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POLYMORPHISMS OF *cagA* AND *vacA* GENES IN *HELICOBACTER PYLORI* ISOLATED FROM GASTRODUODENAL DISEASES PATIENTS IN CENTRAL VIETNAM

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Originality statement

I, Phan Trung Nam declare that the thesis for the award of a Doctor of Philosophy degree in Clinical and Molecular Microbiology of Ph.D School in Biomolecular & Biotechnological Science at University of Sassari, hereby submitted by me, has not been previously submitted for a degree at this or any other university and that it is my original work in design and execution, and that all the reference materials contained therein have been duly acknowledged.

Date:

Signature: …….

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Abstract

Helicobacter pylori is an important pathogen in humans, with infection rates around 50% of the world's population globally but it varies geographically. *H. pylori*, in particular its virulence factors, plays a significant role in the pathogenesis of upper alimentary tract diseases, including peptic ulcer disease, gastric mucosa associated with lymphoid tissue lymphoma and gastric cancer. A successful eradication therapy is important not only to reduce the risk of developing gastric cancer but also to treat other *H. pylori* severe related disorders. Vietnam is a developing country with high prevalence of *H. pylori* infection and an intermediate risk of gastric cancer but there has been a little information about antibiotic resistance and virulotyping of *H. pylori*, especially in the Central region.

The aim of this thesis was to determine the antimicrobial susceptibility patterns and the molecular basis of the resistance pattern; to investigate *cagA* and *vacA* genotypes, the two most important virulence genes, and to determine a clinical relevance of *cagA* and *vacA* polymorphisms of *H. pylori* strains isolated from dyspeptic patients in Central Vietnam.

We examined 127 dyspeptic patients from 7 provinces in Central Vietnam who were referred to Hue University Hospital between July 2012 and January 2014 for upper gastrointestinal endoscopy, to clarify clinical diagnosis and check *H. pylori* infection with respect to age, sex, endoscopic diagnosis and medical history. Two gastric biopsies from antrum were collected, one for rapid urease test during the procedure, and another for culture. A total of 97 *H. pylori* strains were isolated on Columbia agar base plus sheep blood and selective supplement under microaerophilic condition, all of them were

identified by microscopic, biochemistry characteristics and further confirmed by PCR targeting *16S rRNA* and/or *ureA*. Unfortunately, we failed to re-cultivate 5 strains from minus 80^{o}C store, one of them was lost. Finally, on 92 strains antibiogram was performed and 96 extracted DNA samples from 96 strains were available for further analysis.

Antimicrobial susceptibility tests to 4 common antibiotics for *H. pylori* eradication treatment were conducted by E-test and disc diffusion methods. Point mutations related to clarithromycin and levofloxacin on *23S rRNA* and *gyrA* genes were detected by sequencing their amplified PCR products. *vacA* and *cagA* genotyping of 96 strains were carried out using PCR and sequencing to determine their prevalence and polymorphism, then their clinical relevance was further analyzed.

The pattern of antimicrobial susceptibility testing showed that 42.4% were resistant to clarithromycin (primary–34.2%; secondary–73.7%), 41.3% to levofloxacin (primary–35.6%; secondary–63.2%), 76.1% to metronidazole and 1.1% to amoxicillin. Multidrug resistance was observed in 56.5% (primary–50.7%; secondary–79%) of isolates ($p < 0.05$). The rate of resistance to levofloxacin in females was significantly higher than in males ($p < 0.05$) and the resistance to clarithromycin and levofloxacin was increased according to the rise of age.

To define the breakpoints for disk diffusion as a feasible and cheap method for determining qualitative susceptibility based on E-test results as a gold standard test, we found that susceptible strains to clarithromycin had inhibition diameters ≥ 24mm and resistant to clarithromycin with inhibition diameters ≤ 18mm. To levofloxacin, strains with inhibition diameters ≥ 30mm

defined as susceptible, and those with inhibition diameters ≤ 26mm defined as resistant.

Most of the clarithromycin and levofloxacin resistant strains harboured resistance associated mutation with common position at A2143G, T2182C in *23S rRNA* gene and at Asn-87 or Asp-91 in *gyrA* gene. MICs increased in strains carrying quadruple mutations in their *23S* and in strains with Asn-87 *gyrA* mutation ($p < 0.05$). One high level levofloxacin (MIC = 32 mg/L) resistant strain had new mutations with combination of N87A, A88N and V65I.

Determination of virulence factors of 96 strains, *cagA* genotyping showed that *cagA* gene was detected in 85.4% of *H. pylori* strains. In *cagA* negative strains, 57.1% suggested the presence of a deviating cag-PAI. The *cagA* genes were further characterized EPIYA motif to classify *cagA* type showing 9.8% Western *cagA* strains (one EPIYA motif - ABCC and 7 had an EPIYA motif – ABC), whereas all 90.2% East Asian - *cagA* strains had an EPIYA motif – ABD. To confirm *cagA* genotyping by PCR method, sequencing analysis was performed exhibiting the consistent results with previously analysed PCRs. Clinical relevance of *cagA* genotype showing East-Asian *cagA* type was present in all strains isolated from gastric ulcer and gastric cancer patients, which tended to be higher than those from gastritis and duodenal ulcer. On the contrary, *cagA* negative and Western *cagA* type were only in strains from gastritis, duodenal ulcer patients.

Analysis of *vacA* polymorphism revealed that 97.9% of strains had *vacA*s1 genotype; 91.5% had *vacA*i1; *vacA*m1 and *vacA*m2 presented 56% and 44%, respectively. The combination of three regions of *vacA* showed that the

s1i1m1 type had a predominant percentage with 55.7%, followed by s1i1m2, s1i2m2 and s2i2m2 with 35.2%, 6.8% and 2.3%, respectively. All *vacA* s1m1 linked with i1, all s2m2 with i2 and s1m2 linked with i1 or i2. Clinical relevance of *vacA* polymorphism exhibiting *vacA*s1 and *vacA*i1 was present in almost all strains in different gastroduodenal diseases. On the other hand, *vacA*m1 was more predominant than *vacA*m2 in strains isolated from gastric ulcer patients (75% vs 25%) and it showed a higher frequency of *vacA*m1 than that in duodenal ulcer (44%) and gastritis (58.9%). Furthermore, in gastric ulcer strains, *vacA*s1i1m1 was more predominant than s1i1m2 and s1i2m2 polymorphic types (75% vs 12.5% and 12.5%) with a significant difference ($p < 0.05$). Moreover, *vacA*s1i1m1 was present in strains isolated from gastric ulcer patients with a higher frequency than in that from duodenal ulcer (41.7%) and gastritis (59.3%). All s2i2m2 types were present in gastritis and duodenal ulcer strains.

The relation of *cagA* and *vacA* genes showed that *cagA*-positive status was very closely associated with *vacA* s1i1m1 genotype with highest frequency (98%), followed by s1i1m2 (77%) and s1i2m2 (33%), none with s2i2m2. On the contrary, *cagA*-negative status was only present in *vacA* s2i2m2 and was predominant in *vacA* s1i2m2 (67%).

In conclusion, we highlight a very high resistance rate in Central Vietnam with 56.5% of multiple resistance and higher prevalence of secondary resistance versus primary resistance, in particular to clarithromycin and levofloxacin. Data on determinants of resistance to clarithomycin and levofloxacin (mutations in *23s RNA* and *gyrA* genes) are new information in Vietnam. *cagA* positive status, East Asian *cagA* type and *vacA* s1i1m1 type

are the most predominant in gastric ulcer and gastric cancer. The prevalence of *cagA* negative status and Western *cagA* type is higher than that reported in previous studies in different areas of Vietnam.

Publications and Conference proceedings arising from this thesis

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AC	Amoxicillin
cagA	Cytotoxin associated gene A
CH	Clarithromycin
CLSI	Clinical and Laboratory Standards Institute
DU	Duodenal ulcer
EPIYA	Glutamic-Proline-Isoleucine-Tyrosine-Alanine
E-test	Epsilometer Test
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GAS	Gastritis
GC	Gastric cancer
GU	Gastric ulcer
LE	Levofloxacin
MIC	Minimum Inhibitory Concentration
MLST	Multilocus Sequence Typing
MZ	Metronidazole
PCR	Polymerase Chain Reaction
PPI	Pronton Pump Inhibitor
PUD	Peptic Ulcer Diseases
QRDR	Quinolone Resistance Determining Region
vacA	Vacuolating cytotoxin A gene

1. Introduction

1.1. *Helicobacter pylori*

1.1.1. Introduction & history

The presence of spiral shaped bacteria in the human stomach was first described in 1892 by the Italian pathologist Giulio Bizzozero [1]. However, proof for an infectious origin of diseases of the upper gastrointestinal tract was not provided until the early 1980s when Warren and Marshall isolated *H. pylori* from gastric biopsies and established the bacterium's association with gastritis and peptic ulceration and they were awarded the Nobel Prize in Physiology or Medicine for this discovery in 2005 [2]. The bacterium was first named *Campylobacter pyloridis* because of its location and some common properties with *Campylobacter jejuni*. When the difference between *Campylobacter pylori* and *Campylobacter* organisms were confirmed by Goodwin et al in 1989 [3], the name was changed to *Helicobacter* and *Helicobacter pylori* (*H. pylori*) became the first member of the new species. The name *Helicobacter* reflects the two morphological appearances of the organism, often rode-line *in vitro* and helical *in vivo*. More than 30 *Helicobacter* species have been isolated, some infecting occasionally also humans e.g. *H. heilmannii, H. fenelliae* and *H. pullorum* but the primary hosts for the non-*H. pylori* species are animals [4].

Following the discovery of *H. pylori* a large amount of research has been carried out over the last 2 decades, leading to the development of diagnostic tests and antibiotic treatment strategies for *H. pylori* infection. However, despite this intense investigation, *H. pylori* colonisation is still widespread in developing countries. Its route of transmission is poorly

understood and emerging antibiotic resistance has consequences for the efficacy of treatment. Research into *H. pylori* infection and disease is therefore important in developing novel prevention and treatment strategies.

1.1.2. Microbiology

H. pylori is a microaerophilic Gram negative bacterium which is able to colonise and persist in the mucus layer of the human stomach. Cells are from 2 to 4 µm in length and usually curved or spiral shaped with 2 – 6 unipolar sheathed flagella of approximately 3 µm in length which allow rapid motility through the viscous mucus layer. Morphological changes to a viable but non culturable coccoid form occur after prolonged culture or antibiotic treatment [5].

H. pylori is commonly isolated from gastric biopsy samples of infected patients, occasionally from gastric juice, faeces and vomitus [6]. Some studies have reported detection of *H. pylori* in water but the relevance of these studies based on polymerase chain reaction (PCR) remains unclear [7]. *H. pylori* is a fastidious microorganism and requires complex growth media. The optimal environment for growth of *H. pylori* is microaerophilic at 37°C. Although the natural habitat of *H. pylori* is the acidic gastric mucosa because of its ability to produce acid-neutralizing ammonia, a more neutral pH between 5.5 and 8 is the optimal for bacterial grows. The agar plates used to culture *H. pylori* are supplemented with blood or serum and antibiotics such as vancomycin, trimethoprim, cefsoludin, and amphotericin B or polymyxin B. [8]. Isolation of *H. pylori* from biopsy samples is difficult and not always successful. *H. pylori* grows slowly and it may take from 3 to 7 days to achieve a good colony yield.

Prolonged culture is not only unable to increase colony size but may also lead to a transition to the non-culturable coccoid form [8].

Identification of *H. pylori* is based on colonies, microscopic morphology and biochemical characteristics including oxidase, urease and catalase positivity. Colonies of *H. pylori* from primary culture on supplemented blood agar at 37^{o}C under microaerophilic condition usually take 3-5 days, are circular (1-2mm), convex, translucent in appearance. There is slight hemolysis in blood agar around colonies, which are greyish in colour. Microscopic examination of the cultured bacteria may show a morphology different from the bacteria present in the biopsy specimen, i.e., bacilli which are neither spiral shaped nor motile, but straight or curved [8]. Although the bacteria can be stained with common histological stains such as hematoxylin and eosin (H & E), silver-containing stains such as Warthin-Starry or Steiner are strongly recommended, particularly when H & E fails to reveal organisms in a biopsy specimen with chronic active inflammation [9]. Urease enzyme that hydrolyses urea to ammonia and carbon dioxide is one of the most important factors for the survival of *H. pylori* when colonizing the gastric mucosa [10].

The complete genome sequence of *H. pylori* consisting of a circular chromosome with a size of 1,667,867 base pairs and G+C content of 35-40% has been reported and the extent of molecular mimicry between of *H. pylori* and human has been fully explored [11]. *H. pylori* is genetically heterogeneous, which results in every infected individual carrying a distinct strain [12]. Genetic heterogeneity is thought to occur mainly via DNA rearrangement and the introduction and deletion of foreign sequences [13].

1.2. Epidemiology

1.2.1. Prevalence of *H. pylori* infection

Globally, *H. pylori* infection affects 50% of the population but prevalence of *H. pylori* shows large geographical variations [14]. Within geographical areas, the prevalence of *H. pylori* inversely correlates with socioeconomic status, in particular in relation to living conditions during childhood which is a period of major risk for *H. pylori* infection [15].

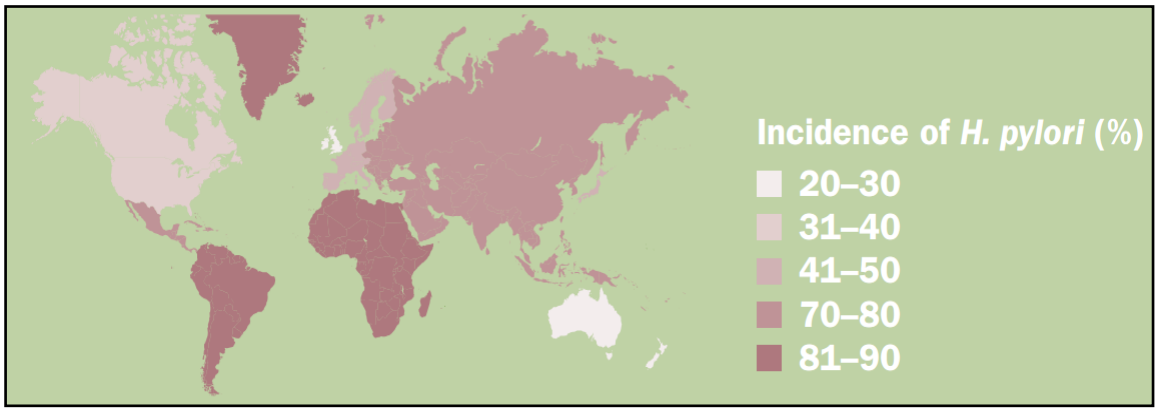


Figure 1.1. Worldwide epidemiology of *H. pylori* infection [16].

Study of the epidemiology of *H. pylori* in the Asia Pacific region reveals that there is a wide variation in the prevalence of *H. pylori* infection, both between countries and within countries. In general, the seroprevalence rates in developing countries are higher than in developed countries. In India, the reported overall seroprevalence rate was 79% [17]. In Vietnam, the *H. pylori* seroprevalence rate was over 75% [18]. On the other hand, the seroprevalence rates in more developed countries in the Asia Pacific region are generally lower. Among North-east Asian countries, the overall seroprevalence rate was 39% in Japan [19], 55% in Taiwan [20] and 60% in South Korea [21]. Among South-east Asian countries, the reported seroprevalence rate was 36% in Malaysia [22],

31% in Singapore [23] and 57% in Thailand [24].

In each country, differences in seroprevalence rates of *H. pylori* infection between different geographic regions and also between different ethnic groups have been reported. A cross-sectional study conducted in children residing in industrial and rural areas of Italy reported that the seroprevalence of *H. pylori* infection was significantly higher in children residing in rural areas compared with those residing in industrial areas [25]. The study also found that, in rural areas, children having shepherd dogs were at greatest risk for *H. pylori* acquisition. In China, seroprevalence rates have been noted to be higher in regions with higher gastric cancer incidence rates [26]. In Malaysia, the seroprevalence rate has been reported to be lower in west Malaysia (26–31%) than in east Malaysia (43–55%) [22]. In Taiwan, the highest seroprevalence rate (63%) has been reported in rural areas where the aborigines live and in which gastric cancer rates are highest. This compares with a prevalence rate of 40.5% in urban areas, where gastric cancer rates were lowest [20]. In Vietnam, differences in *H. pylori* seroprevalence rates have been reported between an urban area (Hanoi; 79%) and a rural area (Hatay; 69%) [18].

Generally, it is agreed that *H. pylori* infection has been declining in industrialized countries. In Japan, the overall seroprevalence rate declined from 73% in 1974 to 55% in 1984 and declined even further to 39% in 1994 [19]. In South Korea, the seroprevalence rate decreased from 67% in 1998 to 60% in 2005 [21]. In particular, lower seroprevalence rates have been observed in the younger population. In Singapore, the seroprevalence rate in those aged less than 3 years has been reported to be 3%, but rose to 71% among those aged more than 65 years [23]. The major decline in *H. pylori* seroprevalence is probably

associated with socio-economic development, including: improvement in public health measures, personal hygiene and living conditions [5]. Consequently, childhood infections have decreased, leading to a lower seroprevalence rate of *H. pylori* in the younger generations, thus lowering the overall seroprevalence rate in the population.

The decline in *H. pylori* infection has been matched by a decline in gastric cancer incidence and gastric cancer mortality. For example, in Japan, with the fall in *H. pylori* prevalence between 1986 and 1996 the fall in gastric cancer mortality has been greater in younger age groups (20–39 years) in comparison with the overall gastric cancer mortality rate [27]. However, a study in Singapore has demonstrated that Singaporean Chinese and Singaporean Indians have similar *H. pylori* seroprevalence rates but have significantly different gastric cancer incidence rates [28]. In this case, bacterial virulence factors, host genetic factors or dietary factors could influence carcinogenesis.

1.2.2. Molecular Epidemiology of *H. pylori*

Population structure analysis based on MLST has revealed seven modern population types of *H. pylori*, which derived from six ancestral human populations. Interestingly, the incidence of gastric cancer is closely related to the distribution of *H. pylori* populations. The different incidence of gastric cancer can be partly attributed to the different genotypes of *H. pylori* circulating in different geographic areas. Furthermore, population genetic studies based on MLST analysis help predict prehistoric human migration ‘‘accompanied’’ by *H. pylori* [29].

Multilocus sequence typing of the seven core housekeeping genes (*atpA, efp, mutY, ppa, trpC, ure1, yphC*) of *H. pylori* isolates from different geographic

regions has revealed seven main geographic strains, termed hpEurope, hpEastAsia, hpAsia2, hpAfrica1, hpAfrica2, hpNEAfrica, and hpSahul [30], [31].

The hpEurope strain is common in Europe and countries colonized by Europeans, while hpEastAsia characterizes strains from East Asia. The hpEastAsia strain has been further classified into hspMaori (Polynesians), hspAmerind (native Americans) and hspEAsia (East Asia) subpopulations. The hpAsia2 strain was isolated from South and South-east Asia. hpAfrica1 includes two subpopulations, hspWAfrica (West Africans, South Africans, and Afro-Americans) and hspSAfrica (South Africans); hpAfrica2 is very distinct and has only been isolated in South Africa. hpNEAfrica is predominant in isolates from Northeast Africa. hpSahul strains are isolated from aborigines of Australia and highlanders in New Guinea. All these modern populations derived from six ancestral populations which were designated ancestral European 1 (AE1), ancestral European 2 (AE2), ancestral EastAsia, ancestral Africa1, ancestral Africa2 [32], and ancestral Sahul [31]. *H. pylori* is predicted to have spread from East Africa over the same time period as anatomically modern humans (58,000 years ago) (Fig 1.2), and has remained intimately associated with their human hosts ever since [30], [31], [33].

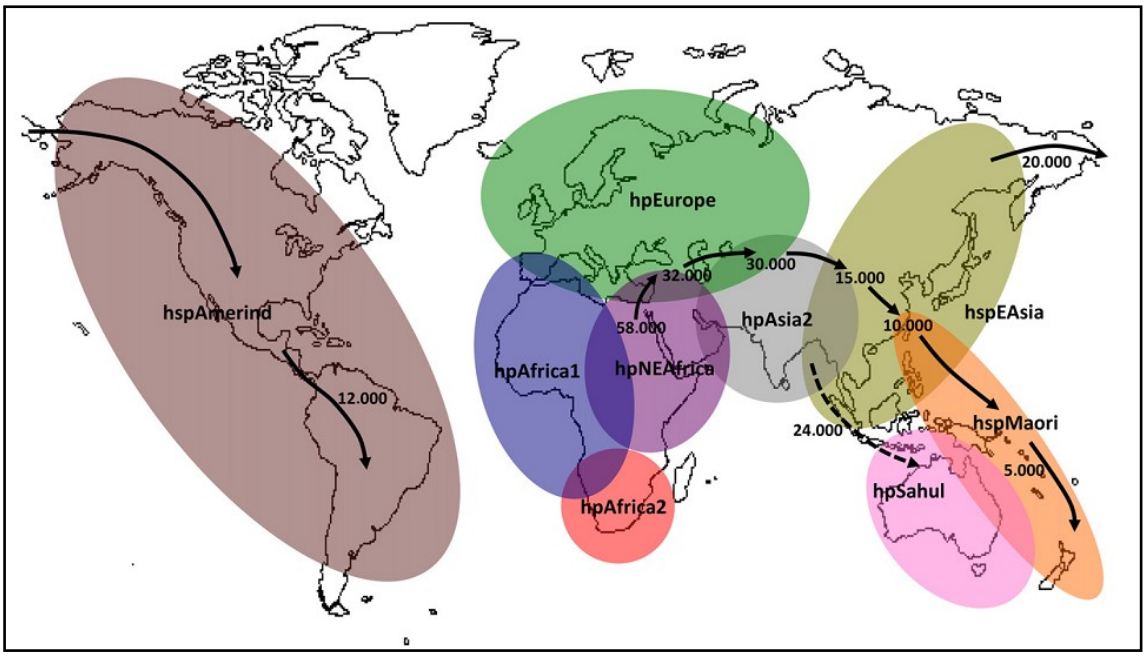


Figure 1.2. Geographic distribution of *Helicobacter pylori* populations and predicted traces of prehistoric human migration [29]
(Colored circles illustrate the putative distribution of H. pylori populations before the ''Age of Exploration.'' Black arrows and numbers represent predicted paths and times of migration)

It has been observed that populations with high gastric cancer rates correspond almost exactly to populations with hpEastAsia strains [34]. In South Asian countries where *H. pylori* seroprevalence rates are high but gastric cancer prevalence rates are low, *H. pylori* strains have been reported to be predominantly hpAsia2. Similarly, in Africa, most strains have been shown to be hpNEAfrica, hpAfrica1 or hpAfrica2 , and the gastric cancer rates are also correspondingly lower than in East Asia [34]. Interestingly, the phylogeny of most cag PAI genes was similar to that of MLST, indicating that cag PAI was probably acquired only once by *H. pylori*, and its genetic diversity reflects the isolation by distance which has shaped this bacterial species since modern humans migrated out of Africa [35]. In addition, strains without the presence of

cagA has been reported to be less virulent regardless MLST, thus, the *cagA* genotype rather than the phylogeographic origin is a better predictive factor of gastric cancer [36].

1.2.3. Transmission of *H. pylori* infection

Up to date, there is still an ongoing debate about the exact mode of transmission of *H. pylori* infection that has been lasting over two decades [37]. The majority of data support the notion that transmission is within families, close contact and level of household sanitation appear to be the important variables [38], [39]. In one study, *H. pylori* status was determined in 41 families; the results revealed that, if the index case (either the mother or father) was positive, the children and spouse in that family were also likely to test positive for *H. pylori*. If the index case was negative, then the children and spouse were likely to be negative as well [40]. The most probable transmission route is through oral-oral, gastro-oral and faecal-oral pathways.

1.2.3.1. Oral-oral transmission

The role of oral cavity as a reservoir of *H. pylori* infection has been controversial. Only one study found *H. pylori* by culture [6]. Several studies using PCR amplification from saliva and dental plaque have demonstrated the presence of *H. pylori* in the mouth [6], [41]. Close mouth to mouth contact has been identified as a risk factor for oral-oral transmission [42], [43]. Cultural and social differences such as pre-mastication of food and sharing chopsticks in Asian countries may be an explanation for oral-oral transmission route. The evidence for this transmission pathway is supported by a study from Bangladesh where the Hindu babies had higher prevalence of *H. pylori* infection as compared to Muslims assumed to be due to Hindu mothers

regularly coating their nipple by saliva before breastfeeding and feeding their babies by pre-mastication of food [43]. In addition, the data from one study conducted by Chow *et al* showed that the infection prevalence of people who used chopsticks to eat from communal dishes was significantly higher than in those who did not (64.8% versus 42.3%) [42].

1.2.3.2. Gastro-oral transmission

Vomitus has been suggested as an important vehicle for *H. pylori* transmission as this organism had been successfully cultured from gastric juice and vomitus [6]. Also, an increased acquisition of *H. pylori* in children during a gastroenteritis outbreak has been reported in one centre [44]. The gastro-oral transmission route seems to occur by either vomitus or regurgitation of stomach contents. Data from some studies have shown that endoscopists had a higher risk of acquiring *H. pylori* infection compared to the general population [45].

1.2.3.3. Faecal-oral transmission

The faecal-oral route is another potential route of transmission through exposure to contaminated food or water.

Firstly, evidence for a faecal-oral transmission route of *H. pylori* has been reported in several studies using DNA to detect *H. pylori* in stool of infected patients and nowadays it's become one of non-invasive tests to diagnose *H. pylori* infection [46]. In addition, the bacterium has been isolated by culture of faecal samples in several studies [6], [47].

Secondly, there is mounting evidence which suggests that the prevalence of *H. pylori* infection has a strong correlation with access to clean water. Several studies found contaminated water to be a risk factor for *H. pylori*

transmission [48], [49]. In a study from India that examined 500 adults, three biopsy samples were collected from each subject to assess *H. pylori* infection [50]. Based on detection by PCR amplification of the gene encoding 16S rRNA from *H. pylori*, the prevalence of infection among people who drank water from wells was 92%, compared with 75% in those who drank tap water ($P < 0.001$). *H. pylori* infection prevalence was found to be higher in people with a low clean water index (88%) than in those with a higher clean water index (33%) ($P < 0.001$). The results of the study suggested that the risk of acquisition and transmission of *H. pylori* can be prevented to a large extent by regular boiling of water used for drinking purposes.

Therefore, faecal-oral transmission has been proposed to commonly occur in developing countries because of limitations in hygiene conditions [5]. The rapid decrease of the infection in developed countries has been speculated to be due to the decrease in gastrointestinal infections in children that are still very common in developing countries [51].

1.3. *H. pylori* virulence and colonisation factors

1.3.1. Cytotoxin associated gene A (*cagA* gene)

CagA protein was discovered in the early 1990s in a study with the strong association between serological responses to CagA and peptic ulcer disease [52]. Then, the cloning of the *cagA* gene [53] as well as identification of cag pathogenicity island (cagPAI) genes and their role in inflammation [54]. Recently, one study have firmly implicated that CagA plays as a bacterial oncoprotein by attenuating apoptosis *in vivo* and *in vitro* [55] and another study demonstrated that transgenic expression of CagA in mice leads to the development of aberrant gastric epithelial proliferation and gastric carcinoma[56].

The *cagA* gene, encodes 120 to 140 kDa CagA protein, is the most studied virulence factor of *H. pylori*, which is located at one end of the cagPAI, an approximately 40 kb region that is thought to have been incorporated into the *H. pylori* genome by horizontal transfer from an unknown source [57]. The cag PAI, contains 27 to 31 genes, encodes a type IV secretion system through which CagA is delivered into host cells (Fig 1.3) and *cagA* is recognized as a marker for the cagPAI region [58], [59].

1.3.1.1. Pathogenicity mechanism of CagA

CagA phosphorylation-dependent host cell signaling:

Following its injection into epithelial cells, CagA undergoes targeted tyrosine phosphorylation by Src and Abl kinases of host cell at motifs containing the amino acid sequence EPIYA, which are located within the 3' terminus of CagA [60]. Within epithelial cells, phosphorylated CagA activates a eukaryotic phosphatase (SHP-2) as well as ERK which results in the impairment of a variety of intracellular signaling systems leading to morphological aberrations (Fig 1.3) [60].

CagA phosphorylation-independent host cell signaling:

Nonphosphorylated CagA also exerts effects within the cell that contribute to pathogenesis. Translocation, but not phosphorylation, of CagA leads to aberrant activation of β-catenin, disruption of apical-junctional complexes, and a loss of cellular polarity [61]. Currently, there are at least 10 known phosphorylation-independent CagA host interaction partners [62]. In addition, CagA itself forms dimers in cells in a phosphorylation-independent manner, and the CagA multimerization (CM) sequence (FPLxRxxxVxDLSKVG) was

identified as the site responsible for dimerization [63]. This sequence is located within the EPIYA-C segment, but is just downstream of the EPIYA-D segment. The CM sequence is also essential for the formation of the CagA–PAR1 (MARK) complex-bound non phosphorylated CagA inhibits the kinase activity of PAR1 to promote the loss of cell polarity, mucosal damage, inflammation and carcinogenesis [63], [64].

The CM sequence has also been named as the MARK2/PAR1B kinase inhibitor (MKI) [65] or named as the conserved repeat responsible for phosphorylation independent activity (CRPIA) [66] that was reported to be responsible for the interaction of CagA with activated c-Met led to the upregulation of β-catenin and nuclear factor κB (NFκB) transcriptional activities which promoted proliferation and inflammation. Therefore, there are currently three acronyms that essentially correspond to the same sequence in CagA [63], [64].

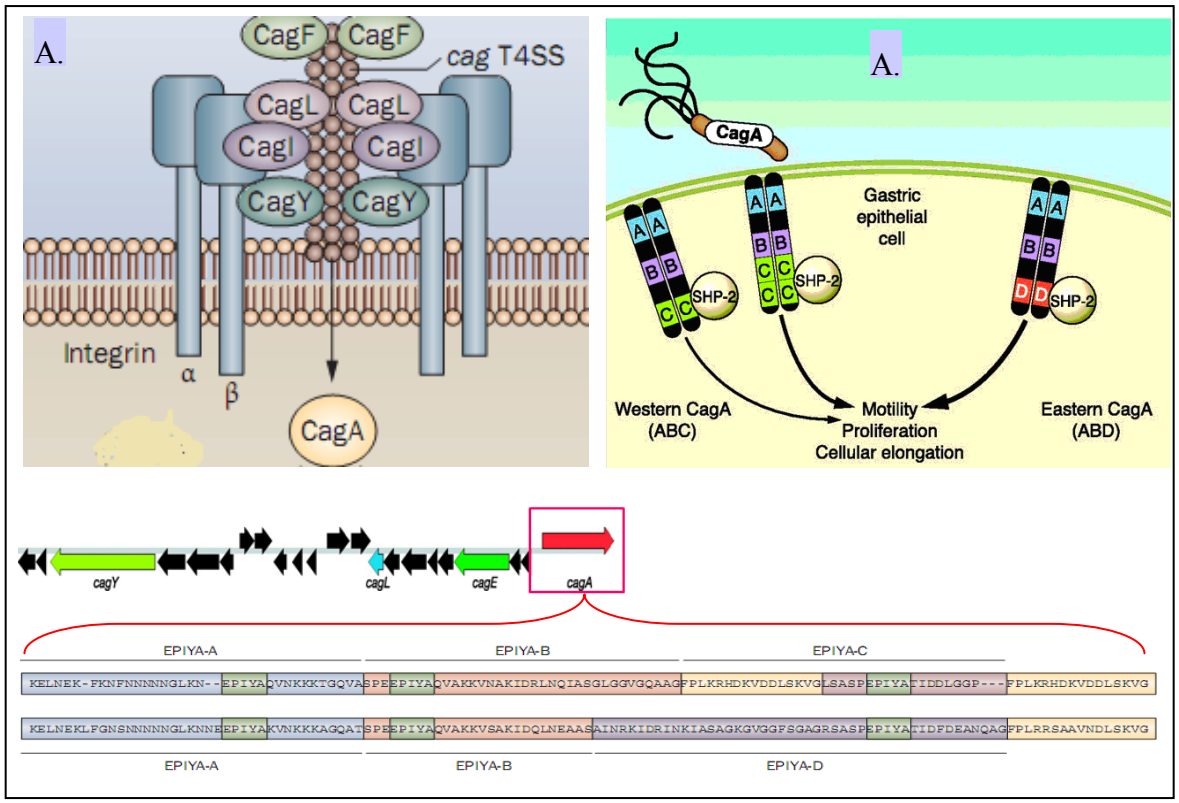


Figure 1.3. CagA structure and its effects in host cell [67].

A: Type IV secretion system, encoded by cag PAI, deliver CagA into the host cells; **B:** CagA phosphorylation motifs and cellular morphogenic alterations induced by intracellular CagA; **C:** Structural polymorphism in CagA. Western-type CagA contain EPIYA-A, EPIYA-B, and EPIYA-C segments. By contrast, East-Asian-type CagA contain the EPIYA-A, EPIYA-B and EPIYA-D segments. The sequence flanking of the EPIYA-D segment is EPIYA-TIDFDEANQAG, but that of EPIYA-C segment is EPIYATIDDLGGP.

1.3.1.2. CagA type: Western versus East Asian

cagA is a polymorphic gene. In particular, there are different numbers of repeat sequences located in the 3' region of the *cagA* gene of different *H. pylori* strains. CagA can be divided in two types, the East-Asian type and the Western type, according to the repeat sequences of the 3' region of *cagA* gene [67].

The repeat regions contain the Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs of CagA in strains harvested from persons residing in Western countries have been termed A, B, or C based on sequences flanking the EPIYA motif. In contrast, phosphorylation sites within CagA proteins from East Asian *H. pylori* strains lack the EPIYA-C motif and, instead, contain a different motif, which is termed D [60]. As such, each CagA is assigned a sequence type consisting of the names of the EPIYA segments in its sequence that is, ABC, ABCC or ABCCC for Western-type CagA and ABD for East-Asian-type CagA due to strong conservation and a lack of duplication in the D region (Fig 1.3). Analyses of the repeat regions of CagA has demonstrated that, although not common, some strains isolated in East Asian countries have a Western-type CagA sequence. By contrast, none of the Western strains studied have an East-Asian-type CagA sequence [68], [69].

In vitro, Western CagA with increased the number of EPIYA-C motifs or East Asian CagA mostly with one EPIYA-D motif is associated with the intensity of CagA phosphorylation, epithelial cellular elongation, and induction of proinflammatory cytokines [70, 71]. CagA of East-Asian type, containing EPIYA-D segments, exhibits a stronger binding affinity for Src homology 2 containing protein-tyrosine phosphatase (SHP-2) and a greater ability to induce morphological changes in epithelial cells than Western-type CagA, which contains one EPIYA-C segments [72].

1.3.1.3. Clinical importance of *cagA*

CagA positive strains were reported to be associated with severe clinical outcomes not only in Western countries [67] but also in East Asian countries, however, the odds ratio in East Asian countries was smaller than that in Western

countries [73]. Additionally, *H. pylori* strains possessing more than three EPIYA-C motifs are more frequently associated with gastric atrophy, intestinal metaplasia, and gastric cancer [74, 75]. Individuals infected with East-Asian type *cagA* strains were reported to have an increased risk of peptic ulcer or gastric cancer than those with Western-type *cagA* strains in Southeast Asian countries, such as Thailand, Malaysia, Singapore [76], [77]. However, in East Asia countries, it is difficult to differentiate between gastritis and gastric cancer simply by considering the number of repeated sequences because almost all strains contain East Asian type CagA with a single EPIYA-D segment [69].

1.3.2. Vacuolating cytotoxin VacA

VacA is the second most extensively studied *H. pylori* virulence factor. Unlike *cagA*, virtually all the *H. pylori* strains have a functional *vacA* which encodes a vacuolating cytotoxin [78].

In 1988, Leunk and colleagues discovered that cell free supernatants from broth cultures of *H. pylori* induced vacuolar degeneration of various cultured epithelial cell lines [79]. This effect was subsequently shown to be caused by a secreted protein toxin, designated VacA [80]. *vacA* gene, which was cloned from several strains of *H. pylori*, encodes the vacuolating cytotoxin, 140-kDa precursor protein [81] [82].

Current models indicate that a 96-kDa VacA protein is secreted, which is then cleaved into an 88-kDa mature protein (p88) and a 10.5-kDa passenger domain (p10). The mature, secreted p88 subunit can undergo further proteolytic cleavage to yield two fragments, p33 and p55 [83], [78], which represent the two functional domains of VacA (Fig.4). Cell binding is mediated by the p55 fragment of VacA [84], but p33 and p55 can also exert multiple other effects.

VacA inserts into planar lipid bilayers to form anion selective membrane channels [85]. The p33 domain contains a hydrophobic sequence, which is involved in pore formation whereas the p55 fragment contains one or more cell-binding domains [84]. Since the p55 subunit contains the m1 and m2 alleles, delineation of protein sequences from unrelated *H. pylori* strains should allow identification of VacA structural features that are important for binding to host receptors [86].

1.3.2.1. Effects of VacA on cellular

In addition to inducing vacuolation, VacA can induce multiple cellular activities, including membrane channel formation, cytochrome c release from mitochondria leading to apoptosis, and binding to cell-membrane receptors followed by initiation of a proinflammatory response (Fig 4). VacA can also specifically inhibit T-cell activation and proliferation [67]. Studies indicate that VacA and CagA can even inhibit at least some of each other's signaling pathways, for example, CagA has been shown to promote the expression of the apoptotic suppressor Mcl1, and inhibit epithelial cell apoptosis caused by *vacA* [55, 87]. These data again emphasize the importance of in vitro infection experiments in which interaction among *H. pylori* virulence factors can be taken into account.

1.3.2.2. *vacA* polymorphism

vacA gene is present in all strains of *H. pylori* and well conserved between strains but there is significant diversity in three distinct regions of the gene: the signal (s) region, the middle (m) and intermediate (i) regions (Fig. 4). Characterisation of these regions has revealed that allelic variation exists. Allelic diversity in *vacA* has allowed strains to be genotyped s1 or s2 for the signal

region and m1 or m2 for the middle region [88].

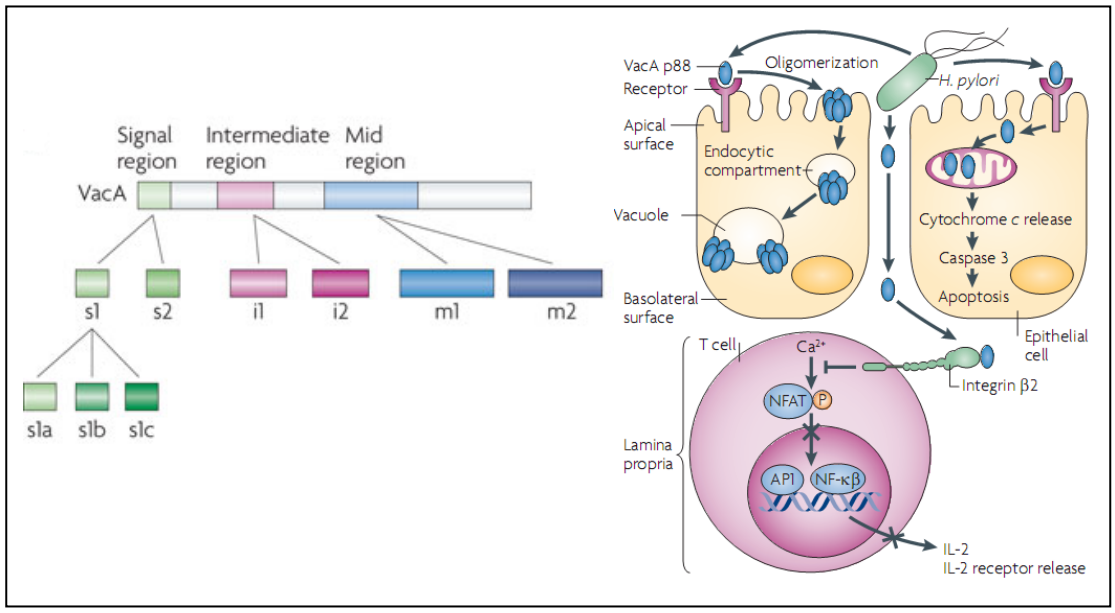


Figure 1.4. *H. pylori* VacA structure and functional effects [89].

The *vacA* genotype is an important determinant of toxicity. Type s2 VacA, carrying a hydrophilic N-terminal, is cleaved a 12 amino acid in the signal peptide sequence and turned into type s1 VacA with a hydrophobic N-terminus. This 12 residue extension to VacA has been shown to be responsible for the loss of vacuolation induction by s2 strains [88, 90]. The *vacA* middle region which encodes part of the p58 region has been shown to be associated with cell specificity, m1 forms vacuolate a variety of cell types but m2 forms are more limited in the cells they vacuolate [88, 91].

The recently identified intermediate region, is located between the signal and mid regions of *vacA*, can also be divided into two types i1 and i2 [92]. *vacA* s1/m1 type strains were nearly always type-i1 and that type s2/m2 strains were always type-i2. The s1/m2 strains varied in their intermediate region type, however, they found that strains that were s1/i1/m2 had vacuolating activity

whereas type s1/i2/m2 strains were non-vacuolating. The i region is thought to be involved in cytotoxin binding [92]. The *vacA* gene may comprise any combination of signal, intermediate and mid region types. Most of the *vacA* combinations have been detected in strains isolated from infected individuals, although the s2/m1 form of *vacA* is rarely found [88].

1.3.2.3. Disease associations of VacA

The clinical significance of VacA has been assessed from studies in animal models and from several observations in humans infected with *H. pylori*. Firstly, large quantities of purified VacA can induce ulcer-like erosions when administered into the mouse stomach [93]. Orally administered toxigenic *H. pylori* sonicates and purified VacA also induce epithelial vacuolation, loss of gastric gland architecture and infiltration of mononuclear cells into the lamina propria [94]. Among *vacA* type, s1m1 strains are the most cytotoxic, followed by s1m2 strains, whereas s2m2 strains have no cytotoxic activity and s2m1 strains are rare [67].

Several studies have investigated the association of *vacA* type and disease outcome. In Western populations such as the USA and Western Europe where *vacA* allelic diversity is common, s1 genotypes are more frequently associated with higher levels of inflammation in the gastric mucosa than s2 types [68, 95]. *vacA* s1m1 and s1m2 strains have been shown to be associated with peptic ulceration and s1m1 type strains have been associated with gastric carcinoma [68, 92, 95, 96]. The *vacA* i1 type is a risk factor for peptic ulcer disease and has been shown to be associated with duodenal ulcer disease as well as gastric cancer [92, 96]. Importantly Rhead *et al* found that although *vacA* s1, i1 and m1 alleles along with *cagA* positive status were all associated with gastric adenocarcinoma in an

Iranian population, the association was most pronounced with i1-type strains. Furthermore, only the i region status was independent of all other alleles studied. They concluded that the *vacA* i region is the best independent marker of toxicity and pathogenicity of a strain in Iranian [92].

In East Asia, however, most *H. pylori* strains have an s1-type, therefore the pathogenic difference cannot be explained by the type of s region present. [68]. Moreover, it is almost all *cagA* positive strains are classified as an *vacA* s1 strain, whereas *cagA*-negative strains are often combined with s2/m2 type [88]. With respect to the m region, however, there is variation within East Asia. Although m1 strains are common in parts of north East Asia, such as Japan and South Korea, m2 strains are predominant in parts of South-East Asia, such as Taiwan and Vietnam [67].

1.3.3. Duodenal ulcer promoting gene (*dupA*)

In 2005, the first disease specific *H. pylori* virulence factor that induced duodenal ulcer and had a suppressive action on gastric cancer was identified, and was named duodenal ulcer promoting gene A [97]. This gene is located within the plasticity zone of the *H. pylori* genome. Initial analysis of 500 *H. pylori* strains from Colombia, South Korea, and Japan showed an increased risk for duodenal ulcer and a decreased risk for gastric cancer in persons carrying *dupA*-positive strains [97]. In vitro, DupA increases IL-8 production [97]. However, a subsequent study focused on strains from Belgium, South Africa, and the United States found no significant relationships between *dupA* expression and duodenal ulcer but a significant association with gastric cancer [98]. Comparison of strains from Iran and Iraq indicates that *dupA* expression is significantly associated with duodenal ulceration in strains isolated from Iraq but not in Iranian isolates [99].

No association was found between *dupA* expression and gastric cancer or duodenal ulcer in strains from Japan [100] or Sweden [101], but correlations were observed between *dupA* and duodenal ulcer disease or gastric cancer in Indian and Chinese [102], [103], [101]. It seems likely that *dupA* may promote duodenal ulceration and prevent gastric cancer in some, but not all, populations.

1.3.4. Adhesins and outer membrane proteins (OMPs)

Adherence of *H. pylori* to the gastric epithelium facilitates initial colonization, persistence of infection, and delivery of virulence factors to host epithelial cells. Approximately 4% of the *H. pylori* genome is predicted to encode outer membrane proteins (OMPs), which is significantly more than that for other known bacterial species. OMPs expression has been associated with gastroduodenal diseases [61].

1.3.4.1. Blood group antigen binding adhesin (BabA)

BabA is encoded by the *babA*2 gene, which binds to fucosylated Lewis^b antigen (Le^b) on the surfaces of gastric epithelial cells and is the most well-described *H. pylori* OMP [104]. Transgenic mice that express Le^b on pit and surface mucous cells demonstrated that *H. pylori* attaches to the surfaces of Le^b expressing cells and induces more severe gastritis than in nontransgenic mice despite a comparable colonization density, suggesting that Le^b mediated colonization may increase the pathogenic potential of *H. pylori* [105]. Analyses of binding specificities of *H. pylori* strains from across the world suggest that the BabA adhesin has evolved in response to host mucosal glycosylation patterns to permit *H. pylori* to adapt to its host and to maintain persistent colonization [106]. The presence of BabA2 is associated with duodenal ulcer disease and gastric cancer, and when found in conjunction with *cagA* and *vacA* s1 alleles, it is

associated with an even greater risk of developing more severe disease [107]. Recently, there are the conflicting data on the usefulness of BabA2 expression in predicting clinical outcome in different countries, for examples, BabA2 expression is associated with the severity of gastric disease in strains isolated from Germany or northern Portugal [107], [108] but not a biomarker for peptic ulcer disease or gastric cancer in ThaiLan strains [109].

1.3.4.2. Sialic acid-binding adhesin (SabA)

SabA is an *H. pylori* adhesin that binds to the carbohydrate structure sialyl Lewis^x antigen expressed on gastric epithelium and is associated with an increased gastric cancer risk but a reduced risk for duodenal ulceration [110]. Sialyl-Lewis^x expression is induced during chronic gastric inflammation, suggesting that *H. pylori* modulates host cell glycosylation patterns to enhance attachment and colonization [111]. Furthermore, SabA is regulated by phase variation, such that SabA expression can rapidly be switched "on" or "off" to adapt to changes exerted by the gastric niche [110].

1.3.4.3. Outer inflammatory protein *(OipA)*

OipA is an inflammation related outer membrane protein which functions as an adhesin and is reported to be involved in the attachment of *H. pylori* to gastric epithelial cells [67]. OipA expression is linked to increased IL-8 production in vitro [112]. Recent a experiment demonstrated a role for OipA in induction of the mucosal cytokines IL-1, IL-17, and tumor necrosis factor alpha (TNF-α) and in gastric mucosal inflammation [113]. OipA is also involved in upregulation of matrix metalloproteinase 1 (MMP-1) and in β-catenin translocation and accumulation in the nucleus that can influence carcinogenesis [89]. *H. pylori* contains either a functional or nonfunctional *oipA* gene, and the presence of a

functional gene is significantly associated with the presence of duodenal ulcers, gastric cancer, and increased neutrophil infiltration [110].

Interestingly, the funtional *oipA* and *cagA* positivity are closely linked with each other and the *cagA* status is also linked to the *vacA* s region type and it is further closely linked to the presence of the *babA* gene. The links among these factors should have a certain biological significance and they may somehow interact with each other. It might be more relevant to hypothesize that these factors interact synergistically with each other and induce serious diseases, rather than to discuss which factor is the most virulent [67].

1.3.5. Induced by contact with epithelium A (IceA)

IceA was highlighted by the isolation of mRNA transcripts from strains associated with peptic ulcer disease [114]. Two polymorphisms were identified: IceA1 for which transcription is up regulated following contact with epithelial cells and the inactive IceA2 which is not. The biological significance of these genes is unclear; IceA1 has been associated with peptic ulcer disease, but this association is not universal [115].

1.3.6. Acid resistance and motility

Acid resistance

Urease, which converts urea into ammonia and carbon dioxide, is essential for the survival of *H. pylori* in the acidic gastric environment [5]. It allows *H. pylori* to maintain a constant periplasmic and internal pH which is required for transmembrane potential difference [5]. Urease is produced by all *H. pylori* strains although the level of urease activity differs significantly between *H. pylori* isolates [116]. Urease has been proposed to participate in tissue damage by

producing ammonia which is thought to be cytotoxic to epithelial cells [117].

Motility

Motility is essential for *H. pylori* colonization [118]. Flagellar motility is thought to be required for the initial stages of infection allowing the bacterium to move from the acidic environment of the gastric lumen into the less acidic mucus layer [119]. Mutants defective in the synthesis in either one of the two flagellins have impaired colonization efficiency and non-motile double mutants are completely non-virulent [120].

1.4. Diseases associated with *H. pylori* infection

Although gastric colonization with *H. pylori* induces histologic gastritis in all infected individuals and most of them has not any symptoms, only a minority develop related diseases of long-term carriage of *H. pylori*. It is estimated that infected patients have a 10 to 20% lifetime risk of developing ulcer disease and a 1 to 3% risk of developing distal gastric cancer [121], [122]. The risk of development of these disorders in the presence of *H. pylori* infection depends on a variety of bacterial, host, and environmental factors that mostly relate to the pattern and severity of gastritis [61].

1.4.1. Gastritis

The association between acute gastritis and *H. pylori* infection was first observed by Warren and Marshall when the developed acute gastritis several days after drinking a pure culture of *H. pylori* [123].

The natural history of *H. pylori* infection can be divided in two phases. The acute phase in which bacteria proliferate and cause gastric inflammation, hypochlorhydria develops and some gastrointestinal symptoms appear such as

fullness, nausea and vomiting. This phase often occurs during childhood and almost difficult to diagnose. After several weeks, the chronic phase begins in which the inflammatory response is reduced and the pH becomes normal, and the infected person becomes asymptomatic [124]. The colonization of *H. pylori* in gastric mucosa leads to infiltration of neutrophilic and mononuclear cells in both the antrum and the corpus that can result in chronic inflammatory [5]. When colonization becomes persistent, a close correlation exists between the level of acid secretion and the distribution of chronic gastritis. The most common phenotype is non-atrophic gastritis with normal acid secretion in asymptomatic subjects. [5]. Another phenotypic antral-predominant gastritis is associated with hyperchlorhydria and duodenal ulcer, whereas a corpus-predominant pangastritis leads to hypochlorhydria, gastric atrophy, intestinal metaplasia and an increased risk of distal gastric cancer [125], [126].

Gastritis is mostly diagnosed, classified and graded based on the histo-pathological criteria [9], [127]. However, endoscopic classification for gastritis is also used by endoscopists to describe the gastric mucosal changes and there are some relationship between endoscopic and histological characterizations [128], [129].

1.4.2. Peptic ulcer disease: duodenal ulcer and gastric ulcer

Peptic ulcers are defined as mucosal defects with a diameter of at least 0.5 cm penetrating through the muscularis mucosa. Gastric ulcers mostly occur along the lesser curvature of the stomach, in particular, at the transition from corpus to antrum mucosa [130]. Duodenal ulcers usually occur in the duodenal bulb, which is the area most exposed to gastric acid.

Both gastric and duodenal ulcer diseases are strongly related to *H. pylori* [2].

Approximately 10% to 20% of infected patients will develop peptic ulcers and ulcer relapse is prevented after cure of the infection [121], [131]. In initial reports from all over the world, approximately 95% of duodenal ulcers and 85% of gastric ulcers occurred in the presence of *H. pylori* infection, furthermore, the lifetime risk for ulcer disease in infected subjects is 3 to 4 times higher than in *H. pylori*-negative subjects [132]. Duodenal ulcer are more prevalent in younger individuals and gastritis in duodenal ulcer was primarily antral and spared the gastric corpus with high levels of acid secretion [133].

Although the same major causative factor (*H. pylori* infection), gastric ulcer is associated with a high risk, but duodenal ulcer with a low risk, of gastric cancer, and patients with gastric ulcers typically have atrophic gastritis and corpus-predominant gastritis leading to decreasing acid secretion, whereas patients with duodenal ulcers have antral-predominant gastritis, but few atrophic changes [134].

1.4.3. Gastric cancer

Gastric adenocarcinoma has been considered an infectious disease since 1994, when the International Agency for Research on Cancer categorized Helico-bacter pylori infection as a class I human carcinogen [135]. The pathogenesis of gastric adenocarcinoma represents a prototype for bacteria induced and inflammation driven malignancies [136].

Gastric cancer is a major cause of global morbidity and mortality. It is the fourth most common cancer worldwide, accounting for 9% of all new cancers, and ranking the second cause of cancer death [137]. Approximately two-thirds of the cases occur in developing countries, with a wide variation in incidence rates worldwide. The highest incidence rates are reported in Eastern Asia, Central and

Eastern Europe, and Central and South America [137].

Typically, the diagnosis of gastric cancer is delayed by a lack of early specific symptoms, and most patients are diagnosed after cancer has invaded the muscularis propria. The 5-year survival rate for gastric cancer is less than 15% even in the United States [138].

Histologically, two distinct variants of gastric carcinoma have been identified: diffuse-type gastric cancer and intestinal-type adenocarcinoma, which progresses through a series of well-defined histological steps and was first described in 1975 [139]. Intestinal-type adenocarcinoma is initiated by the transition from normal mucosa to chronic superficial gastritis; this is followed by atrophic gastritis and intestinal metaplasia, finally leading to dysplasia and adenocarcinoma [140], [141]. This process usually takes decades after persistent infection with *H. pylori*. Intestinal-type gastric cancer affects men twice times than women and commonly occurs in men above 50 years old [142]. Corpus-predominant gastritis predisposes individuals toward gastric cancer, which is thought to be due in part to decreased acid secretion. In contrast, antrum predominant gastritis results in increased acid production and predisposes individuals to duodenal ulcer disease, which is associated with a decreased risk of gastric cancer [126]. The loss of gastric acidity contributes to the promotion of the endogenous formation of N-nitroso compounds by the gastric bacterial flora with non-*H. pylori* species, may be the causative factors of gastric cancer [143]. Moreover, decreased acid in gastric juice results in low levels of ascorbic acid and a diminished ability to block the N-nitrosation process [134].

1.4.4. Gastric MALT lymphoma (Mucosa associated lymphoid tissue)

The gastric mucosa does not normally contain lymphoid tissue, but MALT

nearly always appears in response to colonization with *H. pylori*. In rare cases, a monoclonal population of B cells may arise from this tissue and slowly proliferate to form a MALT lymphoma [5]. MALT lymphomas occur in fewer than 1% of *H. pylori*-positive subjects [144]. Nearly all MALT lymphoma patients are *H. pylori* positive [145]. Low-grade MALT lymphoma accounts for approximately 50% of cases of gastrointestinal non-Hodgkin's lymphoma and in the early stage low-grade MALT lymphoma can be cured by H pylori eradication in 60 to 80% of cases [146], [147].

1.4.5. Extra-gastrointestinal manifestations

H. pylori infection has been associated with the development of a variety of extra-gastric disorders such as iron deficiency anaemia (IDA), idiopathic thrombocytopenia purpura (IPP).

Numerous studies have shown the association between *H. pylori* infection and IDA [148]. The improvement of anaemia after *H. pylori* eradication therapy has been reported [149]. Although the mechanism remains unclear, it is hypothesized that *H. pylori* uses iron in its metabolism which would result in increased iron losses in infected hosts [148]. The role of *H. pylori* in the pathogenesis of IPP was firstly described in 1998 [150]. A correlation between *H. pylori* eradication therapy and the improvement of IPP outcome has been reported [151]. The current concepts in the management of *H. pylori* infection recommend that *H. pylori* infection should be sought for and treated in patients with unexplained IDA and in those with IPP [46].

Interestingly, *H. pylori* has also been linked to other extragastric disorders. These include coronary heart disease, asthma, obesity, dermatological disorders such as rosacea and idiopathic urticaria, autoimmune thyroid disease, Raynaud's

phenomenon, scleroderma, migraine, and Guillain-Barré syndrome. The underlying hypothetical mechanisms include chronic low-grade activation of the coagulation cascade, accelerating atherosclerosis, and antigenic mimicry between *H. pylori* and host epitopes leading to autoimmune disorders [152], [153].

1.5. Diagnosis of *H. pylori* infection

Several methods are currently available to detect the presence of *H. pylori*, each with its own advantages, disadvantages, and limitations. The available tests are generally divided into invasive tests which require gastroendoscopy and taking gastric biopsy specimens for histology, culture, rapid urease activity and noninvasive tests which are based on peripheral samples, such as blood, stools, breath samples, for detection of antibodies, bacterial antigens, or urease activity. Moreover, these tests can be classified according to whether they are used before *H. pylori* treatment or to confirm successful eradication. The choice of a specific test for an individual patient depends on local experience and the clinical setting. In daily clinical practice, use of a single test is generally adequate. In hospital-based care, many patients undergo endoscopy for taking biopsy by invasive test for *H. pylori*. For routine diagnostic purposes, urea breath testing, rapid urease test and histology are most commonly used, whereas the use of serology is most appropriate for large epidemiological studies. The time for testing the success of *H. pylori* eradication after the end of treatment should be at least 4 weeks and PPI should be stopped for 2 weeks before testing by culture, histology, rapid urease test, UBT or stool test. [5], [154], [46].

1.5.1. Invasive diagnostic methods

1.5.1.1. Histology

Since the presence of spiral-shape bacteria was first demonstrated in gastric biopsy specimens by Warthin-Starry silver stain, histology was the original method for detection of *H. pylori* [2]. Presence of *H. pylori* can also be detected by other stains such as modified Giemsa, hematoxylin eosine, Genta, toluidine blue, Hp silver stain, and immuno-staining [155]. The advantage of histology is the possibility to assess the inflammatory process in the gastric mucosa such as the presence of acute or chronic inflammation, lymphoid aggregates, gastric atrophy, intestinal metaplasia, dysplasia and neoplasia. To detect *H. pylori* in biopsy samples, a routine hematoxylin and eosin stain is usually sufficient. Histology for detection of *H. pylori* can be a reliable method but depends on the number and localization of biopsy specimens which can lead to an underestimation of the presence of *H. pylori* if analysis of fewer biopsy samples than recommended due to the patchy distribution of *H. pylori* in the gastric mucosa. The sensitivity and specificity of histology for *H. pylori* diagnosis vary from 53% to 90%, depending on the pathologist's experience and density of colonization [154].

1.5.1.2. Rapid urease test (RUT)

The RUT is inexpensive, rapid, widely available, and highly specific diagnostic method. It can determine the presence or absence of urease activity that is only produced by *H. pylori* in large quantities in gastric biopsies. Therefore RUT can be considered as a proof of the *H. pylori* infection if the test is correctly performed. A biopsy sample is immersed in the medium containing urea and if urease is present, the urea is broken down into carbon dioxide and ammonia, which increases the pH of the medium and causes a subsequent color change in the pH indicator (Fig1.5), often phenol red turn from yellow to

red colour [154]. The sensitivity and specificity of RUT varies from 85% to 95% and 95% to 100%, respectively [155]. Sensitivity of RUT is affected by the number of bacteria in the sample, proton pump inhibitor drugs, bismuth containing compounds, post-treatment and in bleeding patients [154]. The test is recommended to use before eradication treatment in clinical setting [156].

1.5.1.3. Culture

Gastric biopsy is the ideal specimen for culturing *H. pylori* because rarely commensal bacterial flora is expected. Gastric juice samples or the string test can also be used for culture, however, the sensitivity is lower [154]. Culture is considered as the gold standard method for *H. pylori* detection with specific 100% although its sensitivity varies significantly among laboratories [155]. The colonies are Gram negative, urease, oxidase and catalase positive. The accuracy of culture depends on the condition in which the specimens are transported and processed [8]. Because of slow bacterial growth and specific medium condition requirement, it is a complicated and time-consuming procedure and it is not commonly used for the routine diagnosis of *H. pylori* infection. However, this method has the advantage to perform antibiotic sensitivity testing and characterize of *H. pylori* strains [155].

1.5.2. Non-invasive diagnostic methods

1.5.2.1. Urea breath test (UBT)

The UBT using essentially ^{13}C urea remains the best test to diagnose *H. pylori* infection, has a high accuracy, safety and is easy to perform [157]. Currently it is recommended to use not only for the initial diagnosis of *H. pylori* infection but also for the confirmation of eradication after treatment in

clinical setting [46].

The UBT indicates active *H. pylori* infection based on the ability of *H. pylori* to split urea into ammonia and CO_2. The patient ingests the labelled urea, using either ^{13}C or ^{14}C, which is then rapidly hydrolyzed by *H. pylori* urease in the stomach of infected individuals. The labelled CO_2, which diffuses into the epithelial blood vessels, is measured in the exhaled breath within a few minutes (Fig 1.5). ^{13}C is a nonradioactive innocuous isotope, and it can be safely used in children and women of childbearing age [155]. One of the most important advantages of UBT compared to biopsy based tests is its ability to evaluate the whole gastric mucosa, thus not being subject to sampling errors [154]. UBT has been demonstrated to have satisfactory performance with a sensitivity and specificity are more than 95% [158]. A disadvantage of ^{13}C UBT is that the technique requires expensive equipment and is therefore unavailable in many countries.

1.5.2.2. Stool antigen assay

The most recent noninvasive tests are the antigen in stool assays. *H. pylori* antigens are excreted in stool samples and can be detected by ELISA using either polyclonal or monoclonal antibodies. Both types of antigen in stool commercial kits have been evaluated for the primary diagnosis of *H. pylori* infection and for the monitoring of post-eradication therapy [159], [160]. One meta analysis evaluating the antigen in stool test showed a good performance with sensitivity and specificity of 94% and 97%, respectively [161]. Data from the study also showed that the monoclonal test is more accurate than the polyclonal test. Stool samples are easy to obtain from

children without their collaboration while UBT in young children who are not able to collaborate might give false negative results [161], [162]. However, the limit of stool antigen test for developing countries is the high cost of the kits.

1.5.2.3. Serology

Several types of tests have been used to identify antibodies against *H. pylori*. *H. pylori* infection is a chronic condition, therefore almost kits are based on IgG detection. The main immunological methods are enzyme immunoassay (EIA) and immuno-blot (IM) with sensitivity and specificity values ranging from 60% to 100% [154]. The advantages of serological tests are relatively low costs, availability, easy to use and it should be considered in patients with a recent use of antibiotics or PPIs, bleeding ulcers, or gastric atrophy [156]. They are widely used for epidemiology studies. The disadvantage of serology test is its inability to distinguish between active and previous *H. pylori* infection. False negative results may occur in a newly infected patient when the antibody level is insufficiently elevated and false positive results after eradication when the antibody level decreases very slowly. Therefore, this method is recommended to not use for confirmation of eradication after treatment and diagnosis of re-infection [46], [155].

Besides the two main methods mentioned above, which are common in clinical setting, molecular methods, primarily based on Polymerase Chain Reaction (PCR) technique, are mostly used in research. PCR has been used extensively for the diagnosis of *H. pylori* from gastric biopsy specimens, saliva, faeces and archival specimens, as well as for detecting clarithromycin and levofloxacin resistance [155], [8]. Moreover, PCR yields information on the presence of potential virulence markers in the strain, which might have

implications in the development of severe disease.

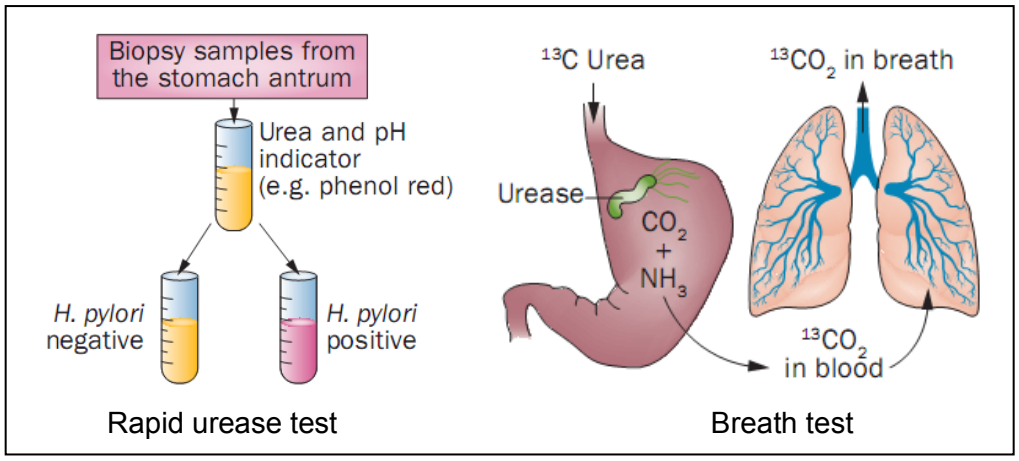


Figure 1.5. Common diagnostic *H. pylori* infection tests [16].

1.6. Treatment of *H. pylori* infection

H. pylori infections are commonly acquired during early childhood [163] and colonization persists lifelong unless antibiotic treatment is administered, as natural clearance of the infection is rare [164]. All consensus statements agree that whenever *H. pylori* is diagnosed it should be cured if possible because *H. pylori* eradication reduces gastric cancer risk. Treatment requires combination of at least two antibiotics to kill the bacteria and anti-acid medications to ensure they are effective in the stomach. For most other infections, culture is available and specfic antibiotics can be chosen. With *H. pylori*, this approach is generally unavailable and the doctor must use other factors to decide, such as knowledge of antibiotic usage in the population, information about the presence of resistance in the region, history of *H. pylori* eradication of patient provide a basis for considering some antibiotics and not

others [165], [16].

It is currently recommended to split first-line empiric therapy into two large groups: populations with low and with high resistance to clarithromycin. For these groups, the acceptable resistance levels are set as < 15% to 20%. The recommendations and therapies available for the eradication of *H. pylori* are mentioned as first-, second-, and third- line treatments, according to clarithromycin resistance [46].

1.6.1. First-line treatment

1.6.1.1. First-line treatment in areas with low clarithromycin resistance

The most frequently used strategy is PPI-based triple therapy which was proposed in 1993 in Italy by Bazzoli [166]. This therapy is composed of a PPI (omeprazole 20mg/12h or other equivalent PPIs), clarithromycin (500mg/12h) and amoxicillin (1g/12h), taken for 7 to 14 days. In cases of allergy to penicillin, metronidazole is an option to replace amoxicillin, as it is equally effective and considered equivalent.

1.6.1.2. First-line treatment in areas with high clarithromycin resistance

a. Bismuth-based quadruple therapy

In areas that have high resistance to clarithromycin, a quadruple therapy can be used. This therapy includes a combination of a PPI, bismuth subsalicylate (525mg, × 4 daily), and 2 antibiotics, metronidazole (250mg × 4 daily) and tetracycline (500mg × 4 daily), for 10 to 14 days. This regimen is safe and well tolerated, patients tend to adhere to the schedule [167]. However, this therapy is not available in all areas. It is recommended that doctors have other alternatives in mind, such as sequential therapy or quadruple therapy without bismuth.

b. Non bismuth-based quadruple therapy

Sequential therapy:

Sequential therapy was proposed by a group of Italian researchers. It involves the combination of a PPI and amoxicillin (1g × 2 daily) for 5 days, followed by a PPI and clarithromycin plus metronidazole or tinidazole (500 mg, × 2 daily) for 5 days. The rationale is to decrease the bacterial load during the first 5 days using amoxicillin, then to eradicate the remaining bacteria by the combination of clarithromycin and metronidazole [168]. Most studies have shown that sequential therapy and bismuth-based quadruple therapy have equivalent success in first-line therapy [169].

Concomitant therapy:

Concomitant therapy is used instead of sequential therapy in areas where the resistance to clarithromycin is greater than 20% and bismuth-based quadruple therapy is not available. Concomitant therapy involves the simultaneous administration of 3 antibiotics (metronidazole, clarithromycin, amoxicillin) and a PPI for 10 days. This therapy is effective, well tolerated and has side effects compared to sequential therapy [170].

1.6.2. Second-line treatment

1.6.2.1. Second-line treatment in areas with low clarithromycin resistance

Options available in areas with a low resistance to clarithromycin include bismuth-based quadruple therapy and therapies with a PPI and levofloxacin plus amoxicillin. However, levofloxacin use has been questioned, based on an increase in levofloxacin resistance [171]. Therefore, susceptibility studies should be performed before starting therapy.

1.6.2.2. Second-line treatment in areas with high clarithromycin resistance

For the case in which bismuth-based quadruple therapy fails, a triple therapy containing a PPI, levofloxacin, and amoxicillin is recommended. Again, the increase in levofloxacin resistance should be taken into account [46].

1.6.3. Third-line treatment

After 2 failed treatments in areas that have either a low or high clarithromycin resistance, it is not advisable to prescribe further antibiotic therapy and treatment should be guided by antimicrobial susceptibility testing whenever possible [46].

1.7. Antimicrobial susceptibility testing

H. pylori is intrinsically resistant to glycopeptides, cefsulodin, polymyxins, nalidixic acid, trimethoprim, sulfonamides, nystatin, amphotericin B, and cycloheximide. Some of these are used as selective agents in isolation media. Wild-type strains are susceptible to β-lactams (except cefsulodin), fosfomycin, macrolides, aminoglycosides, tetracyclines, chloramphenicol, rifampins, fluoroquinolones, 5-nitroimidazoles, and nitrofurans. Because of toxicity of chloramphenicol and lack diffusion of aminoglycosides, all remaining drug groups have been used in *H. pylori* eradication regimes. Bismuth salts also have an 'antiseptic-like' activity and PPI requires a high concentration, which is not achievable in vivo, to have anti *H. pylori* activity [8].

H. pylori, like a few other bacteria, acquires resistance by mutations which are vertically transmitted; rarely involves efflux systems and plasmids which could be horizontally transmitted [172]. A progressive increase in the

resistance rate is due to the selection pressure. The transformation may be possible if two strains are present simultaneously in the stomach [8].

1.7.1. Susceptibility Testing Methods

1.7.1.1. Phenotypic methods

a. Agar dilution method:

The agar dilution method, usually considered the reference method to compare other techniques, has been proposed by the Clinical Laboratory Standard Institute (CLSI) as the method to be used for *H. pylori* susceptibility testing [173].

For clarithromycin, the breakpoint proposed for susceptible strains is <0.25µg/ml, resistant strains is ≥1µg/ml, and that for intermediate strains is from 0.25 to 0.75µg/ml. Excellent predictive values for the success of the clarithromycin amoxicillin-PPI triple therapy were obtained with these breakpoints; For amoxicillin, MICs of ≥1µg/ml are for resistant strains. Strains with MICs of 0.25 to 0.5µg/ml correspond to an decreased susceptibility; The breakpoints commonly used for the other antibiotics are as follows: levofloxacin 1µg/ml, metronidazole 8µg/ml, tetracycline 2µg/ml, rifabutin 1µg/ml [174].

Metronidazole is a special case with a lack of laboratory reproducibility and lack of correlation between the susceptibility results and *H. pylori* eradication. It may be that the intracellular redox potential is not controlled, whereas this parameter is important for metronidazole reduction [8]. Additionally, a pre-incubation of the media in an anaerobic atmosphere has been shown to increase metronidazole activity [175] and strains with a high

MIC can be eradicated possibly due to a variable redox potential inside the stomach.

Recently, according to EUCAST (European Committee on Antimicrobial Susceptibility Testing) in 2013, the *H. pylori* breakpoint for amoxicillin is only 0.12 µg/ml and for clarithromycin is 0.5 µg/ml [176].

b. Broth dilution method:

The broth dilution method has the advantage of being adaptable to automation. However, it has rarely been used for *H. pylori* because of the difficulty of growing this bacterium in broth [177].

c. Breakpoint susceptibility testing:

Breakpoint susceptibility testing is a simplified modification of the agar dilution method. It consists of inoculating a streak of the strain to be tested on an agar plate containing an antibiotic concentration equal to the breakpoint concentration which defines resistance. This test is easy to perform, and theoretically excellent, but the media must be prepared in the laboratory [8].

d. Disk diffusion testing:

The disk diffusion method is the simplest and most economic for routine susceptibility testing. However, it is generally not recommended for slow growing bacteria [8]. Disk diffusion has been validated to only detect macrolide resistance and the breakpoint inhibition zone was 22mm for clarithromycin and 17mm for erythromycin (corresponding to an MIC of >0.5 µg/ml) [178]. This method has not been validated for the other antibiotics, but a good correlation is usually found with the other methods [179].

e. Epsilometer test (E-test):

The E-test method has the advantage of being an easy and reliable quantitative method with a direct expression of MICs and furthermore it is adapted to slow growing bacteria like *H. pylori*. A good correlation has been found between this method and the agar dilution method [180]. Nowadays, E-test has become commonly used in the clinical setting and in the surveillance of antibiotic resistance of *H. pylori* [181].

1.7.1.2. Genotypic detection of resistance

a. Clarithromycin:

Clarithromycin is a drug that belongs to the macrolide family. It acts by binding to 50S bacterial ribosomal subunit at the level of the peptidyl transferase loop of the *23S rRNA* gene and inhibits the protein synthesis. *H. pylori* resistance is the consequence of point mutations at two nucleotide positions 2142 (A2142G and A2142C) and 2143 (A2143G), these two mutations are responsible for more than 90% of clarithromycin resistance in developed countries [182], which lead to a conformational change and a decrease in macrolide binding [183]. Besides two main point mutations were mentioned above, several other point mutations have been identified such as A2115G, G2141A, T2117C, T2182C, T2289C, G224A, C2245T, C2611A with the low frequency and their roles have not been proven. Only T2182C and C2611A have been associated with low resistance levels [184].

Numerous methods have been applied for clarithromycin resistance, either on strains or directly on biopsy specimens, focus on mutations at positions 2142 (A2142G, A2142C) and 2143 (A2143G) of *23S rRNA* gene (Table 1.1).

Table 1.1. Genotypic methods used to detect macrolide resistance

Technique	Reference(s)
Using 23S rDNA amplification	
Sequencing	
Restriction fragment length polymorphism (RFLP)	Versalovic *et al.* (1996); Menard *et al.* (2002)
Oligonucleotide ligation assay (OLA)	Stone *et al.* (1997)
DNA enzyme immunoassay (DEIA)	Pina *et al.* (1998); Marais *et al.* (1999)
Real-time polymerase chain reaction (PCR)	Gibson *et al.* (1999); Chisholm *et al.* (2001); Matsumura *et al.* (2001); Lascols *et al.* (2003); Oleastro *et al.* (2003)
Line probe assay (InnoLipa)	Van Doorn *et al.* (1999)
Genotype HelicoDR	Cambau *et al.* (2009)
Preferential homoduplex formation assay (PHFA)	Maeda *et al.* (2000)
3′ mismatched PCR	Alarcon *et al.* (2000)
3′ mismatched reverse PCR	Elviss *et al.* (2004a)
Pyrosequencing	Hjalmarsson *et al.* (2004); Moder *et al.* (2007)
PCR-based denaturation HPLC	Posteraro *et al.* (2006)
Allele-specific PCR	Furuta *et al.* (2007); Nishizawa *et al.* (2007)
Quadruplex real-time PCR	Burucoa *et al.* (2008)
Oligonucleotide microarray	Chen *et al.* (2008)
Dual-priming oligonucleotide (DPO)-PCR	Woo *et al.* (2009)
Without using 23S rDNA amplification	
Fluorescence *in situ* hybridization (FISH)	Trebesius *et al.* (2000)
Electrocatalytic detection	Lapierre *et al.* (2003)
Microelectronic chip array	Xing *et al.* (2005)

b. Levofloxacin:

It inhibits the A subunit of the DNA gyrase, an essential enzyme for the maintenance of DNA helicoidal structure, encoded by the *gyrA* gene. Point mutations in Quinolones Resistance Determining Region (QRDR) of *gyrA* prevent binding between the antibiotic and the enzyme, conferring antibiotic bacterial resistance [184]. The amino acid positions concerned are mainly 87 (Asn87Lys or Tyr) and 91 (Asp91-Gly or Asn/Ala/Tyr) [185], [184]. Unlike other species, *H. pylori* does not have *parC* or *parE* genes that encode the topoisomerase IV [186]. Therefore, mutation of DNA gyrase, especially in *gyrA* gene, appears to play an important role, so far.

All mutations in the quinolone resistance determining region (QRDR) of the *gyrA* gene in *H. pylori* can be detected by sequencing. Moreover, two main mutations at acid amin position 87 and 91 can be also detected by performing a real time PCR based on the QRDR of *gyrA*. The FRET-MCA (melting curve analysis) principle has also been applied. However, the situation is complicated by the fact that there is a polymorphism, so that all of the mutations are not linked to resistance but generate peaks when the MCA is performed [174].

The allele-specific PCR methodology has also been applied to resistance detection. In this analysis, PCR amplification is performed using a specific primer in which the second nucleotide from the 3′ end is designed to match the site of the point mutation and the third nucleotide is designed to produce a mismatch in order to yield allele-specific PCR amplification. The point mutations can be identified by determining whether or not the PCR amplicons corresponding to the specific primers can be observed [187].

c. Amoxicillin and Metronidazole:

Amoxicillin acts by interfering with the synthesis of the peptidoglycan layer of the bacterial wall, especially by blocking transporters named penicillin binding proteins (PBP). Amoxicillin resistant *H. pylori* strains harbor mutations on the *pbp-1a* gene. The amino acid substitution Ser414Arg appears to be involved [188], leading to a loss of affinity between amoxicillin and PBP-transpeptidase. In addition to an instable amoxicillin resistance has been described in *H. pylori* isolates, the resistance being peculiarly lost upon freezing the culture at -80°C. Such an unusual condition has been defined as ‘amoxicillin tolerance’ rather than resistance leading to a blockage of

penicillin transport and the mechanism proposed was the lack of a fourth PBP, namely PBP-D [189].

Metronidazole inhibits nucleic acid synthesis by disrupting the DNA of microbial cells. This function only occurs when metronidazole is partially reduced. Reduction of metronidazole creates a concentration gradient that drives uptake of more drug, and promotes formation of intermediate compounds and free radicals that are toxic to the microbial DNA. This reduction usually happens only in anaerobic cells, it has relatively little effect upon human cells or aerobic bacteria [190]. An important gene in this respect is rdxA, which encodes oxygen insensitive nitro-reductase. Mutations in rdxA can induce protein ineffective [191]. Other proteins may also be involved in this reduction process, such as the flavin oxido-reductase (frxA), while their role is more controversial [192]. Moreover, a TolC efflux pump appears to play a role in resistance to this group of drugs [193].

Until now, no molecular test has been developed to detect the mutation in pbp1A in amoxicillin resistance and the link between mutations and metronidazole resistance is not strong enough to develop a molecular test.

1.7.2. Relevance of *H. pylori* resistance to antibiotics

Worldwide *H. pylori* antibiotic resistance towards different antibiotics has been increasing because of a widespread use of certain antibiotics in the general population, such as clarithromycin for respiratory infections; levofloxacin for urinary infections or metronidazole for intestinal parasites, periodontal, and gynecologic diseases which are common in developing countries. Primary *H. pylori* resistance towards antibiotics involved in the current eradication regimens affects the therapeutic outcome. Therefore only

recent data are of interest and they vary from place to place [194], [181].

The data of clarithromycin resistance in different countries in 2010 showed that there were fairly high clarithromycin resistance rate, even in some developed countries. In particular, the metronidazole resistance rate in some countries were very high, and this trend has been continuously increasing [194].

Levofloxacin are also not an exception, recently, resistant strains have been increasing, so that more and more levofloxacin-based treatments will likely be ineffective in the future. An example of this unfavorable trend is evident in Asian countries, where the rates of resistance exceed 20%: 20.6% in China [195], and are as high as 63.3% in Pakistan [196]. In Europe, the overall resistance to levofloxacin, detected in a recent multicentric epidemiologic study, is 14.1% [181], with values ranging between 11.7% in Ireland [197] and 29.1% (secondary resistance) in Germany [171]. In Africa, resistant strains vary from 10.2% to 15% [198], [199].

1.7.3. Impact of antibiotic resistance on *H. pylori* eradication

For the triple therapy with PPI plus clarithromycin and amoxicillin, there was a reduction in *H. pylori* eradication of 66% to 70% when the strain was resistant to clarithromycin, versus susceptible strains [182], [200]. For the triple therapy with PPI plus clarithromycin and metronidazole, the decrease in efficacy was 35% when the strain was resistant to clarithromycin versus susceptible strains, but the reduction in efficacy was 18% if the strain was resistant to metronidazole instead of clarithromycin. Moreover, there was 30% efficient reduction in PPI plus amoxicillin and metronidazole therapy when strains were resistant to metronidazole [200]. In the triple therapy with PPI

plus amoxicillin and levofloxacin or gatifloxacin, the eradication will decrease from 75% or 100% of those harboring susceptible strains to 33.3% of those with resistant strains [201], [202].

2. Research objectives - Aims

Vietnam is a developing country, located in Southeast Asia. The prevalence of *H. pylori* infection in this country is very high [18] and gastric cancer represents the second (in males) and the third (in females) most common cancer with aged standardized incidence rate over 24 per 100.000 population [67]. Additionally, there is a difference of gastric cancer rate between North and South Vietnam (Hanoi capital in the North is 1.5 times higher than Ho Chi Minh city in the South) [203]. Moreover, the antibiotic resistance of *H. pylori* has been rapidly increasing leading to failure of eradication treatment.

Until now, there has been little information about antibiotic resistance and virulent genotypes of *H. pylori* in Vietnam, especially in the Central region, therefore, a comprehensive study about these issues is mandatory.

This study aimed to define *cagA*, *vacA* genotype and the status of antibiotic resistance of *H. pylori* isolated from Gastroduodenal diseases patients in Central Vietnam with the following objectives:

1. To determine antibiotic resistance (phenotype & genotype) of *H. pylori* strains isolated from patients in Central Vietnam.
2. To determine *cagA* genotype (East Asian or Western *cagA* type) of *H. pylori* isolates.

3. To investigate *vacA* gene polymorphisms (signal, middle, intermediate regions).
4. To determine clinical relevance of *cagA* and *vacA* genes polymorphisms.

3. Materials and methods

3.1. Study design

This was a cross-sectional study conducted between July 2012 and July 2014, both at the Center of Gastrointestinal Endoscopy, Microbiology Department, Carlo Urbani Centre of Hue College of Medicine and Pharmacy in Vietnam and the Microbiology section of the Department of Biomedical Science of University of Sassari – Italy.

WORKFLOW OF STUDY

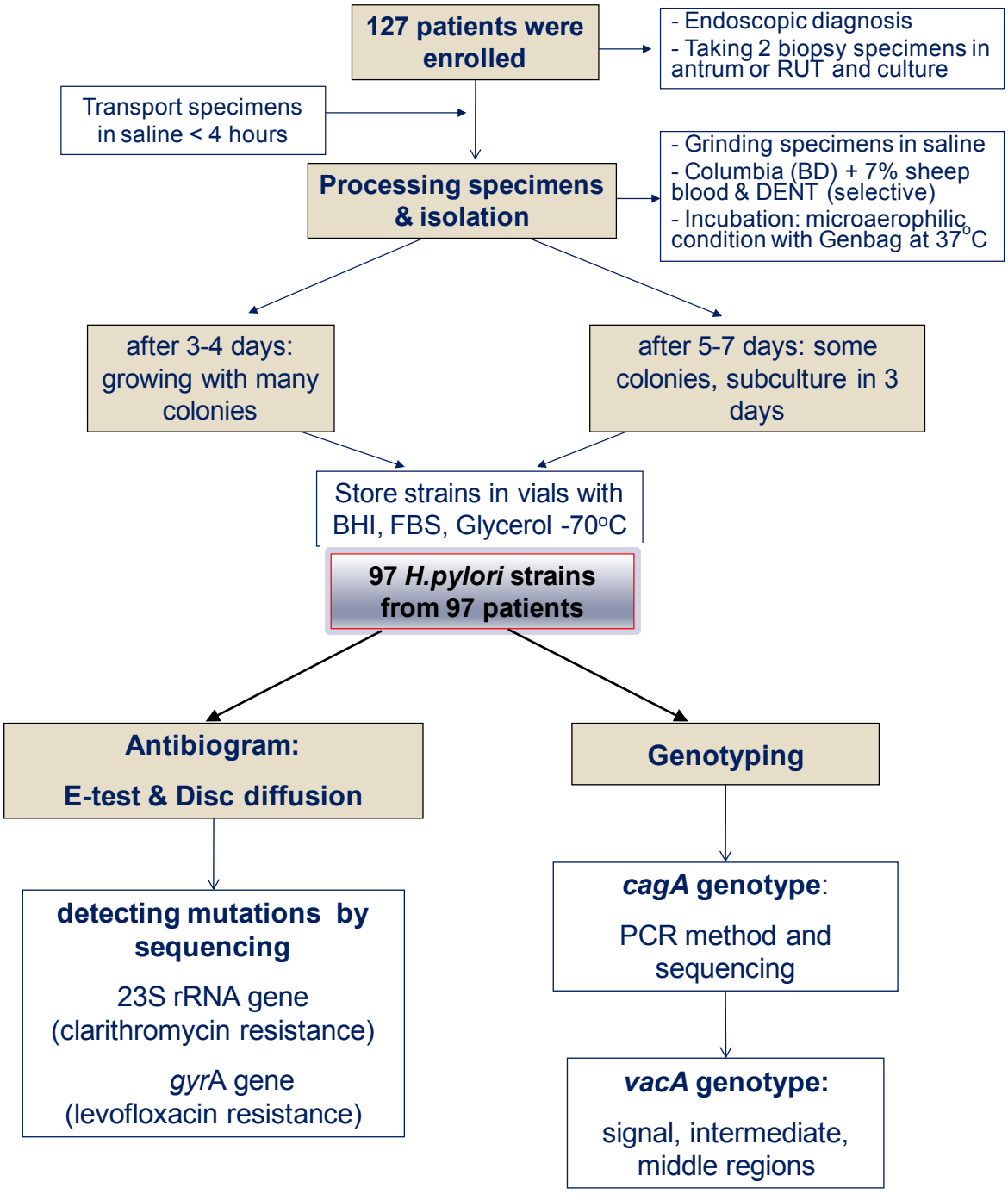


Figure 3.1. Workflow of the study

3.2. Study population

Totally, 127 dyspeptic patients (pts) from seven provinces in Central Vietnam (Fig 3.2) were enrolled in the study, including: Nghe An (1 patient), Ha Tinh (3 pts), Quang Binh (18 pts), Quang Tri (12 pts), Thua Thien Hue (88 pts), Quang Nam (2 pts), Quang Ngai (3 pts). The explanation about the study, endoscopic procedure and written informed consent were made from all the patients. Before endoscopy, participants were interviewed by medical doctor to ascertain their *H. pylori* eradication and medical history.

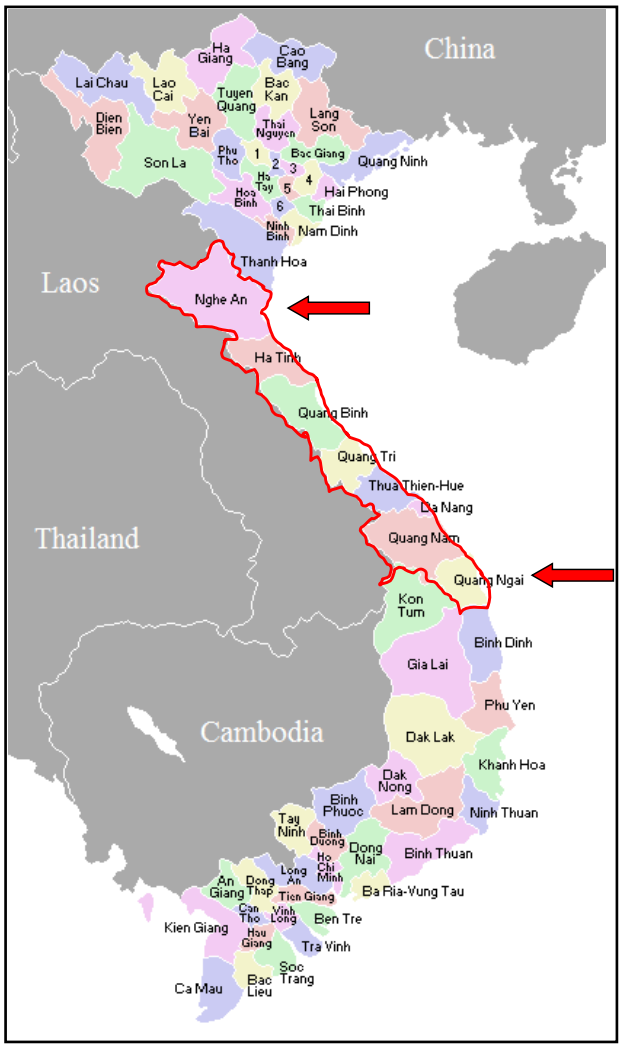


Figure 3.2. Study population in the Central provinces of Vietnam

Each patient underwent gastroendoscopy and two antral gastric biopsies were taken for rapid urease test and culture. Endoscopies were carried out at Hue University Hospital – Vietnam between July 2012 and January 2014. On the basis of endoscopic findings, patients were classified as having gastritis, duodenal ulcer, gastric ulcer, or suspicion of gastric cancer (Fig 3.3) which was confirmed with extra biopsies by pathologist. Exclusion criteria included subjects with the end of eradication therapy for *H. pylori* within 4 weeks, treatment H2-receptor blockers or PPIs within 2 weeks before the study and history of partial gastric resection. The study protocol was approved by the Ethical Committee of Hue University of Medicine and Pharmacy, Vietnam.

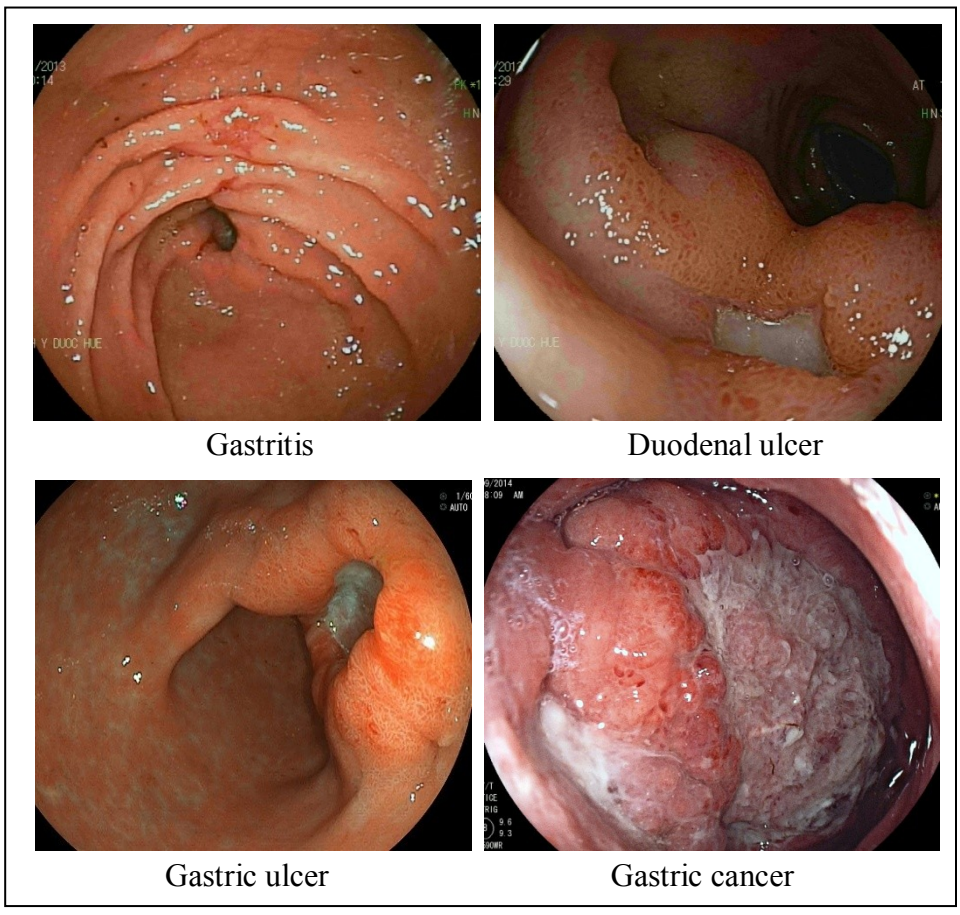


Figure 3.3.
Endoscopic diagnostic features of gastroduodenal diseases

3.3. Methods

3.3.1. Biopsy specimens for culture of *H. pylori*

Pinch biopsies were obtained from each subject during endoscopy. One biopsy was placed into a single tube for culture. Gastric biopsies were sent to clinical laboratory in saline solution. Biopsies should be prepared for culture as soon as possible after endoscopy (4 hours maximum). Using sterile forceps, the biopsy specimens were removed from the transport tubes and placed in sterile 1.5-ml microcentrifuge tube with 0.1 ml saline. The tissue was ground using the sterile disposable pellet pestle. Ten µl or more of the homogenate was inoculated onto a blood agar plate with the appropriate selective antibiotics and spread onto plates with inoculating loop or spreader [204].

3.3.2. Preparation of blood agar plates for growth of *H. pylori*

Successful culture of *H. pylori* requires the use of fresh nutrient blood agar plates. Commercially prepared plates may work, but the freshness of these plates cannot be controlled and they can often be too old or too dry. Columbia Agar Base (Becton Dickinson) was used for growth of this fastidious organisms.

- Weigh-out manufacturer's recommended amount of nutrient agar base for 500 ml medium and add to a bottle containing 500 ml distilled. Swirl until all the powder is dispersed and freely suspended.

- Loosely cover vessels and autoclave under standard conditions for liquid reagents (18 psi/120^{o}C) for 20 minutes.

- Remove containers from autoclave and place in a 56^{o}C water bath. This allows the medium to cool enough for addition of the blood and antibiotics

without denaturing their activity but still be warm enough to prevent the agar from solidifying.

- Add the appropriate amount of the selective antibiotic supplement (DENT-Oxoid) containing vancomycin (10 mg/L), cefsulodin (5 mg/L), trimethoprim (5 mg/L) and amphotericin B (5 mg/L).

- Add 35 ml defibrinated sheep blood, previously warmed to room temperature.

- Remove the flask from the water bath and gently mix until the blood is distributed uniformly.

- Immediately dispense the medium into 100mm petri dishes and remove bubbles. Stack the warm plates into groups of four or five and allow to cool at room temperature (only until agar has hardened).

- Following solidification of the agar, place plates in a plastic sleeve and store at 4^{o}C until needed [204].

3.3.3. Culture of *H. pylori*

- After grinding the biopsy specimen in 0.1ml saline, inoculate 10 µl or more of the homogenate onto a blood agar plate with the selective antibiotic supplement.

- Use a bacterial spreader or inoculating loop to spread the material back and forth across the entire plate from top to bottom. Turn the plate 90^{o} and repeat.

- Cover the plates and place them in an anaerobic jar or plastic bag (Biomérieux, Marcy l'Etoile, France). Keep the plates inverted during incubation and include a water-soaked paper towel in the anaerobic jar to provide a humidified atmosphere.

- Using a catalyst containing a microaerophilic system envelope GENbag or GENbox microaer (Biomérieux, Marcy l'Etoile, France). Seal the top of the anaerobic jar or plastic bag and place in a 37^{o}C incubator.

- After 3 to 4 days, examine the plates for growth. If no growth or only low growth is observed, plates can be incubate for another 2 to 3 days.

- Confirmation of *H. pylori* growth

+ Morphology: Colonies of *H. pylori* from primary culture are circular (1-2mm), convex, translucent in appearance (Fig 3.4). *H. pylori* is Gram-stained negative and has a spiral, comma or gull wing-like shape. In a healthy culture, the bacteria will appear to be "swimming". An overgrown culture may yield an optical density in the desired range, but observation of low or no mobility will indicate lack of viability.

+ Biochemical tests: positive to urease, catalase and oxidase

Urease test: Place a loopful of material into 0.3 ml Stuart's urease test broth. The broth should turn pink within minutes (Fig 3.4).

Catalase test: Place a loopful of material into 0.2 to 0.5 ml of 3% H_2O_2. Viable Helicobacter will rapidly form bubbles. This can be done in a 96 well microtiter plate if multiple samples are to be tested.

Oxidase test: Either place a loopful of material into a drop of distilled water on an oxidase disk or place a disk over a colony or area of growth on a plate with a drop of distilled water. A pink or maroon color within 10 to 20 min, eventually changing to almost black, indicates a positive test [204].

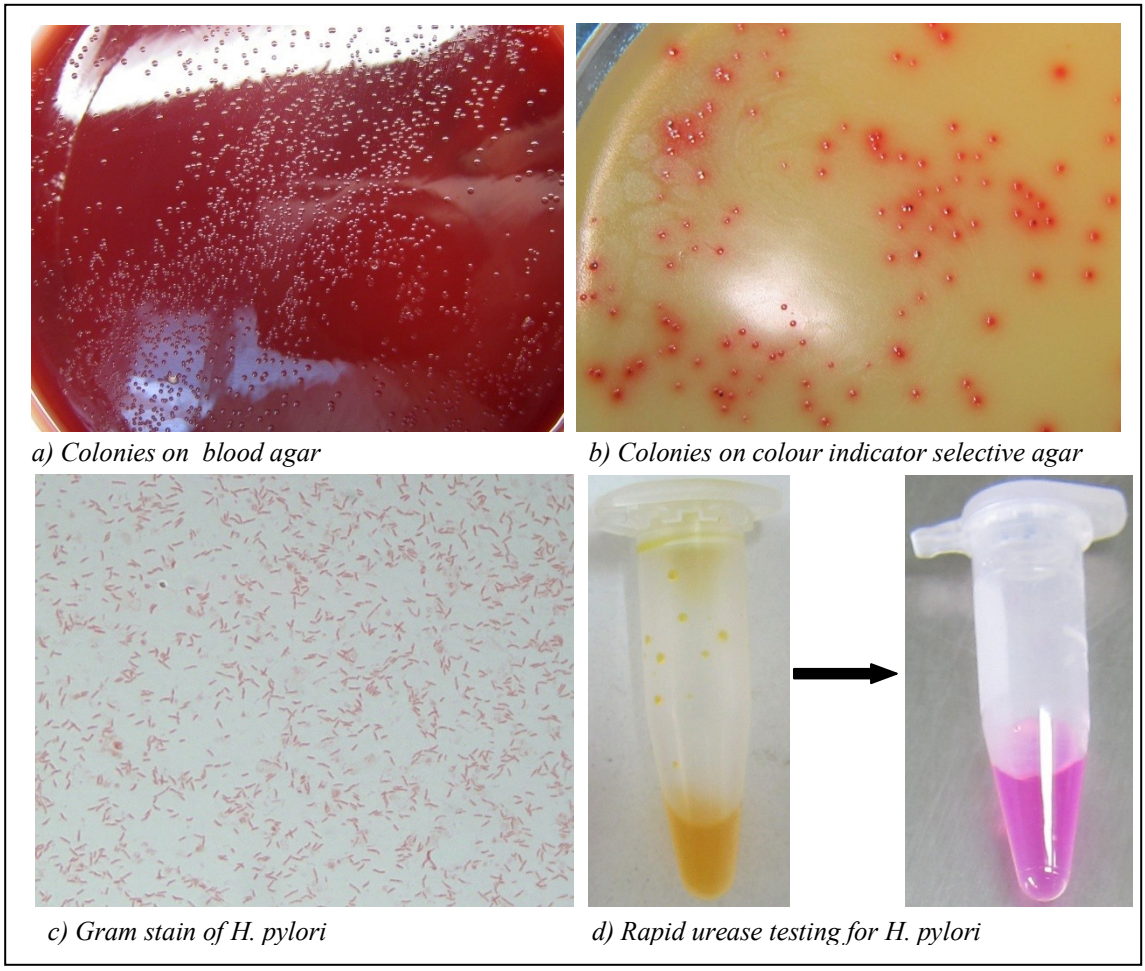


Figure 3.4. Morphology of *H. pylori colonies* and rapid urease testing

3.3.4. Strain storage

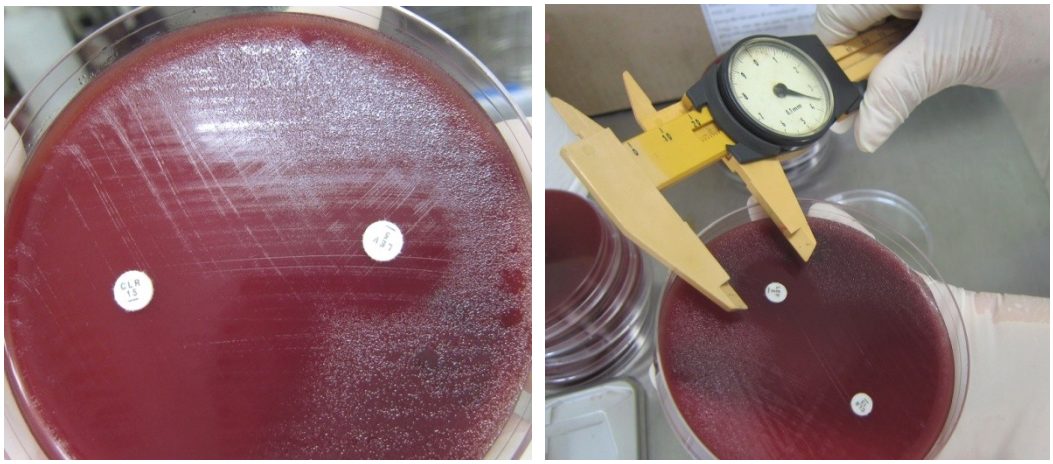
Froze each strain sample in two 2ml cryogenic vials (Nunc) with 1:1 ratio of bacteria suspension (3McF) and BHI broth supplemented with 10% heat-inactivated Fetal bovine serum (FBS) or resuspend few loopfuls of colonies in BHI broth supplemented with 10% heat-inactivated Fetal bovine serum (FBS), then add sterile glycerol to have 10-20% final concentration. Store at -80°C in boxes.

3.3.5. Antimicrobial susceptibility testing

All isolates were obtained at 3 days old of primary culture or 2-3 days old of subculture for setting up susceptibility testing. The culture suspension turbidity adjusted to be equivalent to a McFarland opacity standard of 3.0, taken from many colonies [178], [8].

3.3.5.1. Disk diffusion

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 and allowed to dry for 10-15 minutes. Clarithromycin (15µg) and levofloxacin (5µg) disks (diameter 6 mm; Oxoid) were placed on the plate and pressed gently to ensure complete contact with agar. A distance of at least 20mm was maintained from the edges of the plates to prevent overlapping of inhibition zones (Fig 3.5). Then the plate was incubated in anaerobic jar at 37°C in microaerophilic conditions (GENbag/GENbox microaer, Biomérieux, Marcy l'Etoile, France) for 72 hours. Inhibition zone diameters were measured in millimeters with the caliper.

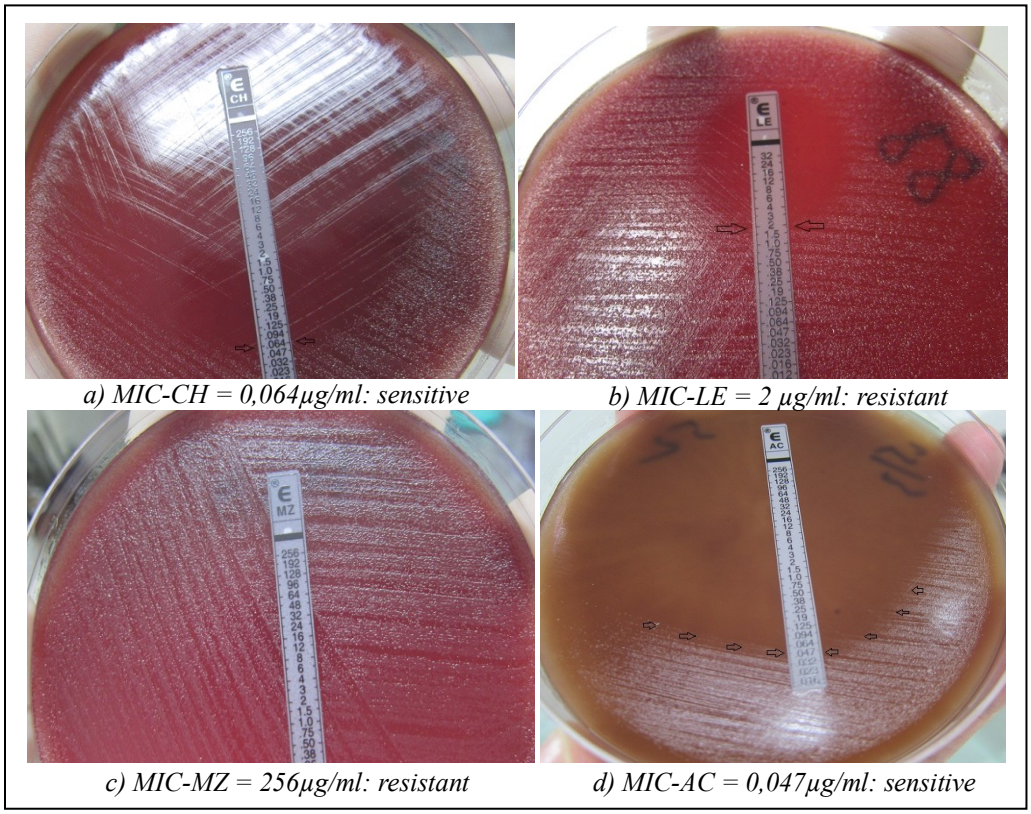


a) Disk diffusion of CH & LE *b) Inhibition diameter of LE =24mm: resistant*

Figure 3.5. Disk diffusion method.

3.3.5.2. Epsilometer test

Epsilometer test (E-test; Biomérieux, Marcy l'Etoile, France) was used to determine the minimum inhibitory concentrations (MICs) of AC, CH, LE, MZ. The bacterial suspensions were spread onto Mueller-Hinton II Agar plates (Becton Dickinson) supplemented with 7% defibrinated sheep blood by sterile cotton swabs. After waiting for drying in 10-15 minute, each E-test strip of the corresponding antibiotic was placed on the separative plate (Fig 3.6) and all plates were incubated in anaerobic jar for 3 days at 37°C under microaerophilic conditions (GENbox microaer, Biomérieux). MICs was defined as the point of intersection of the elliptical inhibition zone with the E-test strip (Figure 3.6). Strains were considered as resistant when the MIC was >1 µg/ml for AC, CH and LE; >8 µg/ml for MZ [174].



a) MIC-CH = 0,064µg/ml: sensitive *b) MIC-LE = 2 µg/ml: resistant*

c) MIC-MZ = 256µg/ml: resistant *d) MIC-AC = 0,047µg/ml: sensitive*

Figure 3.6. Epsilometer test

3.3.6. DNA extraction

Genomic DNA of *H. pylori* was extracted by boiling at 100^{o}C for 10 minutes, 120µl of bacterial suspension equal to 3 Mac Farland obtained resuspending many colonies in sterile distilled water. Samples were then centrifuged at 12,000 rpm for 3-5 minutes, and 100µl of DNA supernatant was transferred to a sterile tube and stored at -20^{0}C for further use.

3.3.7. Detection of point mutations of *23S rRNA* and *gyrA* genes.

3.3.7.1. PCR Amplification

To detect mutations related to clarithromycin resistance in the *23S rRNA* gene, PCR amplification of domain V region with forward primer 5'-GTAAACGG-CGGCCGTAACTA-3' and reverse primer 5'-GACCGAAC-TGTCTCACGACG-3' [205]. PCR conditions are 25µl final reaction volume containing 1x PCR Buffer (Invitrogen Life Technologies), 1.5 mM $MgCl_2$, 0.4 mM each dNTPs, 0.2 µM each primers, 0.5 U Platinum Taq (Invitrogen Life Technologies) and 1 µl DNA; Amplification protocol: 94°C 5 minutes, 35 cycles of 94°C 30 seconds, 52°C 30 seconds, 72°C 45 seconds and finally 1 extension cycle at 72°C for 10 minutes.

To detect mutations related to levofloxacin resistance in *gyrA* gene with amplified primers forward: 5'-TTTRGCTTATTCMATGAGCGT-3'and reverse: 5′-GCAGACGGCTTGGTARAATA-3'[185]. PCR conditions were 25µl final reaction volume containing 1x PCR Buffer (Invitrogen Life Technologies), 1.5 mM $MgCl_2$, 0.2 mM each dNTPs, 0.2 µM each primer, 1.25 U Platinum Taq (Invitrogen Life Technologies) and 1 µl DNA; Amplification protocol: 94°C 4 minutes, 30 cycles of 94°C 1 minutes, 56°C 1 minutes, 72°C 30

seconds and finally 72°C 5 minutes.

3.3.7.2. DNA purification

DNA Clean & Concentrator™-5 - Capped Columns catalog D4014 (Zymo Research) was used to purify DNA fragment of PCR product with the following steps:

- Step1: In a 1.5 ml microcentrifuge tube, add 5 volumes of *DNA Binding Buffer* to each volume of DNA sample. Mix briefly by vortexing.

- Step 2: Transfer mixture to a provided *Zymo-Spin™ Column* in a *Collection Tube*.

- Step 3: Centrifuge for 30 seconds. Discard the flow-through.

- Step 4: Add 200 µl DNA *Wash Buffer* to the column. Centrifuge for 30 seconds. Repeat the wash step.

- Step 5: Add ≥ 6 µl *DNA Elution Buffer* or water directly to the column matrix and incubate at room temperature for one minute. Transfer the column to a 1.5 ml microcentrifuge tube and centrifuge for 30 seconds to elute the DNA.

Ultra-pure DNA is now ready for use.

3.3.7.3. DNA quantification

Two µl purified DNA of each sample plus 1 µl buffer loading and 2 µl water were well mixed and loaded in the 1.2% agarose gel which was prepared in TAE 1x plus a counted volume of GelRed^{TM} Nucleotid Acid Stain 10,000X (Biotium); electrophoresis was performed under 100V direct current in 40 minutes, the bands were observed under the ultraviolet light and DNA

concentration was quantified by comparison with the bands of 2 µl Low DNA Mass Ladder (Invitrogen Life Technologies).

3.3.7.4. Nucleotide sequencing

- DNA Template: the amount of DNA template is dependent on template length, 10-40 ng for 200-500 bp product or 20-50 ng for 500-1000 bp product.

- Sequencing Primer: 3.2 pmol primer was chosen as starting point and worked in the majority of cases.

- Template/Primer Mix: The template/primer mix was provided in 10 mM Tris/Cl, pH 8.5 in a total volume of 7 µl and 200 µl PCR tubes (or 8-strips/plates) were used for preparation of Mix.

- Sanger dideoxy sequencing was performed on both strands by the BigDye Terminator Cycle Sequencing method (v1.1 or v3.1) at the Sequencing Service of LMU, Munich, Germany.

3.3.7.5. Sequence analysis

The resulting DNA sequences were analyzed by Uber Geneious 4.8.4 software (Biomatters LTD) and the obtained nucleotide sequences of *23S rRNA* and *gyrA* genes were compared with the wild type sequences present in GenBank accession no U27270 and L29481, respectively.

3.3.8. PCR for genotyping of *cagA* gene

All the primers used for genotyping *cagA* are listed in Table 3.1.

Firstly, *cagA* status was analysed by PCR using a pair of primers CAGT-F and CAGT-R. In case of negative *cagA*, another pair of primers Luni1 & R5280 was used to confirm cag-PAI empty.

Secondly, genotyping of the C terminus of *cagA* was performed by PCR. Briefly, amplification with primers *cagA*28F or cag2 and *cagA*-P1C or *cagA*-pA-1 identifies an EPIYA-A motif. Amplification with primers *cagA*28F or cag2 and a 1:1 mixture of primers *cagA*-P2TA and *cagA*-P2CG indicates an EPIYA-B motif. Amplification using primers *cagA*28F or cag2 and *cagA*-P3E identifies the presence of either an EPIYA-C motif or an EPIYA-D motif, and an additional amplicon with *cagA*28F or cag2 and the unique *cagA*-pD primer categorizes CagA as having an EPIYA-D motif (Fig 3.7).

In some cases where the PCR amplification was inconclusive and to confirm results of the PCR genotyping, *cagA* was amplified and sequenced with primers CAGT-F and CAGT-R.

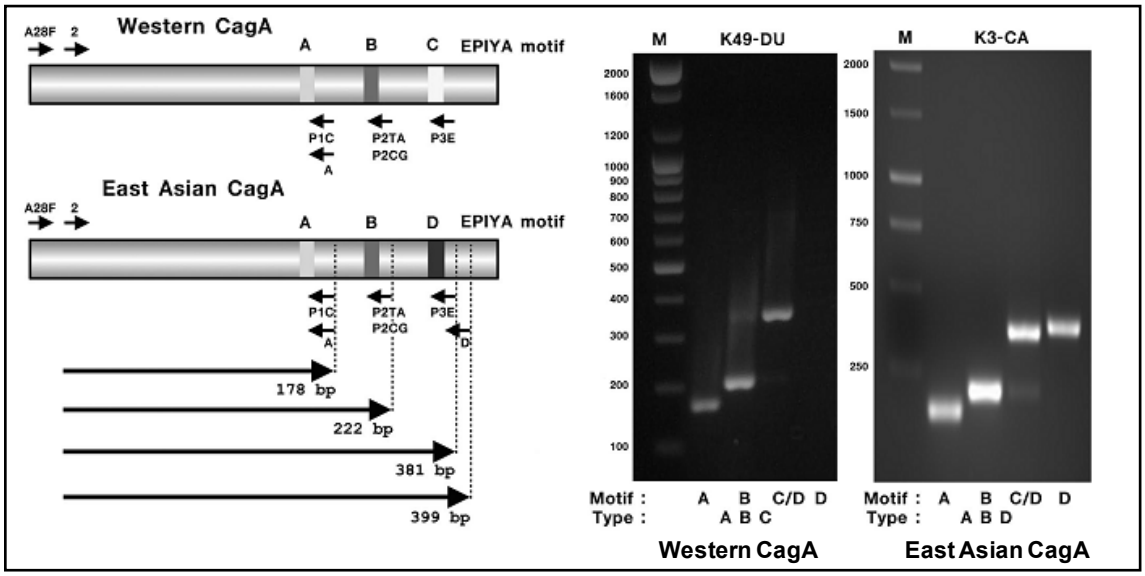


Figure 3.7. Genotyping of *cagA*-EPIYA motifs [206]

Table 3.1. Primers for genotyping *cagA*

primer	Sequence (5'-3')	Condition, protocol	Reference
CAGT-F	ACCCTAGTCGGTAATGGG	1	[207]
CAGT-R	GCTTTAGCTTCTGAYACYGC		
Luni1	ACATTTTGGCTAAATAAACGCTG	2	[208]
R5280	GGTTGCACGCATTTTCCCTTAATC		
cag2	GGAACCCTAGTCGGTAATG	3	[206]
*cagA*28F	TTCTCAAAGGAGCAATTGGC	3	[209]
cagA-P1C	GTCCTGCTTTCTTTTTATTAACTTK AGC	3	[209]
cagA-pA1	CTTGTCCTGYTTTCTTTTTATTAAC	3	[209]
cagA-P2TA	TTTAGCAACTTGAGTATAAATGGG	3	[209]
cagA-P2CG	TTTAGCAACTTGAGCGTAAATGGG	3	[209]
cagA-P3E	ATCAATTGTAGCGTAAATGGG	3	[209]
cagA-pD	TTGATTTGCCTCATCAAAATC	3	[206]

PCR condition and amplification protocols:

- Condition 1: 25µl final reaction volume containing 1x PCR Buffer (Invitrogen Life Technologies), 1.5 mM $MgCl_2$, 0.2 mM each dNTPs, 0.2 µM each primers, 2.5 U Platinum Taq (Invitrogen Life Technologies) and 1 µl DNA; Protocol: 95°C 5 minutes, 35 cycles of 95°C 1 minute 52°C 1 minute, 72°C 1 minute and finally 72°C 7 minutes.

- Condition 2: 25µl final reaction volume containing 1x PCR Buffer (Invitrogen Life Technologies), 1.5 mM $MgCl_2$, 0.25 mM each dNTPs, 1 µM each primers, 1 U Platinums Taq DNA polymerase (Invitrogen Life Technologies) and 1 µl DNA; Protocol: 94°C 3 minutes, 35 cycles of 94^{o}C 1 minute, 55^{o}C 1 minute and 72^{o}C 1 minute and finally 72°C 10 minutes.

- Condition 3: 25µl final reaction volume containing 1x PCR Buffer (Invitrogen Life Technologies), 1.5 mM $MgCl_2$, 0.2 mM each dNTPs, 420 nM primer *cagA*28F or cag2 and 80 nM primers *cagA*-P1C, *cagA*-P2CG, *cagA*-P2TA, *cagA*-P3E, *cagA*-pD, 1.5 U Platinum Taq (Invitrogen Life Technologies) and 1 µl DNA; Protocol: 95°C 5 minutes, 40 cycles of 95°C 30 seconds, 56°C 1 minute, 72°C 30 seconds and finally 72°C 7 minutes.

- All PCR products were loaded on a 1.2% - 1.5% agarose gel stained with GelRed™ Nucleotid Acid Stain 10,000X (Biotium), electrophoresis was performed under 100V direct current in 35-40 minutes, the bands were observed under the ultraviolet light.

3.3.9. PCR for genotyping of *vacA* gene

Four individual PCRs were performed to identify the *vacA* genotype of each strain (Fig 3.8). The s region was identified by amplification with primers VA1-F and VA1-R. The s1 region produced a 259-bp amplicon, whereas the s2 region produced a 286-bp amplicon. The m1 and m2 regions were determined by amplification with primers VAG-F and VAG-R, yielding 567-bp and 642-bp products, respectively. The i region was genotyped by using two independent PCRs with a universal forward primer (VacF1) and different i region type-specific reverse primers C1R and C2R specifically anneal with the i1 and i2 *vacA* alleles, respectively.

All the primers used for genotyping *vacA* are listed in Table 3.2.

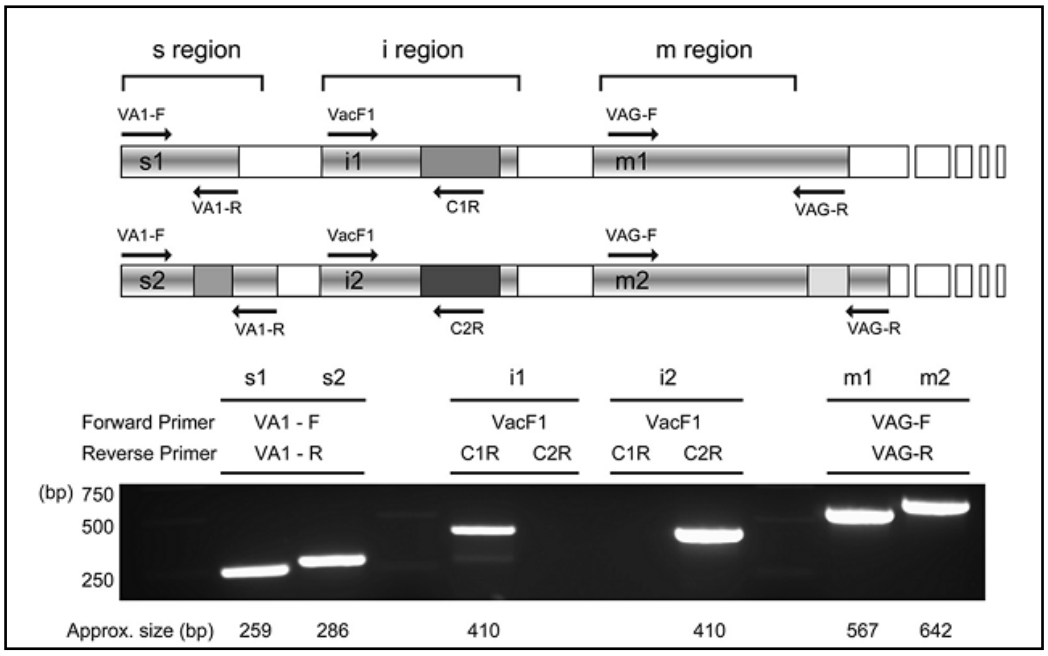


Figure 3.8. Genotyping of *vacA*-s/i/m regions [210]

Table 3.2. Primers for genotyping *vacA*

primer	Sequence (5'-3')	Condition, protocol	Reference
VA1-F	ATGGAAATACAACAAACACAC	4	[88]
VA1-R	CTGCTTGAATGCGCCAAAC		
VAG-F	CAATCTGTCCAATCAAGCGAG	4	[211]
VAG-R	GCGTCAAAATAATTCCAAGG		
VacF1	GTTGGGATTGGGGGAATGCCG	5	[92]
C1R	TTAATTTAACGCTGTTTGAAG	5	
C2R	GATCAACGCTCTGATTTGA	5	

PCR condition and amplification protocols:

- Condition 4: Multiplex PCR with 25µl final reaction volume containing 1x

PCR Buffer (Invitrogen life sciences), 1.5 mM $MgCl_2$, 0.2 mM each dNTPs, 0.3 µM each forward primers and 0.48 each reverse primers, 1 U Platinum Taq (Invitrogen Life Technologies) and 1 µl DNA; Protocol: 95°C 30 seconds, 40 cycles of 95°C 30 seconds, 56°C 1 minute, 72°C 90 seconds and finally 72°C 5 minutes.

- Condition 5: 25µl final reaction volume containing 1x PCR Buffer (Invitrogen Life Technologies), 1.5 mM $MgCl_2$, 0.2 mM each dNTPs, 0.5 µM each primers, 1.25 U Platinum Taq DNA polymerase (Invitrogen Life Technologies) and 1 µl DNA; Protocol: 94°C 5 minutes, 35 cycles of 94°C 30 sec, 52°C 1 minute and 72°C 30 seconds and finally 72°C 5 minutes.

- All PCR products were loaded on a 1.2 - 1.5% agarose gel stained with GelRed™ Nucleotid Acid Stain 10,000X (Biotium), electrophoresis was performed under 100V direct current in 35-40 minutes, the bands were observed under the ultraviolet light.

3.4. Statistical methods

All statistical analyses were performed by SPSS version 19 (SPSS Inc., Chicago, IL). The average age was compared using the unpaired t test or Mann-Whitney U test according to parametric or nonparametric distribution. The univariate association between each factor was quantified using Fisher exact test and $\chi 2$ test. A 2-tailed $p<0.05$ was considered as statistically significant.

4. Results

4.1. Study population

Totally, 127 patients were enrolled in this study. Ninety-seven *H. pylori* strains were isolated (76.4%) and 30 patients (23.6%) resulted culture-negative. The ratio of male and female patients were 1.1 (51:46), mean age: 44.1 ± 13.4, lowest age 18 and highest age 81 years old. Among them, 59 (61%) suffered gastritis, 27 (28%) duodenal ulcer, 9 (9%) gastric ulcer and 2 (2%) had gastric cancer endoscopically diagnosed (Figure 1). Seventy eight strains originated from 78 patients who had never been treated for *H. pylori* infection (primary strains), while the remaining 19 strains were isolated from patients in whom eradication of the infection failed after treatment (secondary strains).

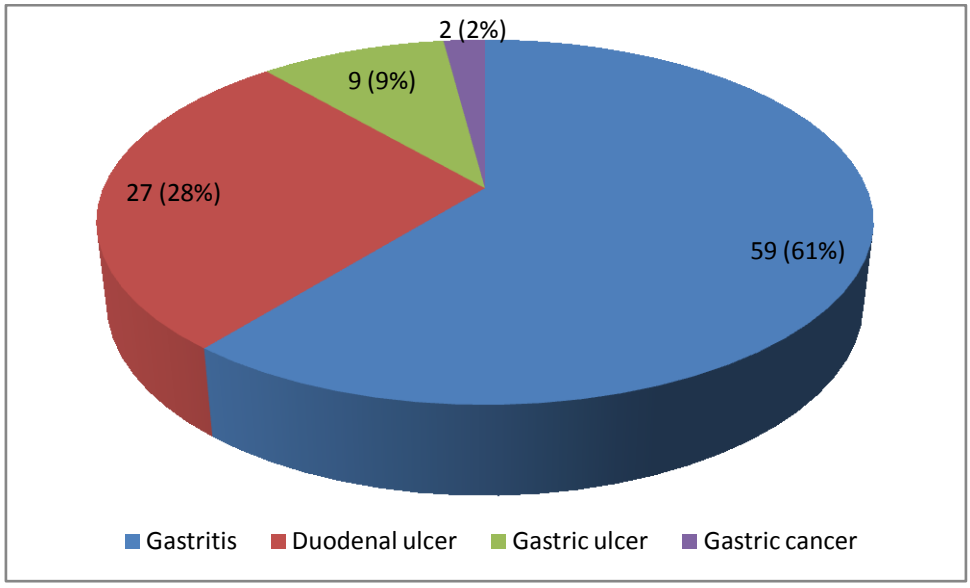


Figure 4.1. Distribution of gastroduodenal disease in 97 patients positive for *H. pylori* culture

4.2. Antimicrobial resistance

4.2.1. Phenotypic methods

4.2.1.1. Prevalence of Antibiotic resistance of *H. pylori* strains

The antibiotic resistance rate of *H. pylori* (regardless of previous eradication history) to MZ was the highest recorded with 71.1%, followed by CH with 42.4%, LE with 41.3% and AC resistance rate was the lowest as 1.1%. The resistance rate of CH and LE tends to be lower in the duodenal ulcer patients than in others, but not statistically significant ($p > 0.05$) (Table 4.1).

Table 4.1. Antibiotic resistance of 92 *H. pylori* strains with E-test in different gastric disorders

Antibiotics	Strains n (%)	GAS n (%)	DU n (%)	GU n (%)	GC n (%)
AC	1 (1.1%)	0	0	1 (11.1%)	0
CH	39 (42.4%)	25 (44.6%)	9 (34.6%)	5 (55.6%)	0
LE	38 (41.3%)	23 (41.1%)	9 (34.6%)	5 (55.6%)	1 (100%)
MZ	70 (76.1%)	38 (67.9%)	23 (88.5%)	8 (88.9%)	1 (100%)
Total	**92**	**56**	**26**	**9**	**1**

AC: amoxicillin, CH: clarithromycin, LE: levofloxacin, MZ: metronidazole, GAS: Gastritis, DU: Duodenal ulcer, GU: Gastric ulcer, GC: Gastric Cancer

4.2.1.2. Age and sex distribution of antibiotic resistance

The resistance to clarithromycin and levofloxacin increased according to the rise of age, especially in patients over 30 years old while resistance to metronidazole was at highest extent in 40 to 49 years old patient group (92%) (Fig 4.2).

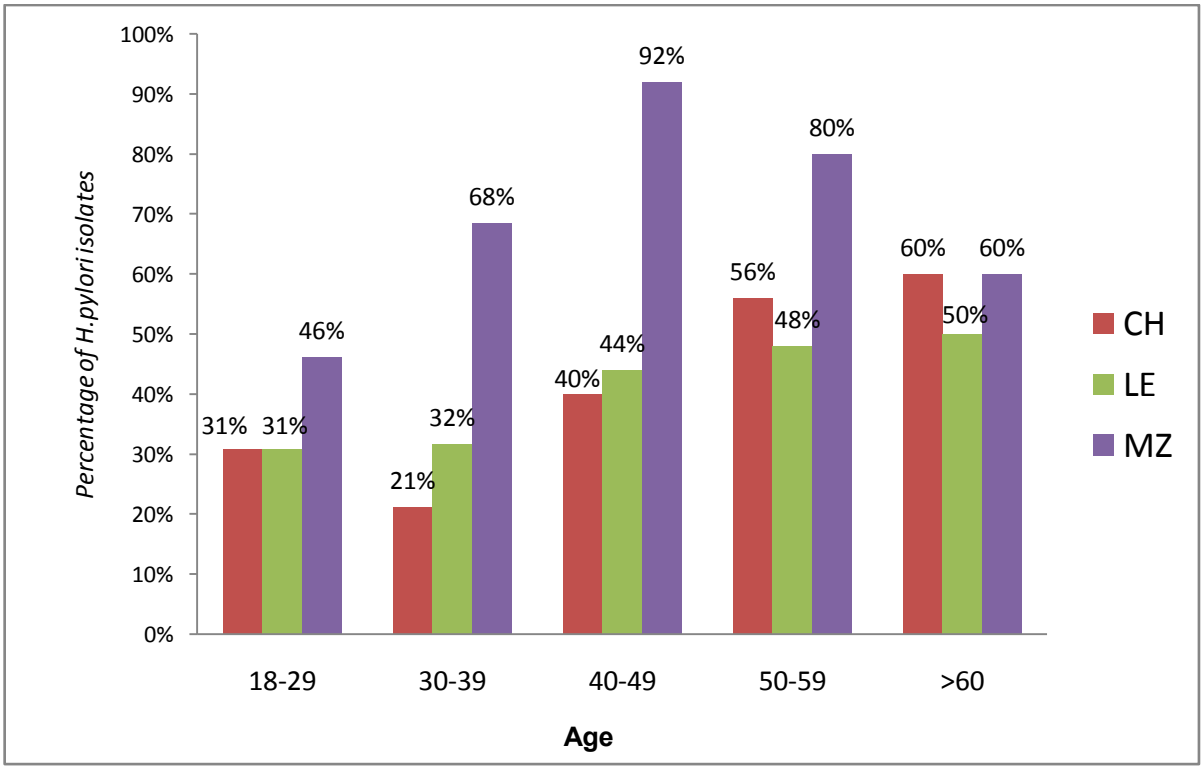


Figure 4.2. Age distribution of antibiotic resistance
(CH: clarithromycin, LE: levofloxacin, MZ: metronidazole)

The levofloxacin resistance rate in female was significantly higher than in the male with $p < 0.05$ (Fig 4.3).

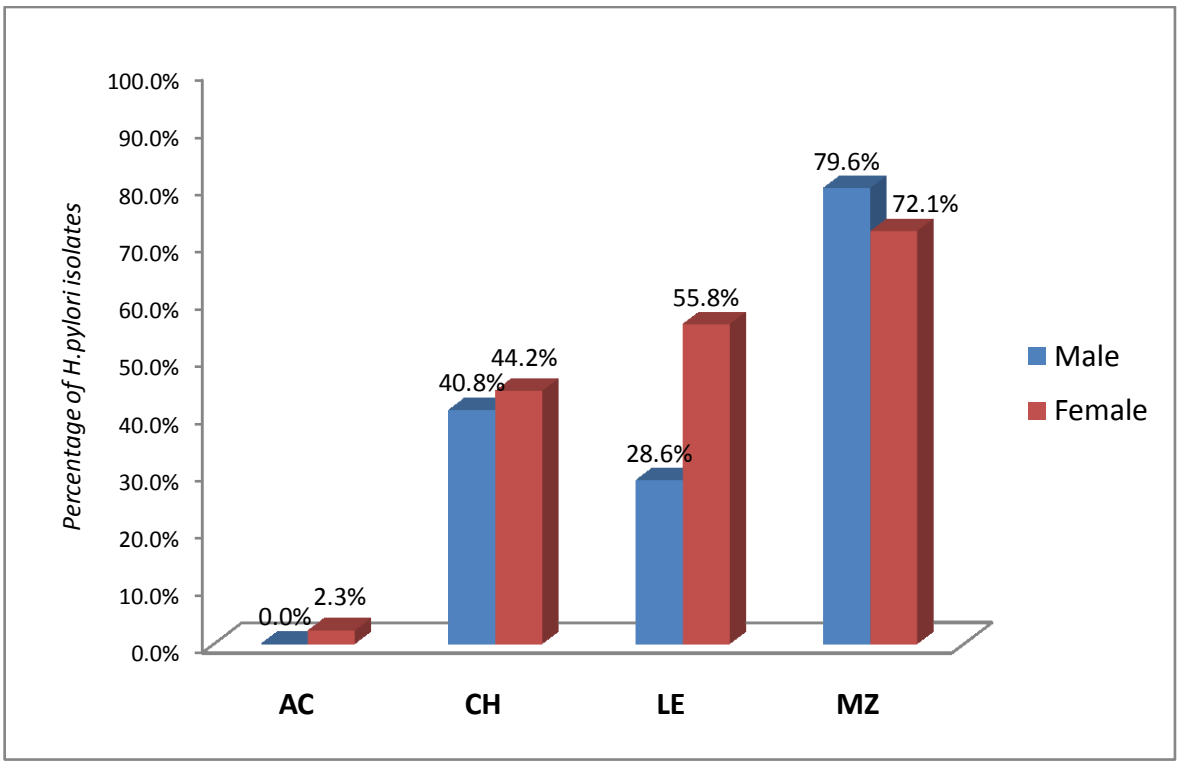


Figure 4.3. Sex distribution of antibiotic resistance
(AC: amoxicillin, CH: clarithromycin, LE: levofloxacin, MZ: metronidazole)

4.2.1.3. Distribution of MICs in 92 *H. pylori* strains

There was only one amoxicillin resistant strain with MIC = 1.5 µg/ml. According to the new guideline of EUCAST (European Committee on Antimicrobial Susceptibility Testing) in 2013, the MIC breakpoint for *H. pylori* resistance to amoxicillin is above 0.12 µg/ml. If applying this criterium for our results, the resistance rate to amoxicillin would be over 6% (6/92) (Fig 4.4a).

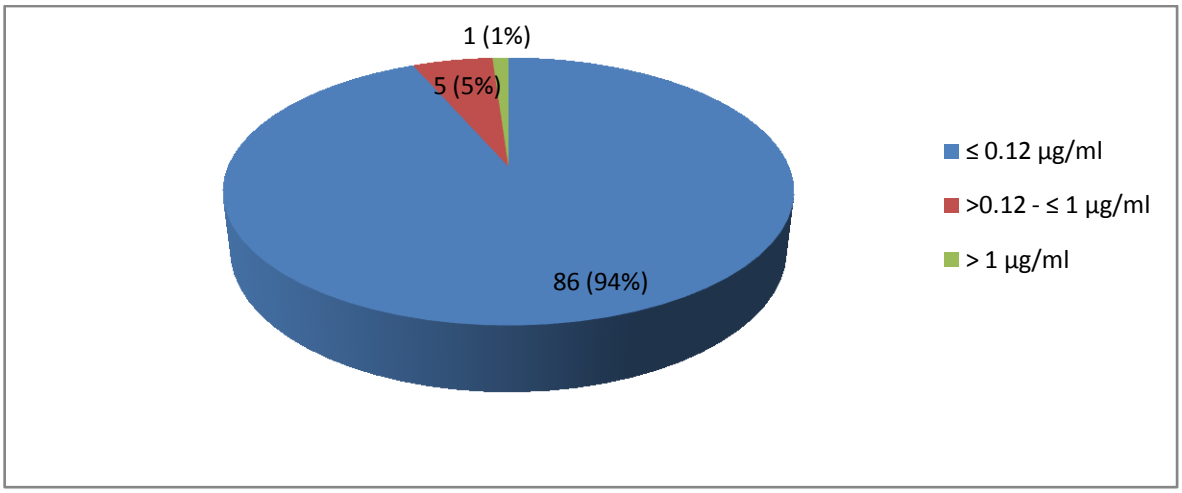


Figure 4.4a. MIC distribution of amoxicillin
(MIC > 1 µg/ml: resistant; EUCAST-2013 MIC > 0.12 µg/ml: resistant)

The high level clarithromycin resistance accounted for over 14% of all strains (14/92) or 35.9% (14/39) of resistant ones with the MIC from 96 µg/ml to 256 µg/ml. There were more than 3% (3/92) intermediate strains with MIC between 0.25 to 1 µg/ml (Fig 4.4b).

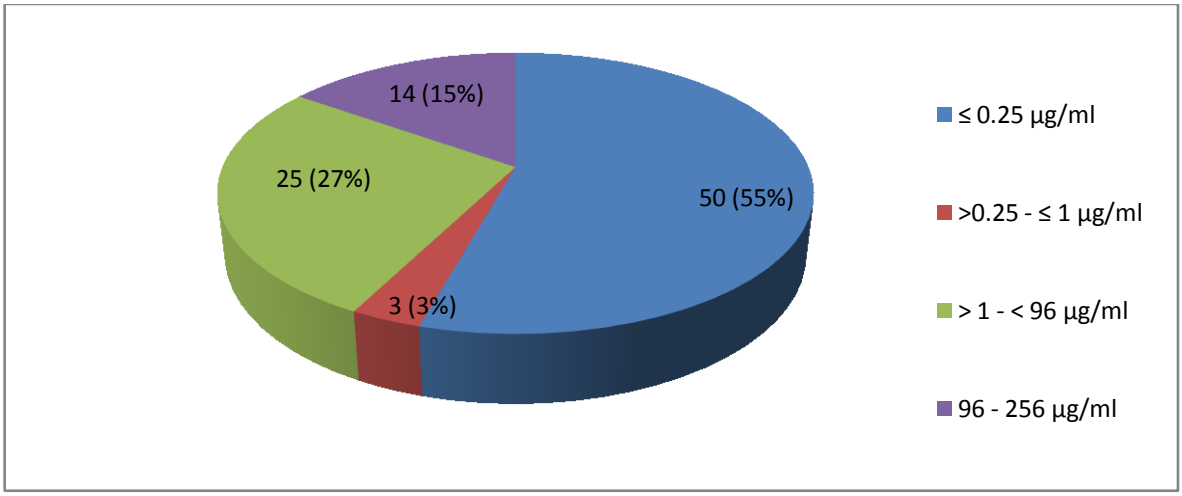


Figure 4.4b. MIC distribution of clarithromycin
(MIC > 0.25 - ≤ 1 µg/ml: intermediate, MIC > 1 µg/ml: resistant, MIC ≥ 96 – 256 µg/ml: high level resistance)

Among levofloxacin resistant strains, the high level resistance accounted for 42.1% (16/38) with the highest MIC in the E-test method being 32 µg/ml (Fig 4.4c).

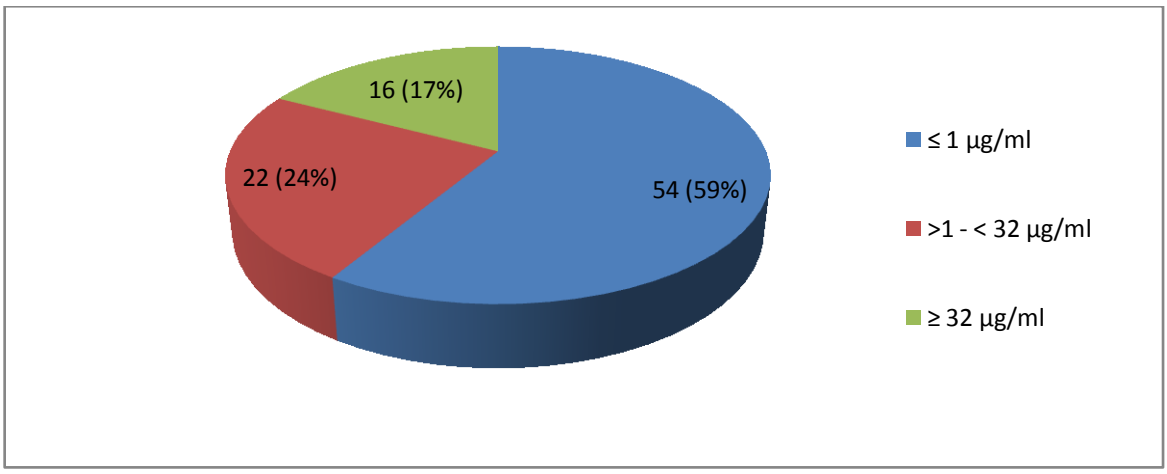


Figure 4.4c. MIC distribution of levofloxacin
(MIC > 1 µg/ml: resistant, MIC ≥ 32 µg/ml: high level resistance)

About 24% (22/92) of all strains and 39.3% (22/56) of resistant ones were high level resistant to metronidazole with the MIC equal to or above 256 µg/ml (Fig 4.4d).

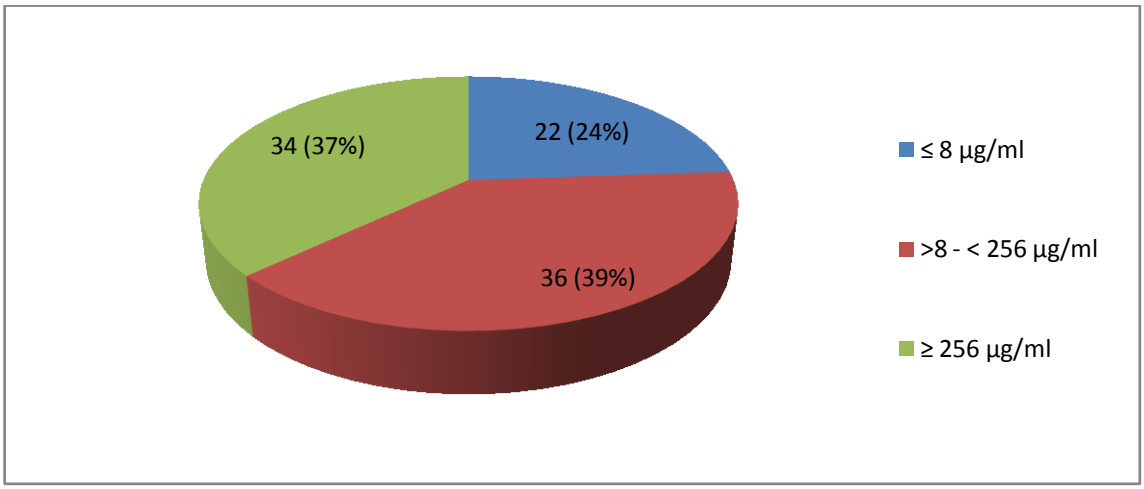


Figure 4.4d. MIC distribution of metronidazole
(MIC > 8 µg/ml: resistant, MIC ≥ 256 µg/ml: high level resistance)

4.2.1.4. Primary and secondary resistance of *H. pylori* strains

The ratio of strains with secondary resistance was significantly higher compared with those with primary resistance, exhibiting: 73.7% (14/19) vs 34.2% (25/73) for clarithromycin ($p < 0.05$) and 63.2% (12/19) vs 35.6% (26/73) for levofloxacin ($p < 0.05$) (Fig. 4.5)..

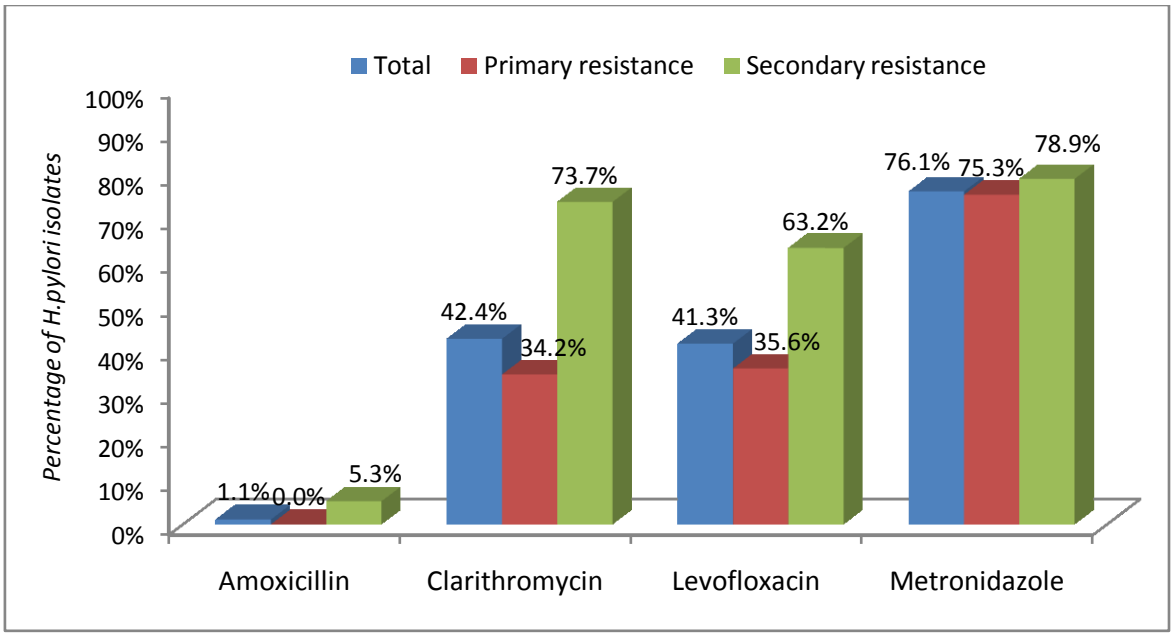


Figure 4.5. Comparison of primary and secondary resistance

4.2.1.5. Resistance patterns of *H. pylori* strains

Altogether, 13% (12/19) strains were susceptible to all antimicrobials tested, while double resistance accounted for a highest rate over 40% (37/92), in which coupled LE and MZ resistances were the most represented with 19.6%, followed by CH & MZ (15.2%) and CH & LE (5.4%) (Table 4.2).

All 38 levofloxacin and 34/39 clarithromycin resistant strains had other additional antibiotic resistance becoming double, triple or quadruple resistant

strains. Multidrug resistance accounted for 56.5% and the rate of MDR from secondary strains was significantly higher than that from primary ones with $p < 0.05$ (79% vs 50.7%).

Table 4.2. Resistance Pattern of *H. pylori*

Resistance	Total (n=92)	Primary strains (n=73)	Secondary strains (n=19)	p - value
No resistance	12 (13.0%)	12 (16.4%)	0	
Single resistance	28 (30.4%)	24 (32.9%)	4 (21%)	
Double resistance	37 (40.2%)	29 (39.7%)	8 (42.1%)	
Triple resistance	14 (15.2%)	8 (11%)	6 (31.6%)	< 0.05
Quadruple resistance	1 (1.1%)	0	1 (5.3%)	
Multidrug resistance[1]	52 (56.5%)	37 (50.7%)	15 (79%)	< 0.05
CH+LE	5 (5.4%)	3 (4.1%)	2 (10.5%)	
CH+ MZ	14 (15.2%)	11 (15.1%)	3 (15.8%)	
LE+MZ	18 (19.6%)	15 (20.5%)	3 (15.8%)	
CH+LE+MZ	14 (15.2%)	8 (11%)	6 (31.6%)	< 0.05
AC+CH+LE+MZ	1 (1.1%)	0	1 (5.3%)	

(AC: amoxicillin, CH: clarithromycin, LE: levofloxacin, MZ: metronidazole;
[1] Multidrug resistance ≥ two antibiotics resistance)

4.2.1.6. Compare two susceptibility testing: E-test and disk diffusion

The 56 strains were plotted according to the MIC values and inhibitory diameters of clarithromycin (Fig.4.6) and levofloxacin (Fig.4.7). All clarithromycin sensitive strains by E-test had the inhibition diameters ≥ 24mm and all clarithromycin resistant strains had the inhibition diameters ≤ 18mm (breakpoint for MIC: 1µg/ml) (Fig. 4.8). For levofloxacin, the breakpoints of inhibition diameters that the best separated for 56 strains into clearly distinguishable susceptible and resistant strains by E-test (breakpoint for MIC: 1µg/ml) were ≥ 30mm and ≤ 26mm, respectively.

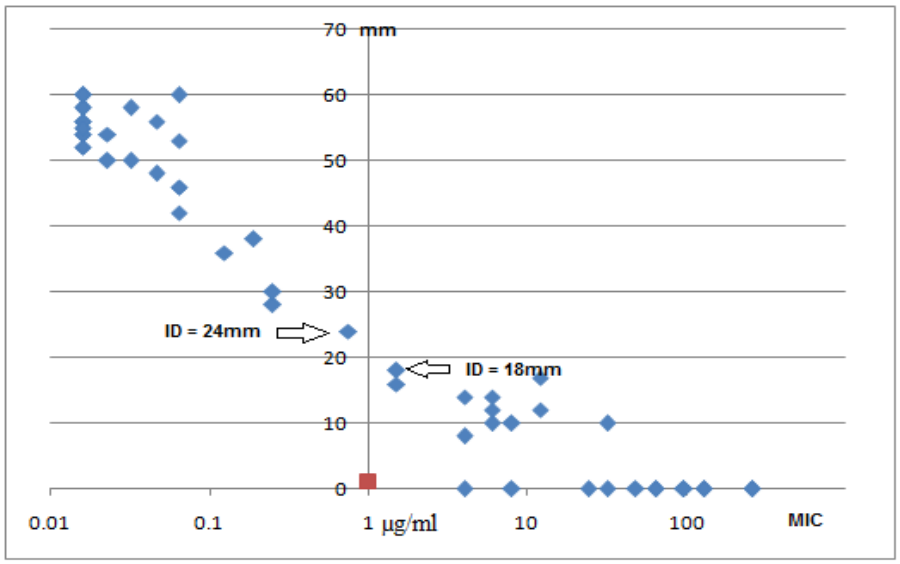


Figure 4.6. Correlation of inhibition zone diameter (ID) and MIC value of clarithromycin in 56 *H. pylori* isolates

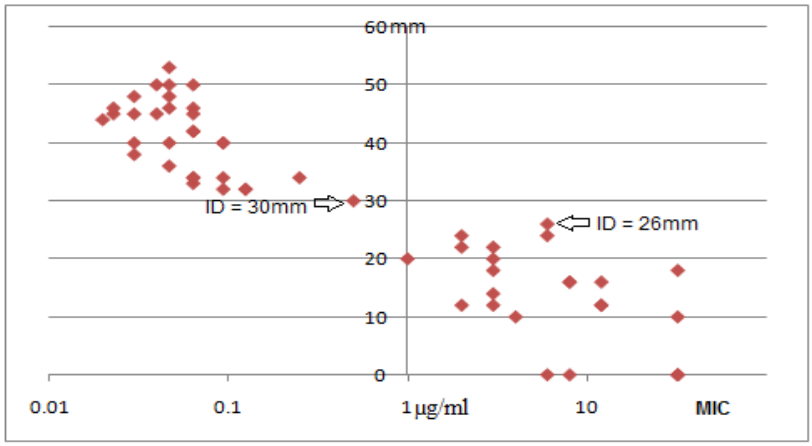


Figure 4.7. Correlation of inhibition zone diameter (ID) and MIC value of levofloxacin in 56 *H. pylori* isolates

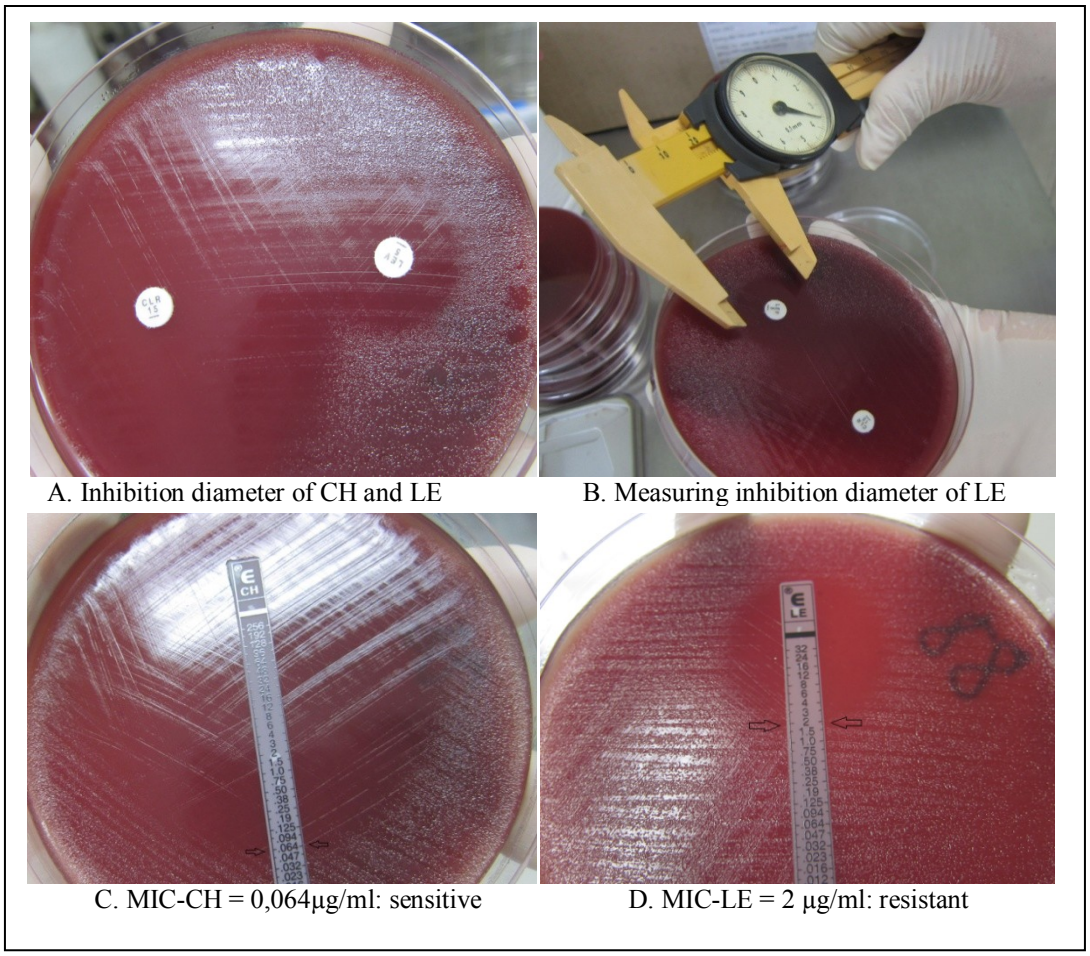


Figure 4.8. Disk diffusion (A-B) and E-test for the same strain (C-D).

4.2.2. Genotypic detection of resistance

4.2.2.1. Sequencing of 23S rRNA gene related clarithromycin resistance

After analysis of all the sequences, four isolates gave mixed results showing simultaneously no mutation at nucleotide position 2143 and A2143G mutation. Different combinations of mutations were found, represented by four or five mutations in 60% of isolates, followed by triple mutations (25.7%), double mutation (8.6%), whereas two strains had one mutation in their *23S* gene (Table 4.3). In addition, all 11 secondary isolates had quadruple mutations. 85.7% of CH-resistant strains showed A2143G mutation whilst none of the CH resistant strains tested showed the mutation at position A2142 (Fig. 4.9). All resistant and sensitive strains had T2244C mutation and 3 intermediate strains with MIC from 0.25 to 0.75 µg/ml had T2182C mutation. The MIC of strains with quadruple mutations were higher than that with triple and double mutation ($p < 0.05$).

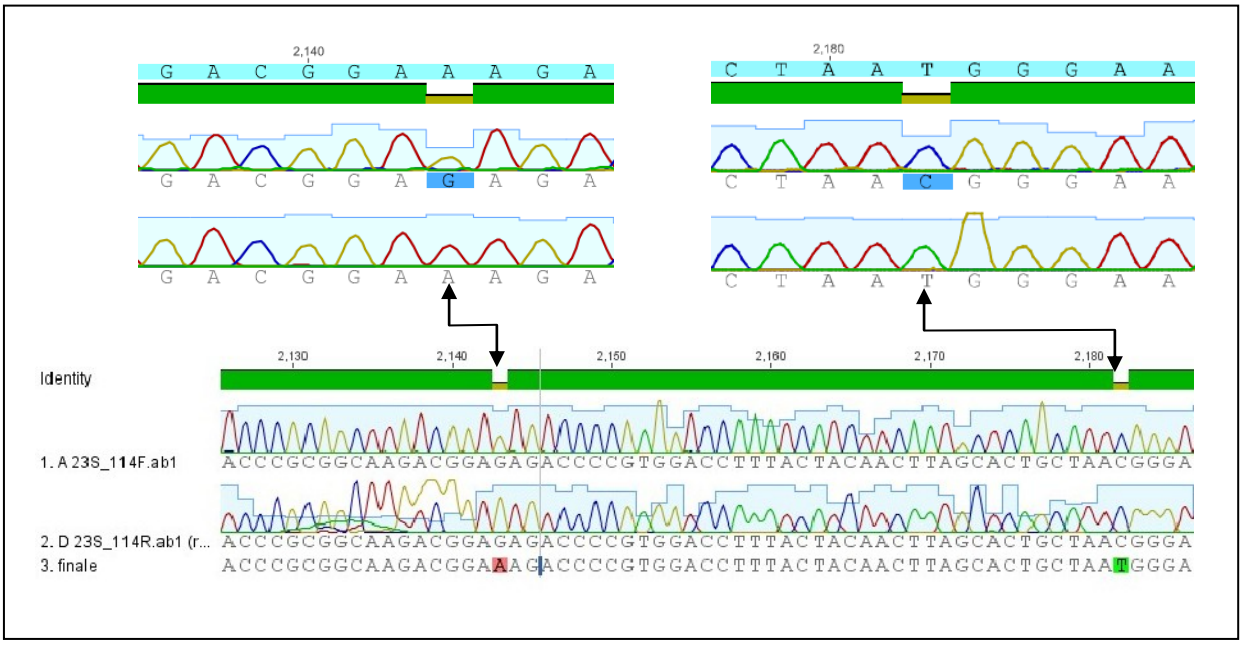


Figure 4.9. Point mutations in *23S rRNA* gene
(A2143G and T2182C nucleotide substitution mutation)

Table 4.3. Mutations of the *23S rRNA* gene in clarithromycin resistant isolates

Mutations in 23S rRNA gene	**No of isolates**	**%**	**MIC (µg/ml)** (min – max)
Resistant strains (n = 35)			
T2244C	2	5.7%	
A2143G + T2244C	1	8.6%	**23.7** (3 – 48)
T2182C + T2244C	2		
A2143G + T2182C + T2244C	8	25.7%	**29.2 ± 10.9** (1.5 – 96)
A2143G + A2223G + T2244C	1		
A2143G + T2182C + A2223G + T2244C	18	60.0%	**79.4 ± 23.0** (1.5 – 256)
A2143G + T2182C + A2302G + T2244C	1		
C2195T + T2182C + A2223G + T2244C	1		
A2143G + T2182C + A2223G + C1953T + T2244C	1		
Sensitive strains (n = 6)			
T2244C	2		**<0,125**
T2182C + T2244C	1		**<0,125**
T2182C + T2244C	3		**0,25 – 0,75**

4.2.2.2. Sequencing of gyrA gene related levofloxacin resistance

More than a half of LE-resistant strains analysed showed a mutation in *gyrA* gene at Aspartic-91(D91) and the 28% at Asparagine-87 (N87), along or in combination with other mutations. Among them, five isolates had mixed results at amino acid position N87 (3 isolates) or D91 (2 isolates) with simultaneously absence of mutation and presence of mutation N87K or D91N (Fig. 4.10). In particular, all 10 secondary isolates had mutations at amino acid D91 or N87. Strains with a mutation at N87 in *gyrA* gene had significantly higher MIC than those with mutation at D91 ($p < 0.05$). There was one high level LE resistant strain (MIC = 32µg/ml) which had new mutations with combination of N87A, A88N and V65I (Table 4.4).

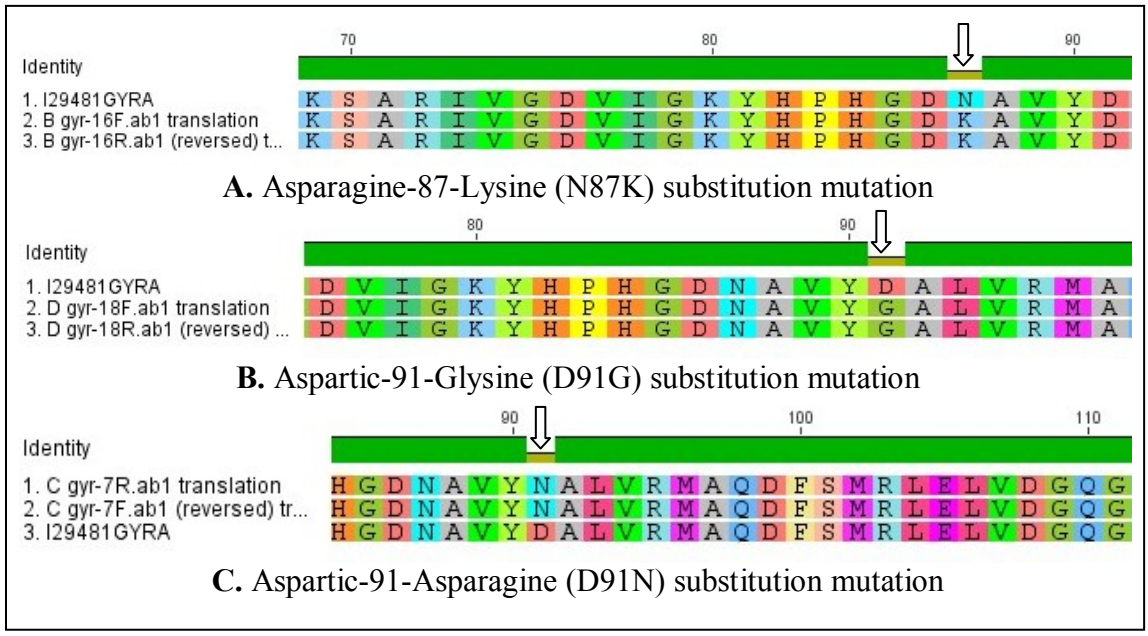


Figure 4.10. Amino acid N87 and D91 mutations in *gyrA* gene

Table 4.4. Mutations of the *gyrA* gene in levofloxacin resistant isolates

Mutations in gyrA gene	**No of isolates (%)**	**MIC (µg/ml)** (min – max)
No mutation	**5 (14.3%)**	
Mutations at Aspartic-91 (D91)	**18 (51.4%)**	**14.3 ± 3.1** (1.5 – 32)
D91N	6	
D91Y	1	
D91G	3	
D91N + L45F	1	
D91N + A55S	1	
D91N + A97V	1	
D91N + R130K	1	
D91Y + L45F	1	
D91G + L45F	2	
D91G + A55S + G60S	1	
Mutations at Asparagine-87 (N87)	**10 (28.6%)**	**25.2 ± 3.5** (8 – 32)
N87K	6	
N87K + L45F	1	
N87K + R130K	1	
N87K + L45F + R130K	1	
N87A + A88N + V65I	1	
Other mutations	**2 (5.7%)**	
A55S	1	
R130K	1	
Total:	**35**	

(**A**: Alanine, **D**: Aspartic acid, **F**: Phenylalanine, **G**: Glysine, **I**: Isoleucine, **K**: Lysine, **L**: Leucine, **N**: Asparagine, **R**: Arginine, **S**: Serine, **V**: Valine, **Y**: Tyrosine)

4.3. Genotyping and clinical relevance of virulent factors

4.3.1. *cagA* gene

4.3.1.1. *cagA* status and *cagA* type

cagA status and *cagA* type were analyzed by conventional PCR methods with different pairs of primers. The *cagA* gene was detected in 83/96 (85.4%) *H. pylori* strains. In 14 *cagA* negative strains, 42.9% (6/14) were positive for cagPAI-empty site indicating completely absence of the cagPAI and 57.1% (8/14) were negative for that by PCR suggesting the presence of a deviating cag-PAI (Fig.4.12). The *cagA* genes from 83 strains were further characterized for EPIYA motif in order to determine *cagA* type. Among 8 (8.3%) Western-*cagA* strains, 7 had an EPIYA motif–ABC and one showed an EPIYA motif–ABCC, whereas all 74 (77.1%) East Asian-*cagA* strains had an EPIYA motif–ABD (Fig 4.11, 4.13).

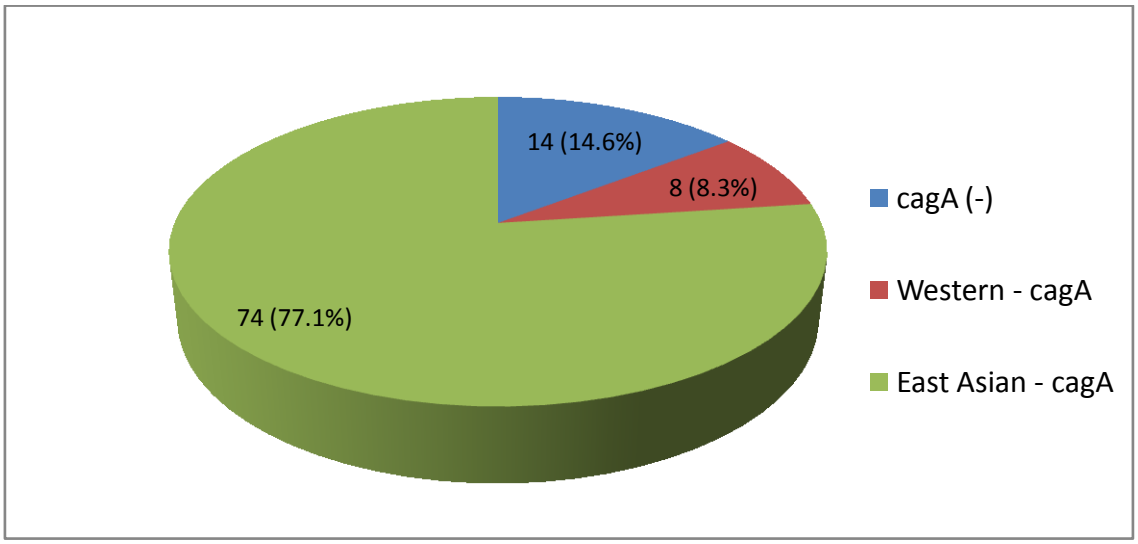


Figure 4.11. Distribution of *cagA* status and *cagA* type in 96 strains

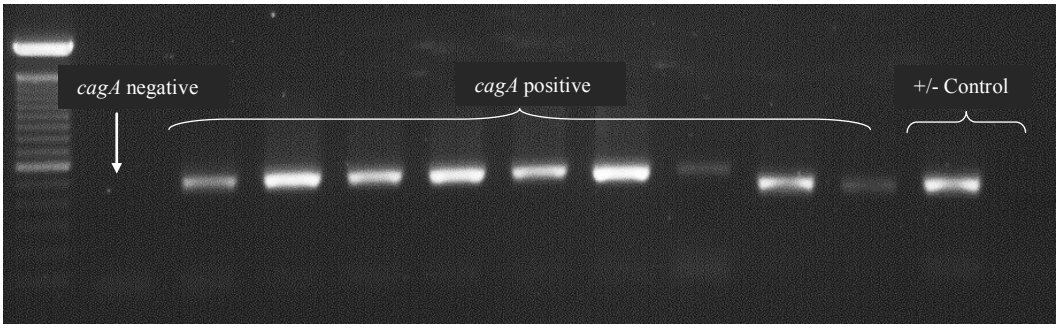


Figure 4.12. *cagA* status by PCR *(M: 100 bp ladder DNA Marker)*

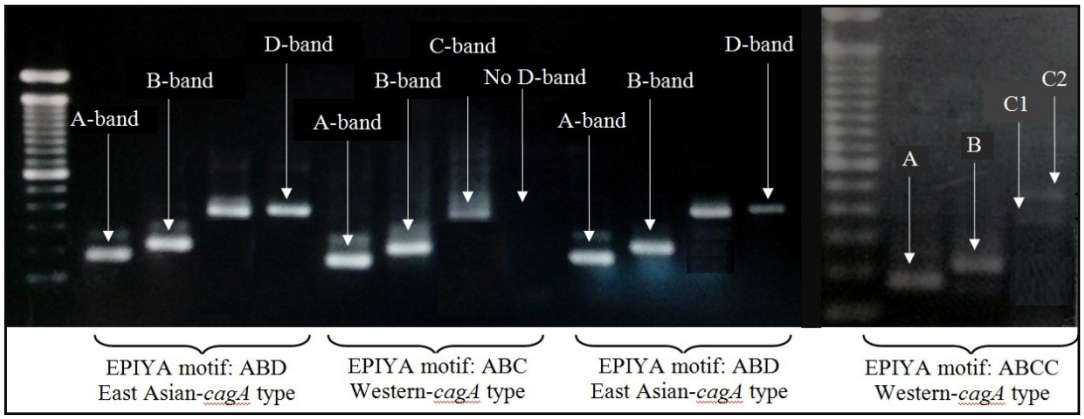


Figure 4.13. Typing *cagA*-EPIYA motif by PCRs
(Electrophoresis on 1.5% agarose gel, M: 100 bp ladder DNA marker. A-band: 178bp, B-band: 222bp, C-band: 381bp, D-band: 399bp, East Asian cagA type has 4 band on the gel corresponding to EPIYA motif A, B, D, Western cagA type has 3 band corresponding to EPIYA motif A, B, C and no D-band)

To confirm the results of *cagA* typing by PCR method, the *cagA* gene of all 8 Western *cagA* strains and 6 selected East-Asian *cagA* strains were sequenced (Fig 4.14). The results of sequencing was consistent with results previously obtained by PCR. All sequenced strains had EPIYA motif A and B, the Western *cagA* had EPIYA motif C with the typical amino acid sequence flanking TIDDLGGP of EPIYA, whereas East Asian *cagA* type possessed EPIYA motif D with the TIDFDEANQAG flanking sequence. There were

some amino acid variations in the sequences among the analysed strains and one Western *cagA* strain had two EPIYA motif C consistent with PCRs results with two C-band on the gel and a bigger amplicon of *cagA* than others.

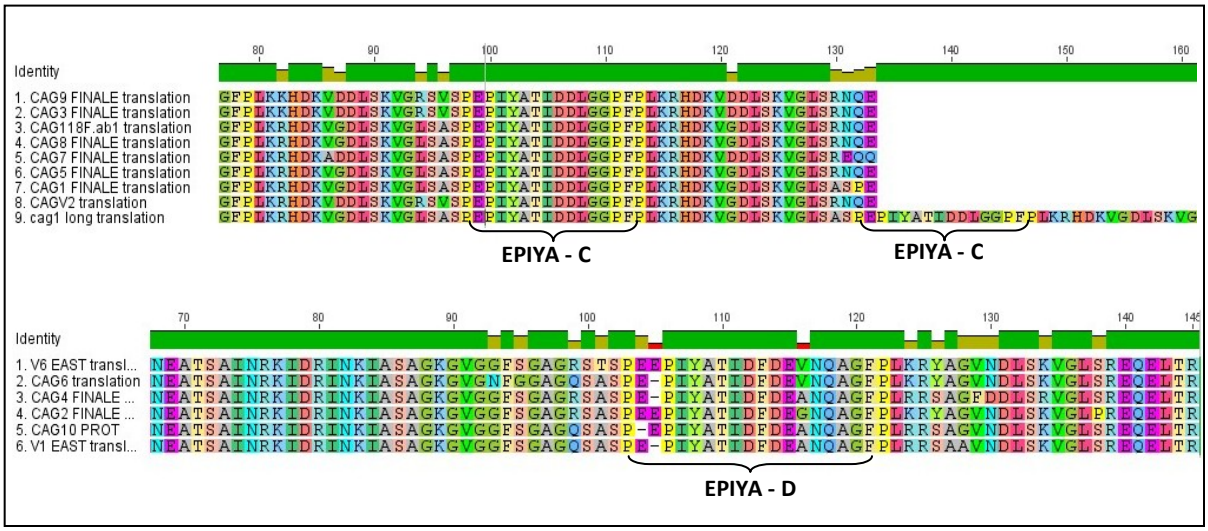


Figure 4.14. Sequences of *CagA* EPIYA motif

4.3.1.2. *cagA* genotype in relation to gastroduodenal diseases

East-Asian *cagA* type was present in all strains isolated from gastric ulcer (9/9) and gastric cancer patients (2/2), which were higher than those from gastritis and duodenal ulcer with 71.2% (42/59), 80.8% (21/26), respectively, but still not statistically significant (p = 0.09) (Fig. 4.15). On the contrary, *cagA* negative and Western *cagA* type were found only in strains from gastritis patients with 16.9% (10/59) and 11.9% (7/59); in duodenal ulcer groups with 15.4% (4/26) and 3.8% (1/26), respectively.

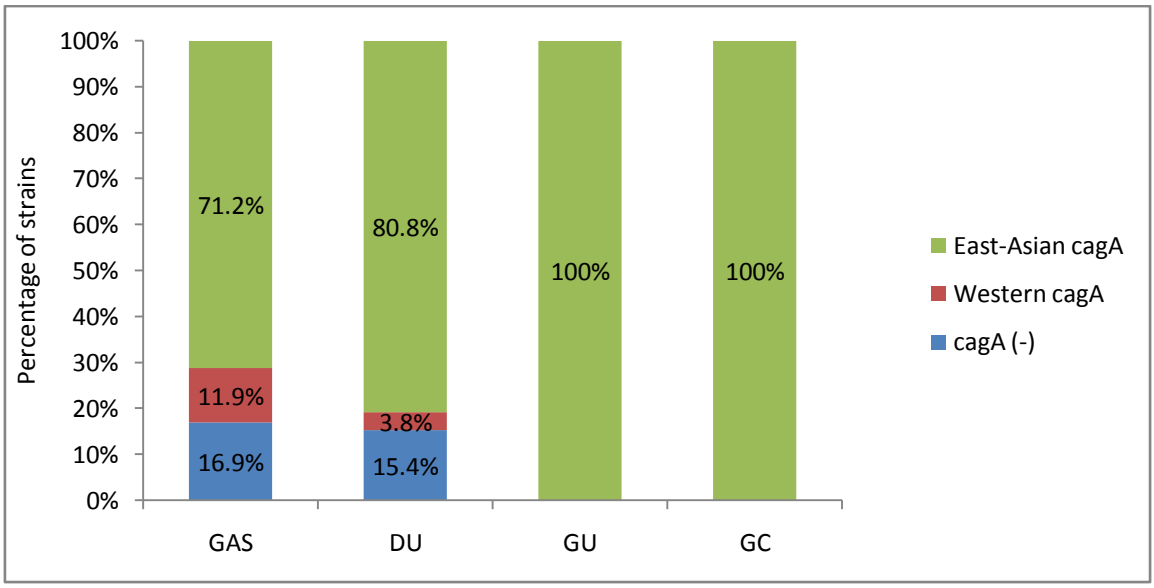


Figure 4.15. *cagA* status and *cagA* type of *H.pylori* strains (n=96) in relation to gastroduodenal diseases

(GAS: Gastritis, DU: Duodenal ulcer, GU: Gastric ulcer, GC: Gastric Cancer)

4.3.2. *vacA* gene

4.3.2.1. *vacA* Genotyping

In total we excluded 8 strains from analysis description: one strain had mixed *vacA* s1/s2 and one was untypeable s-region; one strain with mix i1/i2 and one with untypeable i-region; 2 strains with mixed *vacA* m1/m2 and 3 strains with untypeable m-region to analyse *vacA* s, i, m regions, respectively, in which, one strain had mixed combination of *vacA* s1/s2 and i1/i2.

Most of strains had *vacA*s1 genotype with 97.9% (92/94); 91.5% (86/94) of strains had *vacA*i1; *vacA*m1 and *vacA*m2 presented 56% (51/91) and 44% (40/91) of strains, respectively (Fig.4.16, 4.17, 4.18).

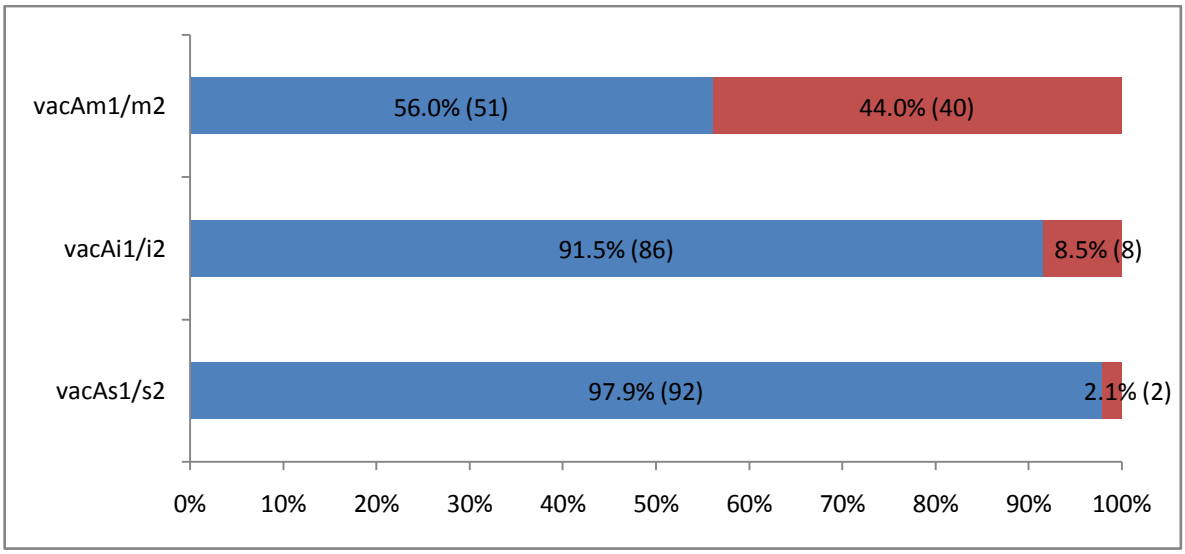


Figure 4.16. Distribution of *vacA* genotype among *H.pylori* strains

(s: signal, i: intermediate, m: midle region)

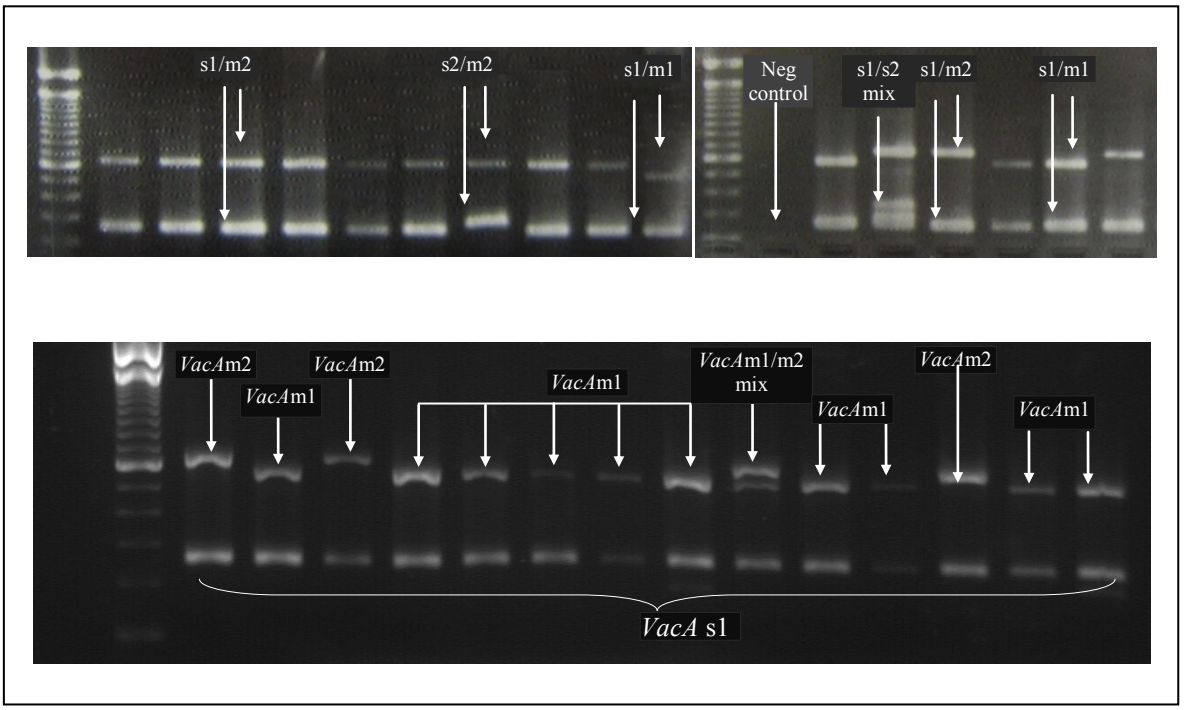


Figure 4.17. *vacA*s/m multiplex PCR

(Electrphoresis on 1.5% agarose gel, 100 bp ladde, vacAs1: 259bp, vacAs2: 286bp; vacAm1: 567bp, vacAm2: 642bp)

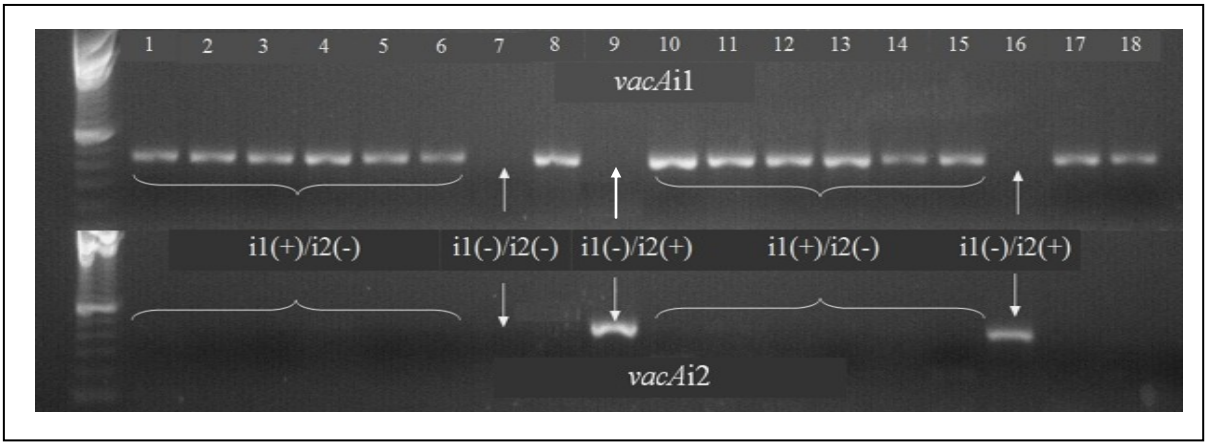


Figure 4.18. *vacA*i PCR-typing
(Electrophoresis on 1.2% agarose gel, 100 bp ladder, vacAi1: lines 1-6, 8, 10-15, 17-18; vacA2: lines 9, 16; untypeable vacAi: line 7)

After excluding 8 mixed or untypeable *vacA* genotype samples from 96 strains (8.3%), altogether, 88 strains were characterised *vacA* gene polymorphism; *vacA* s1i1m1 had a predominant percentage with 55.7% (49/88), followed by s1i1m2, s1i2m2 and s2i2m2 with 35.2% (31/88), 6.8% (6/88) and 2.3% (2/88), respectively (Fig. 4.19). All *vacA* s1m1 combined with i1 and all *vacA* s2m2 combined with i2, *vacA* s1m2 combined with i1 or i2. No strain had the *vacA* type: s2i1m1 or s2i1m2 or s2i2m1 or s1i2m1.

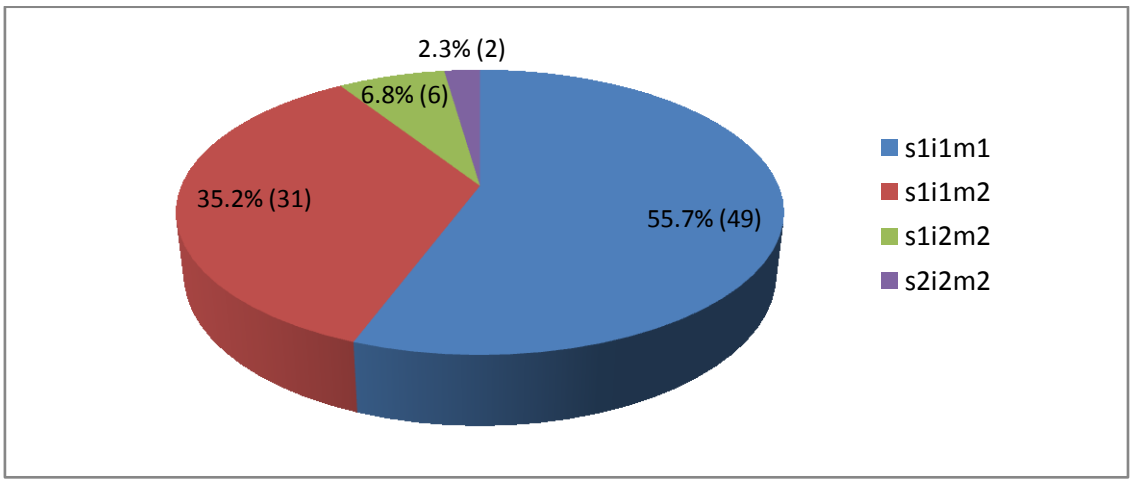


Figure 4.19. Distribution of polymorphic *vacA* gene structure

4.3.2.2. *vacA* genotype in relation to gastroduodenal diseases

*vacA*s1 and *vacA*i1 were present in almost strains in different gastroduodenal diseases. On the other hand, *vacA*m1 was more predominant than *vacA*m2 in strains isolated from gastric ulcer patients (75% vs 25%) and it showed a higher frequency of *vacA*m1 than that in duodenal ulcer (44%) and gastritis (58.9%) ones, but they were not statistically significant ($p > 0.05$) (Table 4.5).

Table 4.5. *vacA* gene in relation to gastroduodenal diseases

***vacA* type**	**Total n**	**GAS n (%)**	**DU n (%)**	**GU n (%)**	**GC n (%)**
s1	94	57 (98.3%)	24 (96.0%)	9 (100%)	2 (100%)
s2		1 (1.7%)	1 (4.0%)		
i1	94	53 (93%)	23 (88.5%)	8 (88.9%)	2 (100%)
i2		4 (7%)	3 (11.5%)	1 (11.1%)	
m1	91	33 (58.9%)	11 (44%)	6 (75%)	1 (50%)
m2		23 (41.1%)	14 (56%)	2 (25%)	1 (50%)

(GAS: Gastritis, DU: Duodenal ulcer, GU: Gastric ulcer, GC: Gastric Cancer)

Among strains in gastric ulcer disease, *vacA*s1i1m1 (6/8) was more predominant than s1i1m2 (1/8) and s1i2m2 (1/8) polymorphic types (75% vs 12.5% and 12.5%) (Fig.4.20) with a significantly difference ($p < 0.05$). Moreover, *vacA*s1i1m1 was present in strains isolated from gastric ulcer patients with a higher frequency than in that from duodenal ulcer (41.7% - 10/24) and gastritis (59.3%-32/54). All s2i2m2 types were present in gastritis and duodenal ulcer strains.

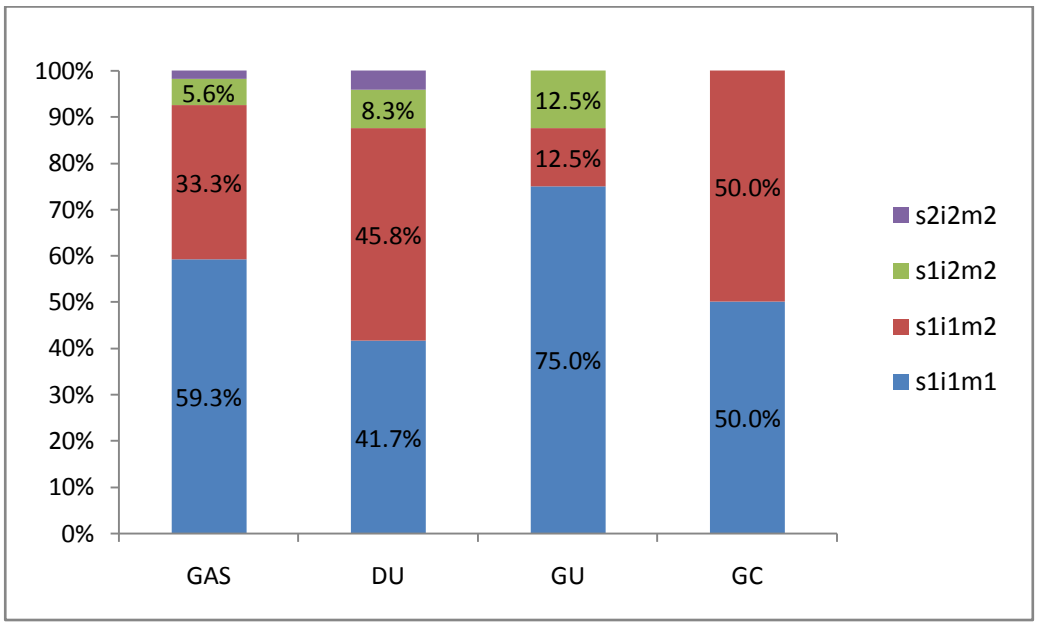


Figure 4.20. *vacA* gene polymorphism in relation to diseases
(GAS: Gastritis, DU: Duodenal ulcer, GU: Gastric ulcer, GC: Gastric Cancer)

4.3.2.3. Association of *vacA* polymorphism and *cagA* status

cagA-positive status was very closely associated with *vacA* s1i1m1 genotype with highest frequency (98%), followed by s1i1m2 (77%) and s1i2m2 (33%), none with s2i2m2 and the difference of those frequency were statistically significant ($p < 0.05$). On the contrary, *cagA*-negative status was only present in *vacA* s2i2m2 and was predominant in *vacA* s1i2m2 (67%).

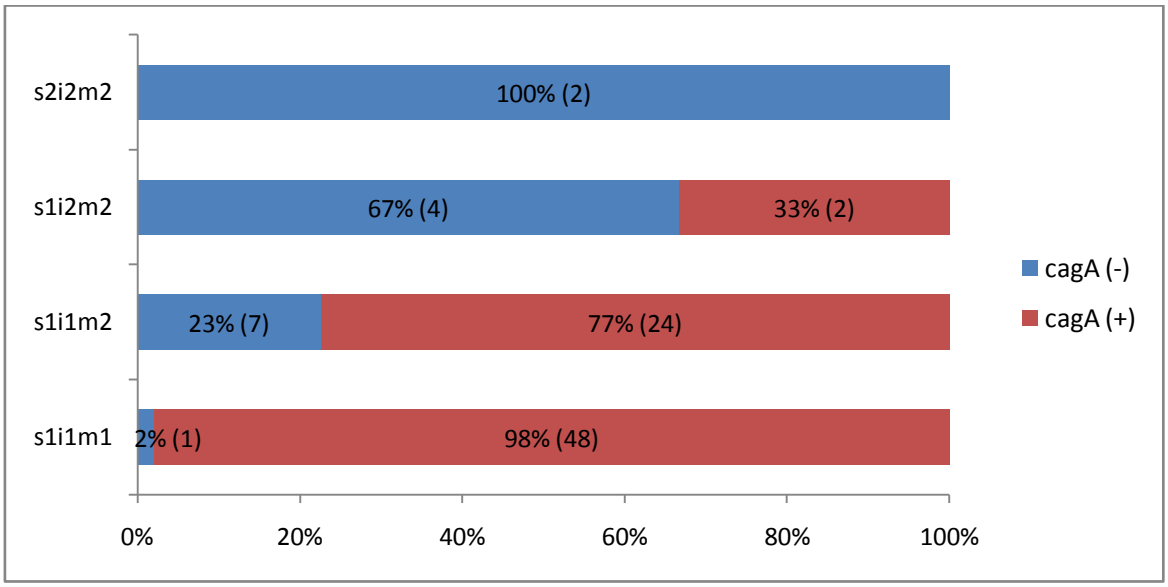


Figure 4.21. *vacA* gene polymorphism in relation to *cagA* status

5. Discussion

5.1. Characteristics of study population and clinical *H. pylori* strains

Vietnam is a developing country, located in Southeast Asia, where the rate of *H. pylori* infection is particularly high [18]. In most of the tertiary care hospitals in Vietnam, including Hue University Hospital which represents one of the largest hospitals in Central region, the diagnosis of *H. pylori* infection has become a routine test during the upper endoscopic procedure by an invasive method consisting in taking gastric biopsies for rapid urease test or histological examination.

Culture to isolate *H. pylori* from gastric biopsy is rarely performed because of the difficulty, sophistication and time-consuming, however, it is considered a gold standard for diagnosis of *H. pylori* infection with nearly 100% specificity [8]. Furthermore, the reason of the culture is mainly the possibility of performing antimicrobial susceptibility testing and the antibiotic resistance prevalence of a given country or region is very important to decide a treatment strategy for *H. pylori* eradication.

In this study, for the first time in Central Vietnam, we systematically performed culture for isolating *H. pylori* from gastric biopsies in gastroduodenal patients. A total of 127 patients from different provinces in Central region were enrolled in this study, 66 males and 61 females with mean age: 44.1 years. Most of them reported some dyspeptic symptoms (upper abdominal pain, fullness, feeling full earlier than expected when eating, accompanied by bloating, belching, nausea, or heartburn) and all of them were indicated to perform upper endoscopy for clarifying clinical diagnosis and checking *H. pylori* infection by rapid urease test during the procedure.

According to endoscopic criteria, they were diagnosed as having gastritis, duodenal ulcer, gastric ulcer and gastric cancer with frequency 61%, 28%, 9% and 2%, respectively. In our results, the prevalence of peptic ulcer (including gastric ulcer and peptic ulcer) was very high when compared with studies conducted in Western countries; in particular, if we compare the prevalence of peptic ulcer, it was only 3% in a report from an Italian survey conducted throughout nationwide endoscopy centers [212].

All patients in our study were positive by rapid urease test but only 76.4% of them were culture-positive, showing that the sensitivity of culture is less than that of rapid urease test and it varies from 58.1% to 85.4% [213], [154]. Our result is fairly good if we consider that this was the first time for culture setting with lack of experience on microbiological technique. The reasons of less culture-sensitive results may be due to an insufficient number of biopsies, delay in transporting, exposition to an aerobic environment or failure to recognise *H.pylori* cultures as a result of microbiological inexperience [155]. In addition, another reason may be linked to the nature of *H. pylori* itself as it is a fastidious micro-organism, it requires complex growth media and a long time for incubation, leading to not always successful isolation. In this study, besides the microbiology and biochemistry criteria for identification of *H. pylori* from the culture, we further confirmed by PCR method with two specific genes for *H. pylori*: *16S rRNA, ureA* genes; DNAs extracted from strains had the specific amplified products of these genes after PCR. Among 97 strains, 78 were isolated from 78 patients who had never been treated for *H. pylori* infection, these are therefore called primary strains, while the remaining 19 strains were isolated from patients in whom

eradication of the infection failed after treatment or had the history of *H. pylori* eradication therapy; these are called secondary strains [174]. In this study, we got information about the history of the *H. pylori* eradication treatment from the patients, but could not obtain the detail of the therapy regimen they underwent.

Unfortunately, we failed to re-cultivate 5 primary strains from minus 80^{o}C store and one of them has been lost, therefore the antibiogram profile of these strains is not available. Altogether, we had the full antibiogram profile of 92 strains and DNAs extracted from 96 strains were available for further analysis.

5.2. Antimicrobial susceptibility testing

5.2.1. Prevalence of Antibiotic resistance of *H. pylori* strains

Considering the high prevalence of *H. pylori* infection in population and an intermediate risk of gastric cancer in Vietnam [18, 67], a successful eradication therapy appears essential not only to reduce the risk of developing gastric cancer but also to treat other severe related disorders such as peptic ulcer disease, gastric mucosa associated lymphoid tissue lymphoma, iron deficiency anemia, hemorrhage idiopathic thrombocytopenia [46].

The increasing rise of antibiotic resistance represents the most important factor responsible for the declining success rate of *H. pylori* eradication therapy [214]. Surveillance of *H. pylori* antibiotic resistance is therefore mandatory in order to adapt the antibiotic combination to local resistance patterns [165]. This issue is of particular relevance with regard to clarithromycin, which can induce virtually a 70% loss of effectiveness in the

standard triple therapy (PPI+CH+AC) in patients infected with clarithromycin resistant strains versus susceptible strains [182]. Unfortunately, we found that the antibiotic resistant strains were common in dyspeptic patients under investigation.

The first relevant finding of our study is that the resistance rate to levofloxacin, which is considered the alternative choice after eradication treatment failure with the standard triple therapy, was remarkably high, being 41,3% in a background of high resistance rate to clarithromycin and metronidazole of 42,4% and 76,1% respectively but to amoxicillin only 1.1% . Furthermore, the resistant strains showing high-level resistance to LE (MIC ≥ 32µg/ml), CH (MIC ≥ 96µg/ml) and MZ (MIC ≥ 256µg/ml) accounted for 42.1%, 35.9% and 39.3% of resistant strains, respectively. According to the new guideline of EUCAST (European Committee on Antimicrobial Susceptibility Testing) published in 2013, the MIC breakpoint for *H. pylori* resistance to amoxicillin is above 0.12 µg/ml and above 0.5 µg/ml for clarithromycin [176]. If applying these criteria to our results, the resistance rate to amoxicillin will be over 6% and 44.6% for clarithromycin. The resistance rate of CH and LE tends to be higher in strains from gastric ulcer patients than others, this might be due to the difficulty of treating gastric ulcer compared to duodenal ulcer or gastritis diseases, so the failure of eradication treatment was encountered more frequently.

Notably, the ratio of secondary resistant strains was significantly greater than that of primary resistant strains, both for clarithromycin (secondary-73.7% vs primary-34.2%) and levofloxacin (secondary–63.2% vs primary–35.6%). The latest consensus recommends that PPI-clarithromycin-containing

triple therapy without prior susceptibility testing should be abandoned when the clarithromycin resistance recorded in the region is higher than 15-20% [46]. Therefore, according to this advice, the use of clarithromycin should be dissuaded in the Central Vietnam as first-line treatment of *H. pylori* infection without a preliminary assessment of drug susceptibility, along with the use of MZ, since the high rate of primary resistance recorded (76.1%), although standard metronidazole susceptibility testing lacks reproducibility in vivo [8]. In addition, considering the high rate of primary resistance (35.6%), levofloxacin should not be considered as an alternative drug for *H. pylori* eradication in this region. Similar to CH, it had about 45% loss of effectiveness in the triple therapy PPI+AC+LE in case of LE resistant strains versus susceptible strains [201]. Comparing with the LE resistance rate in another study in Vietnam, which included *H. pylori* strains isolated in 2008 [215], we observed an increase of the primary resistance rate from 18,4% in that study to 35.6% in our study. Thus, the resistance of *H. pylori* strains to LE was quickly acquired while the resistance to CH and MZ has not changed after nearly 5 years in Vietnam (CH: 33% vs 34.2% and MZ: 69.9% vs 75.3%). This is consistent with some reports of increased LE resistance rate of primary and secondary resistance in the world, *e.g*. increased from 8.6% for primary and 22.9% for secondary resistance in 2006 to 18.8% for primary and 30.6% for secondary resistance in 2011 in Germany [171]; from 4.7% for primary and 16.7% for secondary resistance in 2003-2005 to 28.1% for primary and 50% for secondary resistance in 2009-2012 in South Korea [216].

Interestingly, in our study, the LE resistance rate in females was significantly higher than in males, this probably reflects the increasing use of

fluoroquinolones in females may lead to cross-resistance with LE. Moreover, the resistance to CH and LE increased according to the rise of age. Older age has already been reported as a risk factor for antibiotic resistance [181]. The primary resistance is most likely the consequence of treatments administered to the patient for other types of infection. The abuse of these drugs for self-medication may be the main factor of high resistance rate in Vietnam. In such cases, the antibiotic used as a monotherapy may have selected resistant *H. pylori* mutants, but was not able to lead to eradication. However, the low resistance to amoxicillin in this study indicates that it is probably exceptional (only 1.1%) which is similar to other reports of AC resistance rate lower than 2% [215, 217], [218].

After failure of a CH-based triple therapy, the risk of finding a CH-resistant strain is approximately 65% [182]. It is in the same range after failure of a levofloxacin based triple therapy [201]. Our results showed that the secondary resistance of *H. pylori* to CH and LE was 73,7% and 63,2%, respectively, this was consistent with some studies conducted in Poland and Korea [216, 219]. After several treatment attempts, it was possible to find strains resistant to three, four antibiotics in 15% of the cases [220]. In our study, the resistant strains to three and four antibiotics accounted for 15,2% and 1,1%, respectively. Furthermore, the triple drug and multidrug resistance in secondary resistant strains were significantly greater than that in primary resistant ones ($p<0.05$). Interestingly, all 38 levofloxacin and 34/39 clarithromycin resistant strains displayed double, triple or quadruple resistance, where LE+MZ double resistance was the most frequent (19.6%).

5.2.2. Compare two susceptibility testing: E-test and disk diffusion

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 phenotypic method.

The agar dilution assay proposed by the CLSI [8], is usually considered the reference method compared to other techniques, being the most accurate, 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 with the agar dilution method [180]. However, the E-test is economically impractical for clinical laboratory use when testing individual isolates, particularly in Vietnam. On the contrary, the disk diffusion method is the simplest and the most economic for routine susceptibility testing but it is not well standardized for slow-growing bacteria like *H. pylori* [178]. Therefore, we compared disk diffusion method with E-test for testing susceptibility of *H. pylori* to clarithromycin and levofloxacin which are the key antimicrobial agents for eradication therapy, one for first-line treatment and another for second-line treatment according to the latest guidelines [46].

In this study, the MIC values determined by E-test method provided breakpoint values of inhibition diameters for disk diffusion method. It was recommended that strains with a MIC value of $< 1\mu g/mL$ to be considered susceptible, and those with MICs of $\geq 1\mu g/mL$ to be considered resistant. Correspondingly, strains with inhibition diameters $\geq 24mm$ should be defined as susceptible to CH, and those with inhibition diameters ≤ 18 mm should be defined as resistant to CH. For levofloxacin, strains with inhibition diameters

≥ 30mm should be defined as susceptible, and those with inhibition diameters ≤ 26 mm should be defined as resistant. As a result, to apply the disk diffusion method, we recommended the intermediate diameter zone of inhibition needs to be re-evaluated by E-test when diameters are from 18mm to 24mm for CH and diameters from 26mm to 30mm for LE. According to some studies, strains are defined as sensitive to CH when the inhibition zone diameters is above 22mm (15µg disc) and the inhibition diameter less than 20 mm is defined as resistant and it should be considered to verify it by E-test [178]. Another study conducted in India that used ciprofloxacin (5µg disc) showed that 95% of strains with inhibition diameters greater than 30 mm are sensitive [221]. In the 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 [179]. Compared with the E-test, the disk diffusion method might be more feasible and less expensive, especially in developing countries. In a study conducted in India, Mishra *et al* concluded that the disk diffusion is ten-times cheaper than the E-test (approximately 2.6 cents vs. US$2.60), it is easy to perform and a reliable method for testing *H. pylori* susceptibility to antimicrobial agents in the clinical microbiology laboratory [221].

5.2.3. Point mutation in *23S* rRNA gene related to clarithromycin resistance

Clarithromycin is the most important and common drug recommended as first-line eradication therapy for *H. pylori* infection, and has been often used as standard triple therapy in combination with a proton pump inhibitor and amoxicillin [46]. The reason is that it is able to accumulate intracellularly

where a high concentration can be reached, conferring several advantages. The first advantage is that clarithromycin can act on the few bacteria which have penetrated intracellularly. Even if *H. pylori* is not an intracellular pathogen, recent data have pointed out the occurrence of intracellular bacteria and their importance in the natural history of infection [222]. Second, the slow release of clarithromycin into the mucus allows a sufficient concentration of the antibiotic over time to kill the bacteria. Thus, clarithromycin resistance is regarded as the key factor involved in *H. pylori* therapy failure. This drug belongs to the macrolide family acting by binding to 50S bacterial ribosomal subunit at the level of the peptidyl transferase loop of the *23S rRNA* gene and inhibits the protein synthesis.

In our study, all CH-resistant strains had point mutations in the *23S rRNA* gene with a great diversity, including quadruple mutation being the most represented with different combinations: A2143G + T2182C + A2223G + T2244C (18 strains) followed by A2143G + T2182C + A2302G + T2244C (1 strain) and C2195T + T2182C + A2223G + T2244C (1 strain) and one strain with quintuple mutation: A2143G + T2182C + A2223G + C1953T + T2244C, in particular in secondary resistant strains. Moreover, the MIC of quadruple and quintuple mutations carrying strains were significantly higher than in those with double and triple mutations ($p < 0.05$). Many point mutations in secondary isolates may be a result of exposure to clarithromycin for long periods. The point mutations at two nucleotide positions 2142 (A2142G and A2142C) and 2143 (A2143G) are responsible for more than 90% of clarithromycin resistance in developed countries which lead to a conformational change and a decrease in macrolide binding [183] and A2143G

was reported to be associated with a very low eradication rate [182, 223]. In our results, 85.7% of CH-resistant strains showed A2143G mutation which seems to play a major role in CH resistance. We did not find any A2142G/C point mutation in our isolates. The absence of the A2142G/C mutation in Vietnamese strains may be due to geographical differences. It is similar to many studies conducted in other Asian countries with main point mutation at A2143 and absence at A2142 [224], [225]. All resistant and sensitive strains had T2244C mutation suggesting a minor role in clarithromycin resistance.

Alongside with the resistant strains, T2812C mutation also occurred in 3 intermediate strains with MIC from 0.25 to 0.75µg/ml and one sensitive strain showed that it may have an accessory role or be associated with low level CH resistance in *H. pylori* as already reported elsewhere [224], [226]. The C2195T, A2223G mutations in this study have also been reported in a number of studies around the world and their role has not yet been demonstrated [30, 33]. In addition, we also found that C1953T, A2302G mutations often occurred with the A2143G mutation which have not been reported so far, having a potentially complementary role in increasing the level of resistance.

5.2.4. Point mutation in *gyrA* gene related to levofloxacin resistance

According to the latest guidelines, in case of first-line eradication failure with triple treatment clarithromycin-based, a triple treatment levofloxacin-based (plus PPI and amoxicillin) has been proposed as a second-line and even third-line therapy if quadruple regimen therapy with bismuth-based failed [46]. This combination, first used by Cammarota *et al.* in Italy, resulted in eradication rates between 92 and 95% [227] and has been recommended as the

first choice of salvage therapy. However, similar to clarithromycin, resistance to levofloxacin easily occurs and jeopardizes the success of treatment [181].

Levofloxacin is a drug that belongs to the quinolones family, it acts by inhibiting the A subunit of the DNA gyrase, an essential enzyme for the maintenance of DNA helicoidal structure, encoded by the *gyrA* gene. Point mutations in Quinolones Resistance Determining Region (QRDR) of *gyrA* prevent binding between the antibiotic and the enzyme, conferring antibiotic bacterial resistance [184]. Unlike other species, *H. pylori* does not have *parC* or *parE* genes that encode the topoisomerase IV [11], therefore, mutation of the DNA gyrase, especially *gyrA*, appears to play an important role in fluoroquinolone resistance, so far [185].

In our study, the frequency of *gyrA* gene mutations in LE-resistant strains was 85.7% and 100% in secondary LE resistant strains, which is comparable to a Korean study published in 2011 [228]. The presence of mutations in *gyrA* gene in all LE secondary resistant strains can be explained by an increase in the complexity of mutations after prolonged exposure to this drug. Overall, hot-spot mutations were seen at Asp-91 and Asn-87, which represented the well-known mutations detected worldwide, responsible of fluoroquinolone resistance [185],[228],[186], accounting for 80% of resistant strains with a variation of amino acid substitutions, *e.g.* N87K, D91N, D91Y, D91G. Interestingly, the MIC of levofloxacin in strains harboring Asn-87 mutation was higher than in those with Asp-91 mutation ($p < 0.05$). Our observation is similar to some other studies reporting Asn-87 mutation as an important determinant in failure of fluoroquinolone-containing triple eradication therapy and Asp-91 mutation associated with low-level resistance [186, 228]. We

found one high level LE resistant strain which had a new mutation with a combination (N87A+A88N+V65I) which has not been reported so far. Other mutations coexisted with Asp-91, and Asn-87 mutations may affect accessory roles or may not play any important role in LE resistance. Therefore, further study is needed to clarify the roles of these *gyrA* mutations such as L45F, A55S, G60S, V65I, A88N, R130K. Finally, five LE-resistant strains lacking alterations in *gyrA* gene may be ascribed to mixed infections or to other mutations in QRDR of *gyrB* or other mechanisms [186, 228].

5.3. Genotyping and clinical relevance of virulent factors: *cagA* and *vacA*

5.3.1. *cagA* status and *cagA* type

The *cagA* gene, encoding CagA protein, is a major virulence factor of *H. pylori*, which is located at one end of the cag-PAI, an approximately 40 kb region that is thought to have been incorporated into the *H. pylori* genome by horizontal transfer from an unknown source [57]. The cag-PAI, contains 27 to 31 genes, encodes a type IV secretion system through which CagA is delivered into host cells and *cagA* is recognized as a marker for the cagPAI region which is found in more virulent strains but is typically missing in less virulent *H. pylori* isolates [58], [59]. Moreover, *cagA* is a polymorphic gene, in particular there are different numbers of repeat sequences located in the 3' region of the *cagA* gene of different *H. pylori* strains. According to the repeat sequences of the 3' region of c*agA, H. pylori* strains can be classified in two types, the East-Asian type and the Western type [67].

In the present study, the *cagA* gene was detected in 85.4% *H. pylori* strains and in 14.6% of *cagA*-negative ones in the Central region. Interestingly, the frequency of *cagA*-positive in our results is much lower than that reported

from other studies previously conducted on *H. pylori* strains from different areas of Vietnam; these studies reported an average frequency of 96-97%, in detail: 97% in Hanoi capital (North) and 96% in Ho Chi Minh city (South) [207],[229],[203]. This might be a diversity of the *cagA* status in Vietnam, which is similar to what reported in most of Asian countries, such as in Thailand, Malaysia or China [230],[231]. Conversely, in Europe, *cagA*-positive strains are extremely less prevalent than in Asia, from 19% to 40% in The Netherlands [232] but quite higher in South Europe, such as in Italy with 72% [96], and 62% in Portugal [233].

Among our *cagA* negative strains, only 42.9% were positive with cagPAI-empty site, indicating complete absence of the cag-PAI but the remaining portion was negative by PCR and suggesting the presence of a deviating cag-PAI. This observation is consistent to many studies in the world which showed that the failure to amplify *cagA* with simultaneously cagPAI-empty site negative in these strains were due to point mutation in *cagA* gene or partial deletion of cag-PAI [234], [235]. In particular, according to a study about the cagPAI intactness in *H. pylori* isolates from Vietnam, Nguyen *et al* showed that 11.7% (12/103) of strains contained only a part of cagPAI [236], this might explain our results of cagPAI-empty site negative in *cagA*-negative strains.

Regarding the epidemiological issue, the prevalence of *H. pylori* infections in Japan, Korea, and China is not higher than in South-Asia, Africa and some parts of Europe, but the incidence of gastric cancer is significantly higher [67]. This can be partly explained by the diversity of *H. pylori* strains among these areas, besides *cagA* status, the polymorphisms of EPIYA motifs

in the carboxyl-terminal region of CagA, the Western CagA type (EPIYA-ABC), and East Asian CagA type (EPIYA-ABD) [68],[34].

In our study, *cagA*-positive strains were further characterized EPIYA motif to classify CagA type with frequency of Western CagA and East Asian CagA type as 9.8% and 90.2%, respectively. This is the first report about CagA type in the Central region as far as we know. Among Western C*agA* strains, one had an double EPIYA motif C (ABCC) and 7 had a single EPIYA motif C (ABC), whereas all 74 East Asian C*agA* strains had EPIYA motif -ABD. These results were confirmed by sequencing *cagA* gene of all Western C*agA* strains and selected East-Asian C*agA* strains. All sequenced strains had EPIYA motif A and B, the Western CagA had EPIYA motif C with the typical amino acid sequence flanking of EPIYA was –TIDDLGGP-, whereas East Asian CagA type possessed EPIYA motif D with the –TIDFDEANQAG- sequence flanking. There were some amino acid variations in the sequences among these strains and one Western *cagA* strain had two EPIYA motif C consistent with PCRs results as having two C-band on the gel and a higher amplification of *cagA* than others. Our results of the Western CagA type were significantly higher than those from other studies which examined the strains isolated from different Vietnamese populations with frequencies from 0% to 4% [207],[229],[203]. Along with the *cagA* status, the CagA type in Central region was fairly different to those in Southern or Northern Vietnam, showing that there is a geographically dependent diversity of *cagA* genotype.

Notably, there are about 54 ethnic groups in different areas of Vietnam where, similarly to other Southeast Asian countries, several ethnics groups or populations live together. Recently, Breurec *et al*. reported that five major

types of historical human migration patterns have occurred in Southeast Asia: i) migration from India introducing hpEurope bacteria into Thailand, Cambodia, and Malaysia; ii) migration of the ancestors of Austro-Asiatic speaking people carrying hspEAsia bacteria into Vietnam and Cambodia; iii) migration of the ancestors of the Thai people from Southern China into Thailand carrying of population hpAsia2; iv) migration of Chinese to Thailand and Malaysia within the past 200 years, resulting in the spread of hspEAsia strains; and v) migration of Indians to Malaysia within the past 200 years, distributing both hpAsia2 and hpEurope bacteria. Therefore, both Western and East Asian strains according to EPIYA motif genotyping can be observed in Southeast Asia [237]. Although, having no detailed information about patient's ethnicity or genealogy, this might be a reason for explaining the diversity of *cagA* status and CagA type in Vietnam since it borders to China (in the North), Laos (in the Centre), and Cambodia (in Central and Southern areas) and probably intermarriages of the various races and nationalities with the different ethnic groups contributed to this diversity.

5.3.2. *cagA* genotype in relation to gastroduodenal diseases

CagA protein was firstly discovered in a study with the strong association between serological responses to CagA and peptic ulcer disease [52]. A recent study, moreover, has firmly implicated CagA as a bacterial oncoprotein by attenuating apoptosis in vitro [55] and another study demonstrated that transgenic expression of CagA in mice leads to the development of aberrant gastric epithelial proliferation and gastric carcinoma [56]. Worldwide, a number of studies reported CagA association with severe clinical outcomes not

only in Western countries [67] but also in Asian countries; however, the odds ratio in Asian countries was smaller than that in Western countries [73].

As we know gastric cancer with a predominant intestinal-type adenocarcinoma is a consequence of progresses through a series of well-defined histological steps initiated by the transition from normal mucosa to chronic superficial gastritis, then atrophic gastritis and intestinal metaplasia, finally leading to dysplasia and adenocarcinoma that is triggered by *H. pylori* [140], [135]. Although gastric ulcer and duodenal ulcer, strongly related to *H. pylori*, are classified in the same group (peptic ulcer), they have opposite consequences. Duodenal ulcer is considered a benign disease, instead gastric ulcer is associated with a high risk to advance to gastric cancer typically due to having atrophic gastritis and corpus-predominant gastritis leading to decreasing acid secretion; whereas the antral-predominant gastritis with few atrophic changes and increase of acid production commonly occurs in duodenal ulcer [134]. The loss of gastric acidity contributes to the promotion of the endogenous formation of N-nitroso compounds by the gastric bacterial flora with non-*H. pylori* species, and may be the causative factor of gastric cancer [143].

In our study, all strains isolated from gastric ulcer and gastric cancer patients were East-Asian *cagA* type, which were higher than those from gastritis (71.2%) and duodenal ulcer (80.8%). On the contrary, *cagA* negative and Western *cagA* type were only in strains from gastritis and duodenal patients. Our results are consistent with several studies were the risk of peptic ulcer or gastric cancer is reported higher in patients infected with East-Asian type *cagA* strains, compared to *cagA*-negative and Western-type *cagA* strains in

Southeast Asian countries, such as Thailand, Malaysia, Singapore [76], [77]. These findings were also supported by a number of studies *in vitro* demonstrating that CagA of East-Asian type, containing EPIYA-D segments, exhibits a stronger binding affinity for Src homology 2 containing protein-tyrosine phosphatase (SHP-2) and a greater ability to induce proinflammatory cytokines and morphological changes in epithelial cells than Western-type CagA, which contains one EPIYA-C segment [72], [71]. Furthermore, *H. pylori* strains possessing more than two EPIYA-C motifs are more frequently associated with a number of severe diseases [74, 75]. In our study, only one Western CagA strains had a double EPIYA motif C that was isolated from a duodenal ulcer patient.

Previous studies conducted in different parts of Vietnam did not correlate *cagA* with a pattern of gastroduodenal disease because the majority of the isolates were East Asian CagA type, thus it was difficult to differentiate among these diseases simply by considering the *cagA* status and CagA type [229],[203].

Our findings showed a distribution of different *cagA* status and CagA type in strains from a Central region population and a relation between a pattern of gastroduodenal disease and *cagA* status, CagA type; this highlighted a predominance of East Asian CagA type in severe diseases such as gastric cancer and gastric ulcer, and, conversely, *cagA* negative and Western CagA type in less severe diseases as gastritis and duodenal ulcer.

5.3.3. Genotyping of *vacA* gene

VacA is the second most extensively studied *H. pylori* virulence factor. Unlike *cagA*, virtually all the *H. pylori* strains have a functional *vacA* which encodes a vacuolating cytotoxin well conserved between strains, but there is

significant diversity in three distinct regions of the gene: the signal (s) region, the middle (m) and intermediate (i) regions [67],[78].

In this study, among 96 strains, 8 (8.3%) had mixed or untypeable strains with *vacA* genotype. This was fairly comparable to other studies conducted in developing countries, showing a quite high frequency of mixed strains in a given host examined with several important genetic markers: in detail 10% to 20% revealing mixed *vacA*s/m genotype [238], [235]. Results in these studies suggested that most of the patients acquired *H. pylori* following repeated exposures to this pathogen with different genetic make-up, in particular in developing coutries, which may increase the possibility of super infections and an effect on the severity of the *H. pylori* related diseases.

Most of the strains in this study had *vacA*s1 genotype with 97.9%, whereas *vacA*s2 only in 2.1%; a total of 91.5% strains had *vacA*i1 and 8.5% for *vacA*i2; *vacA*m1 and *vacA*m2 presented 56% and 44% of strains, respectively. Our findings are similar to the majority of the previous studies examining strains from different Vietnamese populations [207],[229],[203]. The genetic characteristics of *vacA* in *H. pylori* strains in Vietnam, as well as in many Southeast Asian countries, are represented in the majority of isolates by *vacA*s1 and *vacA*i1 genotypes, while for what concerns the frequency: in the m1 gene is less frequent from 34% (southern Vietnam) to 58% (northern Vietnam) [230]. Although the prevalence of *vacA*s1 and *vacA*i1 is the same, the *vacA*m1 strains are common (over 95%) in areas of Northeast Asia, *e.g.,* Japan and South Korea [239]. On the contrary, in Europe, there is a less predominant frequency of *vacA*s1, from 45% to 58%, of *vacA*m1 from 21% to 42% and the same range for *vacA*i1 [96],[240].

Characterisation of *vacA* gene polymorphism in our study revealed that *vacA* s1i1m1 was the predominant profile with 55.7%, followed by s1i1m2, s1i2m2 and s2i2m2 with 35.2%, 6.8% and 2.3%, respectively. Although only 2.3% of them had the *vacA*s2i2m2 genotype, this was the first report showing the existence of this genotype not only in Vietnamese strains collected in the Central region but also throughout Vietnam; all previous studies showed that nearly 100% Vietnamese strains possessed *vacA*s1 genotye [68],[203]. Other combinations such as *vacAs2m1* type, s2i1m1, s2i2m1, s2i1m2, s1i2m1 were not present in any of our strains. Our findings were consistent with many studies in the world showing an agreement with the original description of the *vacA* i-region: all s1m1 strains were type i1, s2m2 strains were i2 and s1m2 strains could either be type i1 or i2; in addition the s2m1 strain is rarely found [96],[88],[92].

5.3.4. *vacA* genotype in relation to gastroduodenal diseases

The clinical significance of VacA has been assessed from studies in animal models and from several observations in humans infected with *H. pylori*. The purified VacA can induce ulcer-like erosions when administered into the mouse stomach [93]. Orally administered toxigenic *H. pylori* sonicates and purified VacA also induces epithelial vacuolation, loss of gastric gland architecture and infiltration of mononuclear cells into the lamina propria [94]. Among *vacA* types, s1m1 strains are the most cytotoxic, followed by s1m2 strains, whereas s2m2 strains have no cytotoxic activity and s2m1 strains are rare [88].

In this study, *vacA*s1 and *vacA*i1 were present in almost all strains from different gastroduodenal diseases. However, *vacA*m1 was more predominant than *vacA*m2 in strains isolated from gastric ulcer patients (75% vs 25%) and

it tended to be higher than those from duodenal ulcer (44%) and gastritis (58.9%). Moreover, among strains from gastric ulcer disease patients, *vacA*s1i1m1 was more predominant than s1i1m2 and s1i2m2 polymorphic types (75% vs 12.5% and 12.5%) with a significant difference ($p < 0.05$); in addition, *vacA*s1i1m1 tended to be higher in strains isolated from gastric ulcer than from duodenal ulcer (41.7%) and gastritis (59.3%) patients.

Several studies have investigated the association of *vacA* type and disease outcome. In Western populations such as in America and Europe, where the *vacA* allelic diversity is common, s1 genotypes are more frequently associated with higher levels of inflammation in the gastric mucosa than s2 types [68, 95]. *vacA* s1m1 and s1m2 strains have been shown to be associated with peptic ulceration and s1m1 type strains have been associated with gastric carcinoma [68, 92, 95, 96]. The *vacA* i1 type is a risk factor for peptic ulcer disease and has been shown to be associated with duodenal ulcer disease as well as to gastric cancer [92, 96]. In East Asia, however, most *H. pylori* strains have an s1-type, therefore the pathogenic difference cannot be explained by the type of s region present [68]. With respect to the m region, however, there is variation within East Asia. Although m1 strains are common in areas of Northern-East Asia, such as Japan and South Korea, m2 strains are predominant in areas of South-East Asia, which might explain a higher incidence of gastric cancer in East Asian than others [67]. Our findings showed a higher frequency of high-level cytotoxic *vacA* type strains in the more severe disease. Conversely, all s2i2m2 types, a less or no-cytotoxic active strains types, were only present in patients with gastritis and duodenal ulcer – the less severe diseases.

5.3.5. Association of *vacA* polymorphism and *cagA* status

Many studies indicated that VacA and CagA can even inhibit at least some of each other's signaling pathways; for instance, CagA has been shown to promote the expression of the apoptotic suppressor Mcl1, and inhibit epithelial cell apoptosis caused by *vacA* [55, 87]. These data again emphasize the importance of *in vitro* infection experiments in which interaction among *H. pylori* virulence factors can be taken into account. Moreover, almost all *cagA* positive strains were classified as *vacA* s1 strain, whereas *cagA*-negative strains were often combined with *vacA*s2m2 type [88]. Therefore, it is important to identify both *cagA* and *vacA* genotypes in order to find a correlation of *H. pylori* with clinical outcomes.

In our study, the *cagA*-positive status was very closely associated with the *vacA* s1i1m1 genotype with the highest frequency (98%), followed by s1i1m2 (77%) and s1i2m2 (33%), none with s2i2m2 and the difference of those frequencies were statistically significant ($p < 0.05$). On the contrary, *cagA*-negative status was only present in *vacA* s2i2m2 and was predominant in *vacA* s1i2m2 (67%). These findings showed that a *cagA*-positive tends to link to *vacA*s1i1m1 to become the most cytotoxic-virulent type strain and this synergism might affect the clinical outcomes of the *H. pylori* infection.

As our results showed that each distinct *cagA* positive or *vacA*s1i1m1 type strains were related to a high risk of gastric ulcer. Additionally, *cagA*-positive and *vacA*s1i1m1 type were close linked together. Thus, finally *cagA*-positive and *vacA*s1i1m1 type strains had a relation to gastric ulcer. This was comparable to a number of studies in the world reporting *cagA*-positive/*vacA*s1i1m1 strains strongly associated with high risk of gastric cancer and peptic ulcer [92, 96]. Although gastric cancer, as well as other *H.*

pylori related diseases, may be caused by a combination of different factors, population in a given region is assumed to be exposed to the same environmental factor and partly to the host factor, so that the *H. pylori* virulence may become a significant factor to influence the clinical outcomes.

6. Concluding remarks

The resistance rate is very high in Central Vietnam with 56.5% of multiple resistance and higher prevalence of secondary resistance versus primary resistance, in particular in clarithromycin, levofloxacin.

The disk diffusion can be used as an alternative phenotypic method for testing clarithromycin and levofloxacin susceptbility of *H.pylori*. Compared with E-test, the disk diffusion method may be more feasible and less expensive.

Data on determinants of resistance to clarithomycin and levofloxacin are new information in Vietnam. Most of the clarithromycin and levofloxacin resistant strains harboured resistance-associated mutations with common position at A2143G, T2182C in the *23S rRNA* gene and at Asn-87 or Asp-91 in the *gyrA* gene. MICs were increased in strains carrying quadruple mutations in their *23S* and in strains with an Asn-87 *gyrA* mutation.

cagA positive status, East Asian *cagA* type and *vacA* s1i1m1 type are the most predominant in gastric ulcer and gastric cancer.

The prevalence of *cagA* negative status and Western *cagA* type are higher than those reported in previous studies performed in different areas of Vietnam.

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