Helicobacter pylori Detection and Antimicrobial Susceptibility Testing

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INTRODUCTION

The discovery of *Helicobacter pylori* in 1982 (565) was the starting point of a revolution concerning the concepts and management of gastroduodenal diseases.

It is now well accepted that the most common stomach disease, peptic ulcer disease, is an infectious disease, and all consensus conferences agree that the causative agent, *H. pylori*, must be treated with antibiotics (131a, 295a).

Furthermore, the possibility emerged that this bacterium could be the trigger of various malignant diseases of the stomach and it is now a model for chronic bacterial infections causing cancer. The rare gastric mucosa-associated lymphoid tissue (MALT) lymphoma is the best example for which most of Bradford Hill's criteria of causality have been fulfilled, including remission of the cancer after a successful eradication of

H. pylori (579). While sufficient proof is lacking for the most common gastric cancer, i.e., gastric adenocarcinoma, numerous data have highlighted the essential role of the villain (335).

The public health importance of the discovery of *H. pylori* and its role in stomach diseases was recognized in 2005 by the attribution of the Nobel Prize in Physiology or Medicine to B. Marshall and R. Warren. In the history of Nobel prizes, this is only the third time that the discovery of a bacterium has been acknowledged (358).

For the correct management of peptic ulcer disease and gastric MALT lymphoma, as well as obtaining information on a wide range of diseases associated with *H. pylori* infection, effective diagnostic methods including susceptibility testing are mandatory.

Most of the many different techniques involved in diagnosis of *H. pylori* infection are performed in microbiology laboratories.

The aim of this article is to review the current status of these methods and their application, highlighting the important progress which has been made in the past decade. The traditional division between invasive and noninvasive techniques will be followed.

INVASIVE TESTS

Invasive tests were the first to be used in the diagnosis of *H. pylori*.

The stomach is usually accessed by fiber optic endoscopy, and biopsy specimens are obtained. Unfortunately, with standard technology the endoscopic features of *H. pylori* infection are not specific. Erythema and edema may be seen, but most often, no change is observed (416). In some cases, follicular gastritis may be observed, especially in children and young adults (474), as well as major lesions, e.g., ulcers, polyps, tumors. However, recent progress has been made to magnify the gastric mucosal abnormalities with narrow band imaging (260), endocytoscopy (231), and confocal laser endomicroscopy (257). This last technique enabled detection of *H. pylori* by surface microscopy imaging of living tissue during ongoing endoscopy for the first time. Using two contrast stains, topical acriflavine and intravenous fluorescein, with an endomicroscope (Pentax, Tokyo, Japan), endoscopists were able to see clusters of bacteria as well as single bacterial cells stained by acriflavine both on the surface and in the deeper layer of the gastric epithelium. This report is a real breakthrough in current diagnostic possibilities (257).

To avoid endoscopy, other less invasive paths to the stomach have been proposed. It is possible to obtain gastric juice using a nasogastric tube. Gastric juice allows the detection of *H. pylori* by culture, staining, urease test, and PCR, but it is less reliable than gastric biopsy specimens. The string test can also be used to obtain gastric mucus (443); however, the most attractive method seems to be an extendable oro-gastric brush contained in a plastic tube (Baylor Brush, US Endoscopy, TX). The brush is swallowed, extended into the stomach to brush the mucosa three or four times, retracted in the protective sleeve, and withdrawn from the patient. This method is rapid and appears to be reliable for *H. pylori* infection diagnosis (187).

Culture

Biopsy specimens. (i) Specimen collection. The best specimens to culture *H. pylori* are biopsy samples obtained during endoscopy. Care must be taken to insure that the patients did not receive antibiotics or antisecretory drugs, especially proton pump inhibitors (PPI). Although PPI have no direct antimicrobial effect at the concentration present in the gastric mucosa (360), they indirectly interfere with *H. pylori* distribution in the stomach by changing the pH of its bacterial niche, leading to its disappearance in the antrum. The recommendation is not to consume these drugs 2 weeks prior to endoscopy. Obviously, the impact also depends on the dose and length of treatment; a single dose will not be as detrimental as a substantial acid suppression. Other ulcer drugs, e.g., sucralfate, have not shown any effect.

The potential contamination of biopsy specimens via endo-

scopes was a key issue in the past but now seems to have been resolved, at least in developed countries. Indeed, due to the discovery of prions and viruses, i.e., human immunodeficiency virus and hepatitis C virus (HCV), potentially transmitted by endoscopes, considerable attention has been given to the cleaning and disinfecting of endoscopes. Medical societies have even issued recommendations to use disposable forceps in some cases. A consequence for microbiologists is the elimination of gastric biopsy contaminants, e.g., *Pseudomonas* species and other environmental bacteria and sometimes *H. pylori* itself, which can be transferred from one patient to another. Contamination cases have been documented or suspected in the past, based on postendoscopy acute achlorhydria (534). Contamination of the stomach with oropharyngeal flora, and with bacteria from the intestine in the case of bacterial overgrowth, obviously remains. Pretreatment of the biopsy specimens by washing in saline could improve the recovery of *H. pylori* (239).

The number of biopsies which is necessary to diagnose *H. pylori* infection is a subject of controversy. A single biopsy specimen taken from the antrum (2 cm from the pylorus) gives good sensitivity but is not sufficient for a reliable diagnosis. Indeed, *H. pylori* may have a patchy distribution, and the more biopsy specimens analyzed, the higher the chance of organism detection (27). The recommendation is, therefore, to take two biopsy specimens from the antrum as well as two specimens each from the anterior and posterior corpus. There are some rare cases where the infection lies only in the corpus, but usually, *H. pylori* is present in all sites. After consumption of antisecretory drugs, the corpus may be the only site which remains positive.

Biopsy specimens for culture must be taken before specimens for histological examination, the latter being introduced in a fixative, otherwise there is a risk of transferring small amounts of fixative to the container for biopsy specimens to be used for culture.

(ii) Transport of biopsy specimens. A key point is the transport of the biopsy specimens from the endoscopic suite to the laboratory. Problems at this stage are surely the cause of many culture failures and have led to the negative opinion which some gastroenterologists have of culture. *H. pylori* is a fragile organism. It must be protected from desiccation and contact with oxygen and room temperature. It is mandatory not to expose the biopsy specimens to air and to place them either in a saline solution for short-term transport (4 h maximum) (368) or in a transport medium, usually consisting of semisolid agar, maintained at 4°C. A commercially available medium, Portagerm pylori (bioMérieux, Marcy l'Etoile, France), is effective for this purpose (192, 214). Culture may be delayed by 24 h if such transport media are used, allowing biopsies to be sent by mail in a cool transport container (Sarstedt, Nümbrecht, Germany) (192, 214). If these transport conditions cannot be used, it is better to freeze the biopsy specimens at −70°C or in liquid nitrogen in a dry tube and transport them frozen to the laboratory. Storage at 4°C in a medium containing 20% glycerol also led to *H. pylori* recovery in 81% of the biopsy specimens tested (203).

The proposal of direct biopsy specimen plating in the endoscopy suite is complicated, since biopsy specimen grinding
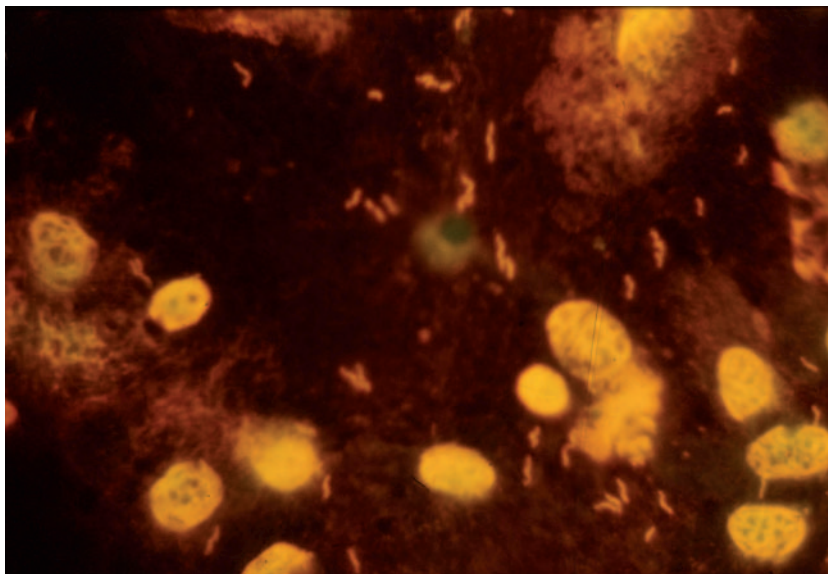
FIG. 1. *Helicobacter pylori* observed on a gastric biopsy smear after acridine orange staining. Magnification, ×1,000.

and a special atmosphere for plate transportation are needed. This procedure is not used.

(iii) Microscopic examination of smears. A standard bacteriology test includes a microscopic examination of a smear prepared from the biopsy specimen or touch cytology (95). Indeed, gastric brushings (43, 59, 77) followed by examination with phase-contrast microscopy in the endoscopy suite allow a quick diagnosis (452) and are an alternative to the urease test (93) (Fig. 1).

Staining can also be performed, using Gram stain (166, 390), rapid Giemsa (598), or the fluorescent acridine orange stain (504). This method has a sensitivity in the range of 80%.

Besides its ability to provide a rapid result, this method allows visualization of inflammatory cells and observation of *Helicobacter heilmannii*, which can be identified by its typical morphology including 6 to 8 spirals (96).

(iv) Grinding of biopsy specimens. In most instances, the bacteria are not distributed homogeneously in the biopsy specimens. Therefore, if they are only streaked on plates, several contiguous organisms will lead to one colony, whereas if the same bacteria were dispersed, several colonies would appear.

Comparison of culture performed with or without grinding showed a higher number of colonies after grinding, although the number of positive specimens did not change (182). For this reason, grinding of the biopsy specimen is mandatory. The recommendation is to use an electrical/mechanical grinder and a small volume of broth. Care must be taken to thoroughly wash and sterilize the probes. Another possibility is to perform manual grinding with disposable material. This solution avoids the risk of possible DNA contamination when performing molecular techniques.

(v) Culture on plates. Due to (i) the difficulty of growing *H. pylori* in broth and (ii) the usual abundance of bacteria, no enrichment has been proposed, only a direct plating is performed.

The media components include an agar base, growth supplements, and selective supplements. Most agar bases are satisfactory for growing *H. pylori*, e.g., brain heart agar, Columbia agar, and Wilkins Chalgren agar. Concerning the growth supplement, it is mandatory to add blood or serum, which includes numerous nutrients (vitamins and oligoelements, etc.) which enhance *H. pylori* growth. The proportion of blood or serum can be 5, 7, or preferably, 10%. Red blood cells can be lysed for these growth substances to be more readily available. Animal blood, e.g., sheep and horse blood, can be added, but human blood, to be used after appropriate testing, seems to confer a slight advantage (570). Other growth supplements such as egg yolk (569), charcoal (177), starch (46), bovine serum albumin, and catalase (208) are used. Cyclodextrins, which are cyclic oligosaccharides produced from starch by enzymatic treatment retaining the same properties as starch, are employed (423). More recently, ferrous sulfate, sodium pyruvate, and swine mucin have been proposed to enhance *H. pylori* growth (235).

Cellini et al. proposed a blood-free medium supplemented with isovitalex (2%) and hemin (10 mg/liter). They also added urea (20 g/liter) and a pH indicator (phenol red) to identify the urease-positive colonies (64). However, our experience does not favor the incorporation of a high urea concentration because the bacteria can be inhibited by the high pH induced. Indeed, pH is an important variable to consider; *H. pylori* grows best at a slightly acidic pH (5 to 6), in agreement with its ecological niche, the mucus layer, where a pH gradient exists (243). Another supplement which may be helpful to readily identify *H. pylori* colonies is 2,3,5-triphenyltetrazolium chloride (40 mg/liter). This compound is reduced by *H. pylori* to insoluble red formazan complexes, resulting in easily distinguished pigmented golden colonies (458).

Selective supplements are also quite important because of the presence of contaminating flora as mentioned before: buccal flora consisting mainly of gram-positive cocci, intestinal flora in the case of duodenal reflux, and bacterial overgrowth. Furthermore, *Candida* species may colonize ulcer craters.

Different selective supplements containing antimicrobial compounds have been proposed: vancomycin or teicoplanin to

inhibit gram-positive cocci; polymyxin, nalidixic acid, colistin, trimethoprim, or cefsulodin to inhibit gram-negative rods; and nystatin or amphotericin B to inhibit fungi. The Dent supplement, a modification of Skirrow's formula in which cefsulodin replaces polymyxin and amphotericin B is added, is commercially available (105).

Several studies performed in the early days of *H. pylori* detection showed the importance of using both a nonselective medium and a selective medium (273, 446) or even two different selective media (13, 514).

A critical point is to use fresh media (less than a week old) which is kept in closed boxes at 4°C to maintain humidity and avoid light exposure.

Helicobacters are microaerophilic and capnophilic bacteria. When their growth was studied according to the oxygen concentration, optimal growth occurred with a pO_2 of 2 to 10 KPa, whereas no growth occurred at a pO_2 of air (243). In contrast to these findings, Xia et al. claimed that most of their *H. pylori* strains grew under aerobic conditions but with reduced cell counts and smaller colonies (585). This discrepancy can be explained by the fact that, at low bacterial concentrations, *H. pylori* is microaerophilic, while at high bacterial concentrations, it can grow aerobically (50). Several systems can be used to achieve a microaerobic atmosphere, from the most sophisticated systems, such as a microaerobic cabinet or an incubator with an adjustable gas level, to jars in which the adequate atmosphere is created with an automatic apparatus (Anoxomat, MART Microbiology BV, Lichtenwoorde, The Netherlands) or with H_2-CO_2-generating packs. Besides their cost, the gas-generating packs take several hours to produce the optimal atmosphere. The atmosphere in jars will vary according to the quantity of bacteria consuming oxygen; therefore, the gas pack should be changed every other day. While *H. pylori* growth is possible in a candle jar (136), it takes a longer time and results in small colonies; therefore, we do not recommend this solution.

The optimal culture temperature is 37°C, testifying to the adaptation of this bacterium to humans. Certain strains can, nevertheless, grow at 30°C or 42°C (359). For primary culture under optimal conditions, colonies may appear after 3 days and are at their optimum on day 4. However, in the case of negative culture, a 7- to 10-day incubation is recommended to ensure that the result is negative; if only a few organisms are present, this time lapse may be necessary to visualize the colonies (544).

In contrast, subcultures only take 2 to 3 days. When few colonies are present, the recommendation is to subculture by plating the colonies on a small area of the agar plate. It is important to remember that once *H. pylori* reaches its growth plateau, it becomes coccoidal and loses its viability, most likely due to a lack of adequate nutriments.

Culture from biopsy specimens has the potential of leading to a high sensitivity, given that only one bacterium can multiply and provide billions of bacteria. However, both strict transport conditions and careful handling in the laboratory are necessary. Culture is extremely dependent on desiccation, contact with air, and temperature, as previously stated, and sensitivity reaches 95% at best. In contrast, its specificity is 100% because, once the bacterium has grown, all phenotypic and genotypic identification tests can be performed.

Quantitative culture of viable bacteria present in gastric mucosa can be performed. However, given the variability in the biopsy specimen size (1 to 10 mg), it is recommended to weigh the biopsy specimen. The bacterial load can be extremely variable, ranging from 5 to 10^5 CFU/mg, in one study (24). Reproducibility is higher in the antrum versus the corpus. A high *H. pylori* density has been associated with bacterial virulence determinants (*cagA*, *vacA*, s1m1), gastric inflammation, and duodenal ulceration, suggesting a central role in pathogenesis (21). Given that quantitative culture is expensive, time-consuming, and not very precise, a semiquantitative evaluation is most often performed.

(vi) Broth culture. Similar media, supplements, and conditions used for agar have been applied to broth culture (492), while brain heart infusion may be preferable for studies on physiology and metabolism (315). A chemically defined broth medium consisting of serum-free Ham's F-12 medium has also been described previously (515). The growth atmosphere is important, and two recommendations have been proposed in this respect: (i) constantly check the tubes in a microaerobic atmosphere (393) or (ii) prepare a biphasic medium with a thin broth layer in a cell culture flask incubated in a microaerobic atmosphere (491). Resulting bacteria are more likely to have a typical morphology and be motile. They can also aggregate. The use of gas-permeable cell culture flasks may improve *H. pylori* growth (490).

To obtain an important bacterial cell mass, subculture in successively increasing volumes from 5 ml to 500 ml is recommended (492). An alternative is to use fermenters.

(vii) Phenotypic identification. The growth of small, circular, smooth colonies observed after 3 to 4 days on the selective media plated with gastric biopsy specimens is an important criterion for *H. pylori* identification. No hemolytic activity is readily observed but may appear after a few days at 4°C.

Microscopic examination of the cultured bacteria may show a morphology different from the bacteria present in the biopsy specimen, i.e., bacilli which are neither spiral shaped nor motile, but straight or curved. Indeed, spiral shape and motility are directly related to the viscosity of the medium (207).

The identification of cultured bacteria consists essentially of testing for the presence of certain enzymes: cytochrome oxidase, catalase, and urease (312) and eventually γ-glutamyl transpeptidase, leucine aminopeptidase, and alkaline phosphatase. Aminopeptidases and esterases (C_4 to C_{12}) are also present (359).

The family *Helicobacteraceae* is comprised of the genera *Helicobacter* and *Wolinella*. *Helicobacteraceae* and *Campylobacteraceae* are in the *Epsilonproteobacteria*, class nov. according to the latest edition of *Bergey's Manual* (157).

Cytochrome oxidase is present in all members of the *Epsilonproteobacteria*. It is usually detected with special reagents on a disk or a strip. Catalase is also present in all helicobacters and most members of the *Campylobacteraceae* and is detected by introducing a loopful of bacteria into a drop of hydrogen peroxide and observing a very abundant production of bubbles. Nevertheless, catalase-negative mutants of *H. pylori* have been described previously (571). Urease is definitely the most important enzyme for identification. To survive in its particular ecological niche, *H. pylori* produces large amounts of this enzyme to buffer the acidic medium and creates a microenvironment (342). Mobley et al. reported that in *H. pylori*, 6% of the

total protein content was urease (381). When a loopful of *H. pylori* is put in contact with a few drops of urease medium, the color change occurs instantaneously regardless of the formulation. Other diagnostic tests are indeed either strictly based on urease, like the rapid urease test and urea breath test, or partially based, like serology and PCR, which may target urease genes.

The ApiCampy strip (bioMerieux, Marcy l'Etoile, France) allows identification of *H. pylori* via positive urease, γ-glutamyl transpeptidase, and alkaline phosphatase and negative nitrate reductase and hippuricase, all located in the first part of the strip, which explores preformed enzymes. The second part of the strip, which explores organic compounds as the only carbon source, cannot be used because growth of the organism cannot be supported by the minimal medium used.

Among tolerance tests utilized to differentiate *Campylobacter* species, *H. pylori* can grow with 2,3,5-triphenyltetrazolium chloride (0.4 and 1 mg/liter), sodium selenite (0.1%), and glycine (1%), but not with glucose (8%) and sodium chloride (3.5%). *H. pylori* is susceptible to cephalothin but resistant to nalidixic acid, with some exceptions (359).

When the bacteria are isolated from gastric biopsy specimens, phenotypic tests are sufficient for a precise identification. However, this is not the case when the bacteria are isolated from other specimens, e.g., stools, saliva, and the environment. Because other known or unknown bacteria sharing the same characteristics may be present, it is mandatory to confirm the identification with molecular methods. If PCR is used, several reactions targeting at least two different genes must be positive simultaneously. Sequencing of the urease gene may be worth performing because data are available for a number of *Helicobacter* species. Another spiral bacterium which we and others (478) identified in the stomach by culture is the urease-negative *Campylobacter jejuni* subsp. *doylei*. It was isolated for the first time from the stomach in 1985 (247).

Culture is an important step to perform further antimicrobial susceptibility testing (see below) and strain typing when necessary (49).

(viii) Question of coccoidal forms. As previously mentioned, when nutrients are lacking, *H. pylori* loses its spiral shape and becomes progressively coccoidal. Most people believe that these forms are both nonculturable and nonviable (278). However, others claim that some of them may be viable but nonculturable and constitute a resistant form of the bacterium (30).

The diagnostic implications are as follows: (i) a discrepancy is possible between the results from culture and from molecular tests as long as the DNA is not degraded and (ii) it is therefore important to subculture the colonies as soon as they reach their optimal size because afterwards they may die.

(ix) Strain maintenance. *H. pylori* is difficult to maintain. Colonies can survive on plates for a week provided they are kept in a microaerobic atmosphere at 4°C. For a long-term conservation, bacteria must be frozen at a low temperature (−70°C freezer or liquid nitrogen). Different broth media have been used, always with a cryoprotective agent, such as glycerol, either in cryotubes or on beads. The freezing-thawing process is always deadly for the bacteria, and only a small proportion survives. For this reason, it is mandatory to use bacteria in their exponential growth phase, as they are more likely to survive.

Frozen *H. pylori* specimens can be maintained for decades at −70°C. Freezing at −20°C is insufficient, and lyophilization appears to be difficult. Loss of viability is noted particularly during the dehydration phase (428). Storage of lyophilized vials at 4°C could help bacterial survival (506).

Other culture specimens. (i) Gastric juice. Gastric juice reflects the whole stomach and is easier to obtain than biopsy specimens but still necessitates an invasive procedure, i.e., introduction of a nasogastric tube. *H. pylori* is found in gastric juice due to the turnover of gastric mucosa, but the sensitivity is much lower than culture from biopsy specimens, with the best results for the former being in the range of 60% (149, 555). It does not seem crucial to buffer the collected liquid.

A less invasive procedure has been proposed by Perez-Trallero et al. involving Enterotest (HDC, San José, CA), also named the string test (443). A gelatin capsule fixed to a nylon fiber is ingested by the patient. After 1 h, the fiber is removed, with the capsule having been digested. The distal part of the fiber can be used for *H. pylori* detection by culture but also by urease test or PCR. This technique was applied by others but with sensitivity rates ranging from 38% to 97% (304, 482, 524). Leodolter et al. proposed to use the string test after the urea breath test as a diagnostic package for the identification of treatment failure and antibiotic resistance without the necessity of upper gastrointestinal endoscopy. The sensitivity of culture from the string test was 87% compared to culture from gastric biopsy specimens (303).

To study the possible transmission of *H. pylori* during vomiting, Parsonnet et al. obtained vomitus after induction of emesis in 16 volunteer subjects. They were able to grow *H. pylori* from all of the samples, often in high quantities (434). In contrast, spontaneous vomitus allowed the isolation of *H. pylori* in only one of four positive cases (306). The delay before performing the analysis on unbuffered gastric juice is critical: the survival rate was 62% after 2 h versus 15% after 24 h (154).

Gastric juice is well adapted for performance of quantitative culture and estimation of the potential for transmission of this organism (153, 593). In the study of induced vomitus from infected subjects, *H. pylori* grew in high quantities ($>10^3$ CFU/ml), while low quantities (50 to 500 CFU/ml) were found in postemesis saliva and cathartic stools (2,000 to 5,000 CFU/ml) in most of the subjects (434).

(ii) Blood. *H. pylori* may reach the blood flow from the gastric mucosa in the case of ulceration and hemorrhage. However, only one isolate was found in a blood culture from a patient with a gastric lymphoma. The strain grew after 5 days in a Bactec NR730 aerobic bottle (403). Indeed, regular blood culture systems may not be optimal for *H. pylori* growth. Conversely, blood culture systems used for fastidious organisms, e.g., brucella broth, led to satisfactory growth when experimentally inoculated (255).

(iii) Liver. Numerous attempts have been made to isolate *H. pylori* or other helicobacters in the liver and bile ducts in different hepatic diseases. Only one strain has been grown from a patient with Wilson disease in Brazil (103).

Gastric culture of other helicobacters. The other *Helicobacter* species possibly present in the human stomach is *Helicobacter heilmannii*, previously known as *Gastrospirillum homi-*

nis. This bacterium is commonly found in the stomachs of pets (dogs and cats) and pigs. It rarely colonizes humans and is considered a zoonosis (365).

This organism is nonculturable on standard media; it can essentially be cultured in vivo by introducing it into the mouse stomach (295). We succeeded in this endeavor three times in a clinical context.

In 1996, Andersen et al. were the first to grow *H. heilmannii* from a 23-year-old patient with dyspepsia on the same plate medium used in their laboratory to grow *H. pylori*, and growth occurred within 5 days in a standard microaerobic atmosphere. From primary culture, only 10 to 15% of the bacteria exhibited a typical morphology contrary to the majority after subculture (10). This is indeed the only positive culture on a plate for this bacterium which was later identified as *Helicobacter bizzozeronii* (233).

In summary, culture is still a valuable tool. It is considered the reference method when comparing the accuracy of noninvasive techniques. Its specificity is maximal, since the availability of colonies allows identification with all techniques, including sequencing of key genes (urease, *vacA* cytotoxin, *cag* pathogenicity island [PAI] open reading frames). Its sensitivity may be inferior, due to the stringent transport conditions required. Theoretically, its sensitivity should be the best because the presence of one organism in the inoculum is enough to give a positive result. However, in studies evaluating noninvasive tests, it is now the rule to consider a specimen negative by culture as *H. pylori* positive if both histology and urease tests are positive. Furthermore, culture is still the ideal method for antimicrobial susceptibility testing and typing, although molecular methods can also be used now directly on specimens for this purpose.

Histopathological Diagnosis

This diagnosis was one of the first to be applied to the detection of *H. pylori*. Historically, it is because of the histological observation of Warren and Marshall that *H. pylori* was looked for and finally cultured (565). This diagnosis is probably one of the most commonly used, at least in countries where endoscopy is frequently performed; gastroenterologists have a long tradition of collaborating with pathologists rather than with microbiologists.

Specimen collection and transport. The usual recommendation derived from the Sydney system (109, 453) is to obtain 2 biopsy specimens from the antrum and 2 specimens from the corpus. While the rationale is essentially to type the gastritis which may be present, *H. pylori* detection benefits from the high number of specimens tested. As indicated before, sensitivity increases with the number of biopsies (27). Bacteria are usually present at both sites even if the lesions occur essentially in the antrum. When topographic studies of *H. pylori* distribution and gastritis were performed, the best site suitable for diagnosis was the lesser curvature of the midantrum, while for the corpus, there was a discrepancy between greater and lesser curvatures (159, 375, 484).

Biopsy specimens are immediately introduced into a fixative of 10% formaldehyde. Consequently, there is no transport problem, since the material can be sent at room temperature by regular mail and is no longer infectious. This fixative main-

TABLE 1. Primary special stains used for histological detection of *Helicobacter pylori*

Stain	Reference	Yr
Warthin Starry	565	1983
Modified Giemsa	190	1986
Acridine orange	562	1986
Cresyl violet	47	1987
Gimenez	354	1987
Half Gram	532	1987
Ziehl-Nielsen	206	1989
Genta	160	1994
H. pylori silver stain	110	1997
Modified Genta	124	2000

tains the morphology of the bacteria, and most stains can be used. However, storage in formaldehyde is limited because, after a week, the diagnosis becomes difficult (142). The Bouin fixative must be avoided because it alters the bacterial morphology.

Preparation of histological slides. Ideally, an orientation of the biopsy specimens should be made before paraffin embedding to have sections which show the surface epithelium where bacteria are essentially located. The use of filter paper before fixation must be discouraged because the absorption of gastric mucus by the filter paper decreases sensitivity (590). It is usually necessary to cut three thin sections (3 to 5 μm) at different levels. The accuracy of this technique is very much dependent on the quality of the histological preparation, which must allow observation of the surface epithelium and the crypts.

There is no stain specific for *H. pylori*. The routine hematoxylin-eosin stain is not well suited for *H. pylori* detection because of the weak contrast between the bacteria and the mucus. There are several special stains (Table 1) which allow better visualization than the standard hematoxylin-eosin stain. This is essentially due to the acidophile characteristic of these stains which allows the staining of *H. pylori* DNA and not of the mucus where the bacteria are present, leading to a good contrast. The conclusion of the presence of *H. pylori* can be made easily, since it is seldom that bacteria other than *H. pylori* with similar morphology occupy this ecological niche.

The Warthin Starry stain allows an excellent visualization of the bacteria, but the procedure is difficult to carry out. This technique is time-consuming and costly and requires extemporaneous preparation of the reagents to be used. The Giemsa stain is probably one of the most popular stains because of its simplicity and good contrast (134, 269). A special stain has been proposed for *H. pylori*: the Genta stain with which both the bacteria and gastritis lesions can be observed (160).

Slide examination. The typical morphology of *H. pylori* in biopsy specimens is a comma or S-shaped bacillus (2.5 to 4 μm long and 0.5 to 1 μm thick). The bacteria are observed on the epithelial surface at high magnification. They adhere to the gastric mucus cells or are free in the mucus and may eventually be present in the intercellular spaces; very few bacteria, if any, are intracellular. Some organisms may be found in the canaliculi of the fundic parietal cells. Others may be present on metaplasic antral cells outside of the stomach, especially in the duodenal bulb (584) or in the esophagus. They do not colonize

the intestinal metaplasia but can adhere to areas of incomplete intestinal metaplasia which consists of a hybrid epithelium with both gastric and intestinal features (426).

There are limits to this technique. First, it is necessary to obtain good quality biopsy specimens, which is not always possible, and from a series of patients, it is common to have biopsy specimens where few epithelial surfaces can be observed. Second, when there is a low number of bacteria present, and in the event that they do not have a typical morphology, it is difficult to draw a conclusion.

The same conditions as previously described for culture (i.e., unsuccessful eradication treatment, consumption of PPI or antibiotics) may also jeopardize the accuracy of histological diagnosis (237). These conditions may trigger the evolution of the bacteria from a typical morphology to a coccoidal form which does not allow a diagnosis to be made. A high proportion of coccoidal forms was noted after gastrectomy in one study (65).

The proportion of cases with a chronic active gastritis but no bacteria present on the slides is currently increasing. The main reason is probably an uncontrolled usage of PPIs. Indeed, omeprazole administered as a monotherapy for 4 weeks decreases the bacterial density in both the antrum and the corpus (185) but at a differing magnitudes from one patient to another. This effect progressively disappears a few weeks after stopping the PPIs. Similar phenomena can be observed with H_2 antagonists but to a limited extent. Antibiotics used as a monotherapy also have a dramatic effect on the bacterial density even if they rarely lead to *H. pylori* eradication. Another entity, namely focally enhanced gastritis, seems to offer a picture similar to *H. pylori* gastritis. This gastritis is frequently observed in patients with Crohn's disease and, according to Pusztaszeri and Genta, is an indication to look for this disease (456).

Finally, the histological diagnosis of *H. pylori* infection is very much dependent on the expertise of the pathologists and on the time devoted to the diagnosis. A comparison of results obtained from experienced pathologists performing routine diagnosis highlights this problem (328, 382). The interobserver reproducibility was tested in 3 series (11, 74, 483) and measured by the Kappa test. A value of >0.75 corresponds to an excellent reproducibility, values of 0.4 to 0.75 correspond to an acceptable reproducibility, and a value of <0.4 is insufficient. The Kappa value was 0.69 and 0.74 in 2 series (11, 483), while in the third it varied from 0.39 to 0.82 according to observer pairs (74). In this last study, the intraobserver value was 0.65 to 0.88. These data cast doubt on the reliability of this diagnosis. Other studies have confirmed this problem (125, 238).

When doubt occurs, pathologists may use the presence of active gastritis as a surrogate, since this condition is almost pathognomonic of *H. pylori* infection. Immunostaining with a specific *H. pylori* antibody (Dako, Glostrup, Denmark) can also be used. In all of the studies performed, immunohistochemistry had the highest sensitivity and specificity. It allows a better interobserver agreement than routine histology and can also be performed with an autoimmunostainer (17, 110, 238, 526). In situ hybridization has been developed using a 16S rRNA gene probe labeled with digoxigenin (246) or with fluorescein (fluo-

rescence in situ hybridization [FISH]). The latter method is now commercially available and will be described in detail later, since it can also be used for antimicrobial susceptibility testing.

Histological results must be reported according to guidelines drawn up in 1990, known as the Sydney system (453). The presence of bacteria in the corpus and in the antrum is expressed semiquantitatively on a scale of 0 to 3. In addition, the histological characteristics of the gastric mucosa (inflammation, activity, atrophy, intestinal metaplasia) are also reported. The recent update of the Sydney system proposes inclusion of biopsy specimens from the incisura, an area where premalignant lesions are commonly found (109).

Furthermore, histology allows the diagnosis of the nonculturable *H. heilmannii*, which is seldomly present and easily identified by its typical morphology: it is longer than *H. pylori* with 6 to 8 spirals, nonadherent to epithelial cells, and commonly localized in aggregates in the crypts' lumen (Fig. 2). It also induces gastritis, which is sometimes transient, and may lead to gastric ulcer and gastric MALT lymphoma (96, 216, 394, 505). The FISH method can also be used to detect this bacterium (528).

In summary, histological detection can reach a sensitivity of 95% under optimal conditions, with the limits being the quality of the material and the pathologist's expertise. The presence of inflammation (lymphocytes) and especially inflammatory activity (polymorphs) is an obvious indication for an in-depth search for helicobacter organisms. The specificity is also in the range of 95%. The presence of other bacteria on the mucosa may be a cause of false-positive results if there are few bacteria with atypical morphology. However, the pathologist can use immunohistochemistry or FISH for confirmation.

Other histological methods. Electron microscopy can be used to detect *H. pylori* on the mucosa (145). However, this technique is too time-consuming and difficult to employ routinely.

Biopsy specimens frozen with a cryostat before an extemporaneous examination have been used as a rapid method, but their interpretation is difficult (244).

Urease Tests

Urease tests have been widely used because they are simple, cheap, and easy to carry out (35, 454). They can be performed readily in the endoscopy suite and give a rapid result.

Principle. The discovery that *H. pylori* was a strong urease producer was made by Langenberg et al. early in the bacterium's history (290). It was first applied to direct rapid diagnosis by McNulty and Wise (356).

To adapt to its special ecological niche where the concentration of urea diffusing from blood to the gastric mucosa is low, *H. pylori* produces large amounts of urease. *H. pylori* urease also has the highest specific activity (36 ± 28 μmol/min/mg of protein) among bacterial ureases (381).

The other urease-positive bacteria present in the gastric mucosa, i.e., streptococci and staphylococci, produce a lower amount of urease, which does not interfere in a short-time detection (<2 h), rendering the method specific to *H. pylori*.

When a biopsy specimen containing *H. pylori* is introduced into a urea-rich medium, the urease breaks the urea down into
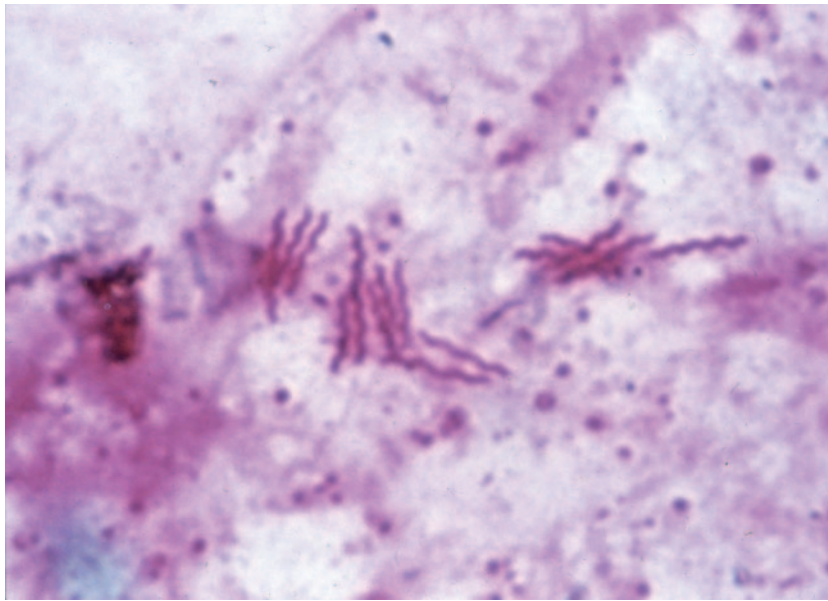
FIG. 2. *Helicobacter heilmannii* observed on a gastric biopsy histological preparation after hematoxylin-eosin staining. Magnification, ×1,000.

carbon dioxide and ammonia. The ammonium ion increases the pH, and a pH indicator, e.g., phenol red, changes color, in this case from yellow to red or violet.

The different urease media commonly utilized in bacteriology, i.e., Christensen medium and urea-indole medium, can be used, but specific media are preferable.

Different ways to improve the sensitivity of the tests include: (i) increasing the urea concentration from 2 to 10% (41), (ii) incubating at a higher temperature (37°C) (287, 595), and (iii) suppression of the buffer (15), while the size of the biopsy does not appear to be important (286, 594). Unbuffered media have a limited shelf life (<5 days at 4°C) (406).

The first-generation commercial kits were agar based, e.g., the CLO test (Kimberley-Clark, Neenah, WI). The new generation kits are strip-based tests with two areas separated by a microporous membrane, one where the urease hydrolyzes urea and the other where NH_3 is trapped and changes the pH (PyloriTek, Serim, Elkhart, IN).

The agar-based tests exhibit a good sensitivity only after 24 h (90 to 95%), compared to 70 to 80% after 1 h. Therefore, they cannot be classified as rapid tests after such a long delay. Furthermore, other urease-positive bacteria from the mouth can decrease the specificity when reading is performed after 24 h (489).

H. heilmannii, which may be present in the stomach, as previously indicated, is also a urease-positive bacterium but may not give a positive result, as the bacterial load is often limited. Therefore, the distinction between *H. heilmannii* and *H. pylori* is made by histological examination as previously indicated.

Attempts to obtain a rapid quantification of *H. pylori* based on the urease produced have been performed using an ammonia electrode measuring the ammonia liberated from samples placed in a urea solution (51).

Factors influencing results of the urease test. A limit to the urease test is the bacterial load necessary to obtain a sufficient sensitivity. A semiquantitative evaluation of the bacteria present by histology clearly showed that false-negative urease tests corresponded to the lowest histological scores for *H. pylori* (38, 522, 597). It seems that at least 10^5 bacteria are necessary for a valid result. This amount may not be present 4 weeks after the failure of eradication therapy, which makes this test less advisable for posteradication follow-up. Treatment with PPI may also jeopardize the result. By changing the milieu where the bacteria are present, especially the antrum, PPI renders it inhospitable and the bacterial load decreases. In addition, PPI themselves may have antiurease properties (533). Another reason for a false-negative test is the presence of intestinal metaplasia, which also corresponds to an inhospitable environment for *H. pylori*.

Per endoscopy, stains such as methylene blue may negate the urease test (198) so the specimens for this test must be taken before employing these stains.

The great progress in this area was the introduction of a strip-based test (PyloriTek) in 1995. In the first study, Rogge et al. compared this new test to the CLO test using histological detection as the reference. PyloriTek showed a 99% sensitivity and 95% specificity after 2 h, which is superior to those of the CLO test (468).

These results have been confirmed since then in several studies (288, 455, 592, 596). Indeed, the sensitivity of the final reading is not significantly different from that of the CLO test, but the last reading can be done after 1 h versus 24 h for the CLO test. For routine use, most endoscopists read the CLO test earlier than recommended, which leads to a marked decrease (20%) in sensitivity (288, 454).

Other specimens for the urease test. The use of the urease test on specimens other than gastric biopsy specimens, e.g., oral specimens, must definitely be discouraged because many other urease-positive bacteria (*Staphylococcus* spp., *Streptococcus* spp., etc.) can be present and give false-positive results.

TABLE 2. Standard PCR for *Helicobacter pylori* detection
in biopsy specimens

Target gene(s)	Yr	Reference	Amplicon size (bp)	Particularities
16S rRNA gene	1990	226		
	1991	222	109	
	1992	336		
	1992	126	500	RT-PCR
	1994	392		Fixed tissue
	1994	567	109	Fixed tissue
	1995	561		
	1995	521	537	
	1995	438	522	
	1996	73	109	
	1997	488	145	Fixed tissue
	1999	418		RT-PCR
ureA	1992	79	411	
	1993	553	411	
	1993	563		Nested PCR
	1995	438	491	RT-PCR
	1996	313		
	1999	196		
glmM (*ureC*)	1991	280	294	
	1995	283	294	
26-kDa antigen (*tsaA*)	1992	201	298	
Random sequences	1991	540	203	
	1996	308	417	

Molecular Methods

The PCR was developed in the 1980s and therefore was quickly applied to the detection of *H. pylori*. This method revolutionized the study of DNA, especially after the introduction of a thermostable DNA polymerase obtained from *Thermophilus aquaticus* (*Taq* polymerase). Its application in the field of *H. pylori* concerns not only the detection of the bacterium but also its quantification and detection of specific genes relevant to pathogenesis (*cagA*) and specific mutations associated with antimicrobial resistance.

Biopsy specimens. (i) Standard PCR for the detection of *H. pylori*. The choice of a target is important in designing the primers which must be specific for *H. pylori* but conserved in all strains of the species. It is therefore necessary to know the DNA sequence of the target in as many strains of *H. pylori* and as many strains of other bacterial species as possible. The first targets used were the genes of the urease operon: *ureA* (78) and *glmM*, formerly named *ureC* (107), or the 16S rRNA gene (222, 226) (Table 2). While highly conserved in bacteria, the 16S rRNA gene exhibits sequences which are specific for different species. However, the specificity of this PCR has been challenged because the 109-bp amplicon obtained with primers targeting the 16S rRNA gene reacted with human tissue samples (73). Other genes with unknown function, e.g., the gene encoding a 26-kDa protein identified as *tsaA* (201), or random sequences were used (540).

Among the drawbacks of PCR are (i) the possible existence of *Taq* polymerase inhibitors which can decrease the sensitivity of the reaction (520) and (ii) the possible contamination of the specimen by exogenous *H. pylori* DNA, which alters the specificity. Fortunately, it is uncommon to find *Taq* polymerase inhibitors in gastric biopsy specimens, so the procedure of DNA extraction can be simplified. The simplest technique consists of grinding the tissue to release the bacteria and bacterial lysis by boiling at 100°C in a lysis buffer.

Theoretically, PCR can detect only one copy of the target DNA when tested in water, but the amplification efficacy is not as good when biological material is concerned. Most studies have shown that standard PCR sensitivity is similar to that of culture for pretreatment diagnosis (31, 132, 297, 386, 540, 553). Several methods have been proposed to improve sensitivity.

To improve DNA extraction, phenol extraction or the use of special extraction kits (QIAGEN, Valencia, CA) to eliminate PCR inhibitors is superior to simple boiling (563). An internal control may help (520) but does not seem to be mandatory.

The use of a nested or seminested PCR was suggested. However, the use of nested PCR increases the risk of airborne contamination by amplicons and must be discouraged (131). Performing a second PCR with the same primer has also been proposed (502).

To increase the sensitivity of amplicon detection, a probe hybridization method can be used instead of the standard detection of the amplicons based on their size by electrophoresis (123, 567). New methods in liquid phase (DNA-enzyme immunoassay) (282, 385) and the reverse dot blot line probe assay (LiPA) (552) have been proposed. In addition, this approach definitely increases the PCR's specificity.

The use of reverse transcription-PCR (RT-PCR) has been reported by several authors (123, 126, 418, 438). Given that it is based on mRNA, it determines the viability of the bacteria present, but no improvement in sensitivity has been shown.

A significant improvement in sensitivity occurred with the introduction of real-time PCR protocols. As is always the case when a new method is more sensitive than the reference method, it is necessary to prove that the results are not falsely positive. One possibility is to conduct a follow-up of the patient over a few months to confirm the reality of the infection.

Specificity is not usually a problem when dealing with gastric biopsy specimens. However, the possibility of false positives arises when endoscopes (470) or grinding apparati in the lab are not correctly cleaned. It is recommended to use strict cleaning procedures in the endoscopy suite and disposable material in the lab to avoid such contamination. An important risk of contamination occurs when the amplicons are analyzed by electrophoresis. For this reason, there are strict rules stating that three independent rooms must be used for performing the different steps of a PCR.

An advantage of PCR is that DNA does not require strict transport conditions, and it has been performed on urease tests sent by mail (108, 314, 316).

(ii) Standard PCR for detection of pathogenic factors of *H. pylori*. An important application of standard PCR is the detection of specific pathogenic factors of *H. pylori*. There are two main pathogenic factors: the *cag* PAI and the polymorphism of the *vacA* gene. Other genes involved in adherence (*babA2*, *sabA*) or in pathogenicity (*oipA*, *dupA*, *iceA*) can also be detected by PCR.

Numerous studies have shown that strains harboring the *cag* PAI are associated with more severe diseases, especially peptic ulcer disease (84, 281) and gastric adenocarcinoma (36), as well as precancerous lesions (130, 450) and extradigestive diseases (148, 448). In contrast, no association was found with

gastric MALT lymphoma except for the *vacA* m2 allele (100, 296), and a negative association exists with gastroesophageal reflux (319). However, these associations have not been found everywhere. In fact, in Asia, most of the strains are *cag* PAI positive, and therefore, this pathogenic association cannot be used in case control studies (497, 501).

The main gene of the *cag* PAI is *cagA*. It was the first to be used as a marker for the presence of *cag* PAI (281). We now know that CagA proteins induce morphological alterations of the gastric epithelial cell, while other genes of the *cag* PAI lead to production of interleukin 8 (86, 588) via the intracellular Nod1 receptor and the nuclear factor κB pathway. The chemokine production is recognized as being responsible for an increased gastric inflammation and subsequent disease development.

While detection of the *cag* PAI has limited consequences on an individual basis, it has interesting implications in epidemiological and pathogenic studies. In addition to the disease association already mentioned, *cagA*-positive strains are easier to eradicate (44, 548).

It is possible to look for antibodies directed against the CagA protein or to detect the *cagA* gene. The first studies were performed on *H. pylori* strains by PCR. The variability in *cagA* sequences favors the successive use of two PCRs (281) or a Southern hybridization (501) to avoid false-negative results. The same PCR can be applied directly on DNA isolated from gastric biopsy specimens and gives similar results with bacterial colonies (67, 283, 433). The former is the preferred technique because it gives a picture of what exists in vivo (194). Material from a positive CLO test as well as from formalin-fixed embedded tissue may allow *cagA* detection (349, 487).

The presence of both *cagA*-positive and *cagA*-negative isolates in the same patient has been described previously (94, 137, 523). Comparison of the isolates by molecular typing showed that they were indeed the same strain (523), indicating the possible deletion of part of or the entire *cag* PAI. By using in situ hybridization with biotylinated probes for an *H. pylori* common gene and an oligonucleotide specific for *cagA*, it was possible to identify *cagA*-negative bacteria in the mucous gel of the apical epithelial surface, whereas *cagA*-positive bacteria colonized the immediate vicinity of epithelial cells or the intercellular spaces (57).

H. pylori also produces a vacuolating cytotoxin, VacA, which has been associated with the more severe diseases, e.g., peptic ulcer disease (144) and gastric adenocarcinoma (371). The gene encoding this cytotoxin is present in all strains but exhibits a mosaicism in the terminal (s) and median (m) regions. There are several alleles corresponding to various amounts of toxin produced: s1m1 corresponds to the highest production, followed by s1m2, while strains with the s2m2 allele do not produce any toxin (19). A PCR has been proposed to identify the genotype (20).

When both *cagA* and *vacA* detection is performed, a strong association exists between the presence of *cagA* and *vacA* s1, corresponding to strains with the highest production of cytotoxin (23, 202, 217, 475, 547, 589) as well as more severe pathology and disease.

A test has been designed to detect *H. pylori* and its main virulence factors: the INNO-LiPA (Innogenetics, Ghent, Belgium). This assay is comprised of a strip containing multiple specific probes for the *vacA* s region, the *vacA* m region and

cagA. Some of the corresponding *H. pylori* genes are amplified with a biotin-labeled PCR primer and subsequently analyzed by a single-step reverse hybridization on the strip (552). Other technical developments include a multiplex PCR for *vacA* and *cagA* genotypes (67) and real-time PCR melting curve analysis using SYBR green I dye (477).

(iii) Real-time PCR. The new real-time PCR technique is a breakthrough in the diagnosis of *H. pylori* because it allows not only a quick and precise detection of *H. pylori* but also its quantification and the detection of point mutations associated with antibiotic resistance.

The principle consists of following the increase in amplicons formed in real time. This can be done by using an intercalating agent, e.g., SYBR green I dye, or the fluorescence resonance energy transfer (FRET) principle (576).

Primers specific for the target gene are designed, as are a biprobe on the amplicon, the acceptor probe (sensor probe) 5′ labeled with LC-Red 640, which hybridizes where the mutation site is located, and a donor probe (anchor probe) 3′ labeled with fluorescein, which hybridizes 3 to 5 nucleotides upstream. When the anchor probe fluorophor is excited, it transfers the energy to the sensor probe fluorophore, which emits a signal. To detect point mutations or a polymorphism, a melting curve analysis is performed.

Since the essential application is not limited to *H. pylori* detection, real-time PCR will be reviewed with susceptibility testing.

(iv) PCR on fixed biopsy specimens. Performing PCR on formaldehyde-fixed paraffin-embedded material is a very attractive possibility because (i) it would allow the study of archive material and (ii) for fresh biopsies, there would be no maintenance problem. Unfortunately, fixed specimens are far inferior to frozen material. The best results are obtained with PCRs producing short amplicons because DNA can be broken by fixatives.

(v) PCR for quantification. PCR is also used for *H. pylori* quantitation first by using a competitive PCR, followed by the development of a real-time PCR which has considerably facilitated the process.

Competitive PCR is based on the coamplification of an internal standard (PCR mimic) and a target DNA sequence of different sizes with the same set of primers. Various amounts of PCR mimic are added to the target, and they compete for primer binding and amplification. A visual comparison allows the detection of bands with the same intensity indicating the amount of target DNA present (389). A colorimetric detection is also possible (430). The quantity of *H. pylori* in gastric mucus correlated with other invasive tests as well as with the urea breath test (UBT) in a study by Furuta et al. (152). They also used this method for patient posteradication follow-up with a high predictive value (153).

Real-time PCR was first used to quantify *H. pylori* DNA in gastric biopsy specimens in 2002. He et al. carried out a noncompetitive PCR on a LightCycler apparatus and FRET technology (209). The detection limit was 10^3 target copies (*ureC*) and there was a linear variation from 10^3 to 10^9 CFU/ml. While this study brings forth interesting data, the presence of target DNA in supposedly *H. pylori*-negative biopsy specimens casts a doubt on the specificity. The authors did not use surrogate markers of infection or follow up in these discrepant cases. The

same method was used by Lascols et al. but with another target, 132 bp of the 23S rRNA gene. A linear correlation was also found with as few as 300 CFU/ml up to 3×10^8 CFU/ml. The correlation with other invasive tests used semiquantitatively (histology, culture) was excellent (r, 0.86). Sensitivity and specificity of the technique were 97% and 94.6%, respectively (292). Another technological tool, the *Taq*Man, was used on paraffin sections of the biopsy samples (264). There was a good correlation with the UBT values.

DNA extraction is an important step in these procedures. It was evaluated in a mouse stomach model. Homogenization with glass beads, followed by use of the QIAGEN DNA mini tissue kit, was found to be the most suitable method (472). The same model was used to test the reliability of real-time RT-PCR to assess the abundance of transcripts in the gastric mucosa. Comparison to infected humans confirmed the value of this tool (469).

Other specimens. (i) Gastric juice. As for culture, PCR targeting mainly *ureA* genes has been applied to gastric juice aspirates with a good sensitivity and specificity (254, 347, 535, 572).

Since the introduction of the string test (442), this method of gastric juice collection has been favored (112, 140, 471, 591). The *cagA* gene, which is highly prevalent in the Far East, has been the target in a study using gastric juice in Taiwan (564).

(ii) Blood. PCR is not performed on blood samples for the detection of *H. pylori*. Interestingly, Dore et al. tested serum samples from a small number of infected patients and controls with genus-specific primers (namely C97-C98), as well as with primers designed on the conserved region of the *vacA* gene; 65% of the *H. pylori*-positive patients were positive with the *Helicobacter* genus primers and 75% of them also resulted in the amplification of *vacA* sequences. Cloning and sequencing of 16S rRNA gene amplicons of 3 samples had 99% identity with *H. pylori*. This surprising finding indicates that *H. pylori* DNA might circulate in peripheral blood (116).

(iii) Specimens from other sites. PCR was used to detect *H. pylori* in various sites along the digestive tract. The bacterium was detected in 2 of 46 appendix specimens from patients with appendicitis (4%) (437), in 4 of 12 ethmoid specimens from patients with chronic rhinosinusitis (33%) (429), and in 7 of 30 tonsil and adenoid tissue samples (30%) (76), probably following reflux.

PCR on liver specimens also yielded positive results for *H. pylori*-like organisms which seem to be associated with more severe diseases (cirrhosis, hepatocellular carcinoma) versus hepatitis alone in HCV-positive patients (467). The low discriminant power of 16S rRNA gene sequencing for species identification in the genus *Helicobacter* limits a definitive incrimination of *H. pylori* (424).

Attempts to detect enterohepatic helicobacters from the intestinal mucosa of patients with inflammatory bowel disease have also allowed the detection of *H. pylori*, probably as a bystander rather than a pathogen. Indeed, *H. pylori* crosses the intestine to be shed in feces but as a nonviable organism (422, 600).

The problem of specificity of PCR becomes fundamental in specimens from sites other than the stomach. At least two PCRs based on different target genes must be positive to conclude an *H. pylori* infection, as indicated before (76, 467).

Gastric PCR for other helicobacters. The tightly spiral *H. heilmannii* organisns are comprised, in fact, of 2 types: *H. heilmannii* I corresponds to a bacterium from the pig (58) and is also known as "*Candidatus* Helicobacter suis"; *H. heilmannii* II corresponds to 3 species, *H. bizzozeronii*, *Helicobacter felis*, and *Helicobacter salomonis*, usually present in cats and dogs. A 16S rRNA gene-based PCR was developed to detect these three species as a group. A small target DNA fragment (78 bp) enabled the study of formalin-fixed paraffin-embedded material (98). A multiplex PCR based on the tRNA intergenic spacers and on the urease gene allowed a discrimination of the three species and, combined with a 16S rRNA gene PCR for "Candidatus *Helicobacter suis*," covers all of the *H. heilmannii* types (25, 99).

A novel PCR targeting 16S rRNA gene has been designed to detect *H. heilmannii*-like organisms which are also pathogenic and could remain undetected by microscopic observation. Screening of 131 gastric biopsy specimens from dyspeptic patients with this assay revealed a prevalence of 2.3% in Southeast England. It was then combined with an *H. pylori vacA* PCR in a multiplex PCR assay allowing the detection of both pathogens (70).

The use of genus-specific primers based on the 16S rRNA gene (positive) in addition to *glmM* primers (negative) allowed the detection of gastric infection by *Helicobacter* species other than *H. pylori*. The 2 cases detected out of 126 were identified as *Helicobacter cinaedi*, an enterohepatic helicobacter, after cloning and sequencing (439).

NONINVASIVE TESTS

Despite the fact that direct diagnosis of *H. pylori* via endoscopy has proved to be a very valuable method and is even considered the reference method by many, especially when culture is performed under optimal conditions, it has several drawbacks which have stimulated the development of noninvasive methods.

The major problem is inherent to the invasiveness of the procedure. Despite the existence of small endoscopes like nasogastric endoscopes, the procedure still causes discomfort, which an increasing number of patients want to avoid. Anesthesia can be used, but it increases the risk of the procedure, which albeit very low, must still be considered. The previously mentioned risk of contamination by viruses such as human immunodeficiency virus or HCV, although theoretically nonexistent, is also a threat to some patients. Moreover, the cost of endoscopy is high, with an additional cost if anesthesia is performed; there is a need for disposable forceps, and the patients lose one working day or more.

Another limit of a direct diagnostic procedure is the possibility to explore only a small part of the stomach, i.e., a few mm^2 of a total surface area of 800 cm^2 (85), leading to possible sampling errors and the subsequent need to do several biopsies.

Therefore, numerous attempts have been made to develop diagnostic methods which avoid endoscopy. Since *H. pylori* is an infectious agent, the first method used was serology. However, due to the difficulty in obtaining an optimal specificity, other methods have been proposed: UBT, stool antigen test,

and most recently, detection of specific antibodies in urine or saliva.

UBT

Historical aspects. The first description of a UBT to detect gastric urease activity was published by Kornberg et al. in 1954 on a cat. After an intravenous injection of $[^{14}C]$urea, they were able to measure the $^{14}CO_2$ in the cat's breath. When antibiotics were administered before $[^{14}C]$urea, the amount of $^{14}CO_2$ decreased. This was the first demonstration of the bacterial origin of a gastric urease (270), now considered to be due to *H. heilmannii* and *H. felis*.

The same principle was applied in 1987 by Graham et al. using urea labeled with ^{13}C, a nonradioactive isotope, to detect *H. pylori* in humans (186). Marshall and Surveyor then described the $[^{14}C]$UBT in 1988 for the same purpose (345).

Principle. A solution of labeled urea ingested by the patient is rapidly hydrolyzed by *H. pylori* urease if this organism is present in the stomach; the labeled CO_2 is absorbed by the blood and exhaled in expired air. If the patient is not infected, no labeled CO_2 is produced and most of the isotope is eliminated in urine without modification.

When $[^{13}C]$urea is used, a specimen collection is performed before and 30 min after the ingestion. The $^{13}C/^{12}C$ ratio is measured in both specimens, and the result is expressed as the difference between the two measurements. The need for a baseline value is due to the various amounts of ^{13}C present in breath according to one's diet. The result is expressed as delta over baseline (DOB) per 1,000.

When $[^{14}C]$urea is used, specimen collection occurs only 20 min after ingestion.

Factors influencing the result and standardization of the $[^{13}C]$UBT. As with the other diagnostic tests, UBT results are dependent on the patient, the bacteria, and the test itself.

Some of these factors have been standardized. To maximize the contact between the labeled urea solution and the bacteria, it is recommended that gastric emptying be delayed by ingestion of a test meal after fasting. The need to fast has been challenged: a 98% agreement was observed comparing fasting and nonfasting protocols (380). However, the nonfasting protocol leads to DOB values which increase the risk of negative results when the DOB is close to the cutoff, even if it is infrequent (128). Eliminating the test meal completely has been advocated (577), but this also leads to lower DOB values and the possibility of false-negative results.

Originally, the test meal consisted of lipids (Ensure; Abbott Laboratories, Abbott Park, IL) to delay gastric emptying (18). This meal was even included in a European standard protocol (320). Later, it became clear that citric acid was a better test meal and was much more practical (113). The accuracy is the same when $[^{13}C]$urea diluted in the citric acid solution is administered (299), and this is now the most highly recommended method. Indeed, citric acid is able to sensitize the test not only by delaying gastric emptying but by a direct effect on intragastric urease activity, possibly on UreI, a proton-gated urea channel, making urea more accessible to the intrabacterial urease (499). Similar results have been found in another study where urease activity increased sixfold between pH 7 and pH 3 (431). Whether the enhanced *H. pylori* urease activity in

vivo is strictly pH dependent or not is still a matter of debate. Indeed, ascorbic acid does not lead to the same result as citric acid (3), which confirms that fruit juices like orange juice are not as good (300).

Another attempt to simplify the procedure concerned the omission of basal samples. However, because the basal DOB is diet dependent, this omission represents a loss in accuracy, especially posttreatment (168, 262).

The dose of labeled urea has also been standardized. It is important that the gastric urea concentration exceed the urease K_{max}. With 100 mg of $[^{13}C]$urea, this concentration is 10 times higher than the K_{max} and, therefore, is completely saturated. The dose has since been decreased to 75 mg, which is now the standard dose for adults. There are attempts to decrease this dose by administering a $[^{13}C]$urea tablet (199, 415) or $[^{13}C]$urea capsule (32, 440). This approach allows an earlier collection of the breath specimens (20 min or less).

With regard to breath collection, the pooled sample collection, which consisted of collecting breath at 5-min intervals for 30 min in the same bag, was quickly abandoned for the one-point sample collection after 30 min (262, 322). The goal is to minimize urea hydrolysis which could occur by contact with oral bacteria and is observed after a few minutes. Nevertheless, the recommendation of brushing one's teeth before performing the test does not seem important. During these 30 min, the amount of $^{13}CO_2$ increases progressively. The patient is advised not to smoke, eat, or drink and to remain seated quietly. It may be good to turn toward one side and then the other to increase the contact between the bacteria and the urea when not many bacteria are present.

There are several methods to measure the $^{13}C/^{12}C$ ratio. The most accurate is isotope ratio mass spectrometry, which requires only a small amount of breath to obtain a result (usually collected by blowing in a tube with a straw) (42). Another method is isotope-selective nondispersive infrared spectrometry (249, 266). While the measurement is less precise, it has no consequence on the result, and the main limitation is the need to collect a large amount of breath air (500 ml) by blowing into a special balloon. Consequently, this measurement must be performed locally rather than being sent by mail. A laser-assisted ratio analysis (399) was also designed but was never developed commercially (62, 485, 543). When the reproducibility of the UBT performed locally or in a central facility was studied, no difference was noted (210). Comparison of the specimen collection made by the patient under medical supervision or at home resulted in a 5% decreased accuracy in the latter (517).

Interestingly, an apparatus (Breath ID; Oridion, Israel) has been developed for online follow-up of the $^{13}C/^{12}C$ ratio, which usually allows the detection of *H. pylori*-positive patients within 20 min (232). The expression of the ratio is recorded in parts per 1,000 using an international standard of calcium carbonate named pee dee belemnite as the reference (88). Basic breath air values are lower than the standard, explaining the negative values obtained. These values increase after $[^{13}C]$urea ingestion in *H. pylori*-positive patients. By convention, the results are expressed as an excess of ^{13}C excretion and not as a percentage of the administered dose or urease activity per minute, which facilitates the comparison between laboratories.

The cutoff used to determine *H. pylori* positivity is still a matter of debate. The standard method of taking the mean

plus 3 standard deviations of the values obtained in *H. pylori*-negative subjects (as determined by invasive tests) gives a cutoff of 4.9 per 1,000. When a comparative gold standard is available, a good method to use is the receiver operating characteristic curve (373), which leads to a cutoff of 3 per 1,000. Other methods have been used, such as the cluster analysis, and a cutoff of 3 per 1,000 was determined (374). A gray zone to separate positive and negative results is not usually proposed but could be a good solution. In fact, there are a limited number of cases falling into this category. Some authors suggest using a lower cutoff for posttreatment follow-up because the negative values seem more homogeneous (3.5 per 1,000).

Given this high standardization, the lack of impact of transport conditions and the independence of the result from human judgment, the UBT is a highly accurate and reproducible test. Among the noninvasive indirect tests, UBT has the best sensitivity, approximately 95%, i.e., in the range of the best invasive tests. Only a few cases with a limited number of bacteria present are missed (173). Specificity is also excellent. However, other urease-positive bacteria present in the stomach may also give a positive result. One example is *H. heilmannii*, but in this rare case, an eradication treatment is also worth prescribing. Enteric bacteria, e.g., *Proteus* sp., can theoretically be present in the stomach in the case of hypochlorhydria and bacterial overgrowth, but they have not been isolated from adults in developed countries. The situation in children, especially those younger than 2 years and from developing countries, needs further investigation (118). False-negative results may also occur in the case of bleeding, i.e., if there is a bleeding ulcer or even after taking gastric biopsy specimens. In a study where a high concentration of citric acid (4 g) was given, false negatives were eliminated when omeprazole or pantoprazole were used but not with lansoprazole or esomeprazole (307). A limitation of UBT is the need for an expensive apparatus, especially the gas chromatography isotope ratio mass spectrometer, but numerous specimens received by mail can be analyzed simultaneously with one apparatus.

Quantitative UBT. Numerous studies tried to correlate UBT values with the quantity of *H. pylori* measured by an invasive method, culture, or histology. A positive correlation was always found, with correlation coefficients ranging from 0.56 to 0.74 (66, 97, 121, 249, 495, 512, 558), except in one study (0.17) (24). High UBT values have also been used as predictors of severe gastritis (350) and low eradication (285). However, in another study, the mean delta values of UBT were not significantly different between patients who later showed eradication success or failure (171).

Other methods not using breath analysis. Measuring labeled bicarbonate in blood instead of measuring $^{13}CO_2$ in breath was first proposed in 1993 by Moulton-Barrett et al. (397). In this assay, the $^{13}C/^{12}C$ ratio is determined in serum by measuring before and 1 h after ingestion of labeled urea. The sensitivity and specificity were lower than that of UBT in one study (259) and equivalent in two other studies, including a United States multicenter trial (69, 89).

The possibility of using another isotope, [^{15}N]urea, and testing $^{15}NH_4$ in urine was also explored (275, 582), but it does not offer any particular advantage over ^{13}C.

Particularities of the [^{14}C]UBT. Most of the characteristics of the [^{14}C]UBT are similar to those of the [^{13}C]UBT. However, for [^{14}C]UBT, the volume of expired CO_2 is critical for the measurement of $^{14}CO_2$. The expired air is first dehydrated by passing it through a solution of $CaCl_2$, and then it is trapped in an alcohol solution of hyamine. A color change indicates that a sufficient volume of CO_2 has been collected. The results are adjusted to the endogenous production of CO_2, which depends on the patient's height and weight. An alternative way to collect $^{14}CO_2$ is to use the Heliprobe breath card and analyzer (Noster System AB, Stockholm, Sweden) (215).

^{14}C is a radioactive isotope with a half-life of 5.7 years. According to Munster et al. (398), approximately 90% of the ^{14}C from a UBT is excreted as CO_2 in expired air or as urea in urine.

Originally, the dose administered was 10 μCi (345), but because of the potential risk, attempts were made to decrease this dose first to 5 μCi (344, 353) and then to 1 μCi (= 37 KBq) (8, 32, 344, 445, 459). To avoid oral hydrolysis, these authors proposed to administer this amount in a capsule (200, 215, 344). The ingestion of citric acid is also used to sensitize the test (462) instead of the traditional test meal.

The ^{14}C measurement is performed on a sample collected after 20 min with a scintillation counter, an apparatus widely available in many settings. The cutoff value to define a positive sample is 2% of the administered dose. The reproducibility of [^{14}C]UBT within 1 week is excellent (508).

Due to the radioactive nature of the isotope, this test cannot be used on children and pregnant women and is forbidden in some countries, but it is a cheap and simple method. A variant of the test where the radioactivity is measured in urine has also been proposed (436).

Stool Tests

Stool tests have the advantage of being direct noninvasive tests because they detect either the bacteria or part of it (DNA, antigen) in a specimen which is easily obtained.

Culture from stools. As everything present in the stomach will be found in the stools, there is no doubt that *H. pylori* is eliminated via this route. However, because the bacterium is susceptible to biliary salts, especially deoxycholic acid (204), and to vital competition from other bacteria, it may take the form of a nonculturable bacterium.

Indeed, the ability to culture *H. pylori* from feces may depend on the transit time. The first successful culture was carried out by Thomas et al. using feces from malnourished children from developing countries with a short transit time (519). By inducing diarrhea with sodium phosphate in *H. pylori*-positive adult volunteers, Parsonnet et al. were able to grow the bacterium in only 50% of the samples (434). Dore et al. also reported the isolation of *H. pylori* from three *H. pylori*-positive patients among 12 explored, by adding cholestyramin, a bile-sequestrating agent, before plating on the culture medium (115). These poor results, obtained despite the special conditions provided, cast doubt on the validity of the results of others who claimed the recovery of *H. pylori* from adult patients (256). However, more recently, Liang and Redlinger reported successful isolation of *H. pylori* in feces of four subjects, highlighting the importance of the antibiotic concentration of the media and the ability to recognize *H. pylori* colonies after 5 to 7 days as well as the timely collec-

tion of the specimens (311).

Indeed, our own experience and that of Namavar et al. (401) indicate that bacteria similar to *H. pylori* may be present in feces, and therefore, it is necessary to carry out a molecular identification before drawing a conclusion. Under no circumstances can *H. pylori* culture from stools be used as a routine diagnostic method.

PCR on stool samples. In contrast to gastric biopsy specimens, where *H. pylori* is usually the only living bacterium, stool samples are very complex material for which the pre-PCR steps are quite important.

The first attempts to detect *H. pylori* DNA in stools using PCR were made by Mapstone et al. in 1993 (337). These results were not reproduced by Van Zwet et al. (553). It became clear that small amounts of target DNA and the presence of substances inhibiting the polymerase reaction were responsible for these failures. Monteiro et al. explored the nature of these inhibitors. They were identified as essentially complex polysaccharides of vegetable origin (384).

Very long, complex, and fastidious methods have been proposed to eliminate these compounds (332). A 48-h vegetable-free diet decreases their amount (388). This observation is in line with superior results obtained with infants who did not have a diversified diet. However, numerous attempts have been made to obtain pure DNA. An immunomagnetic separation (magnetic beads coated with *H. pylori* antibodies) was proposed and was found to be experimentally suitable for young cultures but not for older cultures, probably due to the different antigenicity of the coccoidal forms (127). Polysaccharide concentrations were also significantly reduced using macroporous polypropylene filters (61) and two commercial kits: QIAamp tissue kit and Xtraction DNA extraction kit (QIAGEN) (14). Monteiro et al. also developed a PCR combining *H. pylori* extraction by immunomagnetic separation and an agarose-embedded DNA preparation (387). This method, which was developed to recover intact DNA from pulsed-field gel electrophoresis, provides clean, high quality template DNA. It requires a small number of steps, reducing the risk of DNA loss or contamination and, furthermore, avoids the use of nested PCR and its risk of contamination (131). The sensitivity of this method with *ureA* primers was nevertheless only 81%, with a specificity of 100%. The same proportion of positive samples was obtained by gene capture with a biotinylated oligonucleotide probe targeting the *H. pylori* 16S rRNA gene (326). After incubation, it is harvested on paramagnetic polystyrene beads coated with streptavidin. The recent availability of a DNA extraction kit devoted solely to stools (QIAamp DNA stool kit; QIAGEN) seems to be progress. For detailed information on extraction protocols, see the review by Kabir (241). However, the use of a very specific *H. pylori* target is imperative because other helicobacters may be present in stools, e.g., *H. cinaedi*, *Helicobacter pullorum*, and *H. canis*.

PCR also allows the detection of pathogenicity factors. Sicinschi et al. used an extraction protocol delivering sufficient amounts of DNA, radiolabeled primers, and 80 amplification cycles for the detection of *cagA* and *vacA* genotyping (502). They also compared their genotyping procedure in stool samples to that of biopsy specimens (unfixed and paraffin embedded) and claimed that the stool protocol offered more-accurate results. In another study, *cagA* was also

detected directly in stool specimens (476).

Of even greater interest is the ability to test for antimicrobial susceptibility. A simple DNA extraction method, followed by a seminested PCR and a restriction fragment length polymophism (RFLP) analysis, was used in Italy (146). It is, however, surprising that the two clarithromycin-resistant cases found did not exhibit the usual 23S rRNA gene mutations but had T2717C mutations.

The use of biprobe real-time PCR followed by melting curve analysis is indeed a more promising approach, as described by Schabereiter-Gurtner et al. They obtained a complete agreement for diagnosis, but for specimens with a mixed population of susceptible and resistant strains, only the susceptible type was detected (486). A summary of the publications concerning PCR in stools is presented in Table 3.

The development of a commercial test to detect *H. pylori* and its eventual clarithromycin resistance in stools by PCR will undoubtedly provide an accurate and convenient diagnostic method (457). It is, however, too early to provide figures for sensitivity and specificity.

Detection of *H. pylori* antigen in stools. The first report of successful detection of *H. pylori* antigens in stools was made in 1997. Kozak et al. reported an enzyme-linked immunosorbent assay (ELISA) performed on stools, using polyclonal anti-*H. pylori* antibodies coated on microwells to capture *H. pylori* antigen and peroxidase-conjugated polyclonal antibodies to detect the immune complex (272). This test was named *H. pylori* stool antigen test (HpSA) (Meridian Diagnostics, Cincinnati, OH). Its evaluation on stools obtained from 100 adult dyspeptic patients indicated 88.8% sensitivity and 94.5% specificity (332). A large multicenter study was then carried out in Europe in 11 endoscopy suites. Five hundred patients were included, and sensitivity and specificity were 94.1% and 91.8%, respectively, versus 95.3% and 97.7% for UBT (537). Numerous studies were then performed, and two systematic reviews confirmed the value of HpSA as a pretreatment diagnostic tool (174, 539). The interest of this test for assessing *H. pylori* eradication was also confirmed in 162 patients issued from the multicenter study (538) as well as in others summarized in the systematic reviews (172, 539).

However, recent studies may not be as convincing. Perri et al. compared UBT and HpSA on 458 patients at 4 to 6 weeks posttherapy. They found discrepant or indeterminate results in 8% of the cases. The patients with unclear results underwent endoscopy with biopsy sampling for several invasive tests. On the basis of these further diagnostic methods, the authors concluded that HpSA was less accurate than the UBT (444). In the studies of Bilardi et al. and Matsuda et al., HpSA was also shown to be inferior to UBT, especially with regard to specificity (33, 346).

A new generation of antigen stool tests emerged with the Femtolab (Connex), also known as Amplified-IDEA-Hp-StAR (Dako, Glostrup, Denmark). This new kit was compared to HpSA on stool specimens from 48 pediatric patients (330) and 53 adults (4) and exhibited a comparable accuracy. This kit uses monoclonal antibodies instead of polyclonal antibodies, which ensure a constant quality of reagents and, therefore, an excellent reproducibility rather

TABLE 3. PCR to detect *Helicobacter pylori* in stools[a]

Pre-PCR step and PCR protocol	No. of specimens tested	Target gene	Amplicon size (bp)	Reference method	Sensitivity (%)	Specificity (%)	Reference	Yr
None	24	16S rRNA gene	109	Culture	0	NT	554	1994
	20	16S rRNA gene		Culture	7		401	1995
							409	1996
	71	NI	417	Culture, Histo, Sero	25	90	308	1996
Seminested PCR	100	26-kDa antigen gene	209	UBT, Sero, culture, Histo	93	100	332	1998
IMS	72	*ureA*		Culture, Histo, PCR biopsy	61	100	566	1998
IMS	68	*ureA*	139		60		60	1999
IMS	16	16S rRNA gene		Histo, Sero	68	NT	434	1999
	22	16S rRNA gene	138	Histo, Sero, PCR biopsy	73	100	189	1999
Seminated PCR	49	26-kDa antigen gene	209	UBT, Sero	91	NT	330	2000
IMS + agarose-embedded separation of DNA	104	*ureA*	411	Culture, Histo, UT	81	100	388	2001
Capture probe-IMS	25	16S rRNA gene	204	Histo, PCR biopsy	100	100	500	2002
	100		209		25		7	2002
QIAamp DNA stool kit + seminested PCR	60	*ureA*	155	UBT, UT, Histo	26		575	2002
QIAamp DNA stool kit + seminested PCR	283	23S rRNA gene	783	Culture	100	100	146	2003
Capture probe-IMS	30	16S rRNA gene		UBT, HpSA	75	100	327	2003
QIAamp DNA stool kit modified + 80 cycles	48	*vacA*			92		502	2003
QIAamp DNA stool kit	148	*ureA*		UBT, HpSA	35	98	599	2004
Real-time PCR	79	*ureA*		UT, Histo, culture	100	100	330	2004

[a] IMS, immunomagnetic separation; NT, not tested; NI, not identified; Histo, histology; Sero, serology; UT, urease test.

than intertest variations. The optical densities obtained are also significantly higher than those of HpSA and are far from the cutoff value (330).

Another progress is the use of monoclonal antibodies in an office-based stool test, ImmunoCard STAT HpSA (Meridian) (Table 4), but its accuracy is lower than the standard laboratory ELISA (174).

However, the stool antigen tests have some limitations. The impact of bowel movements has not been studied in detail. In principle, a short transit time should favor elimination of unaltered antigens (unless there is an important dilution factor), while constipation should lead to degradation of the antigens.

TABLE 4. Evaluation of stool antigen tests using monoclonal antibodies performed pretreatment in adult patients for detection of *Helicobacter pylori*[a]

Test (manufacturer)	Country	No. of patients	Reference tests	Sensitivity (%)	Specificity (%)	Reference
HpStAR (Dako)/Femtolab (Connex)	Austria[b]	49	UBT, Sero	98.2	97.5	330
	Germany	53	Histo, UT, UBT, Sero	88.5	96.3	4
	Spain[c]	79	Histo, UBT	78	79	54
	United Kingdom[b]	111	Culture, Histo, UT	88	92.6	12
	Germany[b]	302	Culture, Histo, UT, UBT	98	99	267
	Norway	122	Culture, Histo, UBT	98	94	16
	Spain	122	Histo, RUT	98	76	56
	Spain	86	UBT, Sero	86	100	321
	Turkey	151	Histo, RUT, culture	93	90	129
	United Kingdom	112	Culture, Histo	93.8	100	72
	Italy	104	Histo, RUT	88	100	530
ImmunoCard STAT HpSA (Meridian)	Italy	303	Histo, UT	91.3	93.5	158
	Italy	105	Histo, UT	85	93	530
	United Kingdom	87	Culture, Histo	87.8	89.4	72
	China	53	UT, Histo, culture	92.6	88.5	310
	Germany	50	UT, Histo	73	98	302
	Spain	63	UT, Histo	89–91	86–93	55
	Taiwan	253	UBT	95.8	91.1	581

[a] UT, urease test; Histo, histology; RUT, rapid urea test.
[b] Children.
[c] Cirrhotic patients.

Preservation of the specimen has not been considered important but should be studied. Indeed, in experimentally spiked specimens, the lowest concentration could not be detected after only a 24-h delay (388). Treatment with the mucolytic agent *N*-acetylcysteine has been shown to decrease both the test's sensitivity and specificity (104). As previously stated, determining a cutoff to define a positive sample is crucial, especially for HpSA, and adjustments may have to be made. While these tests are quite specific, it is possible that rare *Helicobacter* species present in stools (enteropathic helicobacters) may also be detected.

Detection of *H. pylori* Antibodies

Serodiagnosis. *H. pylori* infection almost constantly induces a specific systemic immune response which may reflect the antibodies produced at the gastric mucosal level, while only 2% of patients fail to seroconvert (276). This response was used for the diagnosis of this infection immediately following the discovery of *H. pylori* (236, 343). The immune response varies according to the antigens present in the infecting strains and to the host.

(i) Techniques for serodiagnosis. A complement fixation test was the first to be used in 1984 with an 85% sensitivity (236). A commercial complement fixation kit had an insufficient sensitivity (71 to 81%), while the specificity exceeded 90% (183).

A passive hemagglutination test using an *H. pylori* sonicate appeared to have a better sensitivity (343). A latex agglutination was also proposed (220, 568), but the essential technique used for more than 20 years is the standard ELISA and its derivatives, such as rapid immunoenzymatic assays and immunoblotting.

(ii) Antigens used for serodiagnosis. The performance of an ELISA is largely dependent on the nature of the antigens used. They must be highly immunogenic, common to most *H. pylori* strains and absent from other bacteria, and easy to prepare and purify, and they must bind to microwells and be stable upon storage.

The first antigens to be used were whole-cell sonicates, due to the fact that *H. pylori* is quite a unique bacterium in humans. They gave results with high backgrounds and limited sensitivity and specificity.

A partial purification to obtain surface antigens can be achieved by glycine acid extraction. ELISAs with these complex antigens had a sensitivity of 95% with a specificity of 90% according to Newell and Rathbone (404).

The presence of cross-reacting antigens, especially flagellar proteins, between *H. pylori* and campylobacters led to the development of more purified antigen preparations. Attempts were made with purified urease, for example, but the results were not good enough. For this reason, most of the commercially available ELISAs use a mixture of specific antigens for which the exact composition is patented.

A comparison of several kits testing the same sera in several laboratories in Europe indicated that Pyloriset EIA-G (Orion Diagnostics, Espoo, Finland) had the best accuracy (139). Serological tests to detect CagA antibodies have also been designed based on recombinant proteins. Interestingly, when they were used on sera of gastric adenocarcinoma patients, more

cases were detected than with the standard *H. pylori* ELISA, probably because CagA is a highly immunogenic molecule, whose corresponding antibodies persist for longer periods (120).

(iii) Antibodies detected. *H. pylori* infection is a chronic condition and immunoglobulin G (IgG) (subclasses 1 and 4) is the predominant immunoglobulin class, even in children (376). IgG are present at the mucosal level and detected in virtually all blood samples. IgM are rarely observed, merely because acute *H. pylori* infections are seldom available for study. In the experimental infection carried out by Morris et al., an initial IgM response was observed (395). IgA are also elevated in the majority of infected cases but not in all. Therefore, as the relevance of IgM and IgA is limited, commercial kits are primarily designed to detect IgG. It is well known that IgG levels begin to decrease after eradication of *H. pylori*, but this fall in titer may take months or years before returning to baseline values (271).

(iv) Factors affecting the ELISA result. Since *H. pylori* infection is a mucosal infection, the antibody response may be modest, so it is important to have the best antigen and an appropriate cutoff value, but the sensitivity of ELISA serology can be excellent.

As previously stated, *H. pylori* is a very diverse bacterial species, with different clusters found, especially between the Far East and the West (218, 405, 461, 550). Diverse antibody profiles can be seen when different strains are studied by immunoblotting (351). Some authors have emphasized the need to use local strains as the source of antigen rather than foreign strains to obtain the best test performance. This may be especially true in Asia (223, 412). However, others insist on the importance of common antigens present between the strains (9) and recommend pooling antigens from strains representing the different groups (225, 340).

It has often been stated that the cutoff should be validated locally on a pool of reference sera obtained locally rather than using the cutoff values proposed by the manufacturer (234, 318). Indeed, this statement should be revised because some commercial kits propose cutoffs which can be used universally, at least in the West.

False-negative results may occur following a new infection before the antibody level is sufficiently elevated. The specificity of serology is satisfactory (90%). In developed countries, the risk of cross-reactions with other bacteria is limited. In developing countries, the specificity of the test may be altered by the presence of concomitant infections, especially with campylobacters or other related bacteria; furthermore, the host immune response may be lower due to malnutrition. Such cases definitely justify the reevaluation of the tests, and for this purpose, making an receiver operating characteristic curve is the best alternative (527).

The very slow decrease in antibodies after eradication (25% in titer within 6 months or more) is also a cause of a false-positive result. For this reason, the tests detecting active infection are preferable.

Indeed, the negative opinion on the specificity of serology is mainly based on correlations made with poorly performed gold standard methods. Recent data have shown that serology is the best method in difficult situations where bacterial density may

TABLE 5. Detection of *Helicobacter pylori* IgG antibodies in urine with commercial kits (Urinelisa, Rapirun)[a]

Type of test	Type of patient	No. of specimens tested	Reference test	Sensitivity (%)	Specificity (%)	Reference	Yr
ELISA	Adult	100	UBT	99	100	252	1998
ELISA	Adult	132	UBT	86.3	91.5	379	1999
Rapirun	Adult	117	Histo, Sero	92	93.1	587	2000
ELISA	Adult	1,061	Serum ELISA	97.7	95.6	248	2000
ELISA	Adult	238	Culture, Histo, UT	96.2	78.9	248	2000
Rapirun	Adult	142	Culture, Histo, UT, UBT	94.6	89.6	580	2001
ELISA	Child	816	Serum ELISA	85.4	95.5	251	2001
Rapirun	Adult	189	Serum ELISA	93.7	88.9	151	2001
Rapirun	Adult	76	Culture, Histo, UT	100		151	2001
Rapirun	Adult	104	UBT	95.3	96.7	188	2001
Rapirun	Adult	155	UBT	95.9	87.9	378	2001
Urinelisa	Adult	74	UBT	91.4	94.9	378	2001
Rapirun	Adult	74	UBT	85.7	97.4	2	2002
Rapirun	Adult	123	Histo, UT	96.7	95.2	578	2002
ELISA	Child	68	UBT	92.3	76.4	496	2003
ELISA	Child	100	UBT, HpSA	94.4	96.9	419	2004
ELISA	Adult	449	Culture, Histo, UT	90	68	301	2003
Rapirun	Adult	449	Culture, Histo, UT	82	83	301	2003

[a] UT, urease test; Histo, histology; Sero, serology.

be low due to gastric atrophy or due to previous treatment with PPI or antibiotics, as is now frequently the case (334).

(v) Development of point-of-care tests. In contrast to laboratory tests, these tests can be used by the doctor in his/her office. They are also called "office tests," "near-patient tests," or "doctor tests". Their advantage is to be simple, cheap, and able to deliver a quick result. They are essentially based on the diffusion of antibodies from a drop of serum or whole blood obtained by finger puncture through a membrane and an immunoenzymatic reaction. The first test was proposed in 1994 (Quickvue, Quidel, San Diego, CA) with a very promising performance (sensitivity, 92%; specificity, 88%).

Unfortunately, more than 30 studies were later performed with this test as well as others (Helisal, FlexSure, Hp Check, Stat Simple, Accustat, BM test, and Pyloriset Screen, etc.), with sensitivity and specificity values rarely reaching 90% and a mean of 82%. Furthermore, in most of these studies, the tests were not performed under the conditions which should have been applied, i.e., general practitioners carrying out a few tests per month, rather than dedicated laboratory personnel carrying out all of the tests at once, with the latter favoring better performances of the tests. When we compared one of these tests (Pyloriset Screen; Orion, Espoo, Finland) performed by general practitioners to a laboratory ELISA, we nevertheless found a 95% accuracy, which is comparable to the evaluation made in comparison to invasive tests (417).

Another problem is the difficulty in reading the results in 10% of the cases, and the patients do not express a strong preference for a finger puncture versus venipuncture (510).

The final consequence is that the point-of-care tests have not been recommended in the Consensus Conferences (334).

(vi) Immunoblot analysis and detection of CagA antibodies. Immunoblot analysis has proved to be very attractive for the diagnosis of many infectious diseases. While its sensitivity may be equal or inferior to standard ELISA, the resulting antibody profile makes it more specific.

The antigens separated by electrophoresis can correspond to the whole bacterium (351) or to special extracts like cell surface proteins (407). In spite of these potential advantages and the widespread availability of blotting equipment in clinical laboratories, Lepper et al. (305) reported that immunoblot for *H. pylori* antibodies is not common practice and that this was possibly due to discrepant recommendations for interpretation. Indeed, most of the publications concerning this subject are merely descriptive (9, 90, 138, 195, 261, 298, 560), and only four proposed interpretation criteria (219, 407, 527, 559). Lepper et al. compared these criteria on their sera collection. Depending on the aim of performing immunoblotting, the chosen criteria were not always the same. Indeed, the immunoblot is most likely used as a second-step technique to identify false-positive cases detected by ELISA, in which case, the criteria proposed by Nillson et al. (407) and Trautmann et al. (527) leading to 100% specificity must be used. In contrast, when immunoblotting is used for preendoscopy screening, the criteria of Lepper et al., which emphasize sensitivity, are more suitable (305).

A commercial immunoblot test is now available, which is an important advance toward standardization, Helicoblot 2.1 (Genelabs, Singapore) (305, 383, 432).

Interestingly, attempts have been made to correlate the presence of an immune response to a specific antigen with a specific disease. The main antigen in this respect is CagA. The importance of the immune response to CagA as a marker of more severe diseases, i.e., peptic ulcer disease (83, 87), gastric adenocarcinoma (36), and atrophy (29), was indeed discovered by immunoblotting.

However, because the presence of CagA antibodies is not a good predictor of these diseases on an individual basis, its detection has not been included in their management (334). Nevertheless, ELISAs which are specific for CagA antibodies are now on the market.

Serological response to other antigens have tentatively been associated with particular diseases (22, 277, 557).

TABLE 6. Detection of *Helicobacter pylori* IgG antibodies in saliva[a]

Type of test (manufacturer)	Type of patient	No. of specimens tested	Reference test(s)	Sensitivity (%)	Specificity (%)	Reference	Yr
In-house ELISA	Adult	119	Biopsy-based tests	85		435	1994
In-house ELISA	Adult	152	Histo	82	71	324	1995
Helisal (Cortecs)	Adult	86	Biopsy-based tests	88	71	75	1996
Helisal (Cortecs)	Adult	195	Culture, Histo	81	75	503	1996
Helisal (Cortecs)	Adult	106	Histo	66	74	133	1996
Helisal (Cortecs)	Adult	157	Histo	85	58	317	1997
Helisal (Cortecs)	Adult	303	Histo, UT	84	70	463	1997
In-house ELISA	Child	112	Histo, UT	93	82	325	1997
GAP (Bio-Rad)	Adult	70	Culture	84	90	106	1999
In-house ELISA	Adult	81	Culture, Histo, UT	94	85	341	1999
OraSure + ELISA (EPI)	Child	287	UBT	65	95	333	2000
Helori test (Eurospital)	Adult	213	Histo, UT, UBT	81	73	323	2000
ELISA	Child	669	Serum ELISA	64	86	26	2000
Helisal (Cortecs)	Adult	196	UT	74	67	80	2001
Salivette + HM-CAP (Enterics)	Child	477	UBT	80.9	95.3	37	2002
OraSure + oral fluid ELISA (Enterics)	Child	281	Histo	71	90.4	165	2002

[a] Histo, histology; UT, urease test.

Detection of *H. pylori* antibodies in urine. Specific *H. pylori* IgG antibodies are eliminated in urine but at very low concentrations. Alemohammad et al. presented the first data in 1993 using both ELISA and immunoblotting (6) with good accuracy.

Commercial tests have been developed in Japan: a standard ELISA (Urinelisa; Otsuka, Tokyo, Japan) and a rapid point-of-care test based on immunochromatography (Rapirun; Otsuka Diagnostic, Tokyo, Japan) (252). They were evaluated in 14 pretreatment studies, 11 on adults and 3 on children, including 10 in Japan. Sensitivity appeared satisfactory, with no difference between Urinelisa and Rapirun nor between children and adults, but the specificity was not as good (Table 5). Furthermore, most of the studies were performed in Asia, so other evaluations are warranted in the West where *H. pylori* strains appear to be different.

There is a good correlation between the nature of antibodies present in urine and in blood when tested by immunoblotting (248) but not between the antibody titers (251), which are dependent on the urine concentration. The accuracy of the test is not dependent on the pH nor the presence of bacteria in urine. In contrast, a high total IgG may induce false-positive results and a low total IgG may induce false-negative results (251, 496); therefore, patients with urine analysis abnormalities should be excluded. Another critical point is the need to perform the analysis on fresh urine samples and not on frozen samples. Freezing and thawing specimens may lead to protein precipitation, and the test's sensitivity was indeed lower on frozen urine (2, 6). However, once the urine is collected with a preservative, it can be sent by mail and analyzed a few days later. An apparent low specificity was noted in the study of Kato et al. when they compared Urinelisa to biopsy-based tests which was not confirmed because 10 of the 12 false positives were indeed found to be positive by immunoblotting (248).

Detection of *H. pylori* IgG in urine is definitely an easy, rapid, inexpensive, and absolutely noninvasive way to diagnose *H. pylori* infection, and the availability of a point-of-care test exhibiting the same performances is definitely attractive. How-

ever, more studies are needed before recommending them in the West.

Detection of *H. pylori* antibodies in saliva. Salivary antibodies are secreted during the immune response to infectious agents. Surprisingly, detection of specific IgA to *H. pylori* could not distinguish between infected or noninfected individuals (324, 574), but detection of IgG could (324). Following the princeps study by Patel et al. (435), 15 studies have been published (Table 6), six of them using the commercial kit Helisal (Cortecs Diagnostics, Deeside, United Kingdom) for salivary testing and the Oralfluid ELISA (Enteric Products, Stony Brook, NY). Four of the studies were conducted with children. Specimen collection is easy to perform by spitting saliva into a tube. However, another method has been proposed using a special device (OraSure; Epitope, Beaverton, OR), a swab to be rubbed on the gums to obtain a gingival transudate, enriched in IgG (333). Sensitivities and specificities obtained in these studies tend to be low, rarely reaching 90%.

The best results seemingly occur when the serum titer is high, as in patients with duodenal ulcer (156) or in children older than 5 years (165), despite a good correlation found in one of the first studies between titers obtained in sera and saliva (324). The specificity of the test can be improved by immunoblotting (179, 184, 341).

Detection of *H. pylori* in the Oral Cavity

There is a controversy on whether or not the oral cavity is a reservoir for *H. pylori*. In fact, culture from oral specimens has rarely been positive (141, 274, 493).

In a study by Parsonnet et al., low quantities of bacteria were cultured from saliva samples obtained before and after emesis in a small proportion of the subjects: 18% and 56%, respectively (434). The oral cavity is most likely a transitory reservoir for *H. pylori*, following regurgitation or vomiting, and therefore, it is not a site which is suitable for *H. pylori* diagnosis.

A number of studies investigated *H. pylori* in the oral cavity

TABLE 7. Comparison of the performances of diagnostic tests for *Helicobacter pylori* detection in adult patients[a]

Test	Sensitivity (%)		Specificity (%)		PPV (%)		NPV (%)	
	A	B	A	B	A	B	A	B
Culture	98.4	100	100	100	100	100	97.7	100
Histology	96.8	95.6	97.7	100	98.4	100	95.5	96.4
Urease test (24 h)	90.2	88.6	100	100	100	100	87.5	91.5
PCR	96.7	93.2	100	96.2	100	95.3	95.5	94.3
UBT	100	93.3	100	98.1	100	97.7	100	94.6
Serology	98.4	95.6	88.4	92.6	92.4	91.5	97.4	96.2
Immunoblot		95.6		92.6		91.5		96.2
Stool antigen test		88.9		94.4		90.9		90.7

[a] PPV, positive predictive value; NPV, negative predictive value; study A, 105 patients tested (516); study B, 99 patients tested (386).

(saliva or dental plaque) by PCR with numerous positive results. Given the high numbers of bacterial species in the mouth, many yet unidentified, we do not consider these results obtained with only one PCR to be reliable. At least two PCRs based on different target genes should both be positive. Testing the oral cavity by culture or PCR has no practical value for *H. pylori* diagnosis.

HOW TO USE THE TESTS

Since numerous tests are available to diagnose *H. pylori* infection, the question of choice among these techniques arises. We will first describe the comparative properties of the tests before evaluating their use under different circumstances.

Comparative Properties of the Different Tests Used

Sensitivity and specificity. The characteristics dependent on the test are sensitivity and specificity and, when combined, determine the accuracy. In contrast, the positive and negative predictive values, which are important for clinical practice, are very dependent on the prevalence of the infection in the community or on the group of patients considered, as has been highlighted in a number of articles, especially one by Shiotani and Graham for *H. pylori* (498). Another approach which is now more commonly used to evaluate test results is the positive or negative likelihood ratio. The likelihood ratio is the likelihood that a given test result would be expected in a patient with the target disorder compared to the likelihood that the same result would be expected in a patient without the target disorder.

Numerous studies have focused on evaluating the sensitivity, specificity, accuracy, and positive and negative predictive values of the tests used to detect *H. pylori*. They were all faced with the problem of the appropriate gold standard, since none of the tests fulfill this role. To evaluate noninvasive tests, invasive tests are usually considered the reference. As a general rule, *H. pylori* cases are deemed positive when (i) culture is positive or (ii) in the event of a negative culture, both histology and urease tests are positive. This appears to be a good compromise. In a recent study, culture was positive in 88% of the cases and histology plus urease test were positive in 12% (362). Sensitivity can also vary if the test is performed prior to or 4 to 6 weeks after an *H.*

pylori eradication treatment because, in the latter instance, even if the bacteria are still present, there is most likely a much smaller bacterial load.

There are indeed few studies where all of the methods have been performed and, furthermore, under optimal conditions. The results of two studies, one from The Netherlands comparing essentially invasive tests, UBT, and serology (516), and one from our laboratory comparing noninvasive tests to a gold standard based on invasive tests (386) are presented in Table 7.

Availability of the tests. The availability of the different tests described varies considerably from country to country. Histology is probably the most widely available method and, because of the fixative, avoids the problem of transport. It is indeed difficult to find a laboratory performing *H. pylori* culture, and this is further complicated by the strict transport conditions required in sending samples. While urease tests are easy to perform, their use may be limited due to a lack of medical reimbursement in some countries. Molecular methods are only carried out in specialized centers; however, the diffusion of a commercial real-time PCR assay allowing *H. pylori* stool detection should contribute to their increased utilization. UBT can now be performed in most countries worldwide, and the easy transport of breath specimens allows a wider accessibility to this assay. The stool antigen ELISAs could theoretically be performed in all laboratories, but the cost of a kit designed for numerous assays may discourage some who have a limited use to buy it. To the contrary, serology is widely available but the detection of salivary and urinary antibodies is not.

Rapidity in obtaining results. The delay between sampling and receiving the results varies between a few minutes and 2 weeks. If a microscope is available in the endoscopy suite, the direct examination of a slide is the quickest method. A stain can be performed in a few minutes. All of the immunoenzymatic tests (point-of-care tests) to detect antibodies in serum and urine or antigens in stools can be performed within 10 to 15 min in the endoscopy suite. The strip-based urease test can be read within the hour. The UBT requires 30 min to collect the specimens and a few minutes to measure the $^{13}C/^{12}C$ ratio. If an apparatus is available in the vicinity of the patient's room, it also allows a 1-h diagnosis, but this is rarely the case; normally, the specimen must be sent to a central laboratory, often outside the city, which delays the result by at least 2 days. All laboratory ELISAs for antibody detection or antigen detection can be performed within 2 h. However, for organizational reasons, series of tests are usually performed, causing a certain

delay. The same is true for molecular methods, although a real-time PCR protocol can be carried out in 2 h, including both the extraction procedure and detection of mutations associated with antimicrobial resistance. Histology can be performed within a day. Only culture requires from 3 to 10 days depending on the growth. Further susceptibility testing will increase the delay by 3 to 4 days.

Possibility of quantitative tests. None of the tests currently available determine the number of bacteria present in the stomach; only an estimation can be obtained.

Culture, histology, PCR, and UBT provide an approach to quantification, but in routine practice, a semiquantitative evaluation with either histology or UBT is the method employed.

Possibility to detect pathogenic properties. It clearly appears that some *H. pylori* strains are more pathogenic than others, and although routine detection of pathogenic properties associated with severe disease evolution is not yet recommended (334), tests with this potential are definitely of interest for epidemiological studies.

Culture is obviously the best method in this respect because it allows a complete screening of pathogenic properties either by a phenotypic method (VacA) or molecular methods (*cag* PAI genes, *vacA* alleles, *babA2*, *dupA*, *iceA*, and *sabA*, etc.) (296).

Molecular methods can be applied directly to biopsy specimens with the same sensitivity as culture, and the advantage is to get an accurate picture of the strains present, which is not the case after culture, since some strains may grow better than others (67, 194, 283, 433).

The development of FISH has allowed the detection of pathogenic properties on histological preparations. However, probes are not commercially available, in contrast to kits for susceptibility testing. Real-time PCR can also be performed on histological material.

Among the noninvasive tests, serology is the only method which allows the detection of *H. pylori* pathogenic properties, and it applies essentially to the CagA antigen, which is highly immunogenic. CagA serology is even more sensitive than *H. pylori* serology because the antibodies may remain for longer periods after *H. pylori* eradication. This detection can be performed by ELISA or immunoblotting, as previously described.

Globality of the test. Invasive tests do not provide a global picture of *H. pylori* in the stomach because they only explore a small portion of the gastric mucosal surface (85). They are subject to sampling error (27). In contrast, noninvasive tests provide a global picture of *H. pylori* in the stomach, and this may explain the discrepancies between techniques (245, 265). Indeed, the presence of atrophy and intestinal metaplasia corresponds to an inhospitable environment for *H. pylori*. *H. pylori* may only subsist in small areas of the stomach. Serology is the best detection method in such cases, with the bacterial load sometimes being inferior to the cutoff value of UBT and stool antigen tests.

Obtaining gastric juice can be a means of obtaining a global result using direct tests. PCR is then the preferred method because the sensitivity of a bacterial culture is lower than that of gastric biopsy.

Cost of the test. The cost of each test is extremely variable from one country to another. It depends first on the cost of endoscopy, which is a prerequisite for invasive tests, and second on the availability of some apparati which may or may not be present in a given setting.

When endoscopy must be performed, urease test and smear examination are the cheapest tests. Histology and culture require a specific environment and manpower and therefore will always be costly. Molecular tests which are easier to automatize will undoubtedly see their cost decreased in the future.

Among the noninvasive tests, serology is usually the cheapest. UBT and stool antigen tests are often in the price range of histology and culture without the cost of an endoscopy.

Added value of certain tests. In addition to the characteristics previously mentioned, some tests may have a unique property of importance in the choice.

Endoscopy is necessary to obtain biopsy specimens for direct testing, but this examination may provide important information for the patient.

Histology allows an evaluation of the status of gastric mucosa, including the presence of atrophy, intestinal metaplasia, lymphoid follicles, dysplasia, and carcinoma. Observation of activity, which is a surrogate marker for *H. pylori* infection, and disappearance of the activity after eradication can be noted. *H. heilmannii*, which may eventually be present, can be visualized by this technique.

Culture allows antimicrobial susceptibility testing, which is a key issue in patient management (357). It offers the possibility of applying all typing methods to differentiate between recrudescence and reinfection when a relapse occurs.

Molecular methods performed directly on the biopsy specimens can also answer these questions. They include PCR-RFLP for typing and detection of 23S rRNA gene mutations and real-time PCR.

When performed 4 to 6 weeks after the end of the treatment, the UBT and stool antigen test can detect *H. pylori* eradication noninvasively, which is in the patient's interest.

Application in Different Clinical Settings

Before treatment. The approach recommended in guidelines for dyspeptic patients less than 45 years old without alarming symptoms is the so-called "test and treat strategy," i.e., testing the presence of *H. pylori* with a noninvasive test before prescribing an eradication treatment. The value of this approach has been proven in several European countries and may be due to a high proportion of patients with peptic ulcer among the dyspeptic patients treated (352). The cost effectiveness has been evaluated and is of value up to a certain threshold of *H. pylori* prevalence; it is also dependent on medical costs. Patient satisfaction has, however, been questioned (293). The low incidence of gastric carcinoma at a young age is indeed the reason for the relatively high age threshold; however, it may be increased or decreased depending upon the population and particular situation at hand.

The noninvasive test recommended as the first choice is UBT because of its high accuracy, convenience, and availability, and the second choice is the stool antigen test. In contrast to previous guidelines, the Maastricht III 2005 Consensus Report acknowledges that certain highly accurate serology kits can also be used (334). Indeed, serology is the only test for which PPI consumption has a limited impact, and patients taking PPI before consulting a

doctor has become a common situation. Laboratory serology may also be the only accurate test for patients with atrophy or bleeding ulcers. Other antibody tests using urine or saliva and whole-blood doctor tests are not recommended.

For those older than 45 years or those with alarming symptoms, an endoscopy is mandatory and biopsy specimens must be taken. The value of the urease test is that the treatment can be given right away, since a positive result can be obtained within an hour. However, ideally another more sensitive test should be performed, either histology or culture. The choice between the two depends essentially on the prevalence of clarithromycin resistance in the area. In the case of high prevalence (>15 to 20%), it is mandatory to test the patient's susceptibility to this drug before prescribing it (334). Age may also be considered. Generally speaking, younger patients are more likely to have been exposed to this drug, and they are also more likely to experience treatment failure (45, 170) and, therefore, need culture and susceptibility testing. Conversely, older patients are more likely to have atrophy and other mucosal abnormalities, and therefore, histology is recommended. A good alternative to culture is real-time PCR, which allows both detection of the bacterium and clarithromycin susceptibility testing within 2 h.

Posteradication follow-up. Current first-choice 7-day treatment regimens have a limited efficacy, in the range of 70% eradication (101), which can be increased slightly but significantly by increasing the length of treatment to 14 days (147). With such a high failure rate, it is mandatory to have a follow-up 4 to 6 weeks or more after the end of the treatment.

For this purpose, a noninvasive test can be performed, and the UBT has been unanimously approved. If this test is not available, stool antigen test using monoclonal antibodies can be used. Serology cannot be used unless it is possible to compare the quantitative results obtained before and 6 months after the end of treatment. A decrease of at least 25% indicates eradication. This approach is not realistic in clinical practice because serum samples are not normally stored for future use. Under certain circumstances, e.g., gastric ulcer surveillance, endoscopy must be performed to detect an eventual malignancy, and in this case, histology should be performed.

Diagnosis for children. The same diagnostic methods used for adults can be used for children. However, *H. pylori* infection has certain particularities in children which have implications for diagnostic testing. *H. pylori* infection may be established slowly, so it is possible in rare instances to find the bacteria without traces of inflammation. At endoscopy, antral nodularity is common. In young children, the antigen stimulation is limited compared to that of adults. Bacterial overgrowth may be more frequent. In contrast to adults, preneoplastic lesions are exceptional (193), even in high risk areas (465). The infection is seldomly found in infants and young children, which limits the possibilities of test comparisons on large series.

Studies have been numerous over the last 10 years, and consensus conference reports were published in 1999 in Canada (494), in 2000 in the United States (180) and Europe (117), and again in 2005 in Canada (39). They all pointed out that, when faced with a child with recurrent abdominal pain or other symptoms compatible with *H. pylori* infection (e.g., iron deficiency anemia, growth retardation), endoscopy should be performed and biopsy specimens obtained for *H. pylori* diagnosis. The reason is the need to both detect the infection and identify the cause of the symptoms. This identification is primordial despite the fact that endoscopy requires deep sedation or general anesthesia, which increases the risk and cost of the procedure, and also the fact that the probable absence of a malignancy makes noninvasive tests attractive.

However, numerous articles have reported the use of noninvasive tests. UBT has been validated (28, 53, 242, 250, 253, 362, 481, 542) and is the most highly recommended noninvasive test for children. The protocol used in adults can be followed. The dose of labeled urea can possibly be decreased to 2 mg/kg (53) or to a total of 50 mg to make it simpler (28). The citric acid meal can be used if the taste is accepted by the children, otherwise orange juice is an alternative. In contrast, a fatty meal, for example, ice cream, has a negative effect on the test results (473). In very young children, a mask must be used to collect breath air (155). A cutoff of 3.5‰ is usually recommended (28, 53), except for children younger than 2 years. Indeed, a further evaluation of this age group needs to be performed because false positives have been reported (228). Bacterial overgrowth could explain these false-positive results (370). Because of the change in endogenous CO_2 production due to physical activity, stress, or food consumption, a correction in endogenous CO_2 production has been proposed (263) but is not commonly employed.

The stool antigen test (Premier Platinum HpSA) was evaluated for the first time in a multicenter study of children in 2000 (414). The accuracy was excellent with a specific cutoff. Numerous studies have been performed since then because this diagnostic approach is especially convenient for children from whom it is common to collect stools and in whom the tendency of a short transit time is favorable. The stool antigen test using polyclonal antibodies demonstrated variable results and, in general, a less favorable accuracy than the stool antigen test using monoclonal antibodies.

Serology in children younger than 10 years has encountered the problem of a lower antibody titer than in adults, and therefore, a specific cutoff value must be chosen (81, 205, 511). In addition, laboratory serology may not be well accepted by children because, although it is minimally invasive, the child may refuse the serum sampling. Serology has the reputation of a limited accuracy, but authors agree that a nice complement to serology is immunoblotting (377, 420, 460, 466). As in adults, point-of-care tests and salivary or urine tests are not suitable in children.

As an example of a comparative study on noninvasive tests, we report the results of a large multicenter European study performed in 18 centers in 15 countries. Three hundred sixteen children aged 2 to 17 years, including 133 *H. pylori* positive by invasive tests, had complete test results. UBT was determined to be the most accurate test, even in the 2- to 5-year-old age group, and DOB values were far from the cutoff value (Table 8). Serology ranked second in sensitivity with an excellent specificity, indicating the importance in choosing the kit. To the contrary, the polyclonal stool antigen test showed limited sensitivity, which could be slightly improved by modifying the

TABLE 8. Comparison of performances of five noninvasive
diagnostic tests for *Helicobacter pylori* detection in
316 children[a]

Test	Sensitivity (%)	Specificity (%)	PPV[b] (%)	NPV[b] (%)
UBT	96.2	97.3	96.2	97.3
Serology	88.7 (90.2)[c]	93.4 (93.9)[c]	90.8	91.9
Stool antigen test	72.9 (80.3)[c]	97.3 (93.4)[c]	95.1	83.2
Urinelisa	63.2 (72.2)[c]	97.3 (93.4)[c]	94.4	78.4
Rapirun	30.2	98.7	94.7	64.5

[a] Reprinted from reference 362 with permission from Elsevier.
[b] PPV, positive predictive value; NPV, negative predictive value.
[c] Sensitivity and specificity values were obtained after defining the optimal cutoff based on ROC curves.

cutoff value. Unfortunately, we were not able to test the monoclonal antibody-based stool test in this study. Urine antibody testing confirmed its poor accuracy (362).

The problem of transient infection in children from low-prevalence areas has emerged. Based on pepsinogen testing, Nurgalieva et al. showed that a single positive noninvasive test is most likely a false-positive result (411) and must be confirmed by another test.

Diagnosis for the elderly. The same techniques are also used for elderly patients. There are also some characteristics of elderly patients which can influence the diagnostic tests. A high proportion of the elderly population develops gastric atrophy and intestinal metaplasia, which can lead to a hostile environment for *H. pylori*, hence, fewer bacteria and possibly a negative result. Furthermore, elderly people take a number of drugs for chronic diseases and are more likely to be hospitalized. Among the medications prescribed are (i) PPI, which have a negative impact on *H. pylori*, and (ii) antibiotics, which can even eradicate the bacterium. Constipation is frequent and may jeopardize the detection of *H. pylori* antigens in stools. Severe cognitive impairment can alter the capacity to perform the UBT. Due to immune deficiencies, the antibody production may be low.

Endoscopy is frequently performed in elderly patients because of dyspepsia, given the high rate of peptic ulcer disease and gastric cancer in this population, as well as for nonspecific symptoms such as chronic anemia and anorexia. Histology is the preferred method to evaluate the status of the mucosa. It is common to find sequelae of chronic atrophic gastritis and no *H. pylori* for the reasons mentioned before. Both the corpus and the antrum must be explored, and sometimes, *H. pylori* is found only in the corpus. Sampling error is furthermore a limit for culture and urease tests, which also leads to an underestimation of *H. pylori* infection in elderly patients. A study showed that urease tests led to 50% false-negative results in patients older than 60 years (1).

Noninvasive tests have also been evaluated. The UBT is easy to perform, even in elderly patients, except in those with severe cognitive impairment. Pilotto et al. showed good accuracy of UBT in comparison to invasive tests (449). Others focused on possible false positives due to buccal contamination (poor dental hygiene) or bacterial overgrowth (hypochlorhydria) (68, 284). Antecedents of gastric surgery must be sought to adapt the protocol if necessary.

Obtaining stools for the stool antigen test is often more difficult, especially in cases of constipation. In addition, a prolonged period of *H. pylori* stay in the colon leads to antigen degradation and a decrease in sensitivity (388).

Finally, serology can be used. In the case of atrophy, Kokkola et al. showed that positive serological tests corresponded to active *H. pylori* infection (265). It is the only test which remains positive after antibiotic or PPI treatment, but in this case, the results may no longer correspond to an active infection. Underevaluation may also be possible in cases of proteinocaloric malnutrition, leading to humoral and cellular immunodepression (48). As for other populations, immunoblotting tends to be more accurate than serology.

Few comparative studies have been performed on the accuracy of different tests for the elderly. Pilotto et al. compared UBT and serology using invasive tests as the gold standard. They found a high accuracy for UBT, while the serology results showed both low sensitivity (74%) and low specificity (59%) (449). In a study on frail hospitalized elderly patients comparing UBT, serology, and HpSA to invasive tests, we did not obtain such good results. Surprisingly, only about half of the patients exhibited an *H. pylori* infection and, if stringent criteria were applied, the number dropped to only one-fourth. In several patients, only one test was positive, and this was especially true for UBT (480). These results may be explained by the type of population tested, which was considerably older (mean age, 85 years) than that of Pilotto et al. and comprised of hospitalized and frail subjects, half of whom were receiving antibiotics or PPI.

Another study on hospitalized, frail, elderly patients (230) also showed that only half of the patients were *H. pylori* positive. The stool antigen test (Premier platinum HpSA) compared to three tests (histology, serology, and UBT) had a sensitivity and a specificity of 76% and 93%, respectively, with the sensitivity being even higher for those taking PPI.

Diagnosis in the context of upper gastrointestinal bleeding. Recent data have shown that the number of bleeding ulcers has not decreased these last years despite the decrease in *H. pylori* infection. This may be due to a higher consumption of nonsteroidal anti-inflammatory drugs, including aspirin, and anticlotting agents. The diagnosis of *H. pylori* infection is important, but there are limited possibilities to take biopsy specimens, and diagnostic tests are considered less accurate in this context. Urease test, culture, and histology, as well as UBT, lack sensitivity, while the polyclonal stool antigen test has a lower specificity than usual conditions. Serology should remain the preferred method to be used in this context because it is not affected by the local environment.

Gisbert and Abraira recently performed a metaanalysis of the diagnostic accuracy of currently available tests. Sixteen studies were included, totaling 1,417 patients, but not all of the tests were used comparatively in all patients. The results confirmed the low sensitivity but high specificity of invasive tests (urease test, culture, histology), the low specificity of the stool antigen test, and the excellent accuracy of the UBT, but serology showed a surprisingly low specificity (Table 9) (167).

Diagnosis in Developing Countries

The situation is very different in developing countries. Diseases due to *H. pylori*, e.g., peptic ulcer and gastric cancer, are

TABLE 9. Sensitivity and specificity of diagnostic tests for
Helicobacter pylori in patients with bleeding ulcer[a]

Test	No. of studies	No. of patients	Sensitivity (%) (range)	Specificity (%) (range)
Urease test	16	1,417	67 (64–70)	93 (90–96)
Histology	10	817	70 (66–74)	90 (85–94)
Culture	3	314	45 (39–51)	98 (92–100)
UBT	8	520	93 (90–95)	92 (87–96)
Stool antigen test	6	377	87 (82–91)	70 (62–78)
Serology	9	803	88 (85–90)	69 (62–75)

[a] Data from reference 169.

TABLE 10. Distribution of MIC_{90} of various antibiotics against
wild-type *Helicobacter pylori* at various pHs

Agent	MIC_{90} (mg/liter) at pH:		
	7.5	6.0	5.5
Penicillin	0.03	0.5	0.5
Ampicillin	0.06	0.25	0.5
Cephalexin	2	16	32
Erythromycin	0.06	2	8
Clarithromycin	0.03	0.06	0.25
Ciprofloxacin	0.12	0.5	2
Tetracycline	0.12	0.25	0.5
Nitrofurantoin	1	2	2
Metronidazole	2	2	2
Bismuth subcitrate	16	8	

very common. In children, *H. pylori* infection may be a risk factor for diarrheal diseases. Two criteria are crucial in the decision making for diagnosing *H. pylori* infection: the prevalence of the infection and the cost of the tests in relation to the health resources available.

Based on these criteria, it is possible to distinguish between (i) the less advanced countries where the prevalence is extremely high in adults (virtually all of them are infected), where most of the children are also infected by the age of 5 (150, 361), and where the economic situation is not at all favorable, and (ii) emerging countries which still have a high prevalence of infection in adults but have recently benefitted from a socioeconomic evolution, leading to a decreased prevalence in young adults and even more so in children.

In the less-advanced countries, it is questionable whether diagnosis should even be attempted, given the prevalence of the infection and the limits of the tests. If one considers that all patients are infected, money will be saved for the treatment which is the most important. Obviously, in such countries, some wealthy people may be encountered and they can be submitted to diagnosis. In the emerging countries, at this stage the simplest and least costly measure is to perform a urease test if an endoscopy is performed. Performing histology and culture will depend on the availability of these techniques. Since the treatment will not involve expensive antibiotics to which *H. pylori* may be resistant (clarithromycin, levofloxacin) but drugs such as bismuth salts, amoxicillin or tetracycline, and furazolidone, for which resistance is not a major problem, performing susceptibility testing is not as vital. Metronidazole, to which *H. pylori* appears resistant in vitro, may also be used but testing for this drug is not considered to be relevant in clinical practice (334). Histology is probably of greater interest because of its ability to detect premalignant lesions in addition to *H. pylori*. Besides serology, the other tests may not be available or may be too expensive. Unfortunately, posttreatment follow-up may not be possible under such circumstances. Except in special cases, it is not recommended to perform an endoscopy for this purpose. Endoscopy indeed carries a risk of recontamination if the disinfecting procedure has not been carried out carefully.

In some instances the *H. pylori* diagnostic techniques can be adapted, especially to decrease the cost. Instead of kits, urea broth like Christensen medium can be used for the urease test (111), but its sensitivity will decrease. For culture, a candle jar can replace the gas-generating packs (136), but colonies will grow more slowly. To avoid the problem of transport of blood

samples, a dry-plasma collection device can be used (410) and the cutoff should be adjusted (205). Lastly, if the UBT is used in children, the cutoff value must be higher than 5.4 delta per mil (518).

ANTIMICROBIAL SUSCEPTIBILITY TESTING

The current recommended treatment for *H. pylori* eradication includes two antibiotics and an antisecretory drug, essentially a PPI, to which a bismuth salt can be added (334). The most commonly used association worldwide is a double dose of PPI (omeprazole, lansoprazole, pantoprazole, rabeprazole, or esomeprazole) plus clarithromycin (500 mg twice a day [b.i.d.]) and amoxicillin (1 g b.i.d.) for 7 days (treatment 1). Other 7-day regimens include a double dose of PPI plus clarithromycin (500 mg b.i.d.) and metronidazole (500 mg b.i.d.) (treatment 2) or a double dose of PPI plus amoxicillin (1 g b.i.d.) and metronidazole (500 mg b.i.d.) (treatment 3), with the latter being mostly used as a second choice treatment for 14 days in the case of failure of treatment 1.

As any infectious agent, *H. pylori* can acquire resistance to the antimicrobial agents used to treat the infection, and therefore, susceptibility testing is important in the management of the infection.

Phenotypes Observed

H. pylori is intrinsically resistant to glycopeptides, cefsulodin, polymyxins, nalidixic acid, trimethoprim, sulfonamides, nystatin, amphotericin B, and cycloheximide. Some of these are used as selective agents in isolation media.

Wild-type strains are susceptible to β-lactams (except cefsulodin), fosfomycin, macrolides, aminoglycosides, tetracyclines, chloramphenicol, rifampins, fluoroquinolones, 5-nitroimidazoles, and nitrofurans (289, 355). With the exception of chloramphenicol (because of toxicity) and aminoglycosides (because of a lack of diffusion), they have all been used in *H. pylori* eradication regimes. However, active transport of aminoglycosides is not affected by microaerophilic conditions. Bismuth salts and PPI also have an anti-*H. pylori* activity, but these latter compounds require a high concentration which is not achievable in vivo (360).

The MICs are provided in Table 10. When they are determined at an acidic pH (e.g., 5.5 instead of 7.2), MICs increase

TABLE 11. Genes involved by point mutation or other genetic events leading to antibiotic resistance in *Helicobacter pylori*

Antibiotic(s)	Gene(s) concerned
Macrolides	*rrn* 23S
Metronidazole	*rdxA, frxA*
Quinolones	*gyrA*
Rifampins	*rpoB*
Amoxicillin	*plp1*
Tetracycline	*rrn* 16S

markedly. Minimal bactericidal concentrations are rarely determined. They are within a dilution of MICs for most of the antibiotics (181). However, when *H. pylori* is cultured in a chemostat, i.e., under conditions close to those in vivo where the generation time is much longer than in vitro, a poor bactericidal activity is found. Only amoxicillin and bismuth salts are still bactericidal (372). These conditions most likely express the reality of what occurs in an infected patient. A minimal bactericidal concentration study of planktonic versus adherent bacteria also showed that amoxicillin was less effective on adherent bacteria (364).

Resistance Mechanisms

H. pylori, like a few other bacteria such as *Mycobacterium tuberculosis*, acquires resistance by mutation (Table 11). All of the antibiotics which have been proposed in eradication regimens are concerned. The mechanism does not involve plasmids which could be transmitted horizontally but point mutations which are transmitted vertically, while transformation may be possible if two strains are present simultaneously in the stomach. The consequence is a progressive increase in the resistance rate due to the selection pressure.

As in many bacteria, drug efflux proteins can contribute to both natural insensitivity to antibiotics and to emerging antibiotic resistance. In 2000, Bina et al. evaluated the relevance of three putative efflux systems in *H. pylori* resistance to antibiotics and concluded that, in contrast to what is usually described for gram-negative bacteria such as *Escherichia coli* or *Pseudomonas aeruginosa*, efflux systems did not play a role in the intrinsic resistance to antibiotics (34). The same year, DeLoney and Schiller also eliminated the possibility of an efflux mechanism in *H. pylori* amoxicillin-resistant strains (102).

Since then, few data have been published regarding efflux systems in *H. pylori*. Tetracyclines seem to be the only antimicrobial agents in which efflux plays a role. Indeed, Dailidiene et al. suggested that tetracycline resistance could result from an accumulation of changes that may affect tetracycline-ribosome affinity and/or other functions such as porins or efflux pumps (92). In 2006, a putative tetracycline resistance gene, HP1165, which displays identity to the tetracycline efflux gene *tetA* from *Clostridium perfringens* was found to be involved in some strains in the inducible tetracycline resistance in *H. pylori* (309). Several nonspecific multidrug resistance proteins have also been described, but their importance needs to be clarified (279, 396, 479, 541). Apart from resistance to antibiotics, efflux systems in *H. pylori* play a role in maintaining metal homeosta-

sis, which is crucial for the adaptation of this bacterium to the gastric environment (507).

(i) Macrolides. Macrolides act by binding to ribosomes at the level of the peptidyl transferase loop of the 23S rRNA gene. *H. pylori* resistance is the consequence of point mutations at two nucleotide positions, 2142 (A2142G and A2142C) and 2143 (A2143G), which lead to a conformational change and a decrease in macrolide binding (Fig. 3) (413, 556). All macrolides are concerned.

(ii) Amoxicillin. Amoxicillin acts by interfering with the peptidoglycan synthesis, especially by blocking transporters named penicillin binding proteins (PBP). The rare amoxicillin-resistant *H. pylori* strains harbor mutations on the *pbp-1a* gene. The amino acid substitution Ser-414→Arg appears to be involved (163), leading to a blockage of penicillin transport. Tolerance to amoxicillin has also been described, and the mechanism proposed was the lack of a fourth PBP, namely PBP-D (114).

(iii) Tetracyclines. Tetracyclines interfere in the protein synthesis at the ribosome level by binding to the 30S subunit. The change in a nucleotidic triplet (AGA-926 to 928→TTC), cognate of the positions 965 to 967 in *E. coli*, has been associated with resistance to these compounds probably because of a lack of binding to the h_1 loop, which is the binding site of tetracyclines. Tetracycline targets the two *rrn* 16S operons (162, 531).

Single or dual mutations at these positions lead to intermediary MICs. The need to have three mutational events can explain the rarity of tetracycline resistance (91, 161, 408). Tetracycline-resistant strains with no mutation in position 926 to 928 have also been described, and efflux is the mechanism most likely to be involved. These strains, as well as those with the mutations, exhibited a decreased tetracycline accumulation inside the cells (583).

(iv) Fluoroquinolones. Fluoroquinolones inhibit the A subunit of the DNA gyrase, encoded by the *gyrA* gene. Mutations in the quinolone resistance-determining region of *gyrA* are found in *H. pylori* as well as in other bacteria. The amino acid positions concerned are mainly 87 and 91 (391, 513).
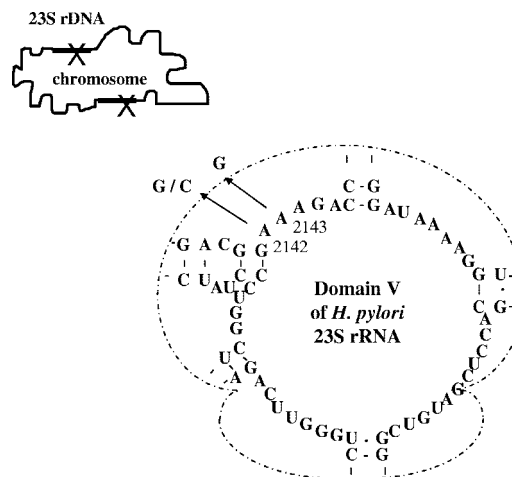

FIG. 3. Mutations arising in domain V of *Helicobacter pylori* 23S rRNA and conferring resistance to clarithromycin.

TABLE 12. Recommended conditions to test *Helicobacter pylori* susceptibility to antibiotics by the agar dilution method

Method	Conditions recommended by (reference):	
	CLSI, United States (402)	European *H. pylori* Study Group (176)
Medium	Mueller-Hinton agar plus sheep blood (5% vol/vol), 2 wk old or more	Mueller Hinton agar + horse blood (10% vol/vol)
Inoculum	1×10^7–1×10^8 CFU/ml (McFarland 2 opacity prepared from a 2-day culture on an agar medium)	0.5×10^9–1×10^9 CFU/ml (McFarland 4 opacity standard prepared from a 2-day culture on an agar medium)
Incubation	35°C, microaerobic atmosphere	37°C, microaerobic atmosphere
Reading	3 days	3 days
Control strain(s)	*H. pylori* ATCC 43504	*H. pylori* CCUG 38770, *H. pylori* CCUG 38771, *H. pylori* CCUG 38772
Quality control availability	Amoxicillin, ciprofloxacin, clarithromycin, metronidazole, tetracycline	Amoxicillin, clarithromycin, metronidazole

(v) Rifampins. Rifampins inhibit the B subunit of the DNA-dependent RNA polymerase encoded by the *rpoB* gene. Mutations have been described for the *rpoB* gene of *H. pylori* at positions 524, 525, and 585, as in *Mycobacterium tuberculosis* and *E. coli* (211). Another mutation at position 149 has also been described previously (213).

(vi) Nitroimidazoles. 5-Nitroimidazoles have to be reduced in the cell to alter bacterial DNA. An important gene in this respect is *rdxA*, an oxygen-insensitive nitroreductase. Mutations in *rdxA* can render the protein ineffective (224). Other proteins may also be involved in this reduction process, like the flavin oxidoreductase (*frxA*), while their role is more controversial (338, 367). In addition, a TolC efflux pump appears to play a role in resistance to this group of drugs (541).

Susceptibility Testing Methods

The usual phenotypic methods of susceptibility testing can be applied to *H. pylori*, but because resistance is essentially due to point mutations, genotypic methods are also used, especially for clarithromycin. They are being developed for the other antibiotics.

(i) Phenotypic methods. *(a) Agar dilution method.* The agar dilution method, usually considered the reference method to compare other techniques, has been proposed by the Clinical Laboratory Standard Institute (CLSI) (formerly National Committee for Clinical Laboratory Standards) as the method to be used for *H. pylori* clarithromycin susceptibility testing (402). In Europe, a working group of the European *Helicobacter pylori* Study Group has also published guidelines which are similar to the CLSI recommendations (176). They are all presented in Table 12.

For clarithromycin, the breakpoint proposed for susceptible strains is 0.25 μg/ml, that for resistant strains is >0.5 μg/ml, and that for intermediate strains is 0.5 μg/ml. Excellent predictive values for the success of the clarithromycin-amoxicillin-PPI triple therapy were obtained with these breakpoints.

While the same standardization work has not been done for other antibiotics, e.g., amoxicillin, tetracycline, rifabutin, and levofloxacin, agar dilution can nevertheless be applied.

For amoxicillin, MICs of >0.5 μg/ml are seldom found. Strains with MICs of 0.25 to 0.5 μg/ml correspond to an intermediate susceptibility, but the clinical impact has not been evaluated.

The breakpoints commonly used for the other antibiotics are as follows: tetracycline, 2 μg/ml; rifabutin, 1 μg/ml; ciprofloxacin (often tested instead of levofloxacin), 1 μg/ml.

Metronidazole is a special case because most of the studies have shown a lack of inter- and intralaboratory reproducibility (176, 363). The reason is not known. It may be that the intracellular redox potential is not controlled, whereas this parameter is important for metronidazole reduction. Indeed, a preincubation of the media in an anaerobic atmosphere has been shown to increase metronidazole activity (63). The lack of correlation between the susceptibility results and *H. pylori* eradication is also of major concern. Strains with a high MIC can be eradicated possibly due to a variable redox potential inside the stomach. The threshold commonly used to define metronidazole resistance is >8 μg/ml.

Because of this lack of clinicobacteriological correlation, the Maastricht III 2005 Consensus Report discourages routine metronidazole susceptibility testing (334).

(b) Broth dilution method. The broth dilution method has the advantage of being adaptable to automation. However, it has rarely been used for *H. pylori* because of the difficulty of growing this bacterium in broth (82). Supplementation of media such as brucella broth or Mueller-Hinton broth led to satisfactory results (197, 447). A good correlation has been found with Etest, except for metronidazole.

(c) Breakpoint susceptibility testing. Breakpoint susceptibility testing is a simplified version of the agar dilution method previously cited. It consists of inoculating a streak of the strain to be tested on an agar plate containing an antibiotic concentration equal to the breakpoint concentration which defines resistance, for example, 1 μg/ml for clarithromycin, or two different concentrations (0.25 and 1 μg/ml) to classify the strains as susceptible, intermediary, or resistant.

This test is easy to perform, and theoretically excellent, but the media must be prepared in the laboratory. It has been used in comparison to the agar diffusion method (Etest and disk) for metronidazole susceptibility testing with a 94% correlation, but reproducibility was not studied (40, 546).

(d) Disk diffusion testing. The disk diffusion method is the simplest and most economic for routine susceptibility testing.

However, it is generally not recommended for slow-growing bacteria.

Disk diffusion has been validated in France to detect macrolide resistance. Due to an important gap between MICs of susceptible and resistant strains, a clear separation is possible by this method. The breakpoint inhibition zone, corresponding to an MIC of >0.5 µg/ml, was 22 mm for clarithromycin and 17 mm for erythromycin. This latter antibiotic is the one recommended for macrolide susceptibility testing (191).

In contrast, discrepant results have been obtained for metronidazole. As previously indicated, it could be due to a lack of standardization of parameters usually not taken into account, e.g., the delay between the medium preparation and the performance of the test, which determines the redox potential (63). This method has not been validated for the other antibiotics, but a good correlation is usually found with the other methods.

(e) Etest. The Etest method has the advantage of being a quantitative method with a direct expression of MICs, and furthermore, it is adapted to slow-growing bacteria like *H. pylori*. A good correlation has been found between this method and the agar dilution method, with the exception of metronidazole, as mentioned before (176).

(f) Which phenotypic method to use? For routine testing, the minimum is to test a macrolide, and the best cost-efficacy ratio is the disk diffusion method, with an erythromycin disk (191). To know the exact MIC, the Etest or agar dilution method can be used.

Given the lack of reproducibility and the low predictive value for treatment efficacy, metronidazole is no longer recommended for routine testing (334). If necessary, the best method is agar dilution or its simplified version, i.e., the breakpoint method with 8 µg/ml of metronidazole.

The agar diffusion method using disks can be used for susceptibility testing of other drugs, tetracycline, rifabutin, and levofloxacin, because of its practical aspect and limited cost. It allows the detection of resistant strains. In the case of resistance, an Etest can be performed.

(ii) Genotypic detection of resistance. As mentioned above, *H. pylori* resistance is essentially due to chromosomal mutations, and for the most part, a limited number of punctual mutations are present which can be easily detected with molecular tests.

(a) Clarithromycin. Given the importance of clarithromycin and the low number of mutations concerned, numerous genotypic methods have been developed to detect this resistance. They are presented in Table 13 and were originally described in a review (427).

The two main methods used are PCR-RFLP and real-time PCR.

The PCR-RFLP method was first applied to the detection of *H. pylori* 23S rRNA gene mutations in 1996 (556). It is based on the fact that mutations reveal restriction sites within the amplicon obtained with primers specific to the *H. pylori* 23S rRNA gene. These restriction sites are recognized by the enzyme BsaI for the A2142G mutation and BsbI for the A2143G mutation, so two bands will be present on the gel if one or the other of these mutations is present. More recently, a third enzyme (BceAI) has been proposed to detect the A2142C

TABLE 13. Genotypic methods used to detect macrolide resistance in *Helicobacter pylori*

23S rRNA-based method type	Method (reference[s])
With gene amplification	Sequencing, pyrosequencing (221, 556)
	RFLP
	Oligonucleotide ligation assay (509)
	DNA enzyme immunoassay (339, 451)
	LiPA (INNO-LiPA) (549)
	Preferential homoduplex formation assay (329)
	3′-Mismatched PCR (5)
	3′-Mismatched reverse PCR (122)
	Real-time PCR
	Microelectronic chip array (586)
	Electrocatalytic detection (291)
Without gene amplification	FISH (529)

mutation (366). This method is simple but has the disadvantages of standard PCR, especially with regard to the delay in obtaining results and the need to manipulate the amplicons obtained.

The real-time PCR method has been mentioned to detect *H. pylori* directly in biopsy specimens as well as point mutations.

The first application including *H. pylori* identification and detection of clarithromycin resistance using the FRET followed by a melting curve analysis was performed on strains in 1999. SYBR green 1, a fluorophore specific for double-stranded DNA was used as the quencher which transfers its energy to a second fluorophore, Cy5, fixed on a probe specific to *H. pylori* to detect the mutations inducing clarithromycin resistance (164). The same method was then applied directly to biopsy specimens (71).

Another approach using a biprobe was subsequently proposed (348, 421). In the method of Oleastro et al., a 267-bp fragment of the 23S rRNA gene was amplified by using specific primers. A sensor probe and an anchor probe hybridizing three bases upstream from the former were used. After amplification using a LightCycler, a melting curve analysis was performed. The wild-type strain's melting peak was 62°C, while that for the A2142C mutant was 58°C and those for the A2143G and A2142G mutants were 53 and 54°C, respectively, given the existence of a nucleotide mismatch between the sequence and the hybridization probe. The 1°C difference between A2143G and A2142G did not allow the separation of two genotypes because of a 1°C maximum variability between runs. This method proved to be specific and is applied directly to gastric biopsy specimens. When tested on 200 *H. pylori*-positive samples, a perfect agreement between the genotype and the clarithromycin phenotypic susceptibility testing was found in 96.4%.

This method represents important progress in the diagnosis of *H. pylori* because the result can be obtained within 2 h, and the possibility of contamination with the amplicons is limited because the reaction is performed in a closed tube. A limitation of this method for *H. pylori* is that few 23S rRNA gene sequences from other *Helicobacter* species are present in the databases. In our experience, *H. heilmannii* is detected with *H. pylori* 23S rRNA gene PCR.

The application of this method on stool samples is a breakthrough in our approach to susceptibility testing (486).

A variation of this method proposed by Lascols et al. consists of a quantitative detection of *H. pylori* in gastric biopsy specimens by real-time FRET PCR and, in the event of a positive result, a different biprobe is used to perform another hybridization followed by a melting curve analysis (292).

The possibility of detecting clarithromycin resistance without performing PCR also exists by FISH. This method has been applied to *H. pylori* and its clarithromycin resistance by Trebesius et al. (529). It consists of an rRNA-based whole-cell in situ hybridization using a set of fluorescent-labeled oligonucleotide probes. Labeling of intact single bacteria is monitored by fluorescence microscopy. This method allows the detection of *H. pylori* with a 16S rRNA probe labeled with the fluorochrome Cy3 (red) and detection of resistant mutants with a 23S rRNA probe labeled with fluorescein (green) simultaneously. This method proved to be sensitive and specific compared to standard methods of culture and susceptibility testing. FISH has the advantage of being independent of a nucleic acid preparation, it is not prone to inhibition like PCR, and it is quick. It also allows visualization of the bacteria, including coccoidal forms, and can be performed on paraffin-embedded biopsy samples (240). A limitation may be the observer-dependent result and sometimes difficulty in reading. It has not been compared yet to the FRET-melting curve analysis protocol.

(b) Fluoroquinolones. The FRET-melting curve analysis method was also applied to detect *H. pylori* resistance to fluoroquinolones. All mutations eventually present in the quinolone resistance-determining region do not translate into resistance. Nevertheless, using two biprobes, it is possible to detect the main mutations at amino acid position 87 or 91 (175).

(c) Tetracycline. A PCR-RFLP has been developed which amplifies part of the 16S rRNA gene. The amplicon is then submitted to restriction by Hinft. The strains which harbor the triple mutation leading to a high tetracycline resistance level exhibit three bands, while only two are present for susceptible strains or for strains with a low resistance level (464).

(d) Metronidazole. The important number of mutations which may arise in the *rdxA* gene, some being independent of *H. pylori* resistance to this antibiotic, did not allow the development of molecular methods for detection of resistance to metronidazole.

An original approach is the detection of the RdxA protein by immunoblotting using rabbit antisera against RdxA. It is possible to visualize a 24-kDa immunoreactive band corresponding to the RdxA band of metronidazole-susceptible strains which it is not seen in resistant strains (294). However, false positives, i.e., the presence of a band in resistant strains, occur in 10% of strains.

This promising method deserves confirmation by studying its predictive value for the eradication in treatments including metronidazole.

(e) Other antibiotics. No molecular method has yet been proposed to detect *H. pylori* resistance to rifabutin, probably because it is seldom encountered.

A more thorough knowledge of the *pbp1* gene mutations should allow the development of a molecular test for amoxicillin.

Relevance of *H. pylori* Resistance to Antibiotics

Significance depends on the prevalence of *H. pylori* resistance and on the clinical impact on the treatment used.

(i) Prevalence of *H. pylori* resistance to antibiotics. Numerous studies have been performed to determine the prevalence of *H. pylori* resistance to antibiotics. However, many of them have drawbacks, especially concerning the number and the representativeness of the strains tested. Most of the studies were performed in referral centers for *H. pylori* treatment where patients who experienced previous treatment failures were explored, increasing the probability of finding resistant strains. These cases are not representative of the patients as a whole and, because these studies are monocentric, the number of patients may be low, leading to wide confidence intervals of the prevalence obtained.

Ideal studies involving patients who are representative of a given region by being selected at random are few. An alternative has been to analyze the prevalence data obtained from clinical trials aiming to evaluate new regimens. Since prevalence is in essence an evolving phenomenon, only the most recent articles (1999 to 2005) have been taken into account in this review (357). However, given the delay in publication, only data from the end of the last decade are essentially available.

(a) Prevalence of H. pylori resistance to clarithromycin. Resistance to clarithromycin is the prevalence which has been the most widely studied so far. It ranges from close to nil to 20 to 25%. In the United States, a prevalence of 10 to 15% was found based on strains isolated during clinical trials, regardless of the region studied (119, 369, 425). In Canada, a systematic review of the studies published before the year 2000 estimated this resistance to be less than 4% (135). A multicenter European study carried out in 1998 in 17 countries found a global prevalence of 9.9% (95% confidence interval, 8.3 to 11.7) with a significant difference between Northern Europe (9.3%) and Southern Europe (18%) (178). A similar range was found in a recently published systematic review (357). The prevalence is even higher in children (24%) because children are more likely to be treated with macrolides when they have respiratory infections (268).

The essential risk factor for clarithromycin resistance is a previous consumption of macrolides, and therefore, a trend for an increased prevalence is observed in most countries.

The prevalence of secondary clarithromycin resistance, i.e., after failure of a treatment including this drug, is extremely high, in the range of 60% (212).

(b) Prevalence of H. pylori resistance to metronidazole. As stated previously, the results concerning *H. pylori* resistance to metronidazole are not reproducible, and on an individual basis, their interest is limited. Nevertheless, the trend of low, medium, or high prevalence rates observed at a population level seems real.

A global resistance in the range of 20 to 40% is observed in the United States (119, 369, 425). as well as in Europe (357). It was 33.1% in the European multicenter study previously cited, with no significant difference between the North and the South (178). In developing countries, the prevalence is much higher (50 to 80%) (400, 525, 573), and it is relatively rare in Japan (9 to 12%) (441).

Again, metronidazole consumption appears to be an impor-

TABLE 14. *Helicobacter pylori* eradication with PPI-based triple therapies according to antimicrobial susceptibility[a]

Regimen[b]	No. of samples tested	Eradication (%)					Metro[s]		Metro[r]	
		Overall	Clari[s]	Clari[r]	Metro[s]	Metro[r]	Clari[s]	Clari[r]	Clari[s]	Clari[r]
PPI-Amox-Clari	1,975	78.2	87.8	18.3						
PPI-Amox-Metro	696	83			89.4	64.4				
PPI-Clari-Metro	593	85.2					97	50	72	0

[a] Data are from clinical trials published between 1999 and 2003 (357). Clari, clarithromycin; Amox, amoxicillin; Metro, metronidazole.
[b] The PPI-Amox-Clari and PPI-Amox-Metro regimens led to eventual resistance to one antibiotic; the PPI-Clari-Metro regimen led to eventual resistance to two antibiotics.

tant risk factor for this resistance.

The prevalence of secondary metronidazole resistance is in the range of 65 to 75% (212).

(c) Prevalence of H. pylori resistance to other antibiotics. The prevalence of *H. pylori* resistance to amoxicillin is very low (<1%), as is the prevalence of *H. pylori* resistance to tetracycline, except in a few countries like South Korea (258).

The prevalence of *H. pylori* resistance to rifampins is virtually absent, given that these antibiotics have a limited use. In contrast, fluoroquinolones, which have shown an increasing consumption over the past few years, are leading to a higher prevalence, e.g., 20% in adults in Portugal (52). A secondary resistance rate of 9% has been found in Germany (212).

(ii) Impact on *H. pylori* eradication. The impact of antibiotic resistance on *H. pylori* eradication must take into account the complexity of the treatment used. Up to 1998, few clinical trials included susceptibility testing, and they have been reviewed previously (227, 545). We performed a systematic review of clinical trials performed between 1999 and 2003, which is reported in the following paragraphs. Clarithromycin and metronidazole are essentially concerned (357).

(a) Clinical impact of clarithromycin resistance. With regard to the PPI-clarithromycin-amoxicillin treatment, a major difference was observed between susceptible strains (87.8% eradication) and resistant strains (18.3% eradication), i.e., a 70% decrease in efficacy if the strain was resistant, with the pooled Mantel-Haenzel odds ratio being 24.5 (95% confidence interval, 17.2 to 35) (Table 14). If the treatment was PPI-clarithromycin-metronidazole for metronidazole-susceptible strains, the corresponding figures were 97% eradication for susceptible strains and 50% for resistant strains.

(b) Clinical impact of metronidazole resistance. When a PPI-clarithromycin-metronidazole regimen was used for clarithromycin-susceptible strains, the eradication rate was 97% for metronidazole-susceptible strains and 72.6% for metronidazole-resistant strains, i.e., only a 25% decrease in efficacy.

With the PPI-amoxicillin-metronidazole regimen, the corresponding figures were 89% for susceptible strains and 64% for resistant strains, i.e., again a 25% decrease in efficacy.

(c) Clinical impact of resistance to both clarithromycin and metronidazole. Very few patients harbored strains with double resistance to both clarithromycin and metronidazole in the trials performed in the past. None of the 14 patients experienced an *H. pylori* eradication.

(d) Clinical impact of resistance to other antibiotics. We can hypothesize that resistance to amoxicillin, tetracycline, rifabutin, and levofloxacin has an impact on treatment success. However, clinical trials performed in the past either did not include resistant strains which are still rare or did not include susceptibility testing.

CONCLUSION

H. pylori detection is not an easy task due to the difficulty in accessing its ecological niche and the fragile nature of the bacterium. Detection has been a topic of great interest since the discovery of the bacterium in 1982, yet even now, a perfect method cannot be proposed. It has been a field of great innovation: the development of the [^{13}C]UBT opened the path to the use of stable isotopes in medicine, the detection of antibodies in urine was one of the first applications in infectious diseases, and molecular methods developed during this period were applied immediately to the diagnosis of this infection. Real-time PCR has become one of the most promising techniques, especially when applied to stool specimens. This rapid noninvasive method allows a direct detection of the bacterium and is also a means to test its susceptibility to macrolides, the most important information currently needed for treatment. The lack of standardization should end with the availability of a kit.

However, many other aspects of *H. pylori* detection not covered in this review concern the host. It is clear that the evolution of *H. pylori* infection depends highly not only on the bacterial strain but also on the host's characteristics. For example, a combination of *cag* PAI-positive strains and certain alleles of interleukin-1β of the host considerably increases the risk of gastric carcinoma (143). Determination of host gastric hormones, i.e., gastrin 17 and pepsinogen 1, in addition to *H. pylori* antibodies included in the Gastropanel (BioHit, Helsinki, Finland), has enabled the diagnosis of precancerous lesions (536). The study of cytochrome P450-2C19 polymorphism which differentiates between high- and low-PPI metabolizers may also be a help in determining treatment success (229). Because of all of these possibilities, it is most likely that, in the near future, *H. pylori* detection will be a pioneer in the field of predictive medicine.

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