An Evaluation of a New In-House Serum and Urine ELISA Test for Detection of *Helicobacter pylori* Infection in Thai Population

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Objective: *Non-invasive tests play significant roles in the test-and-treat approach of Helicobacter pylori management. The detection of Helicobacter pylori antibodies in urine and serum is an easy and inexpensive way to diagnose this infection. In the present study, the authors developed an in-house serum and urine ELISA tests for H. pylori antibodies and evaluated their performance in a Thai population.*
Material and Method: *One hundred thirty eight dyspeptic patients were recruited. All subjects underwent upper endoscopy and one antral biopsy was obtained for rapid urease test, which was used as a standard reference. Urine and serum samples were collected before the procedure to run in-house ELISA test.*
Results: *Thirty (22%) subjects were positive for the rapid urease test and 108 (78%) were negative. Urine and serum optical density were significantly lower in the urease negative group ($p = 0.011$ and $p < 0.001$ respectively), while there were no differences in age, gender, or endoscopic findings between the two groups. Sensitivity, specificity, negative predictive value, positive predictive value, and accuracy of urine and serum ELISA tests were 72% vs. 96.3%, 63.5% vs. 62.7%, 89.6% vs. 98.5%, 33.3% vs. 40.6%, and 64.5% vs. 69.8% respectively.*
Conclusion: *In-house serum ELISA test for H. pylori antibodies yielded a very good sensitivity with acceptable specificity, whereas urine ELISA was unable to produce satisfactory sensitivity or specificity.*

Keywords: *Urine, Serum, Non-invasive test, Helicobacter pylori, ELISA*

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Helicobacter pylori (H. pylori) is one of the most common bacterial infections in humans globally. It is known to play an important role in the development of chronic gastritis, peptic ulcer diseases, gastric adenocarcinoma, and gastric mucosal associated lymphoid tissue (MALT) lymphoma[1,2]. *H. pylori* infection is more prevalent in developing countries with the prevalence of 80-90% compared with 40-50% in developed countries[3,4]. In Thailand, 55% of the population is infected by the third decade of life[5]. Since *H. pylori* eradication reduces recurrence and complication of peptic ulcer diseases[2], accurate diagnosis of *H. pylori* infection is of great value.

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There are a number of diagnostic methods developed for the detection of *H. pylori*, including invasive and non-invasive tests. Invasive tests comprise those that require upper endoscopy, such as histopathology, culture, rapid urease test and polymerase chain reaction, while the non-invasive ones rely on the detection of antibodies (in serum or urine), antigen, or urease activity of the bacteria (urea breath test)[2]. The test-and-treat strategy has been proved to be cost-effective and safe by many studies and is now recommended in current guidelines[2,6]. This approach is to treat uninvestigated dyspepsic patients who are under the age of 55 years with no alarming symptoms. Therefore, non-invasive tests are of great importance in adopting this strategy.

Among non-invasive tests, urea breath test (UBT) is the most accurate for both the diagnosis and the follow-up. However, UBT is a complicated and expensive test and is not widely available. Serological

tests are considered more practical in clinical practice and in epidemiological studies. Due to *H. pylori* heterogeneity and different immune responses in the population living in different areas of the world, the sensitivity, specificity, and accuracy of serological tests vary, depending on which population is being tested[7-21]. Thus, a commercial kit developed outside Thailand might not yield good accuracy if tested in the Thai population. The purpose of the present study was to develop in-house antibody tests for both serum and urine, and evaluate their performance in Thai patients.

Material and Method

Dyspepsic patients who presented at the Gastroenterology Clinic, King Chulalongkorn Memorial Hospital, Bangkok, Thailand between December 2009 and December 2010 were recruited in the present study. Exclusion criteria included *H. pylori* eradication therapy in the past six months, antibiotics treatment in the previous four weeks, preceding history of gastric resection, or consumption of proton pump inhibitors, bismuth containing compounds or sucralfate within two weeks before endoscopy. Written informed consents were obtained from all subjects before participation. The present study protocol was approved by the Institutional Review Board of Faculty of Medicine, Chulalongkorn University.

All patients underwent upper endoscopy and one antral sample was obtained for a rapid urease test. Before the procedure, 5 mL of blood were drawn and 10 mL of urine were collected from each patient. Blood samples were then centrifuged and sera were separated and stored at -80°C until analyzed. Similarly, urine specimens were stored at -80°C until used.

Rapid urease test

An antral specimen was subjected in a rapid urease test (RUT) (CLO test, Ballard Medical Products, Utah, USA). The result was interpreted as positive if the color changed from yellow to pink within 24 hours. Results were used as standard to determine sensitivity and specificity of serum and urine ELISA tests.

Preparation of H. pylori antigens

H. pylori antigens were prepared using pooled *H. pylori* culture collected from gastric specimens of *H. pylori*-infected patients attending King Chulalongkorn Memorial Hospital. Cultured bacteria were harvested in phosphate buffered saline (PBS). Bacterial cells were then broken with a sonicator. The concentration of *H. pylori* extracts was calculated using spectrophotometer at the wavelength of 280 nm.

Preparation of ELISA test kit for the detection of H. pylori antibodies in serum and urine

A 100 μL aliquot of sonicated *H. pylori* antigens in PBS (15 μg/mL and 25 μg/mL for serum and urine kits respectively) were coated onto each well of a microtiter plate (ELISA plates used in the present study were provided by the Department of Microbiology, Faculty of Medicine, Chulalongkorn University). The plate was incubated overnight at 4°C and then washed with PBS-Tween solution (PBS with 0.05% Tween) three times. Then, 200 μL aliquot of blocking agent (PBS-Tween, 0.5% casein, 5% sucrose) was added into each well. After 1-hour incubation at 37°C, the plate was washed three times using PBS-Tween solution and was now ready for use. For urine ELISA kit, 25 μL aliquot of 0.2 M hydroxymethyl aminomethane chloride buffer (0.14 M NaCl, 2% casein, 0.5% bovine albumin, 0.05% Tween 20, 0.001% *E. coli* protein and 0.1% sodium azide) was further pipetted into the plate followed by one hour of incubation at 37°C.

Detection of H. pylori antibodies in serum and urine using in-house ELISA method

A 100 μL aliquot of 1:400 diluted serum or undiluted urine sample was added to each well of the appropriate plate. After incubation for one hour at 37°C, each plate was washed three times with PBS-Tween solution. The plate was then filled with 100 μL of polyclonal rabbit antihuman immunoglobulin (IgG) coupled with horseradish peroxidase (HRP) (diluted 1:4000 in OBS-Tween solution) and were incubated at 37°C for one hour. After thorough washings with PBS-Tween solution, 100 μL of substrate solution (Dako, S2045) was added into each well and the plate was incubated in the dark at room temperature for 20 minutes. A 100 μL aliquot of stopping solution was pipetted into each well. Absorbance was measured at 492 nm using Multiskan® EX plate reader (Thermal Fisher Scientific, NH, USA).

Statistical analysis

Statistical analysis was performed using SPSS for Windows version 16.0. Student t-test and Chi-square test were used for numerical and categorical data respectively. Receiver operating characteristics (ROC) curves were drawn to determine the most appropriate

cut-off values for serum and urine optical density (OD). Using RUT as a standard reference, sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV), and accuracy of serum and urine ELISA tests were calculated. Data are shown as mean ± standard error of mean (SEM.) A p-value of lesser than 0.05 is considered statistically significant.

Results

One hundred thirty eight patients (41 males and 97 females) were enrolled in the present study. Age of participants ranged from 27 to 86 years with mean age of 57.6 ± 1.1 years. According to endoscopic findings, 17 patients (12%) had normal findings, 100 (73%) showed gastritis, 10 (7%) had peptic ulcers, and eight (6%) were found with gastroesophageal reflux disease. From RUT, 108 (78%) were negative for *H. pylori* and the remaining were positive. Further analysis showed no significant difference in endoscopic findings between male and female participants. Patients were classified into RUT positive and RUT negative groups. Table 1 shows selected characteristics and endoscopic findings in each group. While age, gender ratio, and endoscopic findings were not different between the groups, urine and serum OD values were significantly lower in the RUT negative group compared with positive counterpart ($p = 0.011$ and $p < 0.001$ respectively). There was also a weak but significant positive correlation between urine and serum OD values ($r = 0.288$, $p = 0.001$).

Fig. 1 demonstrates a ROC curve for urine OD. The most appropriate cut-off values for urine OD was 0.066, which yielded a sensitivity of 72% and specificity of 63.5%. ROC curve for serum OD indicated a cut-off point at 0.166, and sensitivity and specificity of 96.3% and 62.6% respectively (Fig. 2). Specificity could be increased to 71.6% if the cut-off value went up to 0.1925; however, sensitivity would then decline to 92.6%. As illustrated in Table 2, sensitivity, specificity, NPV, PPV and accuracy of urine ELISA test was 72%, 63.5%, 89.6%, 33.3% and 64.5% respectively (using a cut-off value of 0.066). Additionally, sensitivity, specificity, NPV, PPV and accuracy of serum ELISA test was 96.3%, 62.7%, 98.5%, 40.6% and 69.8% respectively (using a cut-off value of 0.166).

Considering urine ELISA results, the authors found seven patients with false negative and 36 with false positive results. Interestingly, of all 36 false positive results, 14 of them were positive for *H. pylori* antibodies in both urine and serum. There was only one false negative result from serum ELISA test, despite relatively high false positives (38 from 129).

Table 1. Characteristics and endoscopic findings of 138 subjects in RUT positive and negative subgroups

	Urease test result		p-value
	Positive (30)	Negative (108)	
Age	60.3 ± 2.5	56.9 ± 1.2	NS
Male:female ratio	9:21	32:76	NS
Endoscopic findings			
Normal	2	15	NS
Gastritis	23	77	NS
Ulcer	4	6	NS
GERD	1	7	NS
Other	0	3	NS
Urine OD value	0.164 ± 0.03	0.097 ± 0.01	0.011
Serum OD value	0.52 ± 0.06	0.18 ± 0.02	<0.001

NS = no significant difference; OD = optical density

Discussion

With the growing popularity of test-and-treat strategy, non-invasive tests are playing increasing important roles in the diagnosis of *H. pylori*. Despite its great accuracy and reliability, UBT is expensive, complicated, and requires special instruments. Serology is, on the contrary, more advantageous in general practice and epidemiological studies. However, drawing blood is still considered a painful procedure for children. Thus, urine tests would be a good alternative for diagnosing *H. pylori* in young children.

The sensitivity and specificity of antibody tests vary among different ethnic groups and regions of the world[3]. In order to avoid this problem, the authors

Table 2. Sensitivity, specificity, NPV, PPV, and accuracy of urine and serum ELISA tests

	Sensitivity	Specificity	NPV	PPV	Accuracy
Urine ELISA test	72%	63.5%	89.6%	33.3%	64.5%
Serum ELISA test	96.3%	62.7%	98.5%	40.6%	69.8%

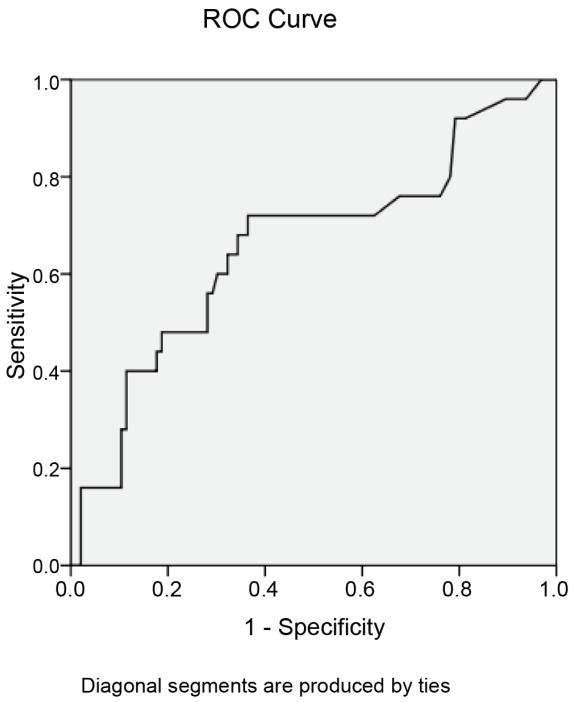

Fig. 1 ROC curve for urine OD

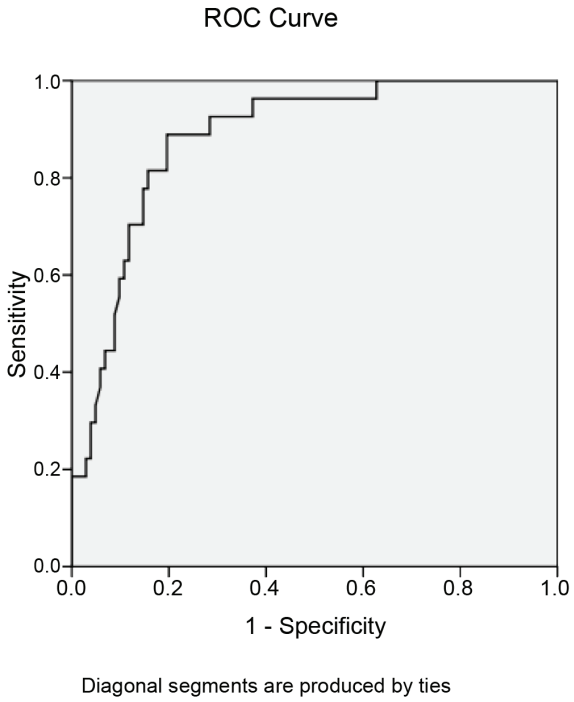

Fig. 2 ROC curve for serum OD

developed an in-house ELISA tests to detect *H. pylori* antibodies in urine and serum using *H. pylori* antigens that are commonly found in Thai populations. The results showed that a serum ELISA test provided satisfactory sensitivity and was comparable with the results from other studies[7,9,13,17,20]. The specificity was, however, relatively low. Comparing with serum study, urine ELISA test yielded lower sensitivity, but similar specificity. The sensitivities of urine tests were highly variable among other studies, ranging from 50% to 96%[8,10,16-19,21].

The lower sensitivity of urine test compared to serology could be due to the fact that IgG concentration in urine is 1/10,000 of that in serum[16]. A more sensitive method of detection is needed to increase the sensitivity of the urine test. Another explanation could be the storage method of urine samples. Adachi K et al[17] showed that the sensitivity of urine tests was extremely low using frozen urine samples. These results improved significantly when unfrozen samples were used (sensitivity increased from 50% to 91.4%). They also stated that urine samples could be kept at 4°C for 18 months and still provide good sensitivity. A possible explanation for this could be the instability of immunoglobulins under frozen condition or the interference with IgG absorbance[15].

A number of studies showed that antibody tests produced relatively low specificity. Marchildon PA et al[13] demonstrated that tests with crude or partially purified antigen preparation yielded lower specificity in relation to purified ones. Furthermore, *H. pylori* antibodies have been shown to cross-react with other intestinal organisms, such as *Campylobacter*[12,13] resulting in low specificity. Test specificity also depends on the type of methods used as a standard reference[19]. Interestingly, using biopsy-based methods as references tends to provide lower specificity.

It was worth noting that 14 of 36 false positive urine ELISA samples were positive for *H. pylori* antibodies in serum. These patients have been infected by *H. pylori* in the past and were cured or that bacterial activity might be too low to be detected by RUT[14]. Alternatively, this might be due to sampling error in obtaining biopsy specimens as *H. pylori* could be localized in an area of the stomach missed by a single biopsy. In order to determine whether these patients truly produced antibodies to *H. pylori*, confirmation tests, such as Western blot study, need to be performed.

Positive predictive values were very low for both urine and serum ELISA tests. These findings went

along with the low prevalence of *H. pylori* (21%) in the present study. It is known that the lower the prevalence the lower the PPV. This prevalence was much lower than what had been previously reported[5]. This might indicate that the prevalence of *H. pylori* in Thailand has been decreasing over time due to better sanitation and improvement in socioeconomic status.

Conclusion

The authors in-house serum ELISA test produced a high sensitivity with acceptable specificity and could be used as a screening test for the detection of *H. pylori.* However, urine ELISA test did not yield adequate sensitivity or specificity to help make a clinical judgment. Future study with new method of antibody detection, more purified antigen preparation, the use of more reliable standard reference, and better urine storage might be warranted in order to make an accurate in-house urine ELISA kit for commercial use.

Potential conflicts of interest

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การประเมินชุดทดสอบซีรัมและปัสสาวะชนิดใหม่ที่พัฒนาขึ้นสำหรับตรวจการติดเชื้อเฮลิโคแบคเตอร์ไพโลไรในประชากรไทย

ดวงพร ทองงาม, มณีรัตน์ ชยานุภัทร์กุล, ปิยะ วงศ์จำปา, อรวดี หาญวิวัฒน์วงศ์

วัตถุประสงค์: *การตรวจการติดเชื้อเฮลิโคแบคเตอร์ไพโลไรโดยวิธีตรวจภูมิต้านทานต่อเชื้อในซีรัมและปัสสาวะนั้นมีบทบาทสำคัญในการวินิจฉัยผู้ป่วยที่มีอาการจุกแน่นลิ้นปี่ โดยไม่ต้องผ่านการส่องกล้องทางเดินอาหารส่วนต้น โดยวิธีนี้ทำได้ง่ายและราคาไม่แพง การศึกษานี้จึงมีขึ้นเพื่อพัฒนา และประเมินความไวและความจำเพาะของชุดตรวจภูมิต้านทานต่อเชื้อเฮลิโคแบคเตอร์ไพโลไรในซีรัมและปัสสาวะที่ผลิตขึ้นเองโดยผู้นิพนธ์*

วัสดุและวิธีการ: *ผู้ป่วยที่มีอาการจุกแน่นลิ้นปี่ทั้งสิ้น 138 ราย ได้รับการตรวจโดยการส่องกล้องทางเดินอาหารส่วนต้นและเก็บชิ้นเนื้อบริเวณแอนทรัม เพื่อตรวจการติดเชื้อโดยวิธียูรีเอสซึ่งใช้เป็นมาตรฐานในการอ้างอิง และทำการเก็บซีรัมและปัสสาวะเพื่อตรวจภูมิต้านทานต่อเชื้อเฮลิโคแบคเตอร์ไพโลไร โดยอาศัยหลักการของเอนไซม์ลิงค์อิมมูโนซอร์เบนท์แอสเส*

ผลการศึกษา: *ผู้ป่วยทั้งหมดได้รับการแบ่งเป็น 2 กลุ่ม คือ กลุ่มที่ผลยูรีเอส เป็นบวก 30 ราย (ร้อยละ 22) และกลุ่มที่ยูรีเอส เป็นลบ 108 ราย (ร้อยละ 78) พบว่าผู้ป่วยในกลุ่มที่ผลยูรีเอสเป็นลบมีค่าการดูดกลืนแสงจากการตรวจภูมิต้านทานต่อเชื้อเฮลิโคแบคเตอร์ไพโลไรในปัสสาวะและซีรัมต่ำกว่ากลุ่มที่ให้ผลบวกอย่างมีนัยสำคัญ ($p < 0.001$ และ $p = 0.011$ ตามลำดับ) โดยไม่พบความแตกต่างในด้านอายุ เพศ และผลการตรวจจากการส่องกล้องระหว่าง 2 กลุ่มนี้ จากการคำนวณทางสถิติพบว่าค่าความไว ค่าความจำเพาะ ค่าพยากรณ์ผลลบ ค่าพยากรณ์ผลบวก และค่าความแม่นยำ ของการตรวจภูมิต้านทานต่อเชื้อเฮลิโคแบคเตอร์ไพโลไร โดยอาศัยหลักการของเอนไซม์ลิงค์อิมมูโนซอร์เบนท์ แอสเสในปัสสาวะและซีรัม มีค่าตามลำดับดังนี้ 72% และ 96.3%, 63.5% และ 62.7%, 89.6% และ 98.5%, 33.3% และ 40.6%, 64.5% และ 69.8%*

สรุป: *ชุดตรวจภูมิต้านทานต่อเชื้อเฮลิโคแบคเตอร์ไพโลไรในซีรัมที่ใช้ในการศึกษานี้มีความไวสูงและมีความจำเพาะอยู่ในเกณฑ์พอใช้ น่าจะสามารถนำไปใช้ในการตรวจคัดกรองผู้ป่วยที่ติดเชื้อเฮลิโคแบคเตอร์ไพโลไรได้ดี อย่างไรก็ตาม ผลการตรวจในปัสสาวะยังไม่เป็นที่น่าพอใจนัก ทั้งในด้านของความไวและความจำเพาะซึ่งต้องมีการพัฒนาต่อไป*