reaction mixture was covered with a layer (50 μL) of inert oil (Sigma) to prevent evaporation during the reaction. The tube was incubated at 60 °C for 1 h. Negative control consisted of all of the same reagents but substituted for the target with an equal volume of ddH_2O that contained no target nucleic acid. The amplification products were analyzed by agarose gel electrophoresis or by tHDA-ELISA detection system (Vincent et al., 2004; An et al., 2005).

2.7. tHDA-ELISA detection system

2.7.1. Coupling protocol for streptavidin-coated microtiter plate

The Nunc Immobilizer™ streptavidin plate was washed with 3× 300-μL/well PBST buffer (phosphate-buffered saline containing 0.05% (vol/vol) TWEEN® 20, pH 7.2) without any incubation step. This was done to ensure improved readouts and a very low coefficient of variation (CV %). Biotinylated probe (HPU P) (0.1 μmol/L, 100 μL) diluted in PBST buffer was incubated in the plate at room temperature for 1 h with gentle agitation. After aspiration, the plate was washed 3 times with 300 μL of PBST to prepare for assay application (Koch et al., 2000).

2.7.2. Colorimetric detection of the DIG-labeled amplicons

Ten microliters of DIG-labeled tHDA product was heated 5 min at 95 °C to denature and added to 100 μL of hybridization solution (50 mmol/L sodium phosphate buffer, pH 7.0). The tHDA product (100 μL) was added to the oligoprobe-coated streptavidin microtiter plate and incubated for 1 h at 37 °C with gentle shaking. Plates were washed 3 times with PBST buffer and incubated again for

Table 1
Nucleotide sequences of oligonucleotide primers and probe used in this study

Primers/probe	Sequence	Location
HPU F	5′-CTTTTAGGGGTGTTAGGGGT-3′	1567–1588
HPU R	5′-DIG AAGCTTACTTTCTAACACTAACGC -3′	1296–1319
HPU P	5′-biotin CGATTGGGGATAAGTTTGTG-3′	1433–1452

30 min at room temperature. One hundred microliters of anti-DIG antibody HRP conjugate (Roche) diluted 1:100 in PBST buffer was added to each well, and microtiter plates were incubated for 30 min at room temperature. After 5 washes with PBST buffer, 100 μL of 2,2-azino-di-(3-ethylbenzthiazolinsulfonate) substrate (Roche) was added to each well and incubated at 37 °C for 15 min. Finally, the developed color was measured at 405 nm in a microtiter plate reader (Gill et al., 2006).

2.8. Validation of the tHDA-ELISA

2.8.1. Sensitivity determination

For the evaluation of the sensitivity of the test, 10-fold serial dilutions of DNA from *H. pylori* (ATCC 49503) were prepared in TE buffer (10 mmol/L Tris, 1 mmol/L EDTA) for tHDA-ELISA procedure described above (Gill et al., 2006; Jean et al., 2002).

2.8.2. Specificity determination

For the evaluation of the specificity, nontarget nucleic acids, notably human genomic DNA and genomic *E. coli* DNA, were added, alone or in the presence of *H. pylori* DNA, and analyzed by the tHDA-ELISA detection system (Gill et al., 2006; Jean et al., 2002).

3. Results

3.1. Specificity evaluation of the tHDA process

The analyses of the tHDA products from *H. pylori* (ATCC 49503) by agarose gel electrophoresis show a unique band corresponding to the expected size of 292 bp (Fig. 3).

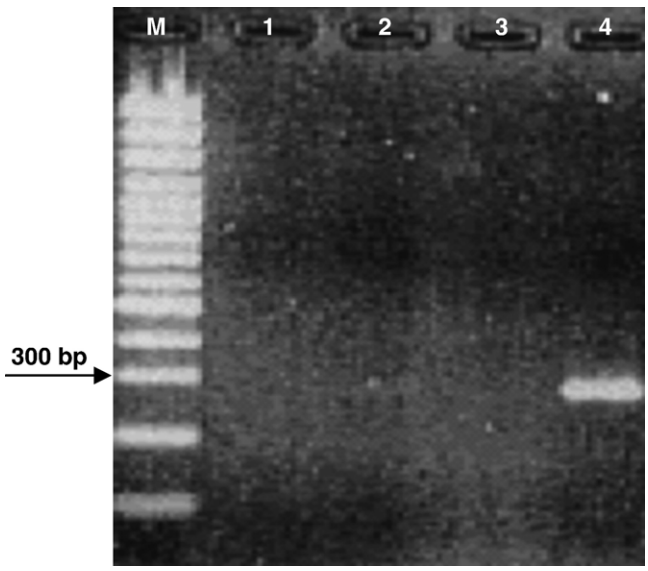

Fig. 3. Analysis of primer specificity of tHDA process. Lane M, 100 bp of molecular marker (Fermentas). Lane 1, no band of tHDA product on negative control. Lane 2, no band of tHDA product on *E. coli* (ATCC 11775). Lane 3, no band of tHDA product on human DNA. Lane 4, 292 bp of tHDA product on *H. pylori* (ATCC 49503).

No band was observed with the negative control, with the *E. coli* DNA and with the human genomic DNA early templates. The results confirmed the effectiveness of the tHDA system developed and the specificity of the selected primer pair for the amplification of the target region in *ureC* DNA.

3.2. Detection of DIG-labeled tHDA amplicons by DIG detection ELISA

As shown in Fig. 4, the efficiency of the tHDA-ELISA was confirmed by analyzing 2-fold serial dilutions of DIG-labeled tHDA amplicons of *ureC* DNA from *H. pylori*.

3.3. Specificity evaluation of the tHDA-ELISA detection system

The specificity of the tHDA-ELISA detection system was confirmed by the analysis of nontarget nucleic acids obtained from *E. coli* DNA and human genomic DNA. The results showed no detection of any target nucleic acid (Fig. 5).

3.4. Sensitivity determination of the tHDA-ELISA detection system

The analytical sensitivity of the tHDA-ELISA assay was determined by testing serial dilutions of *H. pylori* (ATCC 49503) concentration with a detection limit of 10 copies of *H. pylori ureC* gene (Fig. 6). From the results, the cutoff value of the test was calculated 0.3 ± 0.1 according to the optical density in 405 nm for this detection system.

3.5. Clinical performance of assay

The clinical performance of the tHDA-ELISA assay was determined by comparing the tHDA-ELISA results with those of culture and histologic studies. Included in the study were 124 gastric biopsy samples. The overall results presented in Table 2 indicate that tHDA-ELISA has a sensitivity of 90% and specificity of 95.7% in comparison with those of the culture, and sensitivity of 96.6% and specificity of 96.8% in comparison with those of the histologic studies.

4. Discussion

The detection of *H. pylori* is critical because of the high prevalence of this species, its worldwide distribution, and the large number of individuals carrying this organism (Chanto Grettel et al., 2002; Dale et al., 1998; Feldman, 2001; Suerbaum and Michetti, 2002). Molecular methods, in particular, PCR, have the potential to detect more cases of infection because of their greater sensitivity but are technically demanding (Ho and Windsor, 2000). Despite its widespread appeal, the need for temperature cycling in PCR limits its portability because of the significant energy used in such sensitive instrumentation. As a result, several isothermal amplification methods have been developed (Walker

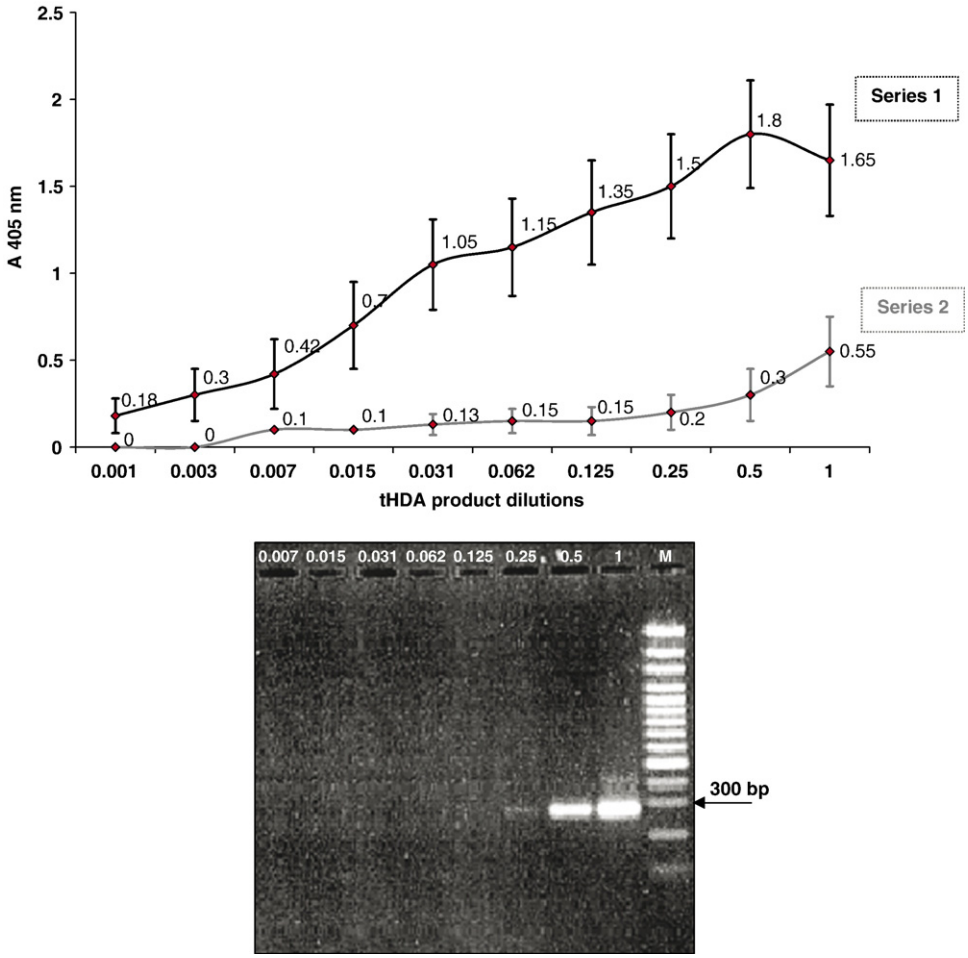

Fig. 4. DIG detection ELISA and gel electrophoresis of 2-fold serial dilutions of DIG-labeled tHDA products. tHDA was performed in the presence of 10^3 copies of *ureC* gene on *H. pylori* (ATCC 49503), and then DIG-labeled tHDA product was 2 fold serially diluted before DIG detection ELISA system (series 1). NSB, DIG detection ELISA of 2-fold serial dilutions of DIG-labeled tHDA product of negative control (series 2). The plotted results are the averages of triplication analysis.

et al., 1992; Dean et al., 2001; Compton, 1991; Notomi et al., 2000; Wharam et al., 2001; Kurn et al., 2005). Recently, a true isothermal DNA amplification technology that incorporates a helicase enzyme into the reaction scheme was reported (Vincent et al., 2004). Helicase-dependent amplification exploits the unwinding activity of a helicase to

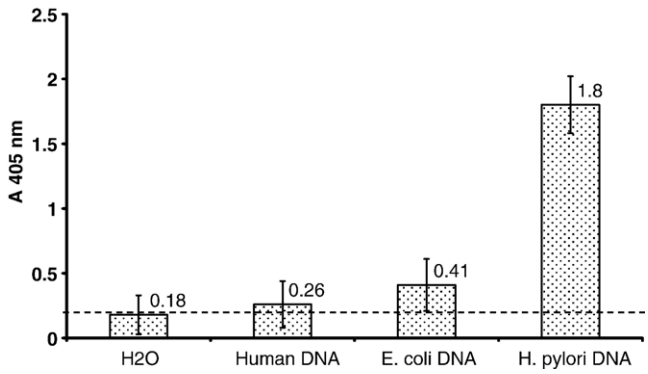

Fig. 5. Specificity of the tHDA-ELISA detection system. tHDA-ELISA was performed with nontarget nucleic acids such as total *E. coli* DNA and human DNA. tHDA-ELISA was performed on DNA of *H. pylori* (ATCC 49503) as a positive control. DIG detection ELISA was performed on ddH_2O as a negative control.

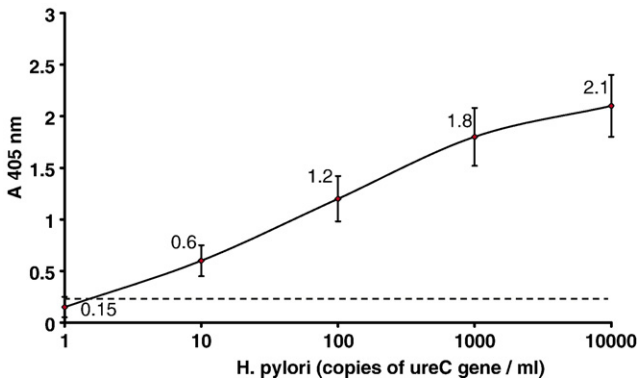

Fig. 6. Sensitivity of the tHDA-ELISA detection system for detection of *H. pylori ureC* gene. Ten-fold serial dilutions of *H. pylori* were prepared and used for DNA extraction and DIG-labeling tHDA by the above protocols. Then 10 μL of the tHDA products was used in DIG detection tHDA-ELISA system. Each dilution was analyzed in duplicate.

Table 2
Comparison of culture results and histologic studies with those of the tHDA-ELISA assay

Test and result	No. of culture results		Sensitivity (%)	Specificity (%)	No. of histologic studies		Sensitivity (%)	Specificity (%)
	Positive	Negative			Positive	Negative		
tHDA-ELISA								
Positive	27	4	90.0	95.7	29	3	96.6	96.8
Negative	3	90			1	91		

separate duplex DNA targets during in vitro DNA amplification, eliminating the need for thermocycling (An et al., 2005). The present study is related to the detection of *H. pylori* in human gastric biopsies using a simple and rapid molecular technique called *tHDA-ELISA detection system*. The assay procedure is composed entirely of transfers, additions, and incubation, and is not technically demanding. However, to avoid potential carryover contamination, careful adherence to the protocol, including bleach decontamination of reaction tubes and work surfaces, is required. Results obtained by testing dilutions of *H. pylori* detected 10 copies of the *ureC* gene. However, Kong et al. have reported 500 copies of target gene in their study for gel electrophoresis tHDA end-point detection (An et al., 2005). The increased sensitivity seen here may be because the detection step is performed with ELISA of DIG-labeled tHDA amplicons.

The clinical sensitivity was 90.0% compared with 98.3% of the culture (Shahamat et al., 2004). This compares with the reported sensitivities for PCR, which range from 34% to 100% (Brooks et al., 2004; Chanto Grettel et al., 2002; Foxall et al., 1992; Tomb et al., 1997; Valentine et al., 1991). In a study of this type, the clinical sensitivity of a test is dependent on 4 major factors: 1) the analytical sensitivity of the assay, 2) the sensitivity of the standard (culture technique), 3) the distribution of positive specimens, from low positive to high positive, included in the study, and 4) the effects of sample heterogeneity, especially in samples with low-positive results. In conclusion, the combination of tHDA with the microplate hybridization system offers several advantages (including optimal sensitivity and specificity) over other molecular techniques. This assay is simple and rapid (4 h) to perform, and allows the analysis of several samples simultaneously. On the other hand, the system is cost-effective because it does not require an expensive thermocycler and the reagents can be obtained with the nearly same budget needed for the PCR from New England Biolabs or Biohelix Corporation.

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