Prevalence of Single Nucleotide Mutation in Clarithromycin Resistant Gene of *Helicobacter pylori*: A 32-Months Prospective Study by Using Hybridization Real Time Polymerase Chain Reaction

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Polymerase chain reaction (PCR) for detection of single nucleotide mutations in 23S rRNA gene of clarithromycin resistance Helicobacter pylori were investigated worldwide. In Thailand, the prevalence of these mutations had not been extensively investigated. The authors conducted a 32-months prospective study to estimate the prevalence of clarithromycin resistant Helicobacter pylori and to compare the sensitivity and specificity of hybridization real time PCR for 23S rRNA gene of Helicobacter pylori detection with the combination of rapid urease test, immunohistochemistry straining and Helicobacter pylori culture. A total of 200 patients with endoscopic examination with gastric biopsy were enrolled from January 2006 to September 2008. Eight gastric specimens were biopsied and performed rapid urease test, immunohistochemistry straining for Helicobacter pylori, Helicobacter pylori culture and hybridization real time PCR for 23S rRNA dectection, as well as single nucleotide mutation detection, such as G2111A, A2115G, A2142G, A2143G and A2144G. The clarithromycin susceptibility of Helicobacter pylori isolated was determined by E-test. The prevalences of clarithromycin, amoxicillin, metronidazole and tetracycline resistance were 13.8%, 21.3%, 55.0% and 8.8%, respectively. Most of clarithromycin resistant Helicobacter pylori (81.8%) had very high minimal inhibition concentration (MIC) more than 256 µg/ml. The resistance to mutation gene at A2142G was found in 4 patients (36.4%). Two patients were found to have multiple mutations at G2111A, A2115G and A2144G (18.2%). The sensitivity, specificity, positive predictive value, negative predictive value, likelihood ratio to test positive and likelihood ratio to test negative of hybridization RT-PCR in detecting 23S rRNA gene of Helicobacter pylori was 95.6%, 94.4%, 93.2%, 96.7%, 17.2 and 4.6, respectively. In conclusion A2142G mutation was most common and associated with high MIC. Hybridization RT-PCR had good sensitivity and specificity to detect 23S rRNA gene, as well as clarithromycin resistance gene mutation of Helicobacter pylori.

Keywords: *Clarithromycin resistance, Helicobacter pylori, 23S rRNA, Real time polymerase chained reaction*

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Helicobacter pylori infection is the common cause of peptic ulcer, worldwide. The diagnosis test for *Helicobacter pylori* infection composed of bacterial culture, immunohistochemistry, rapid urease test of gastric specimens, urea breath test, stool antigen test, serum antibody test and polymerase chain reaction (PCR) of specific gene. Although, *Helicobacter pylori* culture is definite for diagnosis and has advantage in the aspect of determination of the antibiotic susceptibility test. However, the limitation due to many factors such as use of special media, difficult transportation technique, requiring the special incubation and time consuming increased risk of clarithromycin resistance and treatment failure.

In Thailand, prevalence of clarithromycin resistance *Helicobacter pylori* had not been extensively investigated. Due to resource limited setting, most gastroenterologists treated *Helicobacter pylori* by using empirical antibiotics such as clarithromycin plus amoxicillin and proton pump inhibitors instead of routine investigation of antibiotic susceptibility test. Resulting in a higher prevalence of clarithromycin

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resistance *Helicobacter pylori* was approximately 19%[2], when compared to global primary resistance (9.9%; 95% CI 8.3-11.7)[1].

Fortunately, several publications increased the role of real time PCR to detect 23S rRNA gene for *Helicobacter pylori* detection from gastric biopsy specimen. This new technique played a more advantage role by less time consuming and enable to study on genotypic clarithromycin resistance, especially single nucleotide mutations within 23S rRNA peptidyltransferase-encoding regions. Three major point mutations in two positions have been described in which an adenine (A) residue is replaced by a guanine (G) or a cytosine (C) residue at adjacent positions described as A2142G, A2142C and A2143G[3-6]. Moreover, other rare single nucleotide mutations were A2115G, G2141A, T2717A and T2182C[5,7,8] in which the role of clinical resistance was still under debate[5,7,8].

In Thailand, the prevalence of single nucleotide mutation detection, such as G2111A, A2115G, A2142G, A2143G and A2144G have not been extensively investigated. The role of single nucleotide mutation detection may help gastroenterologist to select the right antibiotic(s) and decreased risk of clarithromycin resistance by this investigation.

Objective

The authors conducted a 32-months prospective study to estimate the prevalence of clarithromycin resistant *Helicobacter pylori* and to compare the sensitivity, specificity, positive predictive value, negative predictive value, likelihood ratio to test positive and likelihood ratio to test negative of hybridization real time PCR for 23S rRNA gene of *Helicobacter pylori* detection with the combination of rapid urease test, immunohistochemistry straining and *Helicobacter pylori* culture as the gold standard for diagnosis of *Helicobacter pylori infection*. Moreover, to identify the prevalence of each clarithromycin resistance gene single nucleotide mutations in *H. pylori* from gastric tissue samples.

Material and Method

A total of 200 patients who had indications (dyspepsia) for esophagogastroduodenoscopy and gastric biopsy were enrolled from January 2006 to September 2008. All patients were informed by consent form and study protocol were approved by the ethical committee of Rajavithi Hospital, Thailand. Four gastric biopsy specimens were taken from the antrum (3 centimeters from pylorus) and four biosy specimens from body of the stomach. Each pair of one biopsy specimen from the antrum and body of the stomach were tested for rapid urease test (Pronto Dry®), immunohistochemical stain for *Helicobacter pylori*. Another pair of one biopsy specimen from the antrum and the body of the stomach were placed in normal saline and immediately sent to the microbiological laboratory for culture and susceptibility test, as well as, another pair which were placed into 0.5 ml glycerol-supplemented brain-heart infusion broth in microtubes then freezed at -80°C for hybridization RT-PCR. Gastric infection of *Helicobacter pylori* was defined as (i) positivity of culture, or (ii) positivity for histology plus positive for rapid urease test.

Culture and antibiotic susceptibility testing

Each pair of biopsy specimens from body antrum were cultured under microaerophilic conditions at 37°C for a maximum of 6 days. *Helicobacter pylori* isolates were identified by presence of gram negative spiral shaped bacilli with positive oxidizing test and positive rapidly urease. MICs of clarithromycin, metronidazole, amoxicillin and tetracycline were determined by E-test method (AB Biodisk, Sweden) according to the instructions of the manufacturer by using Mueller-Hinton agar supplemented with 10% horse blood and a cell suspension calibrated at 3 McFarland units. Plated were incubated at 37°C for three days. Cut point of antibiotics resistance were minimally inhibition concentration (MIC) $\geq$ 1 μg/ml for clarithromycin, MIC $\geq$ 16 μg/ml for metronidazole, MIC $\geq$ 4 μg/ml for amoxicillin and MIC $\geq$ 16 μg/ml for tetracycline.

Detection of Helicobacter pylori in gastric biopsy specimens

The technique in detection of *Helicobacter pylori* was real time PCR. Each pair of gastric biopsy specimens from antrum and body were lysed and centrifuged by Magnagreen bead by using Rajavithi gastric biopsy transfer media 400 microliters as the dissolved solution. After tissue preparation to solution, DNA extraction was performed by using automated DNA extraction (Magna pure Compact Nucleic and Isolation Kit-Roche). Finally, DNA was eluted in 200 μl of nuclease-free water and stored at -20°C for subsequent real time polymerase chain reaction (RT-PCR) analysis using Hybridization® probe and primer of (Tibmol® Germany). The forward primers HP23S1 (5′-GGA GCT GTC TCA ACC AGA GAT TC-3′) and

reversed primer HP23S2 (5′-CGC ATG ATA TTC CC [AG] TTA GCA G-3′) being the specific sequence for 23S rRNA gene of H. pylori and the hybridization probes HP23S3 (5′-GGA GCT GTC TCA ACC AGA GA [Red640] T TC-3′) and HP23S4 (5′-GGA ATT TTC ACC TCC ACT ACA ATT TCA CTG [Fluo]-3′) were used for 23S rRNA gene of *Helicobacter pylori* detection. Detection of single nucleotide mutation in the 23S rRNA gene were detect by hybridization PCR by using the probes which composed of G2111A (5'gTggAgATgAAA TTCCTCC TACCg-FL), A2115G (5'gTggAggTgAgAATTCCTCC TACCCg-FL), A2142G (5'-LC610 ggCAAgACgCAAA gACCCC-PH), A2143G (5'-LC610-ggCAAgACgAg AAgACCCC-PH), A2144G (5'-LC610-ggCAAgAC gAAgAgACCCC-H) and 5'-gTggAggTgAAAATTCC TCCTACCCg-FL. Optimized hybridization PCR conditions was amplification for 50 cycles of denaturation (95°C,10s) followed by annealing (55°C, 10s) and extension (72°C, 15s). Melting curve analysis (45-95°C) was performed.

Statistical analysis

SPSS version 17.0 (licensed), StatXact 6 Version 6.2 (licensed) and Stata® Version 6.0 (Licensed) were used for statistical calculations. Descriptive statistic analysis was used for demographic data. Mean and 95% confidence intervals were calculated for continuous variables. Sensitivity, specificity, positive predictive value, negative predictive value, likelihood ratio to test positive (LR^+) and likelihood to test negative (LR^-) were calculated for all diagnostic tools.

Results

Total of 200 patients, 103 male and 97 female, mean age 52.8 years (ranged 18-90) were enrolled in the present study. The prevalence of *Helicobacter pylori* was 46.5% (95% CI: 39.5%-53.4%). Most common gastrointestinal symptoms composed of upper gastrointestinal bleeding (90 patients, 45%), dyspepsia (84 patients, 42%), abdominal pain (11 patients, 5.5%) and anemia (12 patients, 6%).

Gastroscopic findings composed of gastritis (147 patients, 73.5%), gastric ulcer (76 patients, 38%), guodenitis (74 patients, 37%), gastroduodenitis (65 patients, 32.5%) and duodenal ulcer (40 patients, 20%) (Table 1). The Gastric *Helicobacter pylori* infection diagnosed by positive for rapid urease test (Pronto Dry®) and positive for immunohistohistology for *Helicobacter pylori* or positive gastric biopsy cultured for *Helicobacter pylori*. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), likelihood ratio to test positive (LR^+) and likelihood ratio to test negative (LR^-) of Pronto Dry® were 94.6%, 61.7%, 68.2%, 92.9%, 2.5 and 8.8, respectively. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), likelihood ratio to test positive (LR^+) and likelihood ratio to test negative(LR^-) of immuno-

Table 1. General characteristics of the study population

Characters		95% CI
Total, n (%)	200 (100.0)	-
Age mean (Min-Max)	52.8 (18-90)	-
Gender, n (%)		
Male	103 (51.5)	-
Female	97 (48.5)	-
Symptom Presentation, n (%)		
Dyspepsia	84 (42.0)	35.1-48.9
Abdominal pain	11 (5.5)	2.3-8.6
Upper GI bleeding	90 (45.0)	38.5-51.9
Anemia	12 (6.0)	2.6-9.3
Gastroendoscopic findings, n (%)		
Gastritis	147 (73.5)	67.3-79.6
Duodenitis	74 (37.0)	30.2-43.7
Gastroduodenitis	65 (32.5)	25.9-39.0
Gastric ulcer	76 (38.0)	31.2-44.7
Duodenal ulcer	40 (20.0)	14.4-25.5

CI = Confidence Interval, Values are represented as n (%)

histohistology for *Helicobacter pylori* were 92.5%, 94.4%, 93.48%, 93.52%, 16.52 and 7.00, respectively. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), likelihood ratio to test positive (LR^+) and likelihood ratio to test negative(LR^-) of gastric biopsy cultured for *Helicobacter pylori* were 88.2%, 100%, 100%, 90.7% and 11.8, respectively. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), likelihood ratio to test positive (LR^+) and likelihood ratio to test negative (LR^-) of hybridization real time PCR for 23S rRNA gene of *Helicobacter pylori* detection were 95.6 %, 94.4%, 93.2%, 96.7%, 17.2 and 4.6, respectively (Table 2, Fig. 1).

The prevalences of antibiotic resistance to clarithromycin, amoxicillin, metronidazole and tetracycline were 13.8%, 21.3%, 55% and 8.8% respectively (Table 3). The multi-drug resistance was found in 19 patients (39.6%). The most common resistance antibiotic for gastric *Helicobacter pylori* infection was combination of amoxicillin-metronidazole resistance (14.6%) (Table 3). MIC for clarithromycin resistance was greater than 256 in 9 patients (81.8%). All amoxicillin and tetracycline resistance were high resistance, MIC was greater than 256. Forty patients (90.9%) of metronidazole resistance, MIC was greater than 256. Clarithromycin mutation gene was able to identify in 6 patients from 11 patients Mutation gene at A2142G was found in 4 patients (Table 4, Fig. 2). The other two patients were found to have multiple

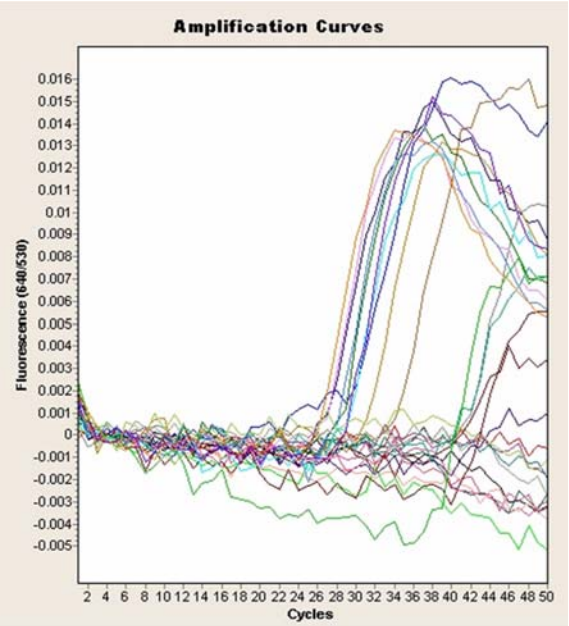

Fig. 1 PCR Detection of *Helicobacter pylori* showed positive amplication curve

Table 2. Sensitivity and specificity of diagnostic tests

		Gold Standard			Sensitivity	Specificity
		positive	negative	total		
1	Pronto dry				94.6%	61.7%
	positive	88	41	129		
	negative	5	66	71		
	total	93	107	200		
2	Histology				92.5%	94.4%
	positive	86	6	92		
	negative	7	101	108		
	total	93	107	200		
3	Culture				88.2%	100.0%
	positive	82	0	82		
	negative	11	107	118		
	total	93	107	200		
4	RT-PCR				95.6%	94.4%
	positive	88	6	94		
	negative	4	102	106		
	total	92	108	200		

Gold standard: (i) positivity of culture or (ii) positivity for histology plus positive for rapid urease test

Table 3. The prevalences with 95% confidence interval of mean antibiotic resistant

	n (%)	95% CI
Single antibiotic resistant		
Clarithromycin	11 (13.8)	6.0-21.4
Amoxicillin	17 (21.3)	12.0-30.4
Metronidazole	44 (55.0)	43.8-66.1
Tetracycline	7 (8.8)	2.4-15.0
Multiple antibiotic resistant		
Amoxicillin-Metronidazole	7 (14.6)	4.2-24.9
Amox-Clarithro-Metro-Tetracycline	5 (10.4)	1.4-19.3
Amoxicillin-Clarithromycin	2 (4.2)	0.0-10.3
Clarithromycin-Metronidazole	2 (4.2)	0.0-10.3
Amox-Metro-Tetracycline	1 (2.1)	0.0-6.2
Amox-Metro-Clarithromycin	1 (2.1)	0.0-6.2
Tetracycline-Metronidazole	1 (2.1)	0.2-6.2

CI = Confidence Interval

Table 4. Clarithromycin mutation Gene

MIC of clarithromycin	Mutations	No. of patients
128	G2111A, A2115G, A2144G	1
>256	G2111A, A2115G, A2144G	1
>256	A2142G	4

mutations at G2111A, A2115G and A2144G (Table 4, Fig. 3). The other five patients were not able to identify point of mutation.

Discussion

Hybridization real time polymerase chain reaction (Light Cycler™) for 23S rRNA gene of *Helicobacter pylori* detection played important roles in both diagnosis of infection and identified clarithromycin resistant gene mutation from gastric specimens biopsy. In the present study, the prevalence of *Helicobacter pylori* infection was very high when compared to a Korean study[10,17] and as much as a Japanese study[1]. The prevalence of clarithromycin resistant *Helicobacter pylori* cultured from gastric specimens was lower than Japanese and Korean previous studies[1,10]. By using gold standard criteria for diagnosis of *Helicobacter pylori* infection, the sensitivity, specificity, PPV, NPV, LR+ and LR- of real time hybridization PCR was higher than other diagnostic tests such as Pronto Dry®, immunohistochemistry and *Helicobacter pylori* culture. Moreover, hybridization

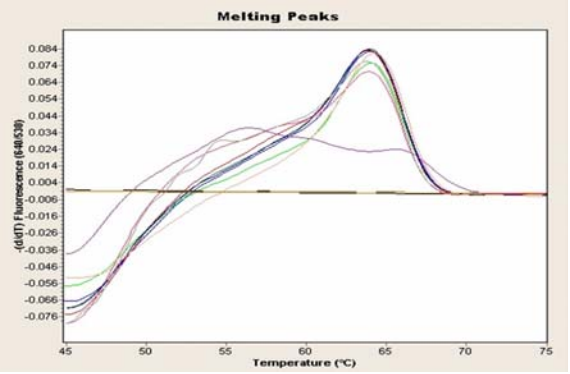

Fig. 2 A2142G Mutation. Peak analysis showed positive amplification curve at 63.4°C

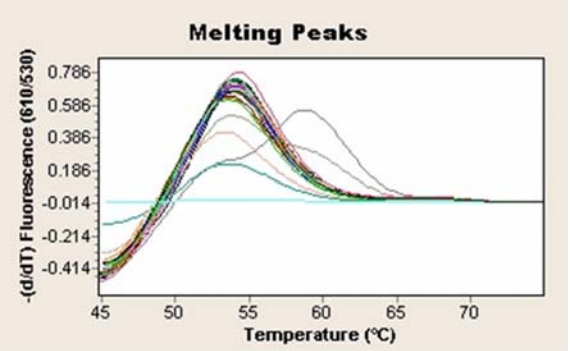

Fig. 3 A2144G Mutation. Peak analysis showed positive amplification curve at 58.2°C

PCR played more advantage than culture by less time consuming, within several hours[9] and may be used instead of screening clarithromycin resistant strain in the future. This technique may provide faster information on clarithromycin resistance before treating the patient when compared to culture. Interestingly, hybridization PCR may have more advantage in resource limits setting that have no need to transfer fresh specimens with viable *Helicobacter pylori* to the referral laboratory center, when compared with transfer viable *Helicobacter pylori* to culture need of special transport conditions. This PCR technique was suitable for a large epidemiologic study on prevalence of *Helicobacter pylori* infection and clarithromycin resistance mutation. Clarithromycin mutation gene was able to identify in 6 patients from 11 patients. Mutation gene at A2142G was found in 4 patients and these corresponded with high MIC (> 256). The other two patients were found to have multiple mutations at G2111A, A2115G and A2144G and the MIC of these two patients was 128 and more than 256. These

resistances were associated with high MIC. A2142G mutation was predominant in the present population which was similar to finding in Brazil, Chile, Saudi Arabia, Korea and Malaysia[10-13]. The other authors have shown A2143G mutation was the most frequently detected mutations[6,15-19]. A2144G was not commonly found to cause the clarithromycin resistance but it had been reported to be the main mutation in Russia, Turkey and Hong Kong[20-23]. The other five patients were not be able to identify point of mutation from the present study. There might be more point of mutations which the authors' design probes were not cover. Many studies have reported more mutation points such asT2190C, C2195T, A2223G from Korea; C2147G, G1939A, T1942C from Chile; A2144T, T2717C domainVI from Italy and T2245C from Brazil[8,11,14,17]. This is an opportunity for a further study; PCR array or microarray is a more appropriate design to detect multiple mutation points[24,25]. In conclusion, real time PCR assay permits accurate, fast detection of *Helicobacter pylori* from gastric tissue specimen. The sensitivity and specificity of RT- PCR was higher than those of rapid urease test, histology and culture despite the fact that isolated PCR positivity was classified as false positivity. A2142G mutation was predominated and multiple mutations were found G2111A+A2115G+A2144G). Thus, RT-PCR technique can be used as diagnostic test and can provide additional data on clarithromycin resistance information.

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Potential conflicts of interest

None.

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การศึกษาไปข้างหน้าเป็นระยะเวลา 32 เดือน เพื่อศึกษาความชุกของการกลายพันธุ์ ของกรดนิวคลิอิกตำแหน่งเดียวในจีนดื้อยาคลาลิโทรมัยซิน ในเชื้อเฮลิโคแบคเตอร์ไพโลรีโดยวิธีปฏิกิริยาลูกโซ่ไฮบริไดซ์ชนิดการวัดค่ารังสีต่อเนื่องตลอดปฏิกิริยา

ปิยะธิดา หาญสมบูรณ์, สิริวัฒน์ อนันตพันธุ์พงศ์, สยาม ศิรินธรปัญญา, กิตติ ชื่นยง, จรินทร์ โรจน์บวรวิทยา

การตรวจหาการกลายพันธุ์ของกรดนิวคลิอิกตำแหน่งเดียวในจีน 23 เอส ไรโบโซมอาร์เอนเอ และการตรวจจีนดื้อยาคลาลิโทรมัยซินในเชื้อเฮลิโคแบคเตอร์ไพโลรี โดยวิธีการตรวจปฏิกิริยาลูกโซ่ได้มีการศึกษาแพร่หลายทั่วโลก ในประเทศไทยการศึกษาความชุกของการกลายพันธุ์ในจีนดื้อยาคลาลิโทรมัยซินยังไม่ได้มีการศึกษาอย่างแพร่หลาย

คณะผู้นิพนธ์ได้ทำการศึกษาไปข้างหน้าเป็นระยะเวลา 32 เดือน เพื่อประมาณความชุกของการกลายพันธุ์ในจีนดื้อยาคลาลิโทรมัยซินในจีน 23 เอส ไรโบโซมอาร์เอนเอ ในเชื้อเฮลิโคแบคเตอร์ไพโลรีโดยวิธีปฏิกิริยาลูกโซ่ไฮบริไดซ์ชนิดการวัดค่ารังสีต่อเนื่องตลอดปฏิกิริยาโดยเปรียบเทียบกับการตรวจ ยูเรียเอสชนิดด่วน การตรวจย้อมชิ้นเนื้อวิธีอิมมูโนเคมีและการเพาะเชื้อ ผู้ป่วยจำนวน 200 ราย ที่ได้รับการส่องกล้องตรวจทางเดินอาหารส่วนต้นได้รับการตัดชิ้นเนื้อส่งตรวจระหว่างเดือนมกราคม พ.ศ. 2549 ถึงเดือนกันยายน พ.ศ. 2551 ชิ้นเนื้อจำนวน 8 ชิ้น ได้ถูกส่งตรวจยูเรียเอสชนิดด่วน การตรวจย้อมชิ้นเนื้อวิธีอิมมูโนเคมีเพื่อหาเชื้อเฮลิโคแบคเตอร์ไพโลรีกลายพันธุ์ในจีนดื้อยาคลาลิโทรมัยซินในจีน 23เอส ไรโบโซมอาร์เอนเอ ในเชื้อเฮลิโคแบคเตอร์ไพโลรีโดยวิธีปฏิกิริยาลูกโซ่ไฮบริไดซ์และการตรวจตำแหน่งกลายพันธุ์ จี2111เอ เอ2115จี เอ2142จี เอ2143จี และเอ2144จีในจีนดื้อยาคลาลิโทรมัยซินในจีน23เอส ไรโบโซมอาร์เอนเอ การตรวจความไวต่อยาคลาลิโทรมัยซินของเชื้อเฮลิโคแบคเตอร์ไพโลรีทำโดยอีเทสต์ ซึ่งพบการความชุกของเชื้อดื้อยาคลาลิโทรมัยซิน อะมอกซีซิลินเมโทรนิดาโซลและเตตราไซคลินเป็นร้อยละ 13.8, 21.2, 55.0 และ 8.8 ตามลำดับ โดยร้อยละ 81.8 ของเชื้อเฮลิโคแบคเตอร์ไพโลรีดื้อยาคลาลิโทรมัยซินมีค่าเอ็มไอซีสูงมากกว่า 256 ไมโครกรัมต่อมิลลิลิตร พบตำแหน่งกลายพันธุ์ เอ2142จี จำนวน 4 ราย คิดเป็นร้อยละ 36.4 พบตำแหน่งกลายพันธุ์ จี2111เอ เอ2115จี และ เอ2144จี ในผู้ป่วยคนเดียวกัน 2 รายคิดเป็นร้อยละ 18.2. ความไว ความจำเพาะ การทำนายค่าผลบวกจริงและการทำนายค่าผลลบจริง ความน่าเชื่อกรณีผลตรวจเป็นบวกและความน่าเชื่อกรณีผลตรวจเป็นลบคิดเป็นร้อยละ 95.6, 94.4, 93.2, 96.7, 17.2 และ 4.6 ตามลำดับ โดยสรุปตำแหน่งกลายพันธุ์ เอ2142จี เป็นตำแหน่งที่พบบ่อยและสัมพันธ์กับค่าเอ็มไอซีที่สูง และการตรวจการกลายพันธุ์ ของกรดนิวคลิอิกตำแหน่งเดียวในจีนดื้อยาคลาลิโทรมัยซินในจีน23เอส ไรโบโซมอาร์เอนเอ และการตรวจหาจีนดื้อยาคลาลิโทรมัยซิน ของเชื้อเฮลิโคแบคเตอร์ไพโลรีโดยวิธีปฏิกิริยาลูกโซ่ไฮบริไดซ์ชนิดการวัดค่ารังสีต่อเนื่องตลอดปฏิกิริยามีค่าความไวและความจำเพาะสูง