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DIAGNOSIS AND TREATMENT OF *HELICOBACTER PYLORI* INFECTION IN VIETNAMESE CHILDREN

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To my family with all my love

ABSTRACT

Aim: The aim of the study was to find the optimal *H. pylori* eradication therapy for children in Vietnam, a developing country. Therefore, we evaluated a non-invasive diagnostic method and antibiotic susceptibility of *H. pylori* strains, the major determinant of treatment outcome, as well as the rate of reinfection after successful eradication, a determinant for the rational of *H. pylori* eradication.

Materials: In a treatment trial, gastric biopsy, blood and faecal samples were obtained from 240 children (age 3-15 years) for various gastrointestinal complaints. *H. pylori* infection status was based on either positive culture or positive monoclonal antigen-in-stool test (Premier Platinum HpSA PLUS) at inclusion and positive monoclonal antigen-in-stool test after treatment and during one year of follow up. For evaluation of specificity of monoclonal antigen-in-stool test, blood and faecal samples from 241 children of similar age with non-gastrointestinal conditions were included.

Methods: In a prospective randomized double-blind treatment trial, children received a combination of lansoprazole and amoxicillin with either clarithromycin (LAC) or metronidazole (LAM). The antigen-in-stool test was used to determine *H. pylori* status in the treatment trial and in the reinfection study. Culture of *H. pylori* from biopsies was performed by standard methods. Susceptibility testing of *H. pylori* to all three antibiotics was performed by Etest using microaerophilic incubation for ≥72h at 35^{o}C.

Results: The sensitivity of Premier Platinum HpSA PLUS was 96.6% (95% CI 93.3-98.5%) and the specificity was 94.9% (95% CI 88.5-98.3%). The per protocol eradication rate was similar in the two treatment regimens, 62.1% for the LAM and 54.7% for the LAC regimens, respectively. The overall resistance to clarithromycin, metronidazole and amoxicillin was 50.9%, 65.3% and 0.5%, respectively. In LAC regimen, eradication was linked to the strains being sensitive (OR 7.23, 95% CI 2.10-24.9, relative to resistant strains). Twice-daily dosage was more effective for eradication of clarithromycin resistant strains than once-daily dosage (OR 6.92, 95% CI 1.49-32.13, relative to once-daily dose). Factual antibiotic dose per kilo body weight were significantly associated with eradication rates (OR 8.13, 95% CI 2.23-29.6). These differences were not seen for the LAM regimen. Low age was the most prominent independent risk factor for reinfection (adjusted HR among children aged 3-4, 5-6, and 7-8 years, relative to those aged 9-15 years, were respectively 14.3 [95% CI 3.8-53.7], 5.4 [1.8-16.3] and 2.6 [0.7-10.4]). Female sex tended to be associated with increased risk (adjusted HR among girls relative to boys 2.5, [95% CI 1.1-5.9]).

Conclusion: The antigen-in-stool assay has a good performance in Vietnamese children. The two triple therapies with methronidazole or clarithromycin gave similar and low eradication rates, likely due to high rates of antibiotic resistance that was unexpected for clarithromycin. The twice-daily medications play an important role in eradication of especially clarithromycin-resistant strains. Age was found to be the main risk factor for reinfection rate in Vietnamese children, with the youngest children running the greatest risk. The high rates of antibiotic resistance imply the need to investigate alternative eradication strategies and the high reinfection rates in the youngest children, if the medical condition permits, to delaying eradication treatment.

LIST OF PUBLICATIONS

I. **Evaluation of a novel monoclonal-based antigen-in-stool enzyme immunoassay (Premier Platinum HpSA PLUS) for diagnosis of *Helicobacter pylori* infection in Vietnamese children.** **Thi Viet Ha Nguyen**, Carina Bengtsson, Gia Khanh Nguyen, Marta Granström. **Helicobacter** 2008;13: 269-273

II. **Evaluation of two triple therapy regimens with metronidazole or clarithromycin for eradication of *H. pylori* infection in Vietnamese children: a randomized, double-blind clinical trial.** **Thi Viet Ha Nguyen**, Carina Bengtsson, Gia Khanh Nguyen, Thi Thu Ha Hoang, Dac Cam Phung, Mikael Sörberg , Marta Granström. **Helicobacter** 2008; 13: 550-556

III. **Eradication of *Helicobacter pylori* infection in Vietnamese children in relation to antibiotic resistance.** **Thi Viet Ha Nguyen**, Carina Bengtsson, Li Yin, Gia Khanh Nguyen, Thi Thu Ha Hoang, Dac Cam Phung, Mikael Sörberg, Marta Granström. (Submitted)

IV. **Age as risk factor for *Helicobacter pylori* reinfection in Vietnamese children.** **Thi Viet Ha Nguyen**, Carina Bengtsson, Gia Khanh Nguyen, Li Yin, Thi Thu Ha Hoang, Dac Cam Phung, Mikael Sörberg, Marta Granström. (Submitted)

CONTENTS

LIST OF ABBREVIATIONS

bid	Twice daily
CagA	Cytotoxin-associated protein
CI	Confidence interval
CLO test	Campylobacter like organism test
ELISA	Enzyme-linked immunosorbent assay
Etest	Epsilometer test
FISH	Fluorescent in-situ hybridization
GERD	Gastro-esophageal reflux disease
HR	Hazard ratio
IDA	Iron deficiency anaemia
ITP	Idiopathic thrombocytopenia purpura
LAC	Lansoprazole + amoxicillin + clarithromycin
LAM	Lansoprazole + amoxicillin + metronidazole
MALT	Mucosa-associated lymphoid tissue
MIC	Minimum inhibitory concentration
OD	Optical density
OR	Odds ratio
PCR	Polymerase chain reaction
PPI	Proton-pump inhibitor
qid	Four times daily
RAP	Recurrent abdominal pain
RUT	Rapid urease test
UBT	Urea breath test

1 INTRODUCTION

Helicobacter pylori (H. pylori) is one of the most common infections in humans, with an estimated 50% of the world's population being infected [1]. This organism has been implicated in the pathogenesis of active and chronic gastritis, peptic ulcer and gastric cancer [2]. Childhood has been identified as the critical time for acquisition of *H. pylori* infection [3-5]. The exact route of transmission remains unclear but the infection clusters in families and familial spread is thought to be the major mode of transmission, both in developed and developing countries [6, 7].

The ideal diagnostic test for *H. pylori* infection would be non-invasive, reliable, low-cost and easily accessible [5, 8]. Diagnosis of *H. pylori* infection generally relies on a combination of invasive and non-invasive methods [5, 8]. Endoscopic examination with sampling of the gastric and duodenal mucosa for histopathology, rapid urease test (RUT) and culture of the biopsies for *H. pylori* are invasive techniques [8, 9]. Culture of *H. pylori* is considered the gold standard for the diagnosis of the infection, but gastroscopy is more complicated in children than in adults [8]. The non-invasive techniques include urea breath tests, serologic assays and antigen-in-stool tests and are very useful for detecting *H. pylori* infection, especially in children [5]. These techniques are widely used and recommended as routine diagnostic tools in high-income countries but are not yet established in most developing countries.

Antibiotic therapy is used to eradicate *H. pylori* infection. The recommended treatment in adults is a triple-drug therapy consisting of a proton-pump inhibitor (PPI) and two antibiotics. Much less is known about the optimal eradication regimen in children. Bacterial resistance to antibiotics has developed all over the world and continues to increase [10, 11]. The prevalence of clarithromycin resistant *H. pylori* strains in children has been reported to vary from 5.9% to 45% [12, 13] and metronidazole resistance ranges from 9% to 95% [12, 13] while amoxicillin resistance ranges from 0% to 59% [12-14].

The high prevalence of metronidazole resistance of *H. pylori* in developing countries complicates the picture [13]. Nothing is known about the rate of metronidazole or clarithromycin resistance of *H. pylori* in children in Vietnam. Antibiotic resistance is increasing mainly because of frequent use of antibiotics for gastroenteritis and respiratory tract infection. The possibility of buying antibiotics over the counter in many developing countries aggravates the problem. Also, very little is known about the risk for reinfection in children in developing countries.

The main objectives of this thesis were to evaluate the performance of a non-invasive method antigen-in-stool test, to find optimal treatment strategies for *H. pylori* infection in relation to antibiotic resistance and to determine the one-year reinfection rate in Vietnamese children.

1.1. THE BACTERIUM

1.1.1. *Helicobacter* species

Spiral bacteria have been observed in gastric specimens of humans and animals over 100 years although the findings were not associated with the presence of gastric diseases [15]. In 1983, after isolation the bacterium was first named *Campylobacter pyloridis* because of its location and some common properties with *Campylobacter jejuni* [16, 17].

When the difference between *Campylobacter pylori* and *Campylobacter* organisms were confirmed by Goodwin et al in 1989 [18], the name was changed to *Helicobacter* and *Helicobacter 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 such as dogs (*H. cani*), cats (*H. felis),* pigs *(H. suis)* and rats (*H. hepaticus, H. rodentum, H. bilis*). In addition to gastric diseases the different non-*Helicobacter pylori* species can cause some other diseases including colitis, hepatic adenoma, adenocarcinoma in animals and liver diseases but also gastroenteritis and diarrhoea in humans [19]. The complete genome sequence of *H. pylori* consisting of a circular chromosome with a size of 1,667,867 base pairs has been reported and the extent of molecular mimicry between of *H. pylori* and human has been fully explored [20].

In 2005, Marshall and Warren were awarded the Nobel Prize in Physiology or Medicine for their discovery of the bacterium *H. pylori* and its role in gastritis and peptic ulcer disease.

1.1.2. Microbiology

H. pylori is a gram-negative, spiral shaped, microaerophilic bacterium, measuring 2 to 4 μm in length and 0.5 to 1 μm in width. It has 2 to 6 unipolar, sheathed flagella of approximately 3 μm in length that end in bulbs and gives the bacterium its motility in the mucus layer overlying the gastric epithelial cells [21]. Although usually in a spiral shaped form, the bacterium can convert to a non-cultivable coccoid form after prolonged *in vitro* culture or antibiotic treatment [22, 23]. *H. pylori* is commonly isolated from gastric biopsy samples of infected patients. Isolations of *H. pylori* can be also made form gastric juice, faeces and vomitus of infected patients [24, 25]. Some studies have reported detection of *H. pylori* in water but the relevance of these studies based on polymerase chain reaction (PCR) remains unclear [26-28].

H. pylori is a fastidious microorganism and requires complex growth media. The optimal environment for growth of *H. pylori* is microaerophilic at 37°C, O_2 levels of 2 to 5%, and the additional need of 5 to 10% CO_2 and high humidity [21]. 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 [29]. The agar plates used to culture *H. pylori* are supplemented with blood or serum and antibiotics such as vancomycin, trimethoprim, cefsoludin, and amphotericin B and polymyxin B [30]. 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. To facilitate optical detection of *H. pylori*, triphenyltetrazolium chloride (TTC) is supplemented in the plates in which *H. pylori* colonies develop a golden shine. Prolonged culture is not only unable to increase colony size but may also lead to a transition to the non-culturable coccoid form [21].

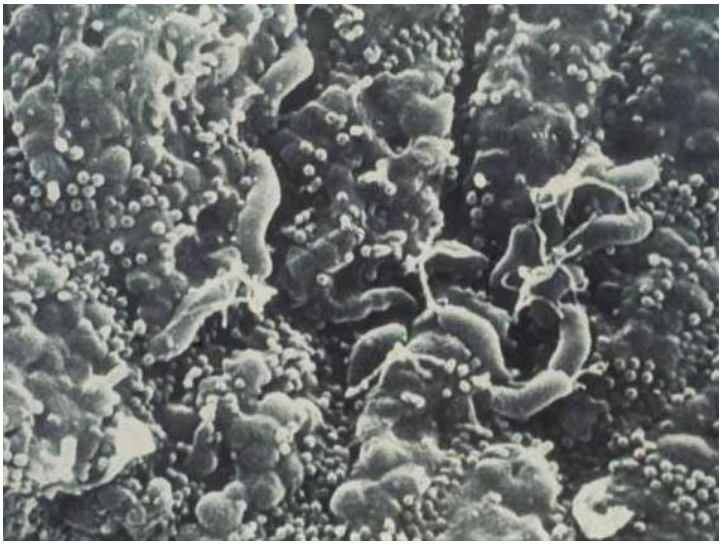

Figure 1. Electron photomicrograph of *H. pylori* colonizing the stomach of a human volunteer who ingested the organism as part of an experimental inoculation (Contributed with full permission from http://www.med.nyu.edu/medicin)

Identification of *H. pylori* is based on microscopic or colony morphology and biochemical characteristics including oxidase, urease and catalase positivity. 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 [31]. 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 [32]

1.1.3. *H. pylori* virulence factors

Disease associated factors include the *cagA* and *vacA* gene, which are the most studied genes. Several other genes have also been identified such as genes encoding outer membrane protein (OMP). These proteins are mostly reported in association with bacterial adherence such as the

blood group antigen-binding adhesion (BabA), outer inflammatory protein (OipA), sialic acid binding adhesion (SabA) [21]. Colonisation factors include enzymes (urease, catalase, oxidase), motility, adhesions and different proteins.

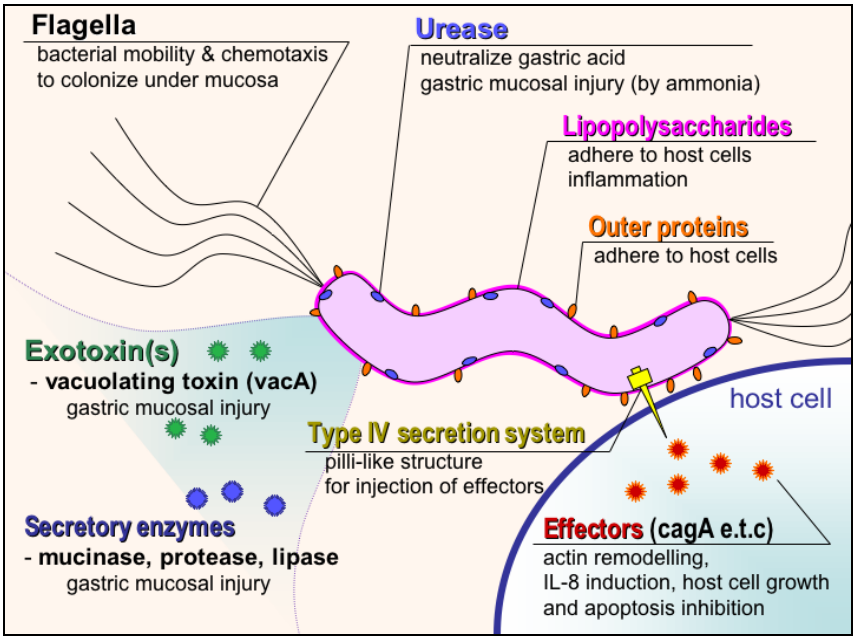

Figure 2. *H. pylori* virulent factors

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The cytotoxin-associated protein (CagA), which is highly immunogenic, is encoded by the *cagA* gene [21]. CagA is involved in binding and perturbing the function of epithelial junction resulted in aberration in junction function, cell polarity and cellular differentiation [33]. The *cagA* gene is located in a large pathogenicity island (*cag*PAI), a region of horizontally acquired DNA that was inserted into the genome of the more virulent *H. pylori* strains [33].

The vacuolating cytotoxin (VacA**),** encoded by the *vacA* gene, is another major virulence factor of *H. pylori* that plays an important role in the pathogenesis of peptic ulcer and gastric cancer [34-37]. Almost all *H. pylori* strains harbour the *vacA* gene, however, genetic variability in the *vacA* gene results in different cytotoxic properties. Approximately 50% of all *H. pylori* strains secrete VacA. It is a highly immunogenic 95-kDa protein that induces massive vacuolization in epithelial cells *in vitro* [21]. Currently, three diversified regions in the gene have been identified. The s region of the gene that encodes the signal sequence (s1a, s1b, s1c and s2) while the middle region of the gene is classified as the m region that encodes part of the 58-kDa domain of VacA (m1, m2a, m2b) [34, 37]. The intermediate i region has recently been described in two variants, i1 and i2, the i1 being associated with gastric cancer [38, 39].

Two distinct types of CagA called Western and Eastern forms have been described. Eastern CagA

is considered being associated with more severe inflammation, peptic ulceration and gastric cancer. In Asia, the incidence of gastric cancer is very high in Eastern Asia (Japan, Korea) with low prevalence rates of *H. pylori* infection whereas the cancer rate is intermediate (Vietnam, Singapore) or low (Thailand, Indonesia, Bangladesh) in highly infected populations. This Asian enigma or paradox has been explained by a combination of different virulence factors, including intact *cag*PAI, Eastern *cagA* and *vacA*s1/m1/i1, in strains infecting different populations [40].

1.2. EPIDEMIOLOGY

1.2.1. Prevalence of *H. pylori* infection

According to World Health Organisation (WHO) report, approximately 50% of the world's population is being infected by *H. pylori* [1, 41]. The prevalence of *H. pylori* infection is significantly higher in developing countries than in developed countries (Tables 1 and 2).

Table 1. Prevalence of *H. pylori* infection in children in developed countries

Country	Author/Year	Number of children	Age (years)	Method	Prevalence (%)
Australia [42]	Moujaber 2008	151	1-4	ELISA (IgG)	4.0
		150	5-9		6.0
		301	10-14		8.3
		300	15-19		10.0
Belgium [43]	Lanciers 1996	883	0-17	ELISA (IgG)	8.2
Finland [44]	Rehnberg-Laiho 1998	337	0-20	ELISA (IgG)	5.6
France [45]	Raymond 1998	623	1-15	ELISA (IgG)	15.8
Germany [46]	Bode 2002	824	9-13	ELISA (IgG)	19.8
Italy [47]	Dore 2002	2810	5-16	ELISA (IgG)	22.0
Japan [48]	Kato 2003	454	0-15	ELISA (IgG)	12.2
Sweden [49, 50]	Granstrom 1997	294	2	ELISA (IgG)	10.0
		294	11		3.0
	Tindberg 2001	695	10	ELISA (IgG)	16.0
Switzerland [51]	Boltshauser 1999	432	5-7	ELISA (IgG)	6.5
UK [52]	O'Donohoe 1996	640	4-13	ELISA (IgG)	16.7
US [53]	Opekun 2000	797	0.5-18	ELISA (IgG)	12.2

Table 2. Prevalence of *H. pylori* infection in children in developing countries

Country	Author/Year	Number of children	Age (years)	Method	Prevalence (%)
Brazil [54]	Parente 2006	303	0.5-12	Antigen in stool	38.0
Cameroon [55]	Ndip 2004	32	0-3		37.5
		106	3-6	Antigen in stool	50.0
		38	7-10		71.0
China [56]	Zhang 2009	1036	8-15	Antigen in stool	31.7
Iran [57]	Alborzi 2006	593	0.75 -15	Antigen-in-stool	82.0
Liban [58]	Naous 2007	414	0-3		28.7
			4-9	Antigen in stool	34.5
			10-17		36.8
Malaysia [59]	Boey 1999	514	0.5-17	ELISA (IgG)	10.3
Pakistan[60]	Jafri 2009	1976	0 - 15	ELISA (IgG)	47.0
South Africa [61]	Pelser 1997	104	0.25-2		13.5
		103	2-5	ELISA (IgG)	48.5
		104	5-10		67.3
		101	10-15		84.2
Tunisia [62]	Siai K 2008	1055	6-7	ELISA (IgG)	51.4
Turkey [63]	Ceylan 2007	275	1-15	ELISA (IgG)	23.6
Vietnam [64, 65]	Hoang 2005	30	0-4		33.3
		59	5-9	ELISA (IgG)	49.2
		52	10-14		69.2
		83	15-19		78.3
	Nguyen 2006	824	0.5 - 15	ELISA (IgG)	34.0

In developing countries, sero-prevalence of *H. pylori* has found to be 50-75% in children with a plateau of 80-90% during adulthood [41] whereas in developed countries, childhood sero-prevalence is 10-20% and increases to 40-60% by 60 years of age [66]. In Sweden the sero-prevalence in the Stockholm population reaches a high of 50% in the age group 80 years and older [67].

As noted previously, childhood has been identified as the critical time for acquiring *H. pylori* infection and thus the increase in infection prevalence reflects the so called cohort effect, which means that older generations were more at risk to be infected in childhood than younger individuals. Identified or proposed risk factors for acquiring the infection include infected family

members [4, 7, 63, 68], large family size [50, 62], crowding [60, 62, 69, 70], poor socioeconomic status of the family [50, 54, 62, 69], nutritional status [71], urban residence [63, 64], institutional residence [72, 73], consumption of raw vegetables [74, 75] and swimming in rivers [26, 74].

1.2.2. Transmission of *H. pylori* infection

The transmission route of *H. pylori* infection has not been clarified. However, the most probable transmission route strongly suggested by epidemiological studies is person-person by oral-oral, gastro-oral and faecal-oral pathways [41].

1.2.2.1. 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 [24]. Several studies using PCR amplification from saliva and dental plaque have demonstrated the presence of *H. pylori* in the mouth [24, 76]. Close mouth to mouth contact has been identified as a risk factor for oral-oral transmission [77, 78]. Cultural and social differences such as pre-mastication of food and sharing chopstick in African and 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 [78]. This report was in line with the result from some other studies about the association between the high prevalence of *H. pylori* infection in children and pre-chewed food feeding [79]. In addition, the data from one study conducted by Chow et al [77] showed that the infection prevalence of people who used chopstick to eat from communal dishes was significantly higher than in those who did not (64.8% *versus* 42.3%).

1.2.2.2. 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 [24]. Also, an increased acquisition of *H. pylori* in children during a gastroenteritis outbreak has been reported in a rehabilitation centre [80]. The gastro-oral transmission route always 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 [81, 82]. The retrospective study in the Netherlands conducted by Langerberg et al found that 1.1% *H. pylori* negative patients became positive after having undergone a gastroscopic examination [83].

1.2.2.3. Faecal-oral transmission

The faecal-oral route is another potential route of transmission. 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 [27, 84, 85] although it is difficult to detect *H. pylori* in faecal samples by DNA methods because of potential inhibitors. In addition, the bacterium has been isolated by culture of faecal samples in several studies [24, 25, 84]. This mode of transmission has been proposed to commonly occur in developing countries because of limitations in hygiene conditions and high risk of diarrheal diseases [21, 41]. 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 [24, 41].

1.3. DISEASES ASSOCIATED WITH *H. PYLORI* INFECTION

H. pylori infection is the cause of chronic gastritis, atrophic gastritis, peptic ulcer disease, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma. *H. pylori* has been classified as grade I carcinogen by the WHO's International Agency for Research on Cancer in 1994 [2].

1.3.1. Gastritis

H. pylori has been accepted to be associated with the development of chronic gastritis. The association between acute gastritis and *H. pylori* infection was first observed by Warren and Marshall when the latter developed acute gastritis several days after drinking a pure culture of *H. pylori* [86]. Recently, the same association has also been reported in a study conducted in 20 volunteer where 90% infected with a CagA-negative *H. pylori* strain from a non-ulcer dyspepsia patient. The infected individuals showed signs of active and chronic gastritis confirmed by UBT, culture and histology [87]. Oberhuber et al had shown the improvement of gastric mucosa after successful eradication of *H. pylori* [88].

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. 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 [1]. 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 or an ulcer process [21]. Although *H. pylori* has been recognized as a cause of chronic gastritis in children [89], only a minority of infected patients present with specific symptoms [1]. Many studies have demonstrated the association of *H. pylori* colonization in gastric mucosa and

chronic gastritis [89-92]. In *H. pylori* infected children, a monocyte and macrophage response was seen in infected mucosa whereas in infected adults both polyphonuclear cells and plasma cells were seen [93]. In this study, Whitney et al also found chronic, macrophage, monocyte inflammatory cell infiltrate in early infected children and a lack of neutrophils compared with the response observed in infected adults. The association between antral nodularity and *H. pylori* infection have been described in adult studies. This significant association has also been found in children [90-92]. There are few studies reporting the presence of atrophic gastritis in *H. pylori* infected children [94-96].

1.3.2. Peptic ulcer disease

H. pylori infection is considered as a major factor in the pathogenesis of peptic ulcer as *H. pylori* was found in 90-100% of duodenal ulcer and 60-100% of gastric ulcer adult patients [97]. Peptic ulcer is defined as a mucosal defect with a diameter of at least 0.5cm penetrating through the muscularis mucosa [21]. Similarly to adults, most of *H. pylori* infected children have asymptomatic infections and only a minority of them develop peptic ulcer. *H. pylori* has been found in 62-91% of children with peptic ulcer diseases [91, 98, 99]. As data concerning peptic ulcer in children are limited, it is difficult to estimate the peptic ulcer prevalence. The prevalence of peptic ulcer in dyspeptic children ranges from 2% in developed countries [98] to 6.8-24% in developing countries [100, 101]. The causative relationship between *H. pylori* infection and peptic ulcer in children is supported by the improvement of duodenal ulcer after *H. pylori* eradication [102, 103].

1.3.3. Gastro-esophageal reflux disease

The association between *H. pylori* and gastro-oesophageal reflux disease (GERD) is still strongly controversial in adult studies [104]. As antral gastritis increases gastric acidity, which might result in increased risk of GERD, *H. pylori* eradication could reduce the risk of acid reflux [105]. In contrast, there are several studies reporting an increased prevalence of GERD after *H. pylori* eradication, suggesting that *H. pylori* might protect against the development of GERD [106, 107]. One meta-analysis in adult showed a lower prevalence of *H. pylori* among patients with GERD, suggesting the protective role of *H. pylori* in GERD [108].

Similar to the studies in adult patients, the relationship between *H. pylori* and GERD in children is still not fully clarified. One study conducted by Nijevitch et al [109] in 42 children with asthma reported the protective effect of *H. pylori* infection in GERD, while a causative association between *H. pylori* and GERD was reported by Gold et al [110]. Children infected with *H. pylori*, especially with cagA positive strains that are considered to be more virulent, had a higher risk of developing serious diseases including gastritis and oesophageal diseases. The study conducted by

Elitsur et al [111] in 150 children showed a lack of significant relationship between *H. pylori* infection and histologic oesophagitis in children before treatment. In contrast, the association between *H. pylori* infection and GERD was reported in two studies [112, 113]. As *H. pylori*-induced hypochlorchydria is rare in children [114] and GERD in children is related to transient abnormality in lower oesophageal sphincter function, further studies are needed to clarify the potential interaction between *H. pylori* and GERD in children [1].

1.3.4. Recurrent abdominal pain

Recurrent abdominal pain (RAP) is defined by the presence of at least three discrete episodes of pain, debilitating enough to interrupt normal daily activities or performance and occurring over a period of ≥3 months during the year preceding clinical examination [1]. The role of *H. pylori* infection in the development of RAP and functional gastrointestinal disorder is still controversial although numerous studies have addressed the association between *H. pylori* infection and RAP. Several studies could not demonstrate the association between *H. pylori* and RAP. Hardikar et al [115] conducted a prospective case-control study in 196 children, reported the negative association between *H. pylori* infection in the development of RAP. Several results were in line with this evidence suggesting that *H. pylori* is unlikely to have an important etiologic role this disorder [116, 117].

There are, however, several studies supporting the association between *H. pylori* and RAP based on the improvement of symptoms after eradication [118-122]. Prevalence of *H. pylori* has been found to be higher among dyspeptic children as compared to asymptomatic ones [121, 123] whereas no or negative association was found in population based studies [124-126]. Since the findings from different studies are inconsistent, the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) consensus statement on *H. pylori* in children concluded that there is no evidence for an association between *H. pylori* and RAP [127]. Further well-designed studies are needed to clarify this isssue.

1.3.5. Gastric cancer and MALT lymphoma

H. pylori has found to be linked with gastric cancer and MALT lymphoma. The association of *H. pylori* and gastric adenocarcinoma has been confirmed by large-scale epidemiological, meta-analysis of case control and experimental studies [2]. One of the most significant studies was conducted by Uemura et al [128]. After a mean follow up of 7.8 years in 1246 *H. pylori*-positive and 280 *H. pylori* negative subjects, gastric cancer was detected in 2.9% of *H. pylori* infection compared with 0% in the uninfected patients. According to the EUROGAST study group report, *H. pylori* infection increases the risk of gastric cancer approximately by six times in infected

populations compared with populations that have no infection [1]. As gastric cancer rarely occurs before the age of 40 years, children are considered not to develop gastric cancer.

H. pylori infection is also associated with the development of lymphoma arising from the MALT of the stomach. The most compelling evidence for the causative relationship between *H. pylori* infection and MALT lymphoma is that 75% of gastric MALT lymphoma regress after *H. pylori* eradication treatment [129]. Chronic antigenic stimulation from *H. pylori* infection acquired during childhood is associated with development of lymphoid follicles, the precursor lesion to MALT lymphoma [130]. Evidence for the causative relationship between *H. pylori* and MALT lymphoma in children has been reported in several studies [131-133]. Patients with MALT lymphoma had gone into remission after *H. pylori* eradication but relapse may occur [130, 134].

1.3.6. Extra-gastrointestinal manifestations

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

Numerous studies have shown the association between *H. pylori* infection and IDA [135-137]. The improvement of anaemia after *H. pylori* eradication therapy has been reported [136-139]. In contrast, there are several studies showing no improvement of *H. pylori* infection in IDA [140, 141]. 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 [142].

The role of *H. pylori* in the pathogenesis of IPP was firstly described in 1998 by Gasbarrini et al [143] in an adult study. A correlation between *H. pylori* eradication therapy and the improvement of IPP outcome has been reported in one meta-analysis in adults [144]. Similarly to adults, normalization of platelet count after *H. pylori* eradication therapy in several studies provided evidence for a causative role in a subset of IPP children [145-147]. Spontaneous improvement in platelet counts makes it however difficult to determine the true benefit of *H. pylori* eradication therapy in IPP [148]. *H. pylori* eradication therapy did not result in an improved outcome of acute IPP in children [149, 150]. In addition, no association between *H. pylori* and IPP was report in one retrospective cohort study conducted by Jaing et al [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 [152].

H. pylori infection has been thought to influence the growth rate in children but this issue is still controversial. Several studies have suggested that *H. pylori* infection might lead to malnutrition and growth retardation in children [139, 153-155] whereas other studies produced conflicting results [156-158]. The potential confounding factor could be poor socioeconomic status

that may contribute to both development of malnutrition and early *H. pylori* infection. The possible mechanism for the association may be secondary malabsorption to the suppression of gastric acid secretion, leading to gastrointestinal infections and diarrhoea and decreased oral intake associated with decreased appetite that would result in reduced growth, decreased immunity and repeated infection [159].

1.4. DIAGNOSIS OF *H. PYLORI* INFECTION

Infection of *H. pylori* can be diagnosed by either invasive techniques requiring endoscopy and biopsy or non-invasive methods. Invasive techniques involve an upper gastroscopy with gastric biopsy samples that can then be used for rapid urease test (RUT), histology, culture, polymerase chain reaction (PCR) and fluorescent in-situ hybridization (FISH) tests whereas non-invasive methods include antibody detection, antigen-in-stool test and urea breath test. The methods all have advantages and disadvantages and may have different indications for use. No single test is currently available to provide a fully reliable method for *H. pylori* detection.

1.4.1. Invasive methods

1.4.1.1. Endoscopy

Endoscopy with biopsy samples is considered as the gold standard in *H. pylori* identification as the bacteria can be demonstrated either by culture or some other direct bacterial detection method. Since most children infected with *H. pylori* are asymptomatic endoscopy is mainly performed in children with gastrointestinal symptoms [8, 127]. Endoscopy is the only reliable method for diagnosis of peptic ulcer and also allows identifying the cause of other upper gastrointestinal disorders e.g. GERD and eosinophilic oesophagitis. Gastric biopsies taken from endoscopy can be used for histology, culture and antibiotic susceptibility testing, which play an important role for treatment therapy in symptomatic children [5, 160, 161]. Endoscopy is not suitable for epidemiological studies due to ethical and practical considerations.

Due to the lack of non-invasive method to diagnose *H. pylori* infection in Vietnam, endoscopy is the only available diagnostic method to obtain biopsy samples for both RUT and histology. The endoscopic procedure is a complicated technique in children thus it is only performed by experienced gastroscopists at the large hospitals with intravenous sedation. For this reason, endoscopy is a time consuming procedure that is costly and causes discomfort to the children.

1.4.1.2. Rapid urease test

Rapid urease test can determine the presence or absence of urease activity that is only produced by *H. pylori* in large quantities in gastric biopsies [9, 162]. Therefore RUT can be considered as proof of the *H. pylori* infection if the test is correctly performed [163]. A biopsy sample is immersed in the medium containing urea and if *H. pylori* is present the urease activity will break down urea resulting in a rise in pH within one hour, which is then detected by a pH indicator, usually phenol red, changing from yellow to red colour [164].

The sensitivity and specificity of RUT varies from 75% to 100% and 84% to 100% [9, 161]. Though sensitivity of RUT is affected by the number of bacteria in the sample and urease enzyme inhibitors such as antibacterial agents, proton-pump-inhibitors and bismuth containing compounds, the test is widely used in clinical practice [1, 9]. The specificity of the assay is the highest when read after one hour. When read after more than one hour, many false-positive results are seen [162, 164]. The sensitivity of RUT has however been reported to be lower in children [8, 165].

Several RUTs are available commercially, e.g. CLO-test, PyloriTek, HUT, Helicocheck and HP-fast [9]. There are two commercial RUTs, CLO-test and PyloriTek, available in Vietnam but not commonly used for patients in hospitals because of costs. Instead, the Division of Enteric bacterial infections, National Institute of Hygiene and Epidemiology (NIHE) produces an in-house urease test that is read after one hour. This RUT was found to have a specificity of 98% in a study of adult Vietnamese peptic ulcer patients [166].

1.4.1.3. Culture

Culture is considered as the most specific method for *H. pylori* detection although its sensitivity varies significantly among laboratories [161, 167]. The colonies are Gram negative, urease, oxidase and catalase positive [9, 162]. The accuracy of culture depends on the condition in which the specimens are transported and processed [168]. Because of slow bacterial growth and specific medium condition requirement, culture is a complicated and time-consuming procedure and not necessary for the routine diagnosis of *H. pylori* infection [8, 167]. Culture is 100% specific, has the advantage of allowing for antibiotic sensitivity testing and for characterization of *H. pylori* strains [9, 162, 168]. In Vietnam, *H. pylori* culture is not available for children with gastrointestinal symptoms. Patients receive eradication treatment based only on endoscopy, RUT and histology.

1.4.1.4. 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* [17].

Presence of *H. pylori* can also be detected by other stains such as modified Giemsa, hematoxylin eosine, Genta, toluidine blue, Romanouski and immunochemical methods [9, 161, 168].

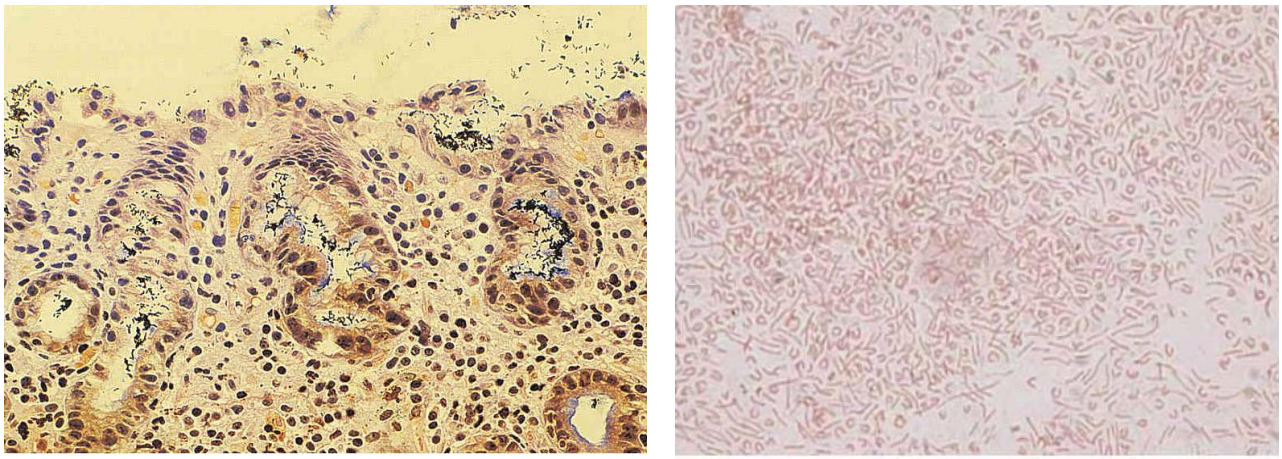


Figure 3. *H. pylori* organisms demonstrated with silver and gram stain (Contributed with full permission from http://www.pathoconsultddx.com and http://www.med.nyu.edu/medicin)

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, intestinal metaplasia and glandular atrophy [32]. Histology for detection of *H. pylori* can be a reliable method but its performance depends on the number and localization of biopsy specimens and on the qualification of the pathologists [1, 164]. The sensitivity and specificity of histology have been reported range from 66% to 100% and 94% to 100%, respectively [21, 161].

In Vietnam, the presence of *H. pylori* in histological examination is usually demonstrated by Giemsa staining and hematoxylin eosine. Recently, brush cytology was evaluated and shown high sensitivity to confirm *H. pylori* infection in Vietnamese children [169].

1.4.1.5. Molecular techniques

Polymerase chain reaction (PCR)-based techniques have been used for detection of *H. pylori* from gastric biopsy samples, gastric juice, stool and saliva [170-173]. The sensitivity and specificity for *H. pylori* infection using PCR-based techniques has been reported at 85% to 100% and 100% respectively when compared to other endoscopy-based tests [171, 173, 174]. The main application for these techniques would be when specimens for culture were compromised by bacterial overgrowth or non-viable *H. pylori* strains due to unfavourable transport conditions [175]. When used as a non-invasive technique, PCR-based methods have not been found to be as sensitive as alternative techniques [176]. PCR is not established in routine clinical practice in most laboratories, and is mainly used for research purposes [177]. In Vietnam, molecular methods to diagnose *H. pylori*

infection are not available.

1.4.1.6. Antibiotic susceptibility testing

Since resistance to antibiotics is considered to be one of the most important variables predicting the outcome of eradication therapy, antibiotics susceptibility testing plays an important role in *H. pylori* eradication treatment. The two methods used to investigate antibiotic resistance are antibiotic susceptibility testing and molecular methods. The agar dilution method is considered as reference method to evaluate other techniques from gastric biopsy specimens [178] whereas broth dilution method has rarely been used because of the difficulty to grow *H. pylori* in broth cultures [179]. The E-test has proven to be an accurate method to be test susceptibility to antibiotics of fastidious organisms, including *H. pylori* [180]. The advantage of Etest is its ability to express MIC values, which is defined as the point of intersection of the zone edge with the graduated scale of the plastic strip carrying the antibiotic. A good correlation between Etest and agar dilution methods for amoxicillin and clarithromycin has been found but not so for metronidazole [180, 181]. As a consequence, the Maastricht III Consensus Report did not recommend routine metronidazole susceptibility testing [152].

The mechanisms of *H. pylori* antibiotic resistance is mainly based on point mutations that can be detected by molecular methods [182]. Molecular tests used to investigate antibiotic resistance are PCR-restriction fragment length polymorphism (RFLP), PCR-oligonucleotide ligation, PCR-dependent preferential homoduplex formation assay (PCR-PHFA), real-time PCR and fluorescence in situ hybridisation (FISH). A majority of mutation inducing clarithromycin resistance will be identified by the most common techniques and can be of value as complement to culture, especially when culture yielded false negative results due to overgrowth of other bacteria or non-viable *H. pylori* in extended transports [175]. Since antibiotic susceptibility testing is based on either culture or molecular methods, it does not exist in Vietnam.

1.4.2. Non-invasive methods

1.4.2.1. Serology

Since *H. pylori* infection produces a systematic antibody response, *H. pylori*-specific antibodies can be detected in serum and plasma. *H. pylori* infection is a chronic condition, IgM only increases in acute phrase and IgA is not elevated in all cases for which reasons most commercial kits are based on IgG detection. The main immunological methods are enzyme-linked immunosorbent assay (ELISA) and immunoblot (IM).

Enzyme-linked immunosorbent assay is the most frequently used method for quantitative determination of IgG antibodies [9]. Antigens used in ELISA must be highly immunogenic,

common to most *H. pylori* strains and absent from other bacteria. They have to be easy to prepare and purify and they must bind to the wells of microplates and be stable upon storage. Many different types of antigen preparation can be used in ELISA such as whole-cell sonicates, glycine acid extraction and purified urease [164]. The performance of ELISA is largely dependent on the antigen used, the clinical context, the reference method and the prevalence of *H. pylori* in the investigated population [164, 168]. In one meta-analysis of ELISA-IgG studies in children, the sensitivity, specificity, positive and negative likelihood ratios are 79.2%, 92.4%, 10.2 and 0.19, respectively [183].

A qualitative antibody assay is based on Western blotting. The specific antigens are separated by gel electrophoresis, transferred to a filter-paper strip and reacted with the patient's serum sample [9]. The main advantage of immunoblot is its ability to detect antibodies to specific *H. pylori* antigens. The most commonly used commercial immunoblot test is Helicoblot and its later version Helicoblot 2.1 (MP Diagnostics, Singapore), found in a meta-analysis of studies in children to have a sensitivity, specificity, positive and negative likelihood ratios of 91.3%, 89%, 8.2 and 0.06, respectively [183].

Since the advantages of serological tests are relatively low costs, accuracy, ease of performance and availability, they are widely used in routine diagnostic laboratories and for epidemiology studies in developed countries. 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 [161, 164]. Most commercial tests have been evaluated in developed countries so the performance of serological tests needs to be validated in the population investigated [152, 184]. In Vietnam, serological tests have been validated for use since 2003 and have been applied for *H. pylori* detection but only in research contexts.

1.4.2.2. Urea breath test

Among non-invasive diagnostic methods, urea breath test (UBT) is considered to be the most accurate test for detection of *H. pylori* infection [185, 186]. 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 [185, 187].

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. UBT can be considered as a gold standard method, especially if endoscopy is not indicated. However, the

performance of UBT is reported to be unreliable in patients recently receiving medications suppressing *H. pylori* such as PPIs or bismuth-containing compounds or antacids [9, 185]. Most experts suggest that PPIs should be stopped for 2 weeks and bismuth or antacids should be withheld for at least four weeks before performing the UBT [185, 186].

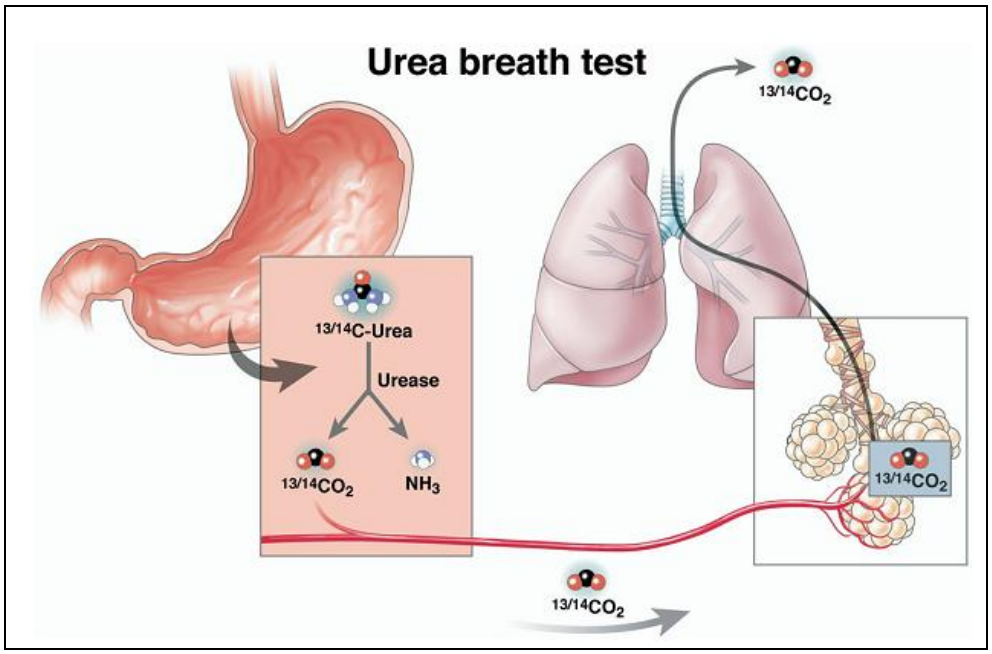


Figure 4. Schematic for urea breath test testing [185]

The ^{14}C UBT is inexpensive and easy to perform since it needs only a scintillation counter to measure radioactivity of breath sample. The sensitivity and specificity of the test are 92.3-97% and 85.5-90%, respectively [188, 189]. Several dose of ^{14}C labelled urea have been used in clinical studies, ranging from 37 kBq (1µCi) to 185 kBq (5µCi) [187]. The disadvantage of this test is the use of the radioactive isotope ^{14}C that has a long half-life and the concern that it causes for the amount of radiation. However, 90% of ^{14}C from urea breath test is eliminated as CO_2 in the breath or urine after 3 days and the amount of isotope retained in the body is negligible. Furthermore, the total cumulative lifetime radiation from each test has been calculated to be equal to the daily background radiation exposure of the radioactive isotope [190]. Thus, the 1µCi ^{14}C dose has been used for general practice in the US without restriction, while ^{14}C UBT has not been recommended in children and pregnant women in European countries [187].

The ^{13}C UBT is similar to the ^{14}C UBT except that ^{13}C is non-radioactive isotope of ^{12}C and its detection requires a mass spectrometer or a non-dispersive isotope-selective infrared spectrometer. Since ^{13}C is the natural and non-radioactive isotope, ^{13}C UBT can be used in children and women without restriction. In children 5 years of age and older, the ^{13}C UBT has been

demonstrated to have satisfactory performance with a sensitivity and specificity of 96-98% and 96-99% [161, 185], respectively but it may be less accurate in the very young children, i.e. less than 2 years of age [127]. ^{13}C UBT has been suggested for monitoring *H. pylori* eradication after treatment [127, 152]. A disadvantage of ^{13}C UBT is that the technique requires expensive equipment and is therefore unavailable in many countries.

1.4.2.3. Stool antigen assay

The most recent non-invasive methods 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. One meta-analysis evaluating the antigen-in-stool test in adults showed a good performance with sensitivity and specificity of 94% and 97%, respectively [191]. Data from the study also showed that the monoclonal test is more accurate than the polyclonal test. The accuracy of antigen-in-stool tests as a method for the primary diagnosis of *H. pylori* infection has been evaluated in many paediatric studies (Table 3)

A recent study suggested that the stool test and the UBT are equally accurate for the initial diagnosis of *H. pylori* infection in children [192]. Consequently, the US Food and Drug Administration (FDA) and the European Helicobacter pylori study group have recommended the use of antigen-in-stool test for this purpose. Furthermore, antigen-in-stool has been suggested as an alternative method in the monitoring of post-treatment outcome if UBT is not available [152, 191]. Stool tests are of special importance in paediatric populations where endoscopy may need either sedation or general anaesthesia. Stool samples can easy be obtained from children without their collaboration while UBT in very young children who are not able to collaborate might give false negative results. However, stool samples are sensitive to storage at room temperature and need to be frozen immediately after collecting. Also, false negative results may occur in patients who are on medications such as antibiotics, proton pump inhibitors or bismuth compounds. A further disadvantage of the antigen-in-stool tests for developing countries is the high costs of the kits.

Table 3. Antigen-in-stool test results in children

First author, year	Type of test	Number of children	Antigen-in-stool		Reference
			Sensitivity %	Specificity %	
Makristathis, 1998	Polyclonal	100	88.9	94.6	[193]
Makristathis, 2000	Polyclonal	49	94.6	97.5	[194]
	Monoclonal	49	98.2	98.1	
Shepherd, 2000	Polyclonal	119	88.0	82.0	[195]
Ni, 2000	Polyclonal	53	93.0	100	[196]
Braden, 2000	Polyclonal	162	91.6	98.6	[197]
Oderda, 2000	Polyclonal	203	100	93.0	[198]
Konstantopoulos, 2001	Polyclonal	274	88.9	94.0	[199]
van Doorn, 2001	Polyclonal	106	100	92.0	[200]
Cardinali, 2003	Polyclonal	210	96.9	100	[201]
Koletzko, 2003	Monoclonal	302	98.0	99.0	[202]
Hino, 2004	Monoclonal	78	97.5	94.7	[203]
Kato, 2004	Polyclonal	123	98.3	98.4	[192]
Elitsur, 2004	Polyclonal	121	67.0	99.0	[204]
Shaikh, 2005	Polyclonal	86	76.0	61.0	[205]
Mégraud, 2005	Polyclonal	316	72.9	97.3	[206]
Sabbi, 2005	Polyclonal	250	97.0	98.0	[207]
Raguza, 2005	Polyclonal	127	94.6	96.5	[208]
Dondi, 2006	Polyclonal	184	93.3	98.7	[209]
Frenck, 2006	Polyclonal	97	71.0	76.0	[210]
	Monoclonal	94	93.0	88.0	
Kolho 2006	Polyclonal	48	95.0	100	[211]
	Monoclonal	102	95.0	90.0	
Rafeey 2007	Polyclonal	96	54.8	79.4	[212]
Yang 2008	Polyclonal	146	94.6	96.1	[213]
Ritchie 2009	Monoclonal	52	55.0	68.0	[214]

1.5. TREATMENT OF *H. PYLORI* INFECTION

Eradication of *H. pylori* infection provides significant benefits in many disease conditions, e.g. peptic ulcer disease and MALT lymphoma. According to the Maastricht III consensus report document, RAP is not an indication for a test and treat strategy for *H. pylori* infection in children [152]. It is important to determine the cause of presenting gastrointestinal symptoms and not the presence of *H. pylori* infection in children with RAP. Children with upper gastrointestinal symptoms should however be tested for *H. pylori* infection after exclusion of other causes for the symptoms and treated if they have the infection [152]. A definite indication for *H. pylori* eradication therapy is children with peptic ulcer disease and *H. pylori* positive status [8]. Iron deficiency anaemia refractory to iron supplementation is another indication for *H. pylori* testing and for eradication treatment if positive after exclusion of other chronic conditions such as celiac and inflammatory bowel diseases.

Triple therapy using two antibiotics and a proton pump inhibitor (PPI) has been shown in a meta-analysis to achieve *H. pylori* eradication in 81-84% of adults [215]. The optimal regimens for eradication of *H. pylori* infection in children have however not been determined. Two major factors influence treatment outcome, i.e. antimicrobial resistance and patient compliance to treatment. A meta-analysis of 80 paediatric studies showed a wide variety of treatment efficacies [216]. As in adults, mono-therapies and dual therapies using PPI with or without an antibiotic resulted in low eradication rates. Eradication rate of mono-therapy regimen using amoxicillin 50mg/kg/day for 4 weeks was 27%. A combination of either bismuth, nitroimidazole, PPI, macrolid, H2-receptor antagonist or ranitidine bismuth subcitrate gave eradication rates of 28.2-83.6%. These results suggested that mono-therapy and dual therapies should not be considered as a treatment option in children because they are not effective and can cause acquired antibiotic resistance [8, 216].

A combination of two antibiotics and PPI gave better eradication rates also in children.
For initial treatment, three first line therapy options have been recommended for use in children. Dosages based on body weight for use in children have been provided in the clinical practice guideline of the North American Society for Paediatric Gastroenterology, Hepatology and Nutrition (Table 4) [5, 8].

One of the most commonly used regimens consists of a combination of a PPI, amoxicillin and clarithromycin that was evaluated in 43 treatment arms and 1771 children with eradication rates of 29-100% [216]. This combination provided 80% eradication rate in European children and 65% in children in developing countries when a UBT was used to assess *H. pylori* status. Efficacy of this combination was not affected by treatment duration.

Table 4. Recommended eradication therapies for *H. pylori* infection in children [8]

Drug regimens	Medications	Dosage
First line options		
1	amoxicillin	50mg/kg/day up to 1g bid
	clarithromycin	15mg/kg/day up to 500mg bid
	PPI: omeprazole or comparable acid inhibitory doses of another PPI	1mg/kg/day up to 20mg bid
2	amoxicillin	50mg/kg/day up to 1g bid
	metronidazole	20mg/kg/day up to 500mg bid
	PPI: omeprazole or comparable acid inhibitory doses of another PPI	1mg/kg/day up to 20mg bid
3	metronidazole	20mg/kg/day up to 500mg bid
	clarithromycin	15mg/kg/day up to 500mg bid
	PPI: omeprazole or comparable acid inhibitory doses of another PPI	1mg/kg/day up to 20mg bid
Second line options		
4	bismuth subsalicylate	1 tablet (262mg) qid or 15ml (17.6mg/ml qid)
	metronidazole	20mg/kg/day up to 500mg bid
	PPI: omeprazole or comparable acid inhibitory doses of another PPI	1mg/kg/day up to 20mg bid
	Plus an addition antibiotic:	
	amoxicillin	50mg/kg/day up to 1g bid
	Or clarithromycin	15mg/kg/day up to 500mg bid
	Or tetracycline [a]	50mg/kg/day up to 500mg bid
5	Ranitidine bismuth-citrate	1 tablet qid
	metronidazole	20mg/kg/day up to 500mg bid
	clarithromycin	15mg/kg/day up to 500mg bid

Initial treatment should be provided in a twice daily regimen for 7 to 14 days

[a]: only for children 12 years of age or older

bid: twice daily, qid: four times daily

Another common treatment regimen includes a PPI, amoxicillin and nitroimidazole and was evaluated in 10 treatment arms and 368 children, providing eradication rates of 74-100% [216]. The eradication rates of regimens that combined a PPI, a macrolid (clarithromycin or spiramycin) and nitroimidazole in 230 children and 10 treatment arms ranged from 51% to 93% [216].

Quadruple therapies are recommended for patients in whom the initial treatment has failed (Table 4) [8]. However, data on a second eradication treatment for children who failed the initial eradication therapy are scarce and the recommended quadruple therapies can only be used in countries where bismuth is available. Quadruple therapies containing bismuth are considered as the treatment option in populations with high clarithromycin and low metronidazole resistance [217].

Sequential therapy, in which the first five days consisted of dual therapy with a PPI and amoxicillin given twice daily, followed by triple therapy consisting of a PPI, clarithromycin and tinidazole or metronidazole twice daily for another five days has been evaluated in adult studies [218, 219]. This combination had a significantly higher eradication rate as compared to standard triple therapy in intention-to-treat analysis (91% *versus* 78%). High eradication rates by sequential therapies also have also been found in preliminary data from two further studies in children [220, 221]. The efficacy of sequential therapy was found to be superior to standard triple therapy in a meta-analysis [222] and this combination seems to be effective in patients with clarithromycin resistant strains [218, 223]. However, since most of studies with sequential treatment regimens have been conducted in Italy, it is currently considered too early to recommend in clinical practice [224] and may not be a good choice for treatment after failed eradication therapies [180, 225].

1.6. ANTIBIOTIC RESISTANCE

The eradication rate of standard triple therapies varies between different geographic regions since it is related to the prevalence of antibiotic resistance. A meta-analysis of adult studies showed the impact of pre-treatment metronidazole and clarithromycin resistance on the outcome of triple therapy [226]. In the presence of clarithromycin resistance an estimated 35% decrease in eradication rate was observed when a clarithromycin-containing regimen was used with a PPI and nitroimidazole. Consequently, in areas with prevalence of clarithromycin higher than 15-20% or when patients have previously received a macrolid, clarithromycin based triple therapy is not advised [224]. The prevalence of clarithromycin resistance in children has been reported to vary from 6% to 45% [12]. A study conducted in Japan showed that the eradication rate of a seven-day triple therapy was significant higher among subjects infected with clarithromycin susceptible strains as compare to those with antibiotic resistant strains (89% *versus* 56%) [227]. The main risk factor for clarithromycin resistance is previous consumption of macrolides such as clarithromycin, azithromycin, and erythromycin, used to treat children with respiratory tract

infections, enterocolitis, otitis media, and chronic sinusitis [10]. Metronidazole resistance is estimated to be higher in developing countries as compared to Europe and the US (50-80% *versus* 20-40%) [10]. Metronidazole has been used as a primary antimicrobial agent to treat *H. pylori* infection because it is actively secreted in the saliva and gastric juice, becoming highly concentrated in the stomach [178]. Resistance to metronidazole was found in a meta-analysis to reduce treatment efficacy by 18% in nitroimidaziole-containing regimens in combination with clarithromycin [226]. In a study of peptic ulcer patients in Vietnam, it was the level of metronidazole resistance that was found to be the most important factor for treatment outcome and the need for twice daily PPI medication [166]. Metronidazole-based regimens are to be considered in patients allergic to penicillin [224].

Recently, strains resistant to both clarithromycin and metronidazole have been identified but the prevalence is still low in Europe 6.9% [11] but much higher in developing countries such as Mexico (17.6%) [228] and Iran (42%) [13]. Treatment efficacy in double resistance was found to be below 50% when these drugs were used [226].

Reported amoxicillin resistance ranges from 0.6 to 59% [11, 13]. The efficacy of amoxicillin-based therapies is controversial since amoxicillin containing regimens have performed better than metronidazole-based regimens in the US while clarithromycin-based therapies were found to be superior in Europe [226]. When amoxicillin was given in combination with clarithromycin, clarithromycin resistance was found to decrease treatment efficacy by as much as 66% [226].

1.7. REINFECTION WITH *H. PYLORI* AFTER ERADICATION THERAPY

H. pylori reinfection after successful eradication is uncommon in adult studies in developed countries such as Europe and the US. The annual reinfection rate evaluated by ^{13}C-Urease breath test was 1.45% in the developed countries whereas it was of 12% in developing countries [229]. Several factors including heavy contamination of the environment, drinking water, institutional patients, medical personnel and *H. pylori* infection within the family have been suggested for high recurrence rate of *H. pylori* in developing countries [230].

Recurrence of *H. pylori* infection is defined as the situation in which the tests for *H. pylori* were negative at least 4 weeks after treatment becomes positive at a later stage. The cause for recurrence could be either recrudescence or true reinfection that is not easily distinguished. Reinfection is defined as the situation where a patient is infected with a new strain that differs from the pre-treatment strains. Recrudescence is defined as a pre-treatment strain, suppressed by treatment and undetected at a control 4 weeks after treatment, recolonizes and becomes detectable at a later stage [231]. Molecular typing has been used to differentiate recrudescence from reinfection, presuming that if the same strain was found that would be a sign of recrudescence. However,

finding of the same strain could also be due to reinfection with the same strain from an infected individual in the environment of the patient [6]. A note of caution is also needed regarding reinfection with a different strain, considered to represent true reinfection, as a patient could have harboured different strains at inclusion, with resurgence of a strain not originally identified [231]. A recent meta-analysis concluded that recurrence within one year in developed countries is most likely to be due to recrudescence while recurrence in developing countries is due to reinfection [230]. *H. pylori* reinfection rate, assessed in a few paediatric studies, has varied from 2.3% to 12.8% (Table 5).

Table 5. Reinfection in children with *H. pylori* after successful eradication therapy

Country	Post-treatment control (weeks)	No. Pts	Age (Years)	Follow-up time (months)	Annual reinfection rate (%)
Developed countries					
Estonia [232]	4-6	18	9 - 15	17.8	6.7
Germany [233]	8	102	1.8 - 18	15.5	2.3
France [234]	4	45	1.2 - 17.6	33.6	5.4
Ireland [235]	4-6	55	1.2 - 16.9	52.0	5.8
Italy [236]	8	52	4.9 - 18	18.0	12.8
Japan [237]	4-8	28	5 - 16	24.0	2.4
UK [238]	6	24	<14	62.4	2.4
Developing countries					
Mexico [239]	4-6	40	5 - 17	21.6	Not specified*

* This study conducted in 141 subjects (40 children aged 5-17 years and 101 adults) and the youngest group to evaluate reinfection rate was aged 5-30 years

2. AIMS

The aim of the study was to identify the best regimen for *H. pylori* eradication in Vietnamese children, a developing country. First, a non-invasive diagnostic method that could be used to follow up eradication was evaluated. Then we also needed to determine the antibiotic resistance in strains infecting Vietnamese children since treatment outcome is mainly determined by susceptibility to the antibiotics used. A major determinant for the rational for eradication treatment in children is the rate of reinfection that if high renders eradication treatment questionable. In order to achieve these goals, the aims of the individual studies were:

1. To compare the non-invasive method of antigen detection in stool with the gold standard method of bacterial culture and serological methods in children (Paper I).
2. To compare two regimens, with or without metronidazole, for eradication of *H. pylori* infection in children (Paper II).
3. To investigate the antibiotic resistance pattern of *H. pylori* in Vietnamese children (Paper III)
4. To investigate risk factors for reinfection in Vietnamese children during one year after successful eradication therapy (Paper IV).

3. MATERIALS AND METHODS

3.1. SUBJECTS

3.1.1. Recruitment of patients

The clinical trial was performed at National Hospital of Paediatrics in Hanoi, Vietnam from May 1st 2005 to March 1st 2007. Patients aged 3-15 years routinely undergoing endoscopy at the Gastroendoscopy Unit, National Hospital of Paediatrics, Hanoi, Vietnam for various gastrointestinal complaints such as recurrent abdominal pain, vomiting, nausea, anaemia, failure to thrive were eligible. The inclusion and exclusion criteria were as follows:

Table 6. Inclusion and exclusion criteria in the treatment trial

Inclusion criteria		**Exclusion criteria**
Symptoms	**Test**	
Severe gastric symptoms Failure to thrive, anaemia Age under 15 years old	Positive rapid urease test	Antibiotic intake in the previous 2 weeks or drug included in this study over the past month Allergy to study drug

All patients were allocated to the treatment groups (Table 7) on the basis of a computer-generated randomization list in blocks of 10, made by the Swedish national pharmaceutics retailer Apoteket, Stockholm, Sweden.

Table 7. Treatment regimens

Therapy	**Regimen LAM**		**Regimen LAC**	
	2 weeks	**2 weeks**	**2 weeks**	**2 weeks**
	13 – 22 kg	**≥23 - ≤45 kg**	**13 – 22 kg**	**≥23 - ≤45 kg**
PPI*	Lansoprazole 15mg once daily	Lansoprazole 15mg x 2	Lansoprazole 15mg once daily	Lansoprazole 15mg x 2
Antibiotic 1	Amoxicillin 500mg x 2	Amoxicillin 750mg x 2	Amoxicillin 500mg x 2	Amoxicillin 750mg x 2
Antibiotic 2	Metronidazole 250mg x 2	Metronidazole 500mg x 2	Clarithromycin 250mg once daily	Clarithromycin 250mg x 2

PPI*: proton pump inhibitor

Patients were offered medication according to weight with one of the above combinations. The medication was to be taken orally for 14 days. Treatment efficacy was evaluated by follow-up monoclonal antigen-in-stool test four weeks after the end of the treatment. If a patient was found to have a peptic ulcer at the first visit, the treatment efficacy was also evaluated by a follow-up endoscopy. If a *H. pylori*-positive RUT was found at the time of the endoscopic follow-up, the patient was offered free of charge re-treatment by the other antibiotic combination and a repeat follow-up by endoscopy after another four weeks.

A questionnaire was used for the interview regarding sociodemographic and family history details, including known risk factors for *H. pylori* infection at the start of the study. Long-term clinical outcome of *H. pylori* infection was evaluated every three months until one year after eradication by antigen-in-stool and endoscopy if motivated by clinical symptoms.

3.1.2. Control patients

As controls in paper I, 98 children with negative serology were selected from 241 children without symptoms of gastrointestinal diseases and of similar age as the *H. pylori*-positive patients. The children received treatment for acute orthopaedic disorders at the Orthopaedic Department, National Hospital of Paediatrics, and other acute diseases such as bacterial pneumonia and viral infections at the Paediatric Department, Bach Mai Hospital. Blood and stool samples were collected at routine checkups for their main disease. Patients were considered *H. pylori* negative when all three serologic tests, an in-house enzyme immunoassay, a commercial Pyloriset EIA-GIII (Orion Diagnostica, Espoo, Finland), and a commercial immunoblot (Helicoblot 2.1, MP Diagnostics, Singapore) were negative.

3.2. SAMPLING

Two hundred forty patients with positive RUT were recruited in the treatment trial at the Endoscopy Unit of the National Hospital of Paediatrics, Hanoi. Upper endoscopic examinations were performed by two experienced endoscopists after sedation with intravenous midazolam. Two biopsy samples were taken from antrum, one used for the RUT followed by histologic examination and the other for culture and one biopsy from corpus was taken for culture. All biopsy samples were stored at -70^{o}C at the National Institute of Hygiene and Epidemiology, Hanoi, Vietnam prior to being transported to Sweden. All patients also provided a 3 mL blood sample and a stool sample that were stored at -20^{o}C at the National Institute of Hygiene and Epidemiology, Hanoi, Vietnam until transport to Sweden. Culture, serological and antigen-in-stool tests were performed at the Department of Clinical Microbiology, Karolinska University Hospital, Stockholm, Sweden.

3.3. QUESTIONNAIRE

Risk factors for *H. pylori* infection such as sociodemographic and family history details were collected in a questionnaire during an interview made by one of the investigators with the co-operation of the parents of every child. Sociodemographic data included age and gender of the index case, number of person living in the same household, number of children in the family classified in 3 groups (1, 2, ≥3 children), birth order of the index child (1, 2, ≥3), breast feeding time in 2 categories (<12 months or ≥ 12 months of age), pre-mastication of food, living area in square meter, divided as above or below the mean national standard ($10m^2$/person), location of the family residence (urban area: district of Hanoi, rural area: others), bed sharing, type of toilet, water sources classified in two main sources: tap and others (family well, streams, rain or collective well). Past history of *H. pylori* infection in the families (diagnosed and treated in hospital) as well as current gastrointestinal symptoms were recorded.

3.4. METHODS

Culture, serology, antigen-in-stool and Etest were performed at the Department of Clinical Microbiology, Karolinska Hospital, Stockholm, Sweden.

3.4.1. Culture

The antrum and corpus biopsies were collected at the endoscopy unit and inserted into separate, labeled and sterile plastic tubes with 0.25mL sterile transport medium containing casamino acid, bactopeptone, yeast extract, NaCl, agar, L-cystein, glucos, Elga water and glycerole (In-house recipe, Department of Microbiology, Linköping University, Sweden.). The sealed tubes were immediately frozen to -70°C in CO_2-ice.

The biopsies were removed from the transport medium, homogenized with some of the transport medium and inoculated onto pre-reduced blood agar and *Campylobacter* agar base with 5% lysed horse blood supplemented with vancomycin, polymyxin B and *para*-2-trimetoprim. The plates were incubated under microaerophilic conditions (CampyGen™, Oxoid Ltd., Basingstoke, UK) and inspected on days 3 and 7. *H. pylori* growth was identified as Gram-negative curved rods producing urease, catalase, and oxidase. *H. pylori* strains were kept in a freezing medium containing 2.5g albumin, 7.4g saccharose, 0.05 kaliumdihydrophosphate (KH_2PO_4), 0.12 g kaliumhydrophosphate (K_2HPO_4) and 0.06 g natrium salt of glutaminic acid per 100ml (In-house recipe, Karolinska University Hospital Solna) at -70^oC.

3.4.2. In-house enzyme-linked immunosorbent assay

The sonicate *H. pylor*i antigen used was based on five clinical isolates from Vietnamese patients with peptic ulcer diseases and the NCTC 11638 strain. The sonicate was used to coat 96-well

microplates at a concentration of 5 μg/ml, as previously described [184, 240]. Sera were diluted 1:1,000, first 1:100 in phosphate-buffered saline and then 1:10 in phosphate-buffered saline containing 70 mg/ml of *C. jejuni* antigen (four clinical isolates) to remove cross-reacting antibodies. Alkaline phosphatase-conjugated antihuman IgG (Euro-Diagnostica, Malmö, Sweden) was used to detect bound antibodies. The method had been established and evaluated previously, giving with a cut-off level of OD 0.22 a sensitivity of 93.9-99.6% and a specificity of 90.7% [240]. Since this type of assay can have a ±10% variability around the established cut-off level, we used the lower cut-off of 0.2 in order to ensure a high sensitivity in the assay.

3.4.3. Pyloriset EIA-G III

The Pyloriset EIA-G III (Orion Diagnostica, Espoo, Finland) was used according to the instructions of the manufacturer. Values of <20 U/mL was to be considered negative, and values of ≥20 U/ml were to be evaluated as positive. The method has previously been evaluated in adult Vietnamese populations, showing a sensitivity of 84.8-98.5% and a specificity of 94.4% [240]. Since this type of assay can have a ±10% variability around the established cut-off level, we used the lower cut-off of 18 in order to ensure a high sensitivity in the assay.

3.4.4. Immunoblot (Helicoblot 2.1)

Sera were also tested by a commercially available immunoblot kit, Helicoblot 2.1 (MP Diagnostics, Singapore) for detection of antibodies against *H. pylori* specific antigen. The kit consists of Western blot membrane strips, made with a surface antigen-enriched preparation of *H. pylori* including CagA (116 kDa), VacA (89kDa) and the urease A subunit (30kDa). All buffers and reagents used were supplied with the kit and used according to the manufacturer's recommendations. The assay was performed with an automated Western blot system (Autoblot system 36; MP Diagnostics). The blots were evaluated as positive or negative according to the criteria supplied by the manufacturer.

3.4.5. Antigen-in-stool test (Premier Platinum HpSA PLUS enzyme immunoassay)

The stool samples were processed and tested by the antigen-in-stool test (Premier Platinum HpSA PLUS, Meridian Bioscience, Inc, USA) according to the manufacturer's instruction. This test utilizes a plurality of monoclonal anti-*H. pylori* capture antibodies adsorbed to microplate wells. Diluted patient sample and peroxidase-conjugated monoclonal antibodies were added sequentially and incubated for one hour at room temperature. Washings were performed to remove unbound material. Substrate was added and the plates were incubated for 10 minutes at room temperature.

Stop solution was added and the colour developed in the presence of bound enzyme was read in a spectrophotometer at 450nm wavelength. According to the manufacturer's instruction, values below OD 0.14 were considered negative, ≥ 0.14 as positive.

3.4.6. Etest

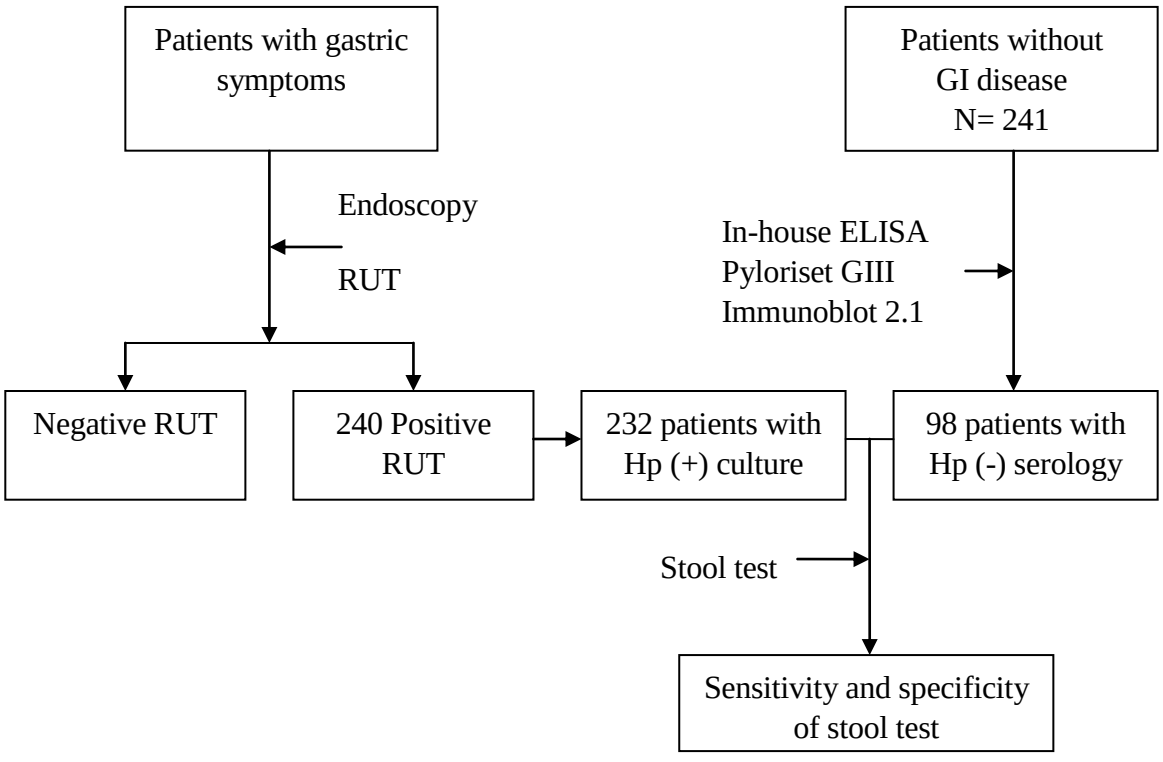
Susceptibility testing of *H. pylori* was performed by Etest (AB bioMérieux, Marcy l'Etoile, France) with agar medium (Müller-Hinton agar + 5% horse blood, aged ≥2 weeks) incubated for 72h (or longer) at 35°C under microaerophilic condition (CampyGen™, Oxoid Ltd., Basingstoke, UK). For metronidazole, microaerophilic incubation for 72h (or longer) was also used to test antimicrobial susceptibility. *H. pylori* strains were classified as resistant to clarithromycin when the MIC (Minimal Inhibitory Concentration) was ≥1 µg/mL and resistant to amoxicillin if MIC was >1 µg/mL. Metronidazole resistance was defined as MIC >4 µg/mL.

3.5. STUDY DESIGNS

3.5.1. Paper I

Evaluation of a novel monoclonal-based antigen-in-stool assay was performed in Vietnamese children prior to applying the assay for evaluation of optimal eradication treatment strategies.

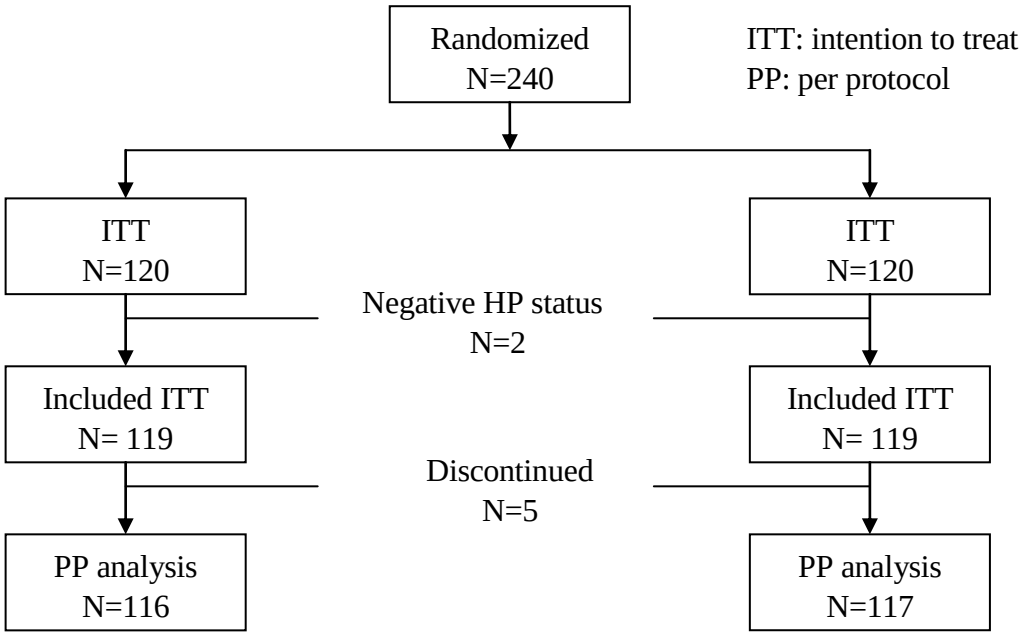
Figure 5. Flow chart of the study



3.5.2. Paper II

A randomized, double-blind clinical trial of two treatment strategies was performed.

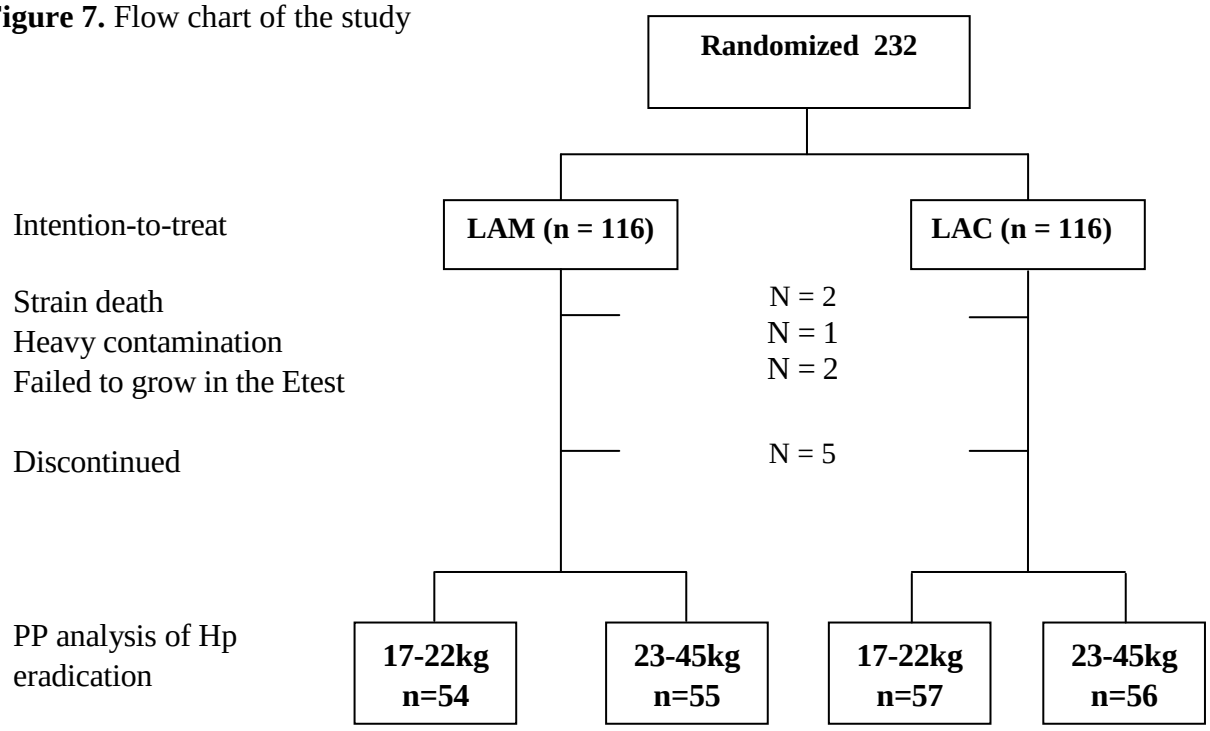
Figure 6. Flow chart of the study



3.5.3. Paper III

All the pre-treatment isolates from children in paper II were tested for resistance to clarithromycin, metronidazole and amoxicillin.

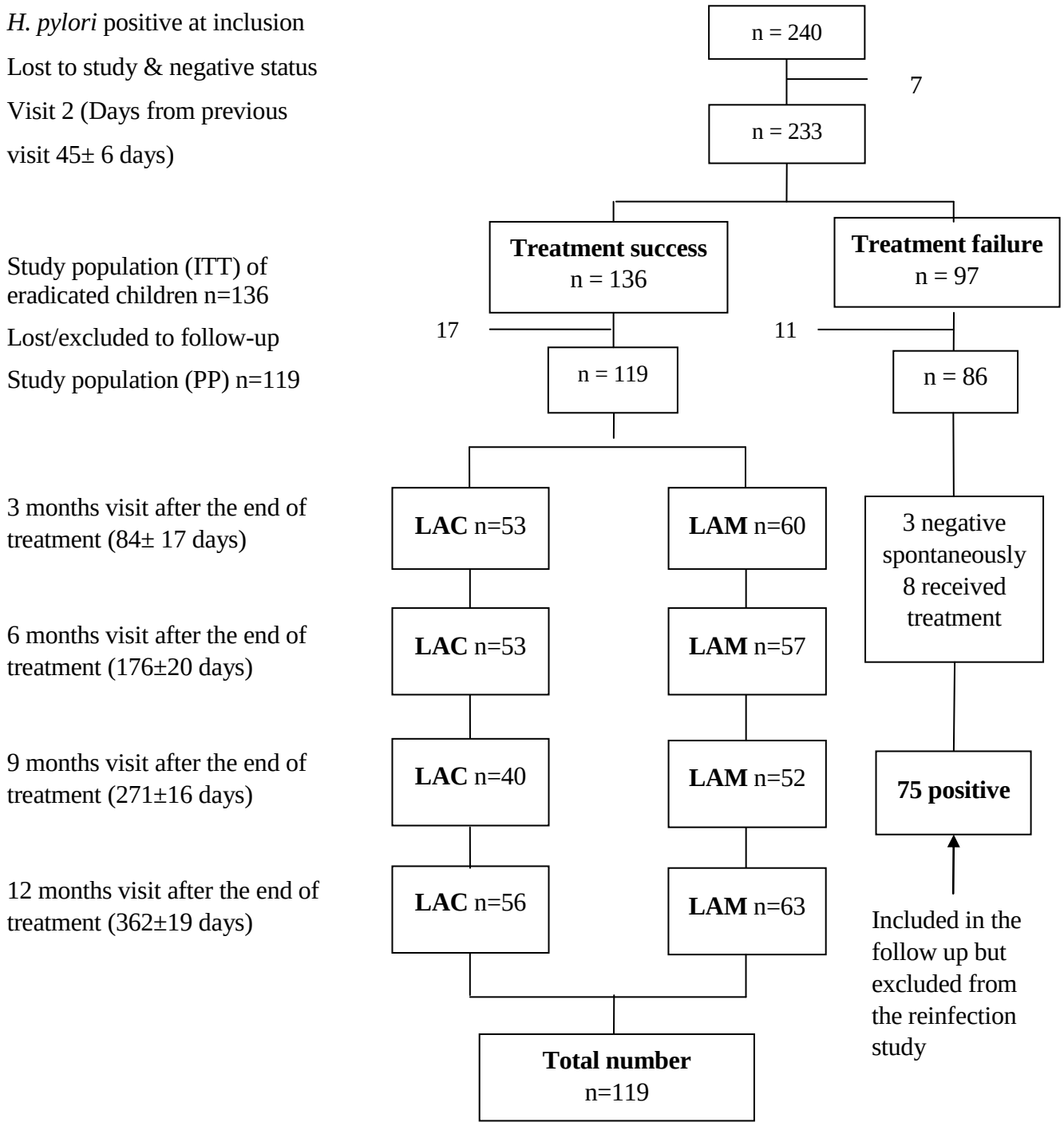
Figure 7. Flow chart of the study



3.5.4. Paper IV

The questionnaire was filled in by all patients (parents) at the first visit. All children in the treatment trial were to be tested every three months until one year after eradication by the antigen-in-stool assay evaluated in paper I.

Figure 8. Flow chart of the study



3.6. STATISTICAL METHODS

3.6.1. Paper I

		Disease		
		Present	**Absent**	
Test outcome	**Positive**	True positive	False positive	→ **Positive predictive value**
	Negative	False negative	True negative	→ **Negative predictive value**
		↓	↓	
		Sensitivity	**Specificity**	

The accuracy of the HpSA PLUS assay can be expressed through sensitivity, specificity, positive and negative predictive value or positive and negative diagnostic likelihood ratios.

$$\text{The sensitivity of the assay} = \frac{\text{True positive}}{\text{True positive} + \text{False negative}} \text{x}100\%$$

$$\text{The specificity of the assay} = \frac{\text{True negative}}{\text{True negative} + \text{False positive}} \text{x}100\%$$

$$\text{The positive predictive value of the assay} = \frac{\text{True positive}}{\text{True positive} + \text{False positive}} \text{x}100\%$$

$$\text{The negative predictive value of the assay} = \frac{\text{True negative}}{\text{True negative} + \text{False negative}} \text{x}100\%$$

The positive likelihood ratio represents the odds ratio that a positive test result will be observed in an infected population compared to the odds that the same result will be observed among a non-infected population. It was calculated as: sensitivity/ (1- specificity)

The negative likelihood ratio presents the odds ratio that a negative test result will be observed in an infected population compared to the odds that the same result will be observed among a non-infected population. It can was calculated as: (1-sensitivity)/ (specificity).

$$\text{Accuracy of the assay} = \frac{\text{True positive} + \text{True negative}}{\text{Total number of patients tested}}$$

Patient and control groups were compared by the Chi-square and the Mann-Whitney U sum rank test, when appropriate. Differences with p values <0.05 were considered significant.

3.6.2. Paper II

The sample size calculation is based on an assumption of 90% eradication rate for the best treatment and allowing detecting a 15% lower performance for the other treatment at the 5% significance level and with a power of 80%. We allowed also for a 20% loss for the possibility of

false positive RUT test and patients not returning for the follow-up. Eradication rate and adverse effects of the two groups were compared by using the Chi-squared test. A p-value of <0.05 was considered to indicate statistical significance.

3.6.3. Paper III

Antibiotic susceptibility in the groups was compared by Mann-Whitney U sum rank test or the Kruskal-Wallis test, when appropriate. The independent association of either clarithromycin or metronidazole resistance with eradication rate was investigated separately for the two treatment regiments LAC and LAM in multivariable logistic regression with adjustment for possible confounding. Relative risk was expressed as odds ratio (OR) with 95% confidence interval (CI) to estimate an association of eradication with various factors. All statistical tests were two-sided.

3.6.4. Paper IV

The cumulative incidence of reinfection was calculated according to the actuarial life-table method [241]. Children who skipped single visits without being lost to follow-up were re-entered in the appropriate intervals. The standard errors were calculated using Greenwood's formula [241]. The independent associations of suspected or previously known risk factors with the incidence of reinfection were modelled using discrete time multivariable Cox proportional hazard models. Hazard ratios (HRs) with 95% CIs, inherently adjusted for possible mutual confounding from other factors in the model, estimated relative risk. The trend for age was tested by the same hazard model, but with age as continuous variable. All statistical tests were two-sided.

4. RESULTS

4.1. PERFORMANCE OF THE ANTIGEN-IN-STOOL TEST (PAPER I)

The sensitivity of the antigen-in-stool test (Premier Platinum HpSA PLUS) was evaluated with stool samples from 232 culture positive children, aged from 3 to 15 years, who participated in clinical trial study and were positive for *H. pylori* infection by culture from biopsies (Paper II). For evaluation of the specificity 98 children of similar age with non-gastrointestinal conditions and who were negative for *H. pylori* infection by serological assays were included with blood and stool samples.

Among the 232 culture positive children, *H. pylori* antigen was detected in 224 stool samples by Premier Platinum HpSA PLUS. In the 98 control children, sero-negative in all three assays, *H. pylori* antigen was not detected in 93 samples. Thus, the results for the Premier Platinum HpSA PLUS kit showed a sensitivity of 96.6% (95% CI 93.3-98.5%) and a specificity of 94.9% (95% CI 88.5-98.3%) (Table 8).

The performance of Premier Platinum HpSA PLUS was also evaluated by age but no differences were found. The sensitivity of the three serological assays was evaluated in the culture-positive children. The sensitivity of the in-house ELISA based on Vietnamese strains was 97.4% (226/232), of the Pyloriset EIA-GIII 92.2% (214/232) and of the immunoblot (Helicoblot 2.1) 97.8% (227/232). The concordance between the in-house ELISA and the immunoblot was 99.6%.

Table 8. The performance of the HpSA PLUS in Vietnamese children

	HpSA PLUS **N = 330**
Sensitivity (%)	96.6
Specificity (%)	94.9
Positive predictive value (%)	97.9
Negative predictive value (%)	92
Likelihood ratio for positive test	18.94
Likelihood ratio for negative test	0.036
Accuracy	96.1
Prevalence of *H. pylori* infection (%)	69.4

4.2. *H. PYLORI* ERADICATION TRIAL (PAPER II)

A total of 240 patients, aged 3 to 15 years, with a history of abdominal pain and *H. pylori* positive by rapid urease test were included and randomized. *H. pylori* infection at inclusion was confirmed in 238 patients, based on positive culture in 231 patients and on positive antigen-in-stool assay in 7 patients. 233 (97%) patients remained for the per protocol analysis. All patients in the per protocol (PP) analysis reported having taken all study medications.

There was no significant difference in the overall efficacy of the two regimens in either the PP or intention-to-treat (ITT) analysis (Table 9). Eradication rate in children who received a once-daily administration of the PPI (and some also a once-daily clarithromycin) *versus* a twice-daily administration was however highly significant, being 45.7% and 70.9% by the PP analysis, the ITT analysis showing similar results (Table 9).

Table 9. Treatment results

Therapy/ Body weight	Eradication rate					
	Per protocol			Intention-to-treat		
	n/N	% (95CI)	p-value	n/N	% (95CI)	p-value
LAM	72/116	62.1 (53.1 - 71.1)	NS	72/119	60.5 (51.5 - 69.5)	NS
LAC	64/117	54.7 (45.7 - 73.7)		64/119	53.8 (44.8 - 62.8)	
< 23 kg*	53/116	45.7 (36.6 - 54.8)	0.0002	53/120	44.2 (35.3 - 53.1)	0.0001
≥ 23 kg**	83/117	70.9 (62.7 - 79.1)		83/118	70.3 (62.0 - 78.6)	
13 – 17 kg	28/47	59.6 (45.5 - 73.7)	0.0221	28/48	58.3 (44.4 - 72.2)	0.0181
18 – 22 kg	25/69	36.2 (24.9 - 47.5)		25/72	34.7 (23.7 - 44.7)	
23 – 33 kg	55/75	73.3 (62.3 - 83.3)	NS	55/76	72.4 (62.3 - 82.5)	NS
34 – 45 kg	28/42	66.7 (52.4 - 81.0)		28/42	66.7 (52.4 - 81.0)	

LAM: Lansoprazole + amoxicillin + metronidazole
LAC: Lansoprazole + amoxicillin + clarithromycin
* lansoprazole once-daily
** lansoprazole twice-daily

For further analyses the group less than 23 kg was subdivided into two groups of 13 - 17 kg and 18 - 22 kg. A significant difference was found between the two subgroups in the PP (and ITT) analysis with the eradication rate being 59.6% and 36.2%, respectively. The heavier group of over 23 kg was also subdivided into two groups, 23-33 kg and 34-45 kg of body weight but here no significant difference in efficacy was noted, being 73.3% and 66.7%, respectively (Table 9).

There was no significant difference in efficacy between the two treatment regimens by weight subgroups (Table 10). Children ≥23 kg of weight that received twice-daily medications had an eradication rate of 69.5% (95% CI 57.8-81.2%) and 72.4% (95% CI 60.9-83.9%) in the metronidazole and the clarithromycin-based treatments, respectively.

Table 10. Eradication rate according to body weight

Therapy		**Body weight group**			
		13-17 kg	**18-22 kg**	**23-33 kg**	**34-45 kg**
Mean age ± SD		4.8 ±1.1	7.3±2	9.6±2.1	12±1.7
Range		3-8	4-11	6-13	10-15
	LAM	68.0 (17/25)	43.8 (14/32)	73.7 (28/38)	61.9 (13/21)
PP % (n/N)	LAC	50.0 (11/22)	29.7 (11/37)	73.0 (27/37)	71.4 (15/21)
	p-value	NS	NS	NS	NS
	LAM	68.0 (17/25)	40.0 (14/35)	73.7 (28/38)	61.9 (13/21)
ITT % (n/N)	LAC	47.8 (11/23)	29.7 (11/37)	71.0 (27/38)	71.4 (15/21)
	p-value	NS	NS	NS	NS

LAM: Lansoprazole + amoxicillin + metronidazole
LAC: Lansoprazole + amoxicillin + clarithromycin

As it has been suggested that a high bacterial load might have a negative effect on treatment outcome, we explored the possibility of using the semi-quantitative results of the antigen-in-stool assay as a proxy for bacterial load. Children with a high level (≥ 4 EU) were found to have a significantly (p=0.007) lower eradication rate of 45.6% than children with a lower antigen load of <4 EU at 69.9%, respectively. Most of the difference came from the clarithromycin-based therapy, with 39.0% *versus* 61.5% (p=0.03) eradication while the metronidazole group had the corresponding eradiation rates of 51.3% and 65% (p=0.2), respectively.

Ulcer healing was evaluated in 30 (12.6%) children with peptic ulcer. There was no significant difference in ulcer healing between the two randomized treatments and the same 83.3% was seen in children <23 kg and ≥23 kg body weight. This finding was in contrast to the significant difference in eradication rate found between the two weight groups.

4.3. ERADICATION OF *H. PYLORI* INFECTION IN RELATION TO ANTIBIOTIC RESISTANCE (PAPER III)

Antibiotic susceptibility testing was performed in a total of 222 pre-treatment isolates. The overall resistance rate to clarithromycin, metronidazole and amoxicillin was 50.9% (113/222), 65.3% (145/222) and 0.5% (1/222), respectively. Double resistance to clarithromycin and metronidazole was detected in 28.8% (64/222) isolates. The rate of clarithromycin resistance decreased significantly with age, 62.7% in 3-6 year-olds, 54% in 7-10 year-olds and 31.7% in 11-15 years-olds (p=0.013 for trend). The rate was also significantly higher in children from urban than from rural areas (71.1% *versus* 26.7% p=0.001). There was no significant difference in the rate of clarithromycin resistance between girls and boys (55.4% *versus* 46.4%, p=0.18). In contrast, metronidazole resistance was high (53.3% - 73.6%), increased with age (p=0.023 for trend) and was higher in rural than in urban areas (74.3% *versus* 57.9%, p=0.011). A higher rate of metronidazole resistance was also found for boys than for girls (71.8% *versus* 58.9%, p=0.044).

Odds for *H. pylori* eradication rate was higher in clarithromycin sensitive strains than in resistant strains (OR 7.23, 95% CI 2.1-24.9) in 113 children who were treated with clarithromycin-based therapy whereas no significant difference in eradication rate was found in the 109 children who received the metronidazole-based therapy by sensitive and resistance strains (OR 2.27, 95% CI 0.64-8.06) (Table 11).

The twice-daily clarithromycin and PPI regimen resulted in an eradication rate that was significant higher than with the once-daily regimen (OR 6.92, 95% CI 1.49-32.13). The improved efficacy of the twice-daily regimen was clear for clarithromycin resistant strains (OR 16.13, 95% CI 1.23-210.53) but not so for the claritromycin sensitive strains (OR 2.90, 95% CI 0.25-34.03). In the metronidazole-based regimen, only lansoprazole was administrated once and twice daily. The twice-daily lansoprazole seemed to be somewhat more effective than the once-daily administration for eradication of both metronidazole resistant (OR 4.64, 95% CI 0.36-59.89) and sensitive strains (OR 3.4, 95% 0.18-63.68) but the differences were not significant (OR 4.12, 95% CI 0.71-24.09).

The overall treatment efficacy was significant higher in rural than in urban areas (OR 3.30, 95% CI 1.45-7.51, adjusted for those variables in table 2 as well as treatment regiments). The lower treatment efficacy in clarithromycin-based regimen among urban children might be explained by a higher antibiotic resistance rate in the urban area (OR 3.44, 95% CI 1.14-10.41). The difference by regions for the metronidazole-based therapy was not significant (OR 2.3, 95% CI 0.65-8.27) (Table 11).

Age was not found to significantly impact eradication rates in relation to antibiotic susceptibility. Factual antibiotic dose per kilo body weight was however significantly associated with eradication rates in the clarithromycin-base regimens (OR 8.13, 95% CI 2.23-29.6). A

tendency towards higher eradication rate was noted in the group receiving the correct dose by weight in the metronidazole-based regimen, but the difference was not significant (OR 2.58, 95% 0.8-8.34) (Table 11).

Table 11. Multivariable logistic regression analysis of association between susceptibility to antibiotics and treatment outcome

Study variables		OR (95% CI)	
Variables	**Categories**	**LAC (n=113)**	**LAM (n=109)**
Age group (years)	3-6	0.17 (0.04 - 0.8)	0.48 (0.1 - 2.2)
	7-10	0.38 (0.1 - 1.53)	0.71 (0.16 - 3.3)
	11-15	1.0 (reference)	1.0 (reference)
Weight group (kg)	13-17	1.17 (0.08 - 17.24)	0.56 (0.03 - 9.75)
	18-22	0.18 (0.02 - 1.9)	0.33 (0.03 - 3.68)
	23-33	2.97 (0.38 - 23.2)	1.86 (0.24 - 14.6)
	34-45	1.0	1.0
Gender	Female	1.60 (0.54 - 4.8)	0.37 (0.12 - 1.17)
	Male	1.0	1.0
Geographic area	Rural	3.44 (1.14 - 10.41)	2.30 (0.65 - 8.27)
	Urban	1.0	1.0
Susceptibility to CH	Sensitive	7.23 (2.1 - 24.9)	1.75 (0.5 - 6.1)
	Resistance	1.0	1.0
Susceptibility to MZ	Sensitive	0.71 (0.23 - 2.21)	2.27 (0.64 - 8.06)
	Resistance	1.0	1.0
Effective dose	High	8.13 (2.23 - 29.6)	2.58 (0.8 - 8.34)
	Low	1.0	1.0

LAM: Lansoprazole + amoxicillin + metronidazole

LAC: Lansoprazole + amoxicillin + clarithromycin

CH: Clarithromycin MZ: metronidazole

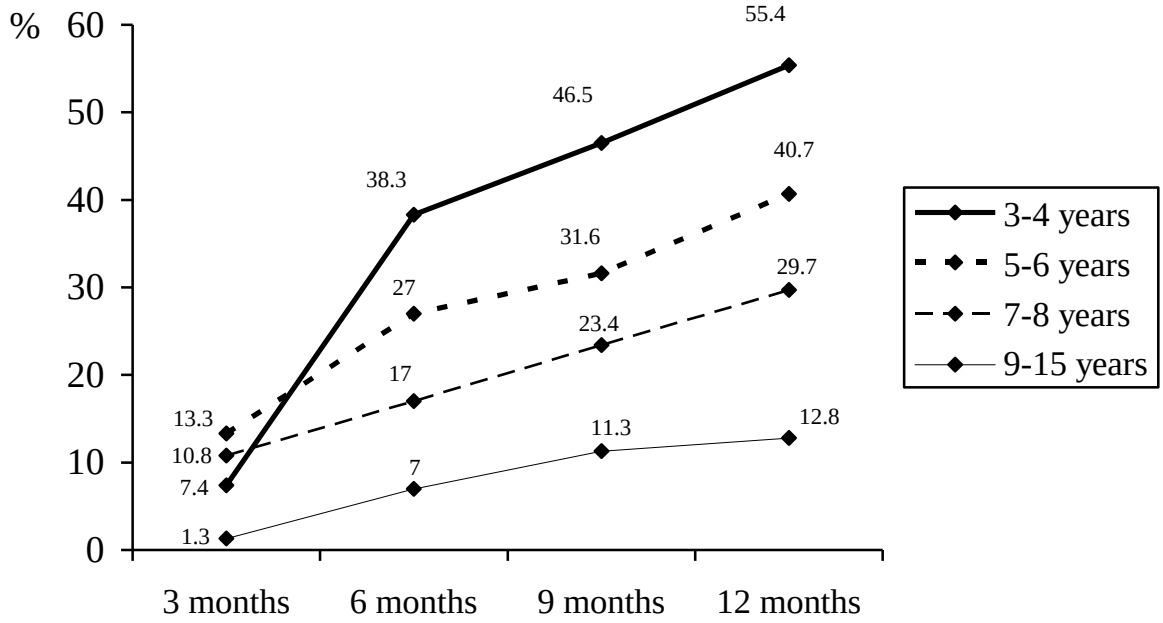
4.4. RISK FACTORS FOR *H. PYLORI* REINFECTION IN CHILDREN (PAPER IV)

All 238 *H. pylori* positive children that participated in the prospective treatment trial were invited to participate in the one-year follow-up study. Of these children 136 were successfully eradicated, 30 children became reinfected with *H. pylori*, while 17 were lost to follow-up after one-year follow-up.

In our final multivariable Cox proportional hazard model with mutual adjustments for possible confounding, low age was the most prominent independent risk factor for reinfection (adjusted hazard ratio (HR) among children aged 3-4, 5-6, and 7-8 years, relative to those aged 9-15 years, were respectively 14.3 (95% CI 3.8-53.7), 5.4 [1.8-16.3] and 2.6 [0.7-10.4]) (Table 12).

As shown in the figure 9, the cumulative reinfection rate at 12 months was 55.4% (95% CI 28-83%), 40.7% (95% CI 21-61%), 29.7% (95% CI 8-52%) and 12.8% (95% CI 5-21%) for children aged 3-4, 5-6, 7-8 and 9-15 years, respectively. Female sex was associated with increased risk (adjusted HR among girls relative to boys 2.5, 95% CI 1.1-5.9) (Table 12). In stratified analyses the association between age and risk of re-infection was more apparent in girls than in boys.

Figure 9. Cumulative probability of reinfection on strata age using an actuarial method



Neither of the factors sibship size, birth order, bed-sharing, sanitary standards, urban/rural habitation, breast-feeding duration, food pre-mastication, or self-reported gastrointestinal disease among family members were found to be statistically significant risk factors for re-infection, but the power to detect small or moderate excesses in risk was poor (Table 12).

The reinfection rate in the two eradication therapy groups was similar, 23.2% in the LAC and 27% in the LAM regimens. The children with a weight of <23 kg had received once-daily treatment with proton-pump inhibitor (PPI) and clarithromycin in the LAC regimen and once-daily PPI in the LAM regimen. Treatment regimen and factual antibiotic dose per kilo body weight in the initial eradication trial were not significantly associated with reinfection risk, contradicting recrudescence as an important mechanism (Table 12).

Table 12. Sociofamilial and demographic data investigated for reinfected and non-reinfected children.

Study variables		Unadjusted HR	Adjusted HR*
Variables	**Categories**	**(95%CI)**	**(95%CI)**
Age group (years)	3-4	6.6 (2.3 - 19.1)	14.3 (3.8 - 53.7)
	5-6	4.0 (1.5 - 10.7)	5.4 (1.8 - 16.3)
	7-8	2.3 (0.7 - 7.6)	2.6 (0.7 - 10.4)
	≥9	1.0 (reference)	1.0 (reference)
Gender	Female	2.3 (1.0 - 4.9)	2.5 (1.1 - 5.9)
	Male	1.0	1.0
Geographic area	Rural	0.8 (0.4 - 1.8)	1.5 (0.6 - 3.9)
	Urban	1.0	1.0
Number of children	1	2.2 (0.4 - 11)	0.7 (0.1 - 5.4)
	2	2.2 (0.5 - 9.9)	1.6 (0.3 - 9.2)
	≥3	1.0	1.0
Order of children	1st	2.9 (0.4 - 22.7)	1.5 (0.1 - 17.3)
	2nd	2.4 (0.3 - 19.1)	0.8 (0.1 - 8.8)
	≥3 rd	1.0	1.0
Bed sharing	No	1.1 (0.2 - 4.9)	2.1 (0.4 - 11.2)
	Yes	1.0	1.0
Pre-masticated food	No	1.2 (0.6 - 2.7)	0.9 (0.4 - 2.1)
	Yes	1.0	1.0
Water source	Tap	1.6 (0.7 - 3.6)	1.8 (0.7 - 4.6)
	Well	1.0	1.0
Effective dose	High	1.2 (0.5 - 2.7)	
	Low	1.0	
Treatment regimen	LAC	1.0 (0.5 - 2.1)	
	LAM	1.0	
GI disease in family	No	1.5 (0.7 - 3.6)	
members	Yes	1.0	

* The final model included the variables with estimates listed in the right-hand column
HR: Hazard ratio
LAM: lansoprazole + amoxicilline + metronidazole
LAC: lansoprazole + amoxicilline + clarithromycin

5. DISCUSSION

5.1. EVALUATION OF THE ANTIGEN-IN-STOOL TEST

The large-scale evaluation of a novel monoclonal-based stool test (Premier Platinum HpSA PLUS) to establish *H. pylori* infection in children showed a sensitivity of 96.6% against culture as gold standard and a specificity of 94.9% with serology as reference. Our results showed a good performance for the novel, monoclonal-based assay used in Vietnamese paediatric population thus it providing a reliable method for evaluation of treatment therapies. The performance of the assay is however dependent on the prevalence of the infection and therefore high in a population with high prevalence of *H. pylori* infection such as Vietnam. If the prevalence of *H. pylori* infection in a country is lower, then the performance of the test will be accordingly lower, i.e. the positive predictive value will decrease.

The accuracy of antigen-in-stool tests as a method for the primary diagnosis of *H. pylori* infection has been evaluated in many paediatric studies [192-211]. Investigations have, however, found a wide range of sensitivity and specificity, from 67% to 100% and from 61% to 100%, respectively [196, 201, 205, 211]. Sabbi et al [207] evaluated a polyclonal enzyme stool antigen assay (Premier Platinum HpSA) in 250 children with histology and rapid urease test as reference methods, finding a high sensitivity (97%) and specificity (98%) for the test. Dondi et al [209] conducted a study in 184 children (median age 2.2 years, range 0.2 to 5.5) with a polyclonal stool test (Premier Platinum HpSA) against culture, histology and rapid urease test, reporting again a high sensitivity and specificity (93.3% and 98.7%, respectively). The performance of the stool test was as good as that of the UBT even in very small children. One study of a polyclonal-based test (Premier Platinum HpSA) in children and adolescents, conducted by Raguza et al [208] reported a high overall sensitivity (94.6%) and specificity (96.5%) but the sensitivity in the younger age group (less than six years of age) was lower than in the older group while specificity was similar in both groups.

In contrast to these favourable data, a large European multicenter study showed a disappointing 72.9-80.3% sensitivity and 93.4-97.3% specificity for a polyclonal-based stool test (Premier Platinum HpSA) in 316 children and adolescents [206]. Low sensitivity (75.6%) was also reported by Shaikh et al [205] who in addition found a low specificity of 61% for a polyclonal-based stool test (Premier Platinum HpSA). A Finnish study comparing three stool assays (one polyclonal and two monoclonal-based) found, on the other hand, excellent performance for all three assays (sensitivity ranging from 96 to 100% and specificity from 90 to 97%) [211]. A monoclonal-based stool test (FemtoLab) was evaluated in 302 untreated children with *H. pylori* status defined by use of invasive methods (rapid urease test, culture and histology) [202]. The sensitivity and specificity of

the test were 98% and 99%, respectively. In this study, as in the Finnish study [211], no relation between OD values and the age of the patients was found. Our data, not showing any variation of test performance by age, are thus in line with the finding of others, as is our finding of a good performance for the novel, monoclonal-based assay used in the present study.

The strength of the material collection was the inclusion of a large number of children and the gold standard, culture of *H. pylori* from biopsies, as a reference for evaluation of the sensitivity of the antigen-in-stool test. A weakness of the study was the reference method used for evaluation of the specificity of the assay, having to be based on serology since UBT is not available in Vietnam. Serology has earlier been considered a less reliable method for diagnosis of *H. pylori* infection and in particular so in children [242, 243]. This negative view has been changed in the most recent Maastricht consensus document [152], but commercial serological assays tend to be less reliable in Asian populations than in Western populations, the tests having been developed for and evaluated in these populations [184, 240]. The combination of the three serological tests with high sensitivity can therefore be claimed with some confidence to have identified the truly sero-negative children in the control group. This does not mean that they may not have had an early infection since antibodies need time to develop. Thus, the children in the control group with negative serology but positive in the antigen-in-stool test might have had an early infection, especially as childhood is known to be the age of acquisition of the infection.

5.2. EFFECT OF ERADICATION TREATMENT

The present study addressed the lack of randomized, double-blind trials in children in developing countries by comparing two triple therapies with lansoprazole and amoxicillin in combination with either metronidazole or clarithromycin for eradication of *H. pylori*. Our result showed that there were no significant differences between the two regimens in either the overall efficacy at 62.1 and 54.7% or for the age groups that received twice-daily medication at 69.5 and 72.4%, respectively.

The trial also illustrated the differences and difficulties of conducting a clinical trial in a paediatric population as compared to adults. The PPI had no pharmacokinetic studies available for twice-daily medication in the younger age groups so in spite of a previous meta-analysis having shown twice-daily medication to be at least 10% more effective than a once-daily administration [166, 244], twice-daily medication with the PPI could not be used. As for many other drugs, paediatric formulations are not readily available so suboptimal dosing could occur with clarithromycin if tablets are not to be broken. For these reasons, although the present four-armed trials was one of the largest conducted in children, the children of 18-22 kg of weight may have received suboptimal doses and/or administration of the proton-pump inhibitor and of clarithromycin.

Another reason for the lower efficacy of treatment seen in the younger (lighter) age groups might be differences in antibiotic resistance patterns in the different age groups. Nothing was known of the resistance profile of *H. pylori* in children in Vietnam but this issue needed clarification. Clarithromycin used to be an expensive drug, not readily affordable in Vietnam, until the patent on the drug expired. Then, a few years ago, 10-times cheaper generic versions appeared on the market. Since then, clarithromycin is prescribed widely for mostly respiratory tract infections and mainly in the younger age groups. Metronidazole is rarely prescribed to children but the drug is cheap and available over the counter. The respective importance of the two possible factors, once-daily *versus* two-daily dosage on one hand and antibiotic resistance on the other, could not be fully discerned in this part of the study.

Because of the possible limitations of our study in the younger age groups, the most reliable efficacy estimates were obtained in the 117 children of ≥23 kg of body weight or ≥10 years of age. The eradication rate for both regimens of some 70% is in line with the rates reported from other studies, especially from developing countries. For the most common treatment regimen, combining a PPI, amoxicillin and clarithromycin, a recent meta-analysis identified 43 treatment arms and 1771 children with a median sample size of 33 children worldwide [216]. The eradication rate varied widely in 18 studies from 29 to 100%, with a mean eradication rate of 80% in Europe but only 65% in seven studies from developing countries. The less common combination of a PPI, amoxicillin and metronidazole in six treatment arms eradicated worldwide 75% of children, with some 10% lower eradication rates being reported for metronidazole-based combinations from developing countries with a higher rate of resistance. Improved eradication rates have been shown in adults with a sequential treatment strategy that could also be an option to test in children [245].

Strength of our study lied in a randomized, double-blind design, the large sample size for a paediatric trial, culture-confirmed infection in 97% of children and a compliance of 97% to the medication and the follow-up. A difficulty of our study is the wide range of body weights in the study children, the lack of paediatric formulations and of pharmacokinetic studies for the youngest age groups allowing more optimal dosing. On the other hand, this is one of the few studies that investigated eradication treatment in children of all age groups over the age of three years. The study could have excluded the youngest age groups but treatment options were needed for them since severe gastrointestinal symptoms are not uncommon also in young children in Vietnam, as illustrated by the youngest child with a peptic ulcer in the study being only three years of age.

5.3. EFFECT OF ERADICATION TREATMENT IN RELATION TO ANTIBIOTIC RESISTANCE PATTERN

The present study showed an unexpectedly high resistance rate to clarithromycin of 50.9%. According to several studies in European and American children, the prevalence of primary resistance to clarithromycin varied between 6% and 45%, with a higher resistance rate in children from countries with a high consumption of macrolid such as Italy, Spain and Portugal [11, 246-248]. Outside Europe and the US, the prevalence of clarithromycin resistance tends to be lower, e.g. a primary clarithromycin resistance of 18% and 36.1% has been reported from Taiwan and Japan, respectively [249, 250]. In the Middle East where the consumption of clarithromycin is still low, the primary clarithromycin resistance rate in Israel [251] and Iran [13] were 15% and 16%, respectively. The high rate of clarithromycin resistance in Vietnamese children found in the present study as compared to the 1% in Vietnamese adults is likely to reflect the increase of clarithromycin consumption in Vietnamese children for respiratory tract infections [252].

The prevalence of primary *H. pylori* resistance to metronidazole in children has been reported in a range of 13 - 57% in Europe and the US [12, 248] and much higher in developing countries, for example 78.4% in Mexico [228] 95% in Iran [13] and 100% in Egypt [253]. Our finding of some 70% metronidazole resistance in older children is thus in line with results from other developing countries and similar to the 76% found in our previous study in Vietnamese adults [252].

Antibiotic resistance to amoxicillin was only 0.5% in our study and consistent with results of several studies reporting primary resistance to amoxicillin in children, ranging from 0.6% to 59%, the highest rate being found in strains from Iranian children [11-13]. Previous studies have reported the prevalence of primary dual resistance to metronidazole and clarithromycin in children to range from 4% to 42% [12, 13]. Our study, with 66 isolates (28.8%) resistant to both clarithromycin and metronidazole, is in line with the previous reports.

Clarithromycin resistance decreased with age in our study, as previously reported from Spain [254]. Also in a European multicentre study a higher rate of clarithromycin resistance was found in children younger than 6 years of age compared to those older than 12 years [11]. In contrast, two studies failed to find a significant difference in antibiotic resistance by age [13, 14]. In a European study a higher rate of clarithromycin resistant strains was found in children of European origin than in those originating from the Middle East and North Africa [11]. This finding seems to be in line with our observation of higher rate of clarithromycin resistance in urban than in rural areas.

The difference in metronidazole resistance rate between urban and rural areas could perhaps be a result of the drug being cheap and easily obtained over the counter. We also found a higher rate of metronidazole resistance in older children as compared to younger ones, as previously reported from Spain [254] while four other studies found no differences by age [13, 14, 255, 256].

The success of treatment in *H. pylori* infection depends mainly on antibiotic sensitivity especially to clarithromycin. The eradication rate in clarithromycin sensitive strains was significantly higher than in resistant strains whereas no such difference was found for methronidazole sensitive and resistant isolates in the metronidazole-based therapy. Our results are agreement with observation from several previous studies that also showed treatment failure when clarithromycin was used against resistant strains [227, 251, 257].

A significantly higher eradication rate was found in the twice-daily clarithromycin and lansoprazole as compared to the once-daily regimen, an observation also made in our previous adult study [252]. This can be explained by a synergistic effect between clarithromycin and PPIs. Although the difference was not significant a trend towards a higher efficacy was also found for both metronidazole resistant and sensitive strains in the metronidazole-based regimen. Our finding is in agreement with the observation from a previous meta-analysis, showing that twice-daily PPI administration resulted in at least 10% more effective eradication than once-daily regimens [244].

Although there was a significant difference in prevalence of antibiotic resistance by age, our findings did not show a significant difference in treatment efficacy in relation to age. The dose of drugs by weight could affect treatment outcome. Exact dosing by weight is excluded in a double-blind paediatric study and paediatric formulations are often lacking. A multicentre, prospective, randomized double-blind control trial conducted in 73 French children reported an eradication rate in both intention to treat and per protocol of 74.2% and 80%, respectively [258]. The low eradication rate in this study was thought to be explained at least in part by low dosages of antibiotics. We analyzed the factual antibiotic dose per body weight in our study and found a significant association with eradication rate for the clarithromycin-based but not for the metronidazole-based therapy.

Strength of our study lies in a randomized, double-blind design and the large sample size for a paediatric trial. Also, including children with a wide age range of 3-15 years renders the study representative for the paediatric population. In addition, Etest, the most appropriate method for testing *H. pylori* antibiotic susceptibility, was used to assess antibiotic resistance. A weakness of the study was the difficulty of exact dosing by weight in a randomized double-blind trial over a wide weight range and the lack of pharmacokinetic data that would have allowed for more optimal dosing in the youngest age groups.

5.4. AGE AS RISK FACTOR FOR *H. PYLORI* REINFECTION IN CHILDREN

The present study addressed risk factors for reinfection with *H. pylori* in children, an area of sparse information in the literature and especially from developing countries. In developed countries, low reinfection rates in children are usually reported. During 15.5 months of follow-up, Feydt-Schmidlt et al [233] found reinfection rate per patient-year of 2.3% in 102 German children aged 1.8-18. Kato et al [237] investigated 23 Japanese children aged 5-16 years after mean followed-up period of 21.2 months. The reinfection rate per patient year of 2.4% was similar to that reported in a study of 24 children in the UK [238]. These findings were in contrast to the higher rates of reinfection per patient-year found in France, Ireland and Italy. Using UBT C^{13} to assessed reinfection with *H. pylori* in 58 patients aged 1.2-18 years during 2.8 years, Halitim et al [234] found 5.4% of French children being reinfected. A study in Ireland reported a reinfection rate per patient-year of 5.8% in 52 children during 24 months of follow-up [235]. Also Magista et al [236] in Italy found a higher reinfection rate per patient-year of 12.8% in 52 children aged 4.9-18 years. In Estonia, where the prevalence of *H. pylori* infection in children aged 9-15 years was 56%, a reinfection rate per patient-year of 6.7% was found in 16 children during a mean of 6.6 years follow-up [232].

Almost no studies have reported *H. pylori* reinfection rates in children in developing countries. Leal-Herrera et al [239] conducted a study in 141 subjects in Mexico (40 children aged 5-17 years and 101 adults) and found an overall reinfection rate of 21.9%. Since the youngest group of this study was aged 5-30 years, reinfection rate of children was not specified. Our study of 136 children revealed a high reinfection rate of 25.2% during the first year after successful eradication of *H. pylori* infection. This finding is in line with the observation of Magista et al [236] who showed that living in a high prevalence area for *H. pylori* infection increases the annual risk of reinfection by approximately fourfold over the annual risk in *H. pylori* low prevalence areas.

Consistent with the findings of four previous studies investigating risk factors associated with paediatric reinfection [232, 234-236], we found no evidence in our study by univariate analysis that the reinfection rate was influenced by geographic location, family size, bed sharing, number and order of children, eating habits, gastrointestinal disease in family member, water source, type of toilet, gestation age at delivery and breastfeeding. The most salient finding in the present study was the gradual decrease of the risk for reinfection from 3-4 years to 9-15 years of age. This finding is in agreement with an observation from Ireland showing that the reinfection rate of 66.6% (4/6) in children less than 5 years of age was significantly higher than the reinfection rate of 4.3% (2/46) in older children [235]. Also the study by Magista et al [236] found that the children below 7 years of age had a significantly greater risk of reinfection than older children (71% versus 22%, $p<0.01$). No previous study has however been large enough to show the gradient of the decrease over a large paediatric age range as in the present study.

Surprisingly, female sex tended in our study to be associated with increased risk for reinfection relative to boys. No similar findings have been reported previously, but since studies do not tend to report reinfection rate by gender it remains unclear whether the association was not found or not looked for.

Fixed dosages had to be administered over a wide range of body weight in the original, double-blind randomized treatment trial, and thus some children may have received suboptimal doses of antibiotics and proton pump inhibitor, raising the possibility that recurrence of *H. pylori* infection might be due to recrudescence rather than reinfection. Factual antibiotic dose per kilo body weight in the initial eradication trial were analyzed and no significant association with risk was found, contradicting recrudescence as an important mechanism. Sensitivity analyses assuming worst and best case scenarios for the 17 children lost to follow up and for the children who skipped one visit but returned later resulted only in marginal changes in the results.

The strength of the present study was the large number of children included and the low rate of dropouts during the follow-up period. Our finding has also implications for eradication strategies in children, indicating that early childhood should be avoided unless motivated by ulcer disease or other medical conditions associated with the infection. A weakness of our study could be the use of an antigen-in-stool assay. This new monoclonal-based assay was used as end-point throughout the entire study but had been evaluated in the study population. Another weakness of the study was the relative small power to detect small excesses in risk in spite of the study being unusually large in a paediatric context.

6. CONCLUDING REMARKS

With the aim to identify the best regimen for *H. pylori* eradication for children in a developing country such as Vietnam we evaluated a non-invasive diagnostic method that could be used to follow up eradication rates in children. We found that the antigen-in-stool assay Premier Platinum HpSA PLUS has a good performance in Vietnamese children. This monoclonal-based antigen-in-stool assay thus represents a choice among non-invasive methods, especially apt for diagnosis of *H. pylori* infection in children.

Our study has shown that two triple therapies with a PPI and amoxicillin in combination with metronidazole or clarithromycin gave similar eradication rates and in line with results obtained in other developing countries. We also found significant differences for both treatments by weight, which could be due to the once-daily PPI and clarithromycin administration and/or more antibiotic resistant strains in the younger children. The twice-daily medications play an important role in eradication of *H. pylori* infection especially for clarithromycin-resistant strains.

We evaluated the antibiotic resistance in strains infecting Vietnamese children since treatment outcome is mainly determined by susceptibility to the drugs used. We found that *H. pylori* clarithromycin resistance was unexpectedly high in Vietnamese children indicating that more rational use of antibiotics should be advocated in Vietnam. Clarithromycin-based therapy is considered as an ineffective regimen when clarithromycin resistance rate is over 20%. The efficacy of sequential therapy was found to be superior to standard triple therapy and this combination seems to be effective in patients with clarithromycin resistant strains. In Vietnam with high rate of clarithromycin resistance, sequential therapy may be a good choice to test for eradication treatment of *H. pylori* in children.

A major determinant for the rational for eradication treatment in children is the rate of reinfection. Our study has found that the reinfection rate in Vietnamese children was high but decreased with age from 55.4% in 3-4 year-olds to 12.8% in the 9-15 year-olds. The finding lends strong support to the notion of early childhood as the main period of susceptibility to the infection. The results also imply that eradication treatment should be postponed, especially in high-prevalence areas, to older childhood ages unless motivated by a medical condition.

Antibiotic susceptibility testing with Etest is not only expensive but also based on culture of bacterial strains which currently does not exist in Vietnam. The mechanisms of *H. pylori* antibiotic resistance is mainly based on point mutations that can be detected by molecular methods. In a situation with a high rate of antibiotic resistance, antibiotic susceptibility testing by molecular methods may need to be developed to improve the efficacy of treatment.

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