CLARITHROMYCIN AND LEVOFLOXACIN SUSCEPTIBILITY TESTING FOR *HELICOBACTER PYLORI* IN CENTRAL VIETNAM: COMPARISON OF E-TEST AND DISK DIFFUSION METHODS

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Abstract

Background: The rate of antibiotic resistance in Helicobacter pylori (H.pylori) is increasing in Vietnam in recent years and has become a main challenge in the management of H. pylori infection. Data about the resistance remains different depending on the time, demography and especially the methods of determining the antibiotics susceptibility. **Aims:** To determine the antibiogram of H.pylori strains isolated from a population in Central Vietnam to clarithromycin, levofloxacin by E-test and disk diffusion, compare two diffusion methods. **Methods:** 56 H.pylori strains were isolated from gastric biopsies of H.pylori infected patients from 7/2012 to 8/2013, of which 13 strains originated from patients in whom eradication of the infection failed after treatment. E-test was used to determine the minimum inhibitory concentrations (MICs) of clarithromycin (CH) and levofloxacin (LE). Disk diffusion was evaluated as an alternative method to determine susceptibility and compared with the E-test results. **Results:** In total, the resistant strains (regardless of previous eradication history) to CH, LE were 42.9% and 44.6%, respectively. The ratio of strains with secondary resistance was significantly greater than that of the strains with primary resistance, CH: 84.6% vs. 30.2%, LE: 61.5% vs. 39.5% ($p < 0.05$). The resistance rate to LE in female was significantly higher than in male ($p < 0.05$). All CH-sensitive strains by E-test had the inhibition diameters of CH was ≥ 24mm and all CH-resistant strains had the inhibition diameters was ≤ 18mm (breakpoint for MIC: 1μg/ml). To LE, the inhibition diameters was ≥ 30mm can determine all LE-sensitive strains and the inhibition diameters was ≤ 26mm can determine all LE-resistant strains by E-test (breakpoint for MIC: 1μg/ml). **Conclusions:** High resistance rate to CH and LE, suggests that standard CH-based triple therapies may not be useful as the first-line treatment and LE-based triple therapy should not use as an alternative therapy in Central Vietnam. The disk diffusion can be used as an alternative phenotypic method to determine the susceptibility of H.pylori, which may be more feasible and less expensive.

Key words: *Helicobacter pylori, levofloxacin, clarithromycin, E-test, disk diffusion, antibiotic resistance.*

1. BACKGROUND

Helicobacter pylori infection is the main cause of chronic gastritis, peptic ulcer, gastric cancer and mucosa associated lymphoid tissue lymphoma [1, 2]. Standard regimens including proton pump inhibitor (PPI) and two of three antibiotics (amoxicillin, metronidazole, clarithromycin) are commonly used in clinical practice to eradicate *H.pylori* [3]. Clarithromycin has been a key drug in the treatment regimens however, the efficacy has significantly decreased (66% - 70%) due to antibiotics resistant [4]. In developing countries, the rate of resistance to metronidazole is high and that to clarithromycin have been rapidly increasing [5]. Recently, levofloxacin base triple therapy is recommended as alternative regimen for treating patients who failed previous treatment [3]. Therefore, information about the resistance rate to clarithromycin and levofloxacin in a specific region or country is very important to select the appropriate and effective treatment regimens.

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To detect phenotypic resistance of *H. pylori*, agar dilution is the standard method used to determine the minimum inhibitory concentration (MIC) of an antibiotic established by Clinical and Laboratory Standards Institute (CLSI) [6]. However, this method is difficult to apply in clinical practice due to requiring much effort and time to perform. Currently, Epsilometer test (E-test) is the recommended phenotypic method for determination of *H. pylori* susceptibility because of a stable pattern of antibiotic release, a tolerance of prolonged incubation and a good correlation with the agar dilution method [7] but the price is very high. On the contrary, the disk diffusion is a simple, easy to perform, and economical method but not well standardized [8, 9]. Thus, definite breakpoints and standard technical conditions for *H. pylori* susceptibility are the pressing need for this method.

Vietnam is a developing country with high prevalence of *H. pylori* infection [10]. Until now, very few data are available on *H.pylori* resistance to antibiotics in Vietnam, particularly in the Central Region. The aims of this study were to determine the resistance rate of *H. pylori* to clarithromycin (CH) and levofloxacin (LE) in Central Vietnam, compare two phenotypic methods E-test and disk diffusion, definite breakpoints of disk diffusion method to determine the resistance of *H. pylori* to clarithromycin and levofloxacin.

2. METHODS

2.1 Study population

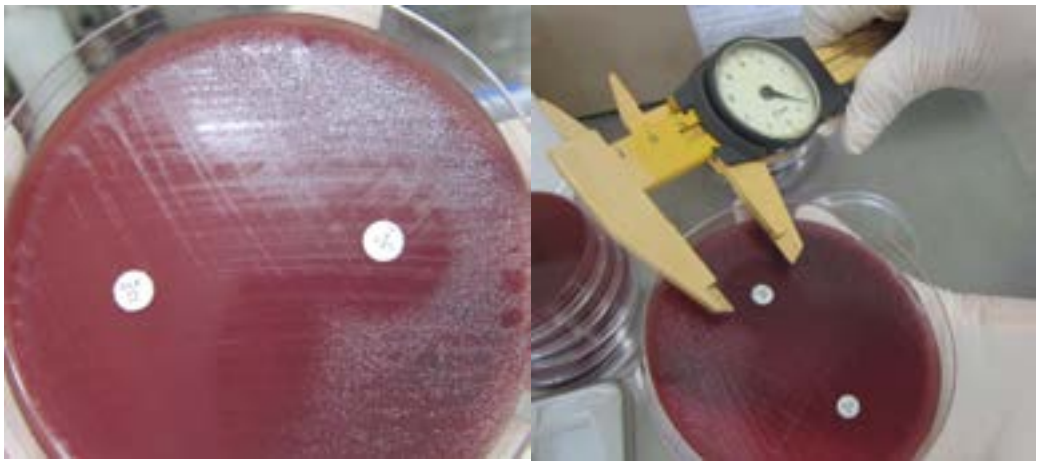
H.pylori strains were isolated from the gastric mucosa biopsies of Vietnamese patients who are from Central region, underwent upper gastrointestinal endoscopy at Hue University Hospital from July 2012 to August 2013. On the basis of endoscopic findings, patients were classified as having gastritis only, ulcerated lesion, or suspicion of neoplasia. Exclusion criteria included eradication therapy for *H. pylori*, treatment with bismuth-containing compounds, H2-receptor blockers or PPIs within 4 weeks before the study, a history of partial gastric resection. Totally, 56 dyspeptic patients were enrolled in the study. Two gastric biopsy specimens were taken from the antrum during upper gastrointestinal endoscopy. One biopsy specimen was tested for rapid urease test, another was cultured for *H.pylori* isolation. The specimen for culture was transported in saline solution to the Microbiology Department of the Hue University Hospital within less than 4 hours. Written informed consent was obtained from all the patients and the study protocol was approved by the Ethical Committee of Hue University of Medicine and Pharmacy – Vietnam.

2.2. *H.pylori* culture and antimicrobial susceptibility testing

The biopsy specimen for culture was homogenized in saline and inoculated onto Columbia agar plate with 7% defibrinated sheep blood and an *H.pylori* selective antibiotic supplement (DENT - Oxoid). The plates were incubated for 3 to 7 days at 37°C in microaerophilic conditions (GENbag microaer, Biomérieux, Marcy l'Etoile, France). *H.pylori* was identified by colony and microscopic morphology, and by positive urease, catalase and oxidase tests.

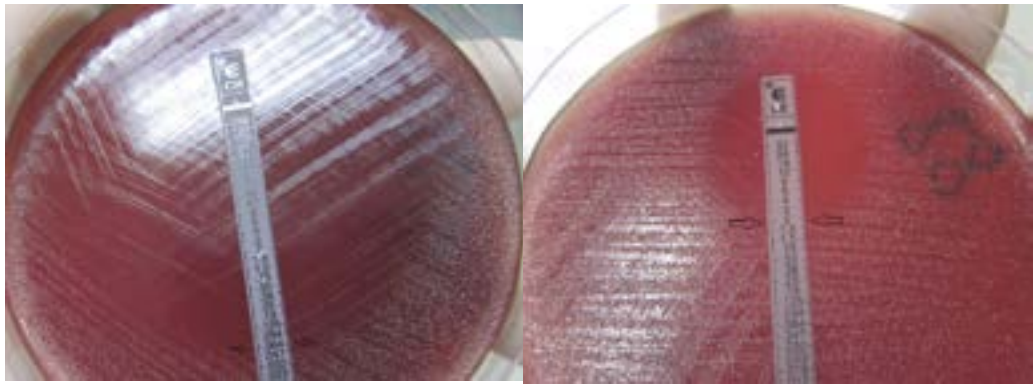
All isolates were obtained at 3 days old of primary culture or 2-3 days old of subculture for setting up susceptibility testing [11]. The culture suspension turbidity adjusted to be equivalent to a McFarland opacity standard of 3.0, taken from many colonies. For the disk diffusion method, the bacterial suspensions were spread onto Mueller-Hinton II Agar plates (Becton Dickinson) supplemented with 7% defibrinated sheep blood by sterile cotton swabs. The disks of 15 µg clarithromycin and 5 µg levofloxacin (diameter 6 mm; Oxoid) were placed on the plates. All plates were incubated at 37°C in microaerophilic conditions for 72 hours. Inhibition zone diameters were measured in millimeters with the caliper (Picture 1).

Epsilometer test (E-test; Biomérieux, Marcy l'Etoile, France) was used to determine the minimum inhibitory concentrations (MICs) of CH, LE. The bacterial suspensions were spread onto Mueller-Hinton II Agar plates (Becton Dickinson) supplemented with 7% defibrinated sheep blood. The E-test strip of the corresponding antibiotic was placed on the plate and incubated for 3 days at 37°C under microaerophilic conditions. MICs was defined as the point of intersection of the elliptical inhibition zone with the E-test strip (Picture 2). Strains were considered as resistant when the MICs was ≥1 µg/ml for both CH and LE [6].



Inhibition diameter of CH and LE *Measuring inhibition diameter of LE and LE*

Picture 1. Disk diffusion method



MIC-CH = 0.064µg/ml: sensitive *MIC-LE = 2 µg/ml: resistant*

Picture 2. Epsilometer test of the same strain on picture 1

Data Analysis

The linear regression method was applied to compare MICs with inhibition zone diameters and to establish disk diffusion breakpoints. The chi-square test for trend was used to compare rates of resistance to antibiotics during the study period. P values of ≤0.05 were considered statistically significant. All analyses were performed by SPSS version 13 (SPSS Inc., Chicago, IL) and Microsoft software excel 2007.

3. RESULTS

3.1. The resistance to clarithromycin and levofloxacin of *H.pylori* strains

Totally, 56 *H.pylori* strains were isolated from 56 patients (28 males and 28 females, mean age: 45.9 ± 14.2, lowest age 19 years old and highest age 72). Among them, 33 (58.9%) had gastritis, 22 (39.3%) had peptic ulcer diseases and 01 (1.8%) had gastric cancer. Forty three strains originated from 43 patients who had never been treated for *H.pylori* infection (primary strains), while the remaining 13 strains were isolated from previously treated patients (secondary strains).

Antibiotic resistance rate of *H.pylori* (regardless of previous eradication history) to CH and LE was 42.9% (24/56) and 44.6% (25/56), respectively (Table 1). The rate of resistance to LE in female was higher than in male (P<0.05). The distribution of age and antimicrobial resistance is shown in Fig 1. The resistance rate of LE in the age group from 40 to 49 years old was higher than other groups and CH resistance was greatest in the age group above 60 years old. The ratio of strains with secondary resistance was significantly greater than that of the strains with primary resistance, CH: 84.6% (11/13) vs 30.2% (13/43) and LE: 61.5% (8/13) vs 39.5% (17/43) (p < 0.05) (Fig 2).

Table 1. Antibiotic Susceptibility of 56 *H. pylori* strains

	No. (%) Resistance isolates in patients					
Antibiotics	**All patients (n=56)**	**GAS (n=33)**	**PUD (n=22)**	**GC (n=1)**	**Male (n=28)**	**Female (n=28)**
CH	24 (42.9%)	13 (39.4%)	11 (50%)	0	12 (42.9%)	12 (42.9%)
LE	25 (44.6%)	15 (45.5%)	9 (40.9%)	1 (100%)	**8 (28.6%)**	**17 (60.7%)**
CH: clarithromycin, LE: levofloxacin, GAS: gastritis, PUD: peptic ulcer disease, GC: gastric cancer						

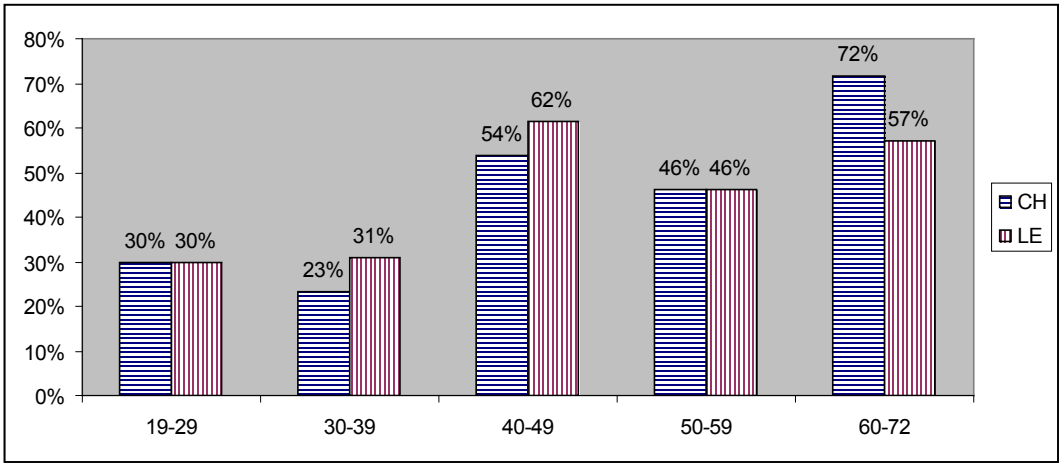


Figure 1. Age distribution of antibiotic resistance

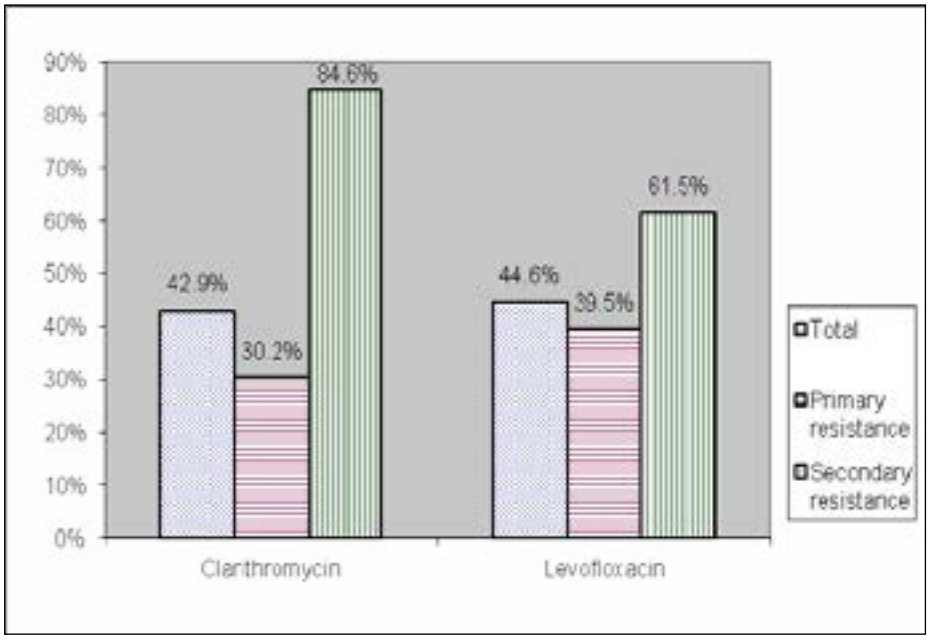


Figure 2. Comparison of primary and secondary resistance

3.2. Distribution of MICs and diameters of inhibition zones to clarithromycin and levofloxacin

The 56 strains were plotted according to the MIC values and inhibitory diameters of clarithromycin (Fig.3), the MIC values and inhibitory diameters of levofloxacin (Fig.4). All CH-sensitive strains by E-test had the inhibition diameters was ≥ 24mm and all CH-resistant strains had the inhibition diameters was ≤ 18mm (breakpoint for MIC: 1µg/ml). For LE, the breakpoints of inhibition diameters that the best separated for 56 strains into clearly distinguishable susceptible and resistant strians by E-test (breakpoint for MIC: 1µg/ml) were ≥ 24mm and ≤ 18mm, respectively.

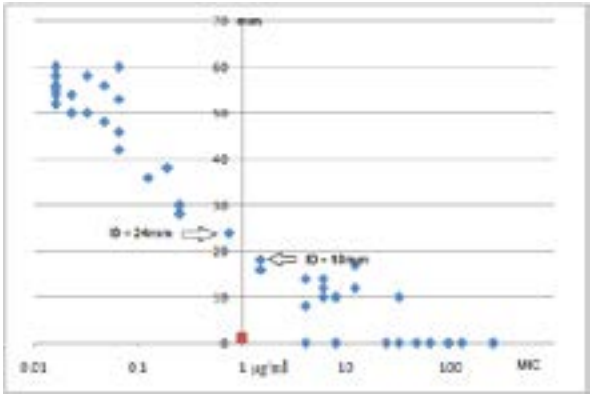


Figure 3. Distributions of MICs and diameters of inhibition zones (ID) determined by 15-mg clarithromycin disk diffusion testings for 56 clinical isolates.

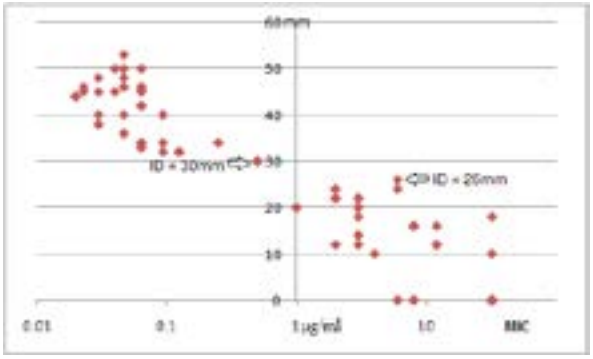


Figure 4. Distributions of MICs and diameters of inhibition zones (ID) determined by 5-mg levofloxacin disk diffusion testings for 56 clinical isolates of *H. pylori*

4. DISCUSSION

4.1. The resistance to clarithromycin and levofloxacin of *H.pylori* strains

Vietnam is a developing country with high prevalence of *H.pylori* infection in population [10]. therefore. successful eradication therapy is important not only to reduce the risk of developing gastric cancer but also to treat other severe *H.pylori*-related disorders [3]. Antibiotic resistance is the most important factor responsible for reducing success rate of *H.pylori* eradication therapy. Surveillance of *H.pylori* antibiotic resistance is mandatory in order to adapt the antibiotic combination to local resistance patterns [12. 13]. This issue is of particular relevance with regard to clarithromycin. which can induce a virtually 70% loss of effectiveness in the standard triple therapy when the strain is resistant to clarithromycin versus susceptible strains [4]. Unfortunately. we found that the antibiotic resistant strains were common in dyspeptic patients. Our present study showed that the rate of resistance (regardless of previous *H.pylori* eradication history) to CH. which is the most important antibiotic in the first-line therapies. was 42.9%. Especially. the resistance rate to LE. which is considered as an alternative choice after failure of *H.pylori* eradication with the standard triple therapy. was very high with 44.6%. The ratio of secondary resistant strains was significantly greater than that of primary resistant strains. namely. CH: 84.6% vs 30.2% and LE: 61.5% vs 39.5%. The latest consensus recommends PPI-clarithromycin-containing triple therapy without prior susceptibility testing should be abandoned when the clarithromycin resistance rate in the region is more than 15-20% [3]. Therefore. according to the above data. CH should not be used in the first-line treatment of *H. pylori* infection without a preliminary assessment of drug susceptibility. In addition. with the high rate of primary resistance. LE should not be considered as an alternative part for *H.pylori* eradication in this region. Similar to CH. it was about 45% loss of effectiveness in the LE-based triple therapy in case of LE resistant strains versus susceptible strains [14]. Comparing with the LE resistance rate in another study in Vietnam including *H.pylori* strains which were isolated in 2008 [15]. the primary resistance rate increased from 18.4% (in that study) to 39.5% (in our study). Thus. the resistance of *H. pylori* strains to LE is quickly acquired while the resistance to CH has not changed after nearly 5 years (33% vs 30.2%) in Vietnam. This is consistent with many studies in the world. the LE resistance rate increased from 8.6% for primary resistance and 22.9% for secondary resistance in 2006 to 18.8% for primary resistance and 30.6% for secondary resistance in 2011 in Germany [16]. from 4.7% for primary resistance and 16.7% for secondary resistance in 2003-2005 to 28.1% for primary resistance and 50% for secondary resistance in 2009-2012 in South Korea [17]. Interestingly. in our study. the LE resistance rate in female was significantly higher than in male. this probably reflects the increasing use of fluoroquinolones in female may lead to cross resistance with LE.

4.2. Distribution of MICs and diameters of inhibition zones to clarithromycin and levofloxacin

Currently, the determination of the resistance of *H.pylori* by culture and susceptibility test is still the basic and accurate method, although some molecular techniques have appeared and replaced for phenotypic method. In phenotypic methods,

the agar dilution usually considered the reference method to compare other techniques, being the most accurate, has been proposed by CLSI [6], but it is difficult to perform routinely. The E-test method has the advantage of being a quantitative method, it is adapted to slow-growing bacteria like *H.pylori* and has a good correlation between this method and the agar dilution method [7]. However, the E-test is economically impractical for clinical laboratory use when testing individual isolates. In contrast, the disk diffusion method is the simplest and most economic for routine susceptibility testing but it is not well standardized for slow-growing bacteria like *H.pylori* [11]. Therefore, we compared disk diffusion method with E-test for testing susceptibility of *H. pylori* to CH and LE. In this study, the MIC values determined by E-test method provide breakpoint values of inhibition diameters for disk diffusion method. It was recommended that strains with an MIC value of < 1µg⁄mL were considered susceptible, and those with MICs of ≥ 1µg⁄mL were considered resistant. Correspondingly, strains with inhibition diameters ≥ 24mm should be defined as susceptible to CH, and those with inhibition diameters ≤ 18 mm should be defined as resistant to CH. To LE, strains with inhibition diameters ≥ 30mm should be defined as susceptible, and those with inhibition diameters ≤ 24 mm should be defined as resistant. As a result, to apply disk diffusion method, we recommended the intermediate diameter zone of inhibition need to be re-evaluated by E-test when diameters from 18mm to 24mm with CH and diameters from 26 mm to 30mm with LE. According to some studies, strains are defined as sensitive to CH when inhibition zone diameters above 22mm (15µg disc) and the inhibition diameter less than 20 mm is defined as resistant and considered to verify by E-test [11]. Another study in India used ciprofloxacin (5µg disc) showed that 95% strains with inhibition diameters greater than 30 mm is sensitive [18]. In present study, the recommendations of *H.pylori* susceptibility testing were closely followed with age of cultures, the opacity of inoculum suspension and culture medium, therefore the results might be reliable [19].

5. CONCLUSION

- High resistance rate to CH and LE suggests that standard CH-based triple therapy may not be useful as the first-line treatment and LE-based triple should not use as an alternative therapy in Central Vietnam.

- The disk diffusion can be used as an alternative phenotypic method for testing CH and LE susceptbility of *H.pylori*. Strains are considered resistant to CH when the inhibition diameter is ≤18 mm and sensitive to CH when the inhibition diameter is ≥ 24mm. With LE, strains are considered resistant when the inhibition diameter is ≤ 26mm and sensitive when the inhibition diameter is ≥ 30mm. Compared with E-test, disk diffusion method may be more feasible and less expensive.

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