Development of a Lateral-flow Immunochromatographic Strip Using Gold Nanoparticles for *Helicobacter pylori* Detection

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Helicobacter pylori (H. pylori) plays an important role in the development of chronic gastritis, peptic ulcer diseases, gastric adenocarcinoma, and gastric mucosal associated lymphoid tissue (MALT) lymphoma. The standard methods of bacterial staining, bacterial culture, and urease test for the detection of H. pylori are time consuming and invasive. Non-invasive testing plays a significant role in the test-and-treat approach to H. pylori management. Lateral flow immunoassay (LFIA) is a promising method for pathogenic detection that is fast, easy to use, and low cost. In the present study, the authors developed an H. pylori LFIA strip using gold nanoparticles for H. pylori detection. The results reported that 20 μg of anti-H. pylori antibody mixed with 1 mL AuNPs solution and incubated for 2 hours was the best concentration preparation for application coverage over the gold nanoparticle surface. The limit of detection observable by the naked eye was 15 μg of H. pylori protein lysate. The findings of this study suggest the possible future development of an H. pylori LFIA strip for fast, easy to use, and low-cost diagnostic testing.

Keywords: *Helicobacter pylori, Lateral flow immuno-assay, Gold nanoparticles*

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Helicobacter pylori (*H. pylori*) is a gram-negative bacterium that attaches itself to mucus-secreting cells of the intestinal epithelium. *H. pylori* secretes urease enzyme, which changes urea to ammonia. The ammonia by-product decreases the acidity of the stomach, making conditions more suitable for *H. pylori* bacterium. The helical shape of *H. pylori* allows it to burrow into the mucus layer of the stomach. *H. pylori* is a major cause of duodenal and peptic ulcer disease[1,2]. It is also associated with gastric cancer. Diagnostic testing for *H. pylori* includes both invasive and non-invasive techniques, based on the need for endoscopy. Direct diagnostic methods include culture and histopathology demonstration of the organism, whereas indirect methods use urease testing or the detection of antibody response as a marker for disease. Factors that may influence testing or test results include cost, availability, clinical situation, population prevalence of infection, pretest probability of infection, and the use of proton pump inhibitors or antibiotics. The standard methods for *H. pylori* diagnosis have been bacterial staining, bacterial culture, and urease test. However, these methods are invasive and time consuming. In addition, physicians have to biopsy a small piece of gastric epithelium using a gastroscope, with subsequent staining or culture; a procedure that can take several days to complete[3]. Currently, the detection of specific IgG or IgA in serum, antigen detection, and diagnosis using polymerase chain reaction testing (PCR) from non-invasive sampling from sources like saliva and gastric secretion is promising[4].

Lateral flow immunoassay (LFIA) is one type of rapid detection system that aims to detect specific antigens or antibodies, using immunochromatographic techniques. A LFIA strip is composed of a test line and

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a control line positioned on a nitrocellulose membrane (Fig. 1). The test line and control line contain a different specific antibody that is grafted onto the nitrocellulose membrane. A test sample is then dropped onto the sample pad, where it mixes with the conjugate (specific antibody of interest attached to gold nanoparticles). The target antigen bound with the conjugate will be sandwich bound with the specific antibody at the test line, while the excess conjugate will be trapped with another specific antibody at the control line. Several reports have supported the LFIA platform as a practical platform for screening tests, as evidenced by clinical success in pregnancy testing and influenza virus detection[5,6].

The present study was the first to establish a suitable concentration and combination of gold nanoparticles and *H. pylori* antibody or antigen conjugation that produced a positive band result on a lateral flow strip. From the findings and information described in the present study, the future development of a new lateral flow immunoassay test for *H. pylori* detection will provide an alternative *H. pylori* bacterium, detection method that is easy, fast, and low cost.

Material and Method

Gold nanoparticle synthesis

Sixty nm gold nanoparticles were synthesized using the citrate reduction method[7]. In a typical standard citrate reduction procedure, 250 µL of 1% $HAuCl_4$ in 25 mL of deionized water was heated to 100°C on a stirrer hot plate under reflux conditions. Stirring vigorously, 500 µL of 1% trisodium citrate, a reducing agent, was quickly added, resulting in a color change from yellow to grey, purple, and red, respectively. The solution containing gold nanoparticles was kept at 100°C for 15 minutes and subsequently cooled to room temperature. The solution was then stirred for 1 hour. Observation of a deep red color indicated the formation of gold nanoparticles. In completed form, the gold nanoparticles were near spherical and had a negatively charged surface. Particle size and zeta potential of the gold nanoparticles was measured by zetasizer machine (Malvern Instruments, Ltd., Worcestershire, UK). Light absorption was measured by UV spectrophotometer at wavelength 400-800 nm.

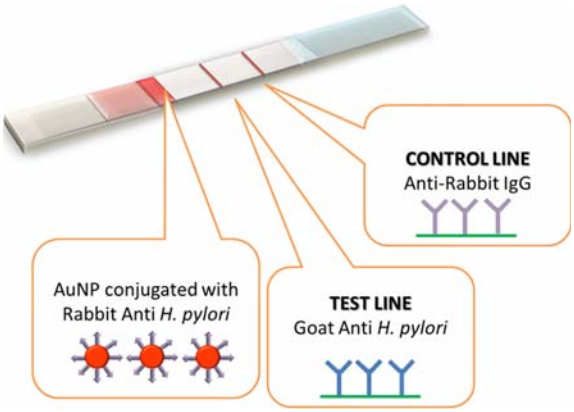

Fig. 1 Diagram illustrating the principle of lateral flow immunoassay (LFIA) and thecore components of the LFIA platform, including sample pad, conjugate pad, conjugate probe, test line, and control line.

Gold nanoparticles conjugated with H. pylori protein (AuNP-HP)

This protocol was previous described by Zhang et al[5]. In a brief overview, 60 nm diameter gold nanoparticles (AuNPs) were pH-adjusted to 9.0 using 0.01 M K_2CO_3. The conjugate solution comprised 20 µg of anti-*H. pylori* antibody (Dako Denmaek A/S, Glostrup, Denmark) mixed with 1 mL AuNPs solution and incubated for 2 hours at 37°C. The conjugate solution was then added to 200 µL of 10% BSA and incubated for 1 hour. The solution was then centrifuged at 6,000 rpm for 10 minutes and the supernatant liquid was removed. In the last step of the process, the conjugate solution was resuspended in 200 µL conjugate buffer (20 mM borax, 1% BSA, and 0.05% sodium azide).

Lateral flow strip test preparation

Nitrocellulose membrane preparation:

Each nitrocellulose membrane (Millipore HF240, EMD Millipore, Bellerica, MA, USA) was cut to 3x25 mm (width x length). Undiluted anti-*H. pylori* antibody and anti-rabbit IgG antibody were dropped onto the nitrocellulose membrane at the test line and control line, respectively. After drying for 1 hour, the membrane was blocked by soaking in 1% BSA for 5 minutes, rinsed with distilled water, and dried for 1 hour. The membrane was then stored in a cool place with silica gel to absorb moisture. The arrangement of the lateral flow components is described in Fig. 2.

Sample assay procedure

H. pylori cell lysate samples (500 µL of $1x10^9$ cells of *H. pylori*) from stock colony culture, were mixed with 250 µL cold RIPA buffer (Thermo Scientific, Waltham, MA, USA), then sonicated for 30 seconds at 30% amplitude, and incubated on ice for 30 minutes. The mixture was then centrifuged at 14,000 rpm for 10

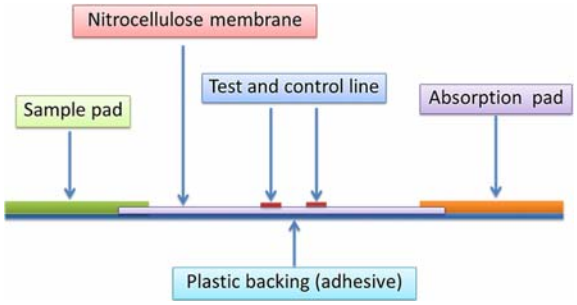

Fig. 2 Illustration of platform components and arrangement layout of the module.

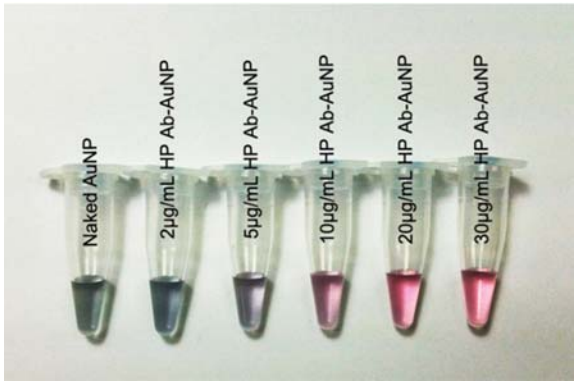

Fig. 3 Optimum antibody concentrations suspended in colloidal gold solution: After incubating gold nanoparticles and antibody for 30 minutes, 10% NaCl was added to each tube. Naked gold nanoparticles immediately changed color from red to yellow and the low antibody concentration groups (2, 5, and 10 µg/mL) aggregated in the same manner. The 20 and 30 µg/mL antibody groups did not aggregate.

minutes. Supernatant fluid was collected and its concentration was measured using NanoDrop. Lateral flow running procedure was performed by mixing gold nanoparticle conjugate and cell lysate protein together. After incubation at 37°C for 5 minutes, the solution was pipetted onto the sample pad of the lateral flow strip, followed by 30 µL of running buffer (0.1xPBS supplemented with 0.1% SDS). The results were then observed after 30 minutes.

Results

Gold nanoparticles conjugated with H. pylori protein (AuNP-HP)

Sixty nm diameter gold nanoparticles (AuNPs) were pH-adjusted to 9.0 using 0.01 M K_2CO_3 and stored at 4°C. Optimum antibody concentration to colloidal gold solution ratio was determined prior to *H. pylori* antibody conjugation using the Hill et al[8] method for excellent outcome. *H. pylori* antigens were added to 1 mL AuNPs solution to create concentrations of 2, 5, 10, 20, and 30 µg/mL, followed by incubation for 30 min at room temperature. One hundred (100) µL of 10% NaCl solution was then added to each mixture. Each mixture concentration was observed for aggregation with the naked eye and the aggregated and non-aggregated groups were distinguished (Fig. 3). The results suggested that anti-*H. pylori* 20 µg/mL was the optimal concentration for overall gold nanoparticle surface coverage. This concentration was used in the further examination of 10 repeated experiments.

Lateral flow strip test assembly and detection

The lateral flow strip was assembled and tested with 1 µL of $1x10^9$ cell/mL *H. pylori* cell suspension. After running for 30 minutes, the control line band appeared in both strips, but the test line band was observed only in the positive strip.

A detection limit experiment was performed. This experiment used *H. pylori* protein lysate instead of *H. pylori* cell suspension for the convenience of calculating concentration. Three different concentrations of *H. pylori* cell lysate were tested in the lateral flow strip; 15, 4.5, and 1.5 µg, respectively. For the negative control group, we applied 100 µg of BSA to simulate the non-target protein. The assay procedure was then undertaken with the desired concentration of *H. pylori* cell lysate. Fifty (50) µl of 0.5xPBS supplement with 0.1% Tween-20 was also added. After running for 15 minutes, the lateral flow strip was observed by naked eye and recorded by digital camera for the analysis of signal levels using Image J (public domain) software. The limit of detection observed by naked eye was 15 µg and showed a weak signal at 4.5 and 1.5 µg of *H. pylori* cell lysate.

Discussion

Helicobacter pylori (H. pylori) is one of the most common globally occurring human infections. 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 a prevalence rate of 80-90%, as compared with 40-50% in developed countries[10]. In Thailand, 55% of the population is infected with *H. pylori* by the third decade of life[11]. Since *H. pylori* eradication reduces recurrence

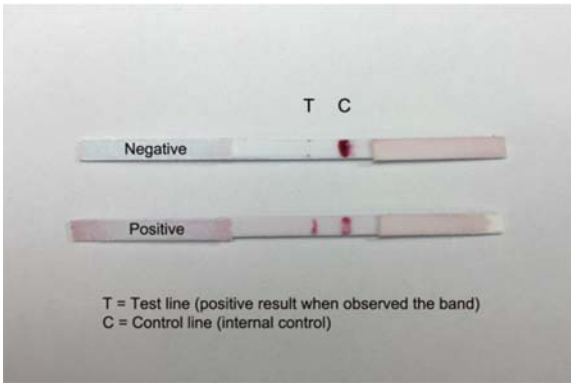

Fig. 4 Specificity test for lateral flow platform using *H. pylori* cell suspension. The negative strip was run without *H. pylori* suspension. After 30 minutes, a red band appeared at the control line only. In contrast, a red band appeared in both the test and control lines on the positive strip.

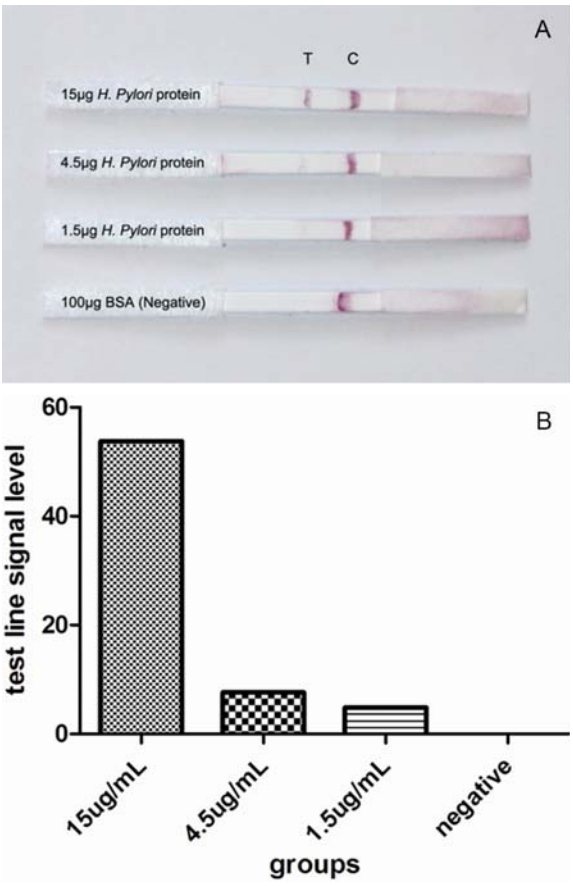

Fig. 5 Limit of detection experiment: A) The lateral flow strip was run with varied concentrations (from 15 to 1.5 µg) of *H. pylori* cell lysate, with the negative using 100 µg BSA. After applying the protein sample and running with 0.5x PBS buffer supplement with 0.1% Tween-20 for fifteen minutes, the lateral flow strips showed a positive band at 15 µg (T), and a weak positive band at 4.5 and 1.5 µg. B) Test line signal from Image J software showed a signal after corrected baseline background signal from the negative group.

and the complications associated with peptic ulcer diseases[1], fast and accurate diagnosis of *H. pylori* infection is meaningful and necessary.

There are a number of diagnostic methods that have been developed for the detection of *H. pylori*, including both invasive and non-invasive tests. Invasive tests like histopathology, culture, rapid urease test, and polymerase chain reaction require upper endoscopy. Non-invasive methods rely on the detection of antibodies (in serum or urine), antigens, or the urease activity of the bacteria (urea breath test). The test-and-treat strategy has proven to be cost-effective and safe by many studies and is now recommended in the current guidelines[1,2]. This approach involves testing and, if necessary, treating uninvestigated dyspeptic patients who are under the age of 55 years that present with no alarming symptoms. Therefore, non-invasive tests will be of great value and importance in the adoption of this strategy.

Among non-invasive tests, the urea breath test (UBT) is the most accurate, both for diagnosis and for 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 differing immune responses in different ethnic populations, the sensitivity, specificity, and accuracy of serological tests vary, depending on the population being tested[3,12-15].

The authors demonstrated that *H. pylori* antibody conjugated gold nanoparticles were a promising method for detecting *H. pylori* infection. This system proved fast and easy to use, required only 10-15 minutes of running time, and required only a centrifuge and pipettes. Previous reports have shown this method to be practical and convenient for the high sensitivity screening of different bacterial diseases[5,6,9]. However, the sensitivity of these assays was shown to be dependent on platform design and experiment conditions. The present study reports the development of the *H. pylori* LFIA platform using gold nanoparticles and demonstrates the possibility for its practical usage

in the future. A phase two study is recommended to accelerate continued and further study of this promising clinical testing advancement.

Funding disclosure

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What is already known on this topic ?

The standard methods for *H. pylori* diagnosis have been bacterial staining, bacterial culture, and urease test. However, these methods are invasive, time consuming, and require endoscopy.

What this study add ?

The authors have developed a lateral flow immunoassay using gold nanoparticles for the fast and easy detection of *H. pylori* infection.

Potential conflicts of interest

None.

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การพัฒนาชุดตรวจวินิจฉัยการติดเชื้อเฮลิโคแบคเตอร์ ไพลอไรโดยอาศัยวิธีอนุภาคทองคำระดับนาโนเมตร

ดวงพร วีระวัฒกานนท์, มงคล พงษ์สุชาติ, อมรพันธุ์ เสรีมาศพันธุ์, อรวดี หาญวิวัฒน์วงศ์, ประสงค์ ศิริวิริยะกุล

เฮลิโคแบคเตอร์ ไพลอไรเป็นสาเหตุสำคัญของกระเพาะอาหารอักเสบเรื้อรังแผลทางเดินอาหาร รวมถึงการเกิดมะเร็งกระเพาะอาหาร วิธีมาตรฐานที่ใช้วินิจฉัยการติดเชื้อเฮลิโคแบคเตอร์ ไพลอไรคือการตรวจชิ้นเนื้อ เพื่อนำมาย้อมหาตัวแบคทีเรีย การเพาะเชื้อหรือการทดสอบวิธียูรีเอส จัดเป็นวิธีที่ invasive ซึ่งแพทย์จะต้องตัดชิ้นเนื้อ จากกระเพาะอาหารออกมาโดยผ่านทางการส่องกล้องทางเดินอาหาร ซึ่งกระบวนการตรวจย้อมชิ้นเนื้อ และการเพาะเชื้อต้องใช้เวลาหลายวันจึงจะได้ผลวิธี non-invasive จึงมีบทบาทเพิ่มขึ้นในการวินิจฉัย การศึกษานี้ได้พัฒนาชุดตรวจวินิจฉัยการติดเชื้อเฮลิโคแบคเตอร์ ไพลอไรโดยอาศัยอนุภาคทองคำระดับนาโนเมตร ซึ่งเป็นอนุภาคของโลหะในระดับนาโนที่มีความเสถียรสูงเป็นตัวรายงาน (reporter) ใน Lateral flow strip test โดยอาศัยหลักการ immunochromatography สามารถอ่านผลได้ด้วยตาเปล่าจากแถบสีชมพูของอนุภาคทองคำนาโนที่เกิดขึ้นที่ตำแหน่งควบคุม (control line) และตำแหน่งทดสอบ (test line) โดยถ้าแถบสีชมพูเกิดขึ้นที่ตำแหน่งทดสอบและตำแหน่งควบคุมเท่ากับผลเป็นบวก แต่หากมีแถบสีชมพูเกิดขึ้นที่ตำแหน่งควบคุมเพียงตำแหน่งเดียว เท่ากับผลเป็นลบ ผลการศึกษาแสดงให้เห็นว่าความเข้มข้นของเฮลิโคแบคเตอร์ ไพลอไรแอนติ้บอดี้ 20 ไมโครกรัม ร่วมกับ 1 มิลลิลิตร ของอนุภาคทองคำนาโน เป็นความเข้มข้นที่เหมาะสมในการทำให้เกิดปฏิกิริยา ซึ่งเป็นการศึกษาเริ่มแรกก่อนการพัฒนาในระยะที่สองต่อไปเพื่อให้ได้ชุดตรวจวินิจฉัยที่ง่าย รวดเร็ว แม่นยำและราคาถูก เหมาะสำหรับวินิจฉัยการติดเชื้อเฮลิโคแบคเตอร์ ไพลอไรได้ในอนาคต