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A Multiplex-Urease PCR Assay for Detection of *Helicobacter pylori* Infection Directly from Gastric Biopsy Specimens and Comparison of Multiplex-Urease PCR Results with Rapid Urease Test and Histopathology

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Abstract

Purpose: To develop a multiplex-urease PCR assay specific for *ure*A and *ure*B genes directly from gastric biopsy specimens and compare the results of multiplex-urease PCR assay with rapid urease test (RUT) and histopathology.

Methodology: This study was conducted on 109 patients. A RUT was run; histopathological staining and multiplex urease PCR were applied to biopsy specimen to detect *H. pylori*. Multiplex-urease PCR, RUT and histopathology results were compared by calculating Cohen’s kappa coefficient.

Results: A multiplex-urease PCR assay specific for *ure*A and *ure*B genes was developed to detect *H. pylori* directly from biopsy specimens. Cohen's kappa coefficient results of histopathological staining and multiplex-urease PCR indicate a substantial agreement. There was a moderate agreement between the results of histopathological staining and RUT results. There is a fair agreement between the multiplex urease PCR and the RUT results. Furthermore, the multiplex-urease PCR can detect *H. pylori* in some samples that are identified as negative by the rapid urease test and histopathological staining method. Moreover, in some patient samples *ure*A could not be detected while *ure*B detected.

Conclusion: Multiplex-urease PCR assay was developed to detect *ure*A and *ure*B genes of *H. pylori* directly from gastric biopsy specimens. Comparison results indicated that detection rate of *H. pylori* with multiplex-urease PCR and histopathological staining is higher than the RUT. Moreover, it is critical to apply RUT test to biopsy specimens taken from both antrum and corpus part as in multiplex urease PCR assay. Furthermore, developing a multiplex PCR for both *ure*A and *ure*B is essential to detect active *H. pylori* infection.

Keywords: *H. pylori*; Urease A and B; Multiplex-PCR; RUT; Histopathology

Background

Helicobacter pylori (*H. pylori*) is a Gram negative, spiral-shaped, microaerophilic bacterium that is colonized primarily in the stomach of more than half of the world’s population during their life span. While most of the infected individuals remain asymptomatic, bacteria have a significant pathogenic role in peptic ulcer, chronic gastritis, mucosa-associated lymphoid tissue lymphoma and gastric cancer in 20% of infected individuals [1].

There are both invasive and non-invasive diagnostic tests for *H. pylori* infection. Invasive tests require upper gastrointestinal endoscopy (gastroscopy) and are based on the analysis of gastric biopsy samples. Non-invasive techniques such as serology, urea breath test (RUT), urine/blood test, or detection of *H. pylori* antigen in stool specimen [2].

Rapid urease test (RUT) is a widely used in clinical practice as a biopsy-based method, to detect *H. pylori* urease enzyme which promotes the survival of bacteria in the acidic stomach environment breaking down the urea into carbon dioxide and ammonia allowing *H. pylori* to survive in the acidic medium. RUT method detects the active *H. pylori* infection in a gastric biopsy specimen using an agar gel or a reaction strip containing urea. A biopsy specimen is taken from the antrum of the stomach is placed into a medium containing urea and an indicator such as phenol red. The urease produced by *H. pylori* hydrolyzes urea to ammonia, which raises the pH of the medium, and changes the color of the specimen from yellow (negative) to red (positive). It is a simple and a rapid test but its sensitivity and specificity can be decreased in patients with acute ulcer bleeding and usage of bismuth containing compounds and antibiotics [3].

Urease enzyme is pivotal for decreasing stomach acidity by generating ammonia and carbonate from urea. Urease enzyme is necessary for *H. pylori* colonization on the gastric mucosa layer and a potent immunogenic that elongates a vigorous immune response. The active site of the urease enzyme is found in the UreB subunit. The UreA subunit of Helicobacter species is different from the other bacterial species because of its amino acid sequence that is encoded by the single *ure*A gene [4].

For the genetic identification of *H. pylori* several PCR assay methods have been developed [5]. In a study *vac*A and *cag*A were investigated by multiplex PCR in directly from gastric biopsy specimens, for genotyping [6]. In another study *H. pylori* was detected by using one step multiplex PCR targeted to urease A gene, 16S ribosomal RNA and *hpa*A gene [7]. To best of our knowledge there are no multiplex PCR assay for *ure*A and *ure*B for the detection of *H. pylori* previously published.

The aim of the present study was to develop a multiplex-urease PCR assay specific for *ure*A and *ure*B directly from gastric biopsy specimens and compare the results with rapid urease test (RUT) and histopathology results to determine its sensitivity and specificity.

Methods

Ethical approval

Each patient included in this study provided a written informed consent to participate to the study. This study was approved by the ethical committee of the Acıbadem University and Istanbul Technical University.

Patients selection

Biopsy specimens were obtained from the patients who underwent endoscopy because of gastroduodenal diseases at the Gastroenterology Department of Acibadem Hospital Groups, in Istanbul, Turkey. In total, 109 patients were selected for the study.

DNA region(s) amplified	Primer Name	Sequence (5'→3') PCR	Product Size (bp)
*ure*A	*ure*A-F	TGATGGGACCAACTCGTAACCGT	244
*ure*A	*ure*A-F	CGCAATGTCTAAGCGTTTGCCGAA	244
*ure*B	*ure*B-F	AGTAGCCCGGTAGAACACAACATCCT	645
*ure*B	*ure*B-F	ATGCCTTTGTCATAAGCCGCTTGG	645

Table 1: Multiplex urease PCR primers designed for the amplification of *H. pylori* urease genes.

Gastric biopsy specimens

During endoscopy two gastric biopsy specimens were taken from the antrum and corpus parts of stomach for the DNA isolation. Fresh biopsy specimens were placed into the RNA later solution (Ambion, RNAlater® RNA Stabilization Solution) and kept at +4°C for overnight then put at −80°C deep freezer until DNA isolation. Also biopsy specimens were obtained for the routine RUT and histopathological examination.

DNA extraction

To study multiplex urease PCR, DNA was extracted by using a DNA isolation kit (Quick g-DNATM, ZYMO RESEARCH) following the manufacturer's description. All DNA samples were quantified using NanoDrop (ND-2000, ROCHE) and maintained in −80°C deep freezer.

Primer design

Primers, obtained from metabion international AG, Germany, were used in this study. For the multiplex urease PCR assay primers, GenBank entries were searched for the selected urease genes sequences including *ure*A, *ure*B. The primers were designed by using Primer 3 software (Table 1). A BLAST search was performed to confirm the specificity of the DNA sequences of all the primers (http://www.ncbi.nlm.nih.gov/BLAST/).

Multiplex urease PCR assay

For rapid identification of *H. pylori* with urease (urea) gene-based PCR assay, genomic DNA from several biopsy specimens and primer sequences were evaluated. *ure*A and *ure*B genes are the targets for the urease activity of *H. pylori*. To optimize the multiplex urease PCR genomic DNA isolated from *H. pylori* G27 strain was used as a positive control that includes both *ure*A and *ure*B genes. The amplification reactions were carried out in a total volume of 25 µl and the multiplex urease PCR assay mixture consisted of 0.65 U of Dream *Taq* DNA polymerase (Thermo Scientific), 2.5 µl from 10X Dream *Taq* Buffer (includes 20 mM $MgCl_2$), 20 µM of forward and reverse primers, 200 µM of each dNTP and 3 µl DNA. Amplification programme included an initial denaturation step at 95°C for 3 min followed by 45 cycles of denaturation at 95°C for 45s, primer annealing at 60°C for 45s and primer extension at 72°C for 2 mins, with final extension step at 72°C for 5 mins.

The PCR products were subjected to electrophoresis on agarose gels and stained with SYBR Gold (*In vitro* gene). The specificity of the urease primer pairs was confirmed by employing a positive and several negative controls.

Results

Detection of *H. pylori* by multiplex urease PCR

Two subunit genes, *ure*A and *ure*B encode urease, which is the enzyme that contributes to the survival of bacteria in the acidic environment of the stomach. To determine the presence of *H. pylori* in human gastric biopsy specimens, we assessed the expression status of *ure*A and *ure*B by multiplex-PCR assay. We utilized *H. pylori* strain G27 with a known complete genome sequence [8] to optimize multiplex-PCR to detect *H. pylori* virulence factor genes, *ure*A and

*ure*B genes. As a result of optimization studies, *ure*A and *ure*B genes were detected in a single PCR assay (Figure 1).

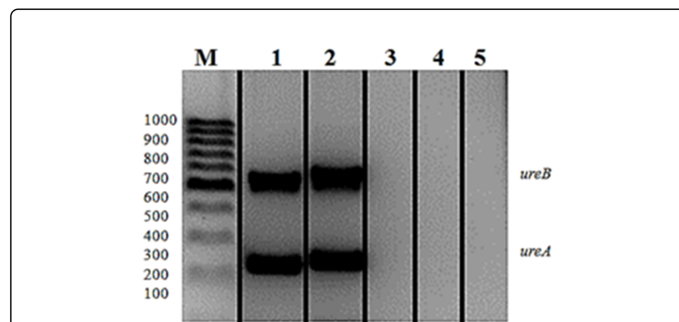
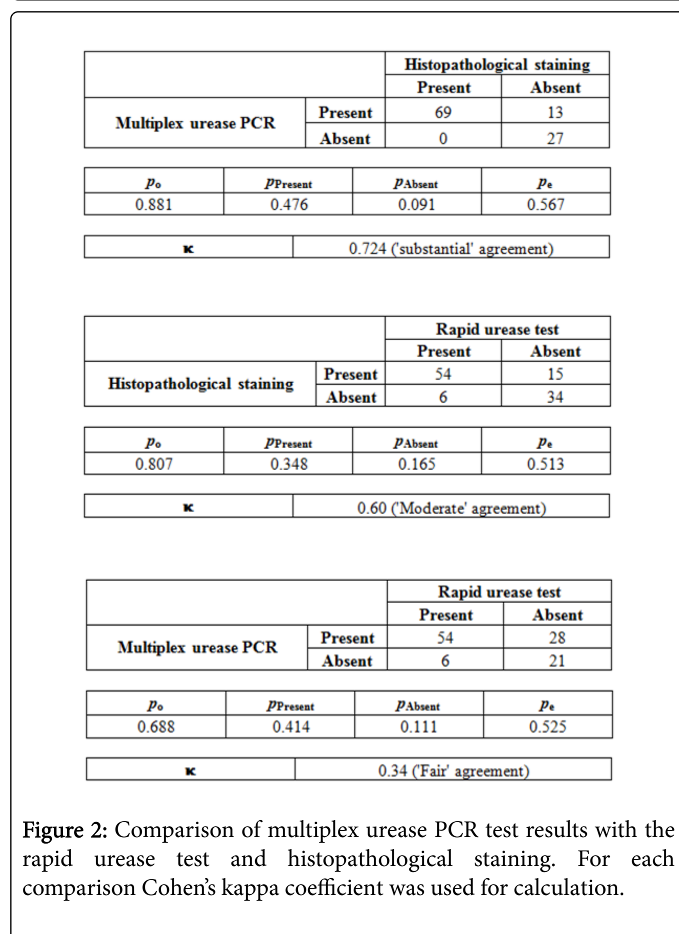


Figure 1: Multiplex urease-PCR assay to detect the *H. pylori* positive and negative samples; Lane M: 100 bp ladder-marker (ThermoSCIENTIFIC, Gene Ruler); Lane 1: amplification of *ure*A and *ure*B genes of *H. pylori* positive control strain G27; between Lane 2 to 5 are from randomly selected patients. Lane 2 is *H. pylori* positive patient sample. Lane 3 to 5 are *H. pylori* negative samples.



		Histopathological staining	
		Present	Absent
Multiplex urease PCR	Present	69	13
	Absent	0	27

p_o	$p_{Present}$	p_{Absent}	p_e
0.881	0.476	0.091	0.567

κ	0.724 ('substantial' agreement)

		Rapid urease test	
		Present	Absent
Histopathological staining	Present	54	15
	Absent	6	34

p_o	$p_{Present}$	p_{Absent}	p_e
0.807	0.348	0.165	0.513

κ	0.60 ('Moderate' agreement)

		Rapid urease test	
		Present	Absent
Multiplex urease PCR	Present	54	28
	Absent	6	21

p_o	$p_{Present}$	p_{Absent}	p_e
0.688	0.414	0.111	0.525

κ	0.34 ('Fair' agreement)

Figure 2: Comparison of multiplex urease PCR test results with the rapid urease test and histopathological staining. For each comparison Cohen's kappa coefficient was used for calculation.

Next, we used multiplex-urease PCR assay to detect the presence of *H. pylori* in gastric biopsy specimens of 109 patients. In total 80 of these patients were detected as *H. pylori* positive.

Furthermore, multiplex-urease PCR results were compared with methods used in routine, the rapid urease test and histopathological staining. To analyses the efficiency, specificity and accuracy of multiplex-urease PCR assay compared with the rapid urease test and histopathological staining, Cohen's kappa coefficient value which measures inter-rater agreement for categorical items, was calculated for each comparison (Figure 2). If the raters are in complete agreement then κ=1. The κ value can be interpreted as follows; κ<0 "poor agreement", κ=0.00-0.20 "slight agreement", κ=0.21-0.40 "fair agreement", κ=0.41-0.60 "moderate agreement", κ=0.61-0.80 "substantial agreement", κ=0.81-1.00 "almost perfect agreement" [9]. There was a substantial agreement between the results of multiplex-urease PCR and the histopathological staining. Cohen's kappa coefficient for the comparison of histopathological staining and rapid urease test results indicate a moderate agreement. There is a fair agreement between the multiplex urease PCR and the rapid urease test results in which the κ is between 0.21-0.40 (Figure 2). Furthermore, the multiplex-urease PCR can detect *H. pylori* in some samples that are identified as *H. pylori* negative by the rapid urease test and histopathological staining method.

Discussion

There are many different tests available to identify *H. pylori*, each of which has certain advantages and disadvantages. However, due to poor sensitivity or specificity, none of them can be considered as gold standard. Rapid urease test and pathological staining are the most useful methods in the clinical practice to detect *H. pylori* [10]. In this study, multiplex urease PCR assay was developed to detect *ure*A and *ure*B virulence genes of *H. pylori*. Comparison of the results of the rapid urease test with histopathological staining and the multiplex urease PCR indicated that detection rate of *H. pylori* with multiplex urease PCR and histopathological staining is higher than the rapid urease test. The possible reason for the lack of sensitivity of rapid urease test might be the region of biopsy specimens that is taken to be only from the antrum part of the stomach where the most of the bacterial colonization can be seen. However, localization of *H. pylori* colonies in patients can vary as being only in antrum, only in corpus or in both antrum and the corpus part of the stomach. In the multiplex urease PCR and histopathological staining method two biopsy specimens, one from antrum and one from corpus part of stomach are taken to increase the detection rate of *H. pylori*. Additionally, we identified that sensitivity of multiplex urease PCR assay was higher than the histopathological staining in this study. The reason for this may be because PCR may detect the DNA of the bacteria that has just began to colonize and the histopathological staining may not detect these newly colonized bacteria. Using the combinations of rapid urease test, histopathological staining and multiplex urease PCR assay, may provide quite satisfactory results in the diagnosis of *H. pylori* infection. Moreover, one-step multiplex-urease-PCR molecular technology acts as a diagnostic safety net in the detection of *H. pylori*.

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