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***Helicobacter pylori* infection – recent developments in diagnosis**

Lopes AI *et al.* *H. pylori* diagnosis advances

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**Abstract**

Considering the recommended indications for *Helicobacter pylori* (*H. pylori*) eradication therapy and the broad spectrum of available diagnostic methods, a reliable diagnosis is mandatory both before and after eradication therapy. Currently, only highly accurate tests should be used in clinical practice and the sensitivity and specificity of an adequate test should exceed 90%. The choice of tests should take into account clinical circumstances, the likelihood ratio of positive and negative tests, the cost-effectiveness of the testing strategy and the availability of the tests. This review concerns some of the most recent developments in diagnostic methods of *H. pylori* infection, namely the contribution of novel endoscopic evaluation methodologies for the diagnosis of *H. pylori i*nfection, such as magnifying endoscopy techniques and chromoendoscopy. Also, the diagnostic contribution of histology and urea breath test was recently explored in specific clinical settings and patient groups. Recent studies recommend enhancing the number of biopsy fragments for the rapid urease test. Bacterial culture from the gastric biopsy is a gold standard technique, which is recommended for antibiotic susceptibility test. Serology is used for initial screening and stool antigen test is particularly used when urea breath test is not available, while molecular methods gained attention mostly for detecting antibiotic resistance.

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**Key words**: *Helicobacter pylori;* Diagnosis; Endoscopy; Histology; Culture; Urea breath test; Stool antigen test; Serology; Molecular methods

**Core tip:** Considering the importance of a reliable diagnosis in the setting of current recommendations for *Helicobacter pylori* (*H. pylori*) eradication therapy, recent developments in both invasive and non-invasive methods, may further contribute to improve *H. pylori* detection. The manuscript presents an extensively referenced overview about major advances in endoscopy, histology, culture, urea breath test, serology, stool tests and molecular methods, emphasizing their major contributions and potential shortcomes.

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**INTRODUCTION**

A reliable primary diagnosis and control of treatment success of *Helicobacter pylori* (*H. pylori*) infection is crucial for patients with a wide spectrum of *H. pylori*-related conditions, including uncomplicated or complicated ulcer disease, mucosa associated lymphoid tissue (MALT) lymphoma, atrophic gastritis, previous partial gastric resection for gastric cancer. Accurate diagnosis of *H. pylori* infection involves the combined knowledge, effort and research of laboratories, gastroenterologists and pathologists. Traditional diagnosis is made using a combination of tests, both invasive and noninvasive. Considering the broad spectrum of diagnostic methods, only highly accurate tests should be used in clinical practice under specific circumstances and currently, the sensitivity and specificity of an adequate test should exceed 90%. The choice of tests to use usually depends on clinical circumstances, the likelihood ratio of positive and negative tests, the cost-effectiveness of the testing strategy and finally of the availability of the tests. The present paper aimed to present an overview of the most recent advances in both biopsy and non-biopsy based diagnostic methods for *H. pylori* infection (Table 1).

**ENDOSCOPY**

Considering that accurate prediction of *H. pylori*infection status on endoscopic images can improve early detection of gastric cancer, especially in some geographic areas, the contribution of both conventional and novel endoscopic evaluation methodologies, has continuously gained increased attention, particularly in specific clinical settings. A summary of the latest endoscopic studies is presented below. Watanabe *et al*[1] studied the diagnostic yield of endoscopy for *H. pylori*infection at three endoscopist career levels - beginner intermediate and advanced. For this study, 77 consecutive patients who underwent endoscopy were analyzed for *H. pylori*infection status by histology, serology and urea breath test (UBT). The diagnostic yield was 88.9% for *H. pylori*-uninfected, 62.1% for *H. pylori*-infected, and 55.8% for *H. pylori*-eradicated. Intra-observer agreement for *H. pylori*infection status was good (*k* > 0.6) for all physicians, while inter-observer agreement was lower (*k* = 0.46) for beginners than for intermediate and advanced (*k* > 0.6). For all physicians, good inter-observer agreement in endoscopic findings was seen for atrophic change (*k* = 0.69), but the accuracy was lower for beginners.

In 496 asymptomatic Japanese middle-aged men, a prospective evaluation (mean follow-up period of 54 years), of gastric cancer development was performed in non-atrophic stomach with highly active inflammation identified by serum levels of pepsinogen and *H. pylori* antibody together with a specific endoscopic feature: endoscopic rugal hyperplastic gastritis (RHG) (reflecting localized highly active inflammation)[2]. Cancer incidence was significantly higher in patients with RHG, high *H. pylori* antibody titers and low PGI/II ratio than in patients without. Significantly, no cancer development was observed in these high-risk subjects after *H. pylori* eradication. This relevant study emphasizes the high risk of cancer development in subjects with *H. pylori-*associated highly active non-atrophic gastritis and the usefulness of the two serological tests and endoscopic RHG for their identification.

Considering that *H. pylori* eradication is essential for metachronous gastric cancer prevention in patients undergoing endoscopic mucosectomy (EMR), for early gastric cancer, as reported by Fukase *et al*[3], Lee *et al*[4] aimed to determine the optimal biopsy site for *H. pylori* detection in the atrophic remnant mucosa of 91 EMR patients. Three paired biopsies for histology were taken at antrum, corpus lesser (CLC), and greater curve (CGC). Additional specimens were obtained at antrum and CGC for rapid urease test (RUT). *H. pylori* infection was defined as at least two positive specimens on histology and/or RUT. Serologic atrophy was determined by pepsinogen levels. The authors concluded that CGC is the optimal biopsy site for *H. pylori* diagnosis in EMR patients with extensive atrophy and that antral biopsy should be avoided, especially in serologically atrophic patients.

Although gastroscopic biopsy-based tests such as the RUT, histological examination, and culture have been widely used to diagnose *H. pylori* infection, many investigators have attempted to categorize the endoscopic findings characteristic of a *H. pylori*-infected stomach.

In 2002, Japanese endoscopists[5] found that collecting venules, seen as numerous minute red dots in the gastric corpus, were a characteristic finding in the normal stomach without *H. pylori* infection using both standard and magnifying endoscopy (identification of micromucosal patterns). This finding was termed “regular arrangement of collecting venules” (RAC). However, these findings are not a reliable method of diagnosis because of their low sensitivity and specificity.

Though magnifying endoscopy provides more precise information concerning abnormal mucosal patterns[6,7], it is not available in all endoscopy units. Moreover, its use requires training under an experienced supervisor and expertise. In addition, magnifying endoscopy is not necessarily appropriate for routine clinical practice as it is time-consuming and only a few facilities carry out this technique on a routine basis. On the other hand, endoscopic features corresponding to Sydney System pathological findings have not yet been identified and the diagnosis of *H. pylori* infection in gastric mucosa by endoscopic features has not yet been established (Figure 1). In this setting, the Study Group of Japan Gastroenterological Endoscopy Society for Establishing Endoscopic Diagnosis of Chronic Gastritis performed a prospective multicenter study enrolling 275 patients[8], investigating the association between endoscopic findings (conventional findings and indigo carmine contrast) and histological diagnosis of *H. pylori* (antrum and corpus). It was shown that specific endoscopic findings (such as diffuse redness, spotty redness and mucosal swelling assessed by conventional endoscopy and swelling of *areae gastricae* by the indigo carmine contrast method, were useful for diagnosing *H. pylori* infection.

Cho *et al*[9] aimed to establish a new classification for predicting *H. pylori*-infected stomachs by non-magnifying standard endoscopy alone. A total of 617 participants who underwent gastroscopy were prospectively enrolled and a careful close-up examination of the corpus at the greater curvature maintaining a distance of 10 mm between the endoscope tip and the mucosal surface. Despite concerning a monocenter study in which standard endoscopy was not directly compared with magnifying endoscopy, these results suggest two important contributions for prediction of *H. pylori* infection status: (1) the observation of gastric mucosal patterns using standard endoscopy and proposal of a new endoscopic classification including a normal RAC and three abnormal mucosal patterns; and (2) an accuracy of prediction of *H. pylori* positivity at least similar to that reported in magnifying endoscopy studies (sensitivity of 95.2% and specificity of 82.2%)[10]. In the future, multicenter trials comparing standard endoscopy against magnifying endoscopy, including the changes of mucosal patterns after *H. pylori* eradication and including endoscopists with different levels of expertise are needed to confirm the reliability of these data.

Chromoendoscopy has also recently regained attention as an additional methodology for detection of *H. pylori* in gastric mucosa. A multicenter Japanese study involving 275 patients, evaluated the possibility to diagnose *H. pylori* by conventional endoscopy and chromoendoscopy using indigo carmine in comparison with histology performed according to the Sydney System[7]. Based on several indices the authors obtained a sensitivity of 94% in the corpus and 88% in the antrum. However, the specificity in the corpus and in the antrum was low (62% and 52%, respectively). Another study concerning a Cuban adult population[11], also aiming to evaluate the diagnostic yield of chromoendoscopy with red phenol at 0.1% for the detection of *H. pylori* infection against histology, reported a sensitivity of 72.6% (95%CI: 64.9-79.2) and a specificity of 75.5% (95%CI: 61.9-85.4). The authors concluded that it might be a useful method in the diagnosis of *H. pylori* infection in the gastric mucosa, potentially with some specific advantages (topographic localization, avoidance of contamination and fast and immediate reading).

**HISTOLOGY**

Although histology has been considered to be the gold standard for *H. pylori*detection, the influence of clinical practice on the histopathological detection of *H. pylori*infection has been yet insufficiently explored. Recognizing that the number and distribution of *H. pylori* organisms vary in patients taking proton pump inhibitors (PPIs), it has been recommended to discontinue PPIs two weeks before endoscopy and to take biopsies from both the body and antrum.

In a representative study, Lash *et al*[12] aimed to evaluate the yield of different gastric sampling strategies and to determine the adherence to the Sydney System guidelines in a nationwide sample of endoscopists in United States. Using a database of biopsy records diagnosed at a single pathology laboratory, the results of gastric biopsies taken to evaluate gastric inflammatory conditions in patients with no endoscopic lesions were reviewed. The *incisura angularis*, rarely sampled, yielded minimal additional diagnostic information and the acquisition of at least two biopsy specimens from antrum and corpus, essentially following the Sydney System recommendations, confirmed to be a sensible strategy that guarantees the maximum diagnostic yield for the most common gastric inflammatory conditions.

In a Canadian study[13], electronic patient records were evaluated for the sites of gastric sampling and PPI use at endoscopy, collecting 150 cases with biopsies taken from both antrum and body, which were randomly selected for pathological re-review with special stains. The gastric regions sampled, *H. pylori* distribution and influence of clinical factors on pathological interpretation were assessed. This study confirmed that, despite national and international guidelines for managing *H. pylori*infection, these guidelines are infrequently adhered to, with PPIs frequently contributing to false diagnosis and sampling only one region increases the likelihood of missing active infection by at least 15%.

Considering that atrophy of the stomach mucosa develops in about 50% of *H. pylori* infected individuals by the age of 65, and is considered a pre-malignant lesion for gastric cancer[14-16],*H. pylori* eradication is recommended in the presence of atrophy[17], since atrophy may reverse after successful eradication therapy. It is critically important and challenging, therefore, to determine the presence or absence of *H. pylori* in patients with atrophic gastritis. In the course of atrophy progression, however, the density of *H. pylori* in the stomach mucosa decreases, and during the late stages of atrophy the infection may completely disappear[14,16] . This may explain the markedly lower sensitivity of biopsy-based tests (RUT, histology, culture) in the presence of atrophy. Similarly, UBT and antigen stool detection can also give false-negative results in these circumstances. In contrast, serology is not influenced to such an extent by a lower density of the microorganism, and is reliable even in advanced gastric body atrophy[14,16]. Maastricht guidelines updates have reserved serology for special situations, including extensive atrophy of the stomach mucosa on the basis that other tests might be misleading at a low bacterial density. Thus the debate continues regarding the most appropriate *H. pylori* diagnostic method in atrophic gastritis.

Lan *et al*[18] aimed also to evaluate the site and sensitivity of biopsy-based tests in terms of degree of gastritis with atrophy. Biopsy-based tests (*i.e*., culture, histology Giemsa stain and RUT) and non-invasive tests (anti-*H. pylori* IgG) were performed in 164 uninvestigated dyspepsia patients. The sensitivity of biopsy-based test decreased when the degree of gastritis with atrophy increased regardless of biopsy site and in moderate to severe antrum or body gastritis with atrophy, additional corpus biopsy resulted in increased sensitivity to 16.67%, as compared to single antrum biopsy. These results confirm that in moderate to severe gastritis with atrophy, biopsy-based test should include the corpus for avoiding false negative results in *H. pylori* detection.

Since the discovery of *H. pylori*, pathologists have used different diagnostic techniques, including immunohistochemical (IHC) methods and special stains such as Giemsa and Warthin-Starry on an institution- and laboratory-dependent basis (with variable sensitivities and specificities for identifying *H. pylori*). On the other hand, it is clear that IHC staining is highly sensitive and specific for *H. pylori*, with the lowest rate of inter observer variation and much less consuming than conventional histology[19]. However, the necessity for routine special stains and/or IHC stains has been debated in recent years. A recent study by Wang *et al*[20] confirmed what is assumed by many pathologists: routine special stains, specifically IHC stains, are not cost-effective or necessary. Recently, Smith *et al*[21], in a retrospective study involving 200 consecutive gastric biopsy specimens, further confirmed that *H. pylori* is easily observed in the majority of cases with H&E (sensitivity 91% and specificity 100%), remaining the most expedient and least expensive test for identifying *H pylori* in gastric biopsies.

An institutional quality assurance study of a conventional method for the diagnosis of *H. pylori* -associated gastritis has been performed by Hartman *et al*[22] in United States, based on head-to-head evaluation by four methods, H&E stain, Giemsa stain, Warthin-Starry stain, and *H. pylori* immunostaining of 356 gastric biopsy specimens. About 83% of *H. pylori* gastritis identified were diagnosed on the initial H&E-stained slides, further supporting the use of routine ancillary stains to diagnose *H. pylori* infection in gastric biopsy specimens. Usually, the use of special stains is only recommended for biopsy specimens with moderate to severe chronic active or inactive gastritis in which *H. pylori* is not identified by HE staining, for post-treatment biopsy specimens and in cases in which structures “suspicious” but not definitive for *H. pylori* are observed by H&E staining[23].

Both routine conventional histology-based methods and novel methods for *H. pylori* detection have been increasingly foccused on specific clinical settings and patient groups (bleeding peptic ulcer, gastric cancer). False-negative results may occur when using histological and RUT to detect *H. pylori* in biopsy specimens obtained during peptic ulcer bleeding episodes (PUB). Choi *et al*[24] evaluated different diagnostic methods in the specific setting of peptic ulcer, concluding that histology was the most accurate test, regardless of bleeding, as compared toculture, serology and RUT.Ramirez-Lazaro *et al*[25], have found that IHC and real-time PCR methods might improve the sensitivity of biopsy-based diagnosis in this specific setting (PUB).

In patients submitted to subtotal gastrectomy due to gastric cancer, the identification and treatment of *H. pylori* are the key points in the prevention of cancer recurrence. Xu *et al*[26] have evaluated the predictive value of neutrophil infiltration, a hallmark of active inflammation (updated Sydney system), as an histological marker of *H. pylori* infection, in 315 dyspeptic patients undergoing upper gastrointestinal endoscopy, including patients with subtotal gastrectomy. The diagnosis of *H. pylori* infection was based on UBT and on anti-*H. pylori* immunoglobulin G (IgG) antibody if subtotal gastrectomy status. Although neutrophil infiltration of gastric mucosa was strongly associated with overall *H. pylori* infection, in patients with subtotal gastrectomy, the diagnostic accuracy of neutrophil infiltration in *H. pylori* infection was low.

De Martel *et al*[27]using data from a large Venezuelan cohort of 1948 adults, compared the gastric detection of *H. pylori* by polymerase chain reaction (PCR) of the *vacA* gene in one antral biopsy, to the detection of *H. pylori* by histopathology (HE and Giemsa staining) in five biopsies (antrum and corpus). Overall, *H. pylori* was detected in 85% and 95% of the subjects by PCR and histopathology, respectively, thus confirming that histopathology on five biopsies is an accurate tool for *H. pylori* detection in most subjects, compared to the PCR method on one biopsy. However, in subjects with the most severe precancerous lesions (intestinal metaplasia type III and dysplasia), PCR displayed elevated sensitivity for detecting the bacteria (significantly more often than histopathology on a single biopsy), thus suggesting its potential usefulness in this setting.

Tian *et al*[28] reported a meta-analysis evaluating *H. pylori* diagnostic methods in patients with partial gastrectomy. The pooled sensitivity and specificity were 93 and 85% for histology, 77 and 89% for UBT, and 79 and 94% for RUT, respectively, thus leading to the conclusion that histology was the most reliable test in this setting. Lee *et al*[4] evaluated 91 patients requiring endoscopic mucosal resection for early gastric cancer (GC), obtaining three pairs of biopsies from the antrum, corpus lesser curvature (CLC), and corpus greater curvature (CGC). The sensitivity of histology in detecting *H. pylori* was significantly higher in the CGC than that in the antrum or CLC, suggesting that the CGC might be the optimal biopsy site for *H. pylori* in patients with extensive atrophy.

The utility of routine biopsy of gastric ulcer margin (currently performed to exclude malignancy) in diagnosing *H. pylori* infection, has recently been re-assessed by Lin MH *et al*[29], by prospectively examining a cohort of 50 patients with gastric ulcer (54% uninfected). Histology, RUT and UBT were compared; six biopsied specimens from the margin of the gastric ulcer and one each specimen from antrum and body of non-ulcer part were obtained for histology using HE staining. The diagnostic accuracy of the histological examination of the ulcer margin was quite good and importantly, the addition of one specimen from the antrum or body did not increase its diagnostic yields, thus emphasizing its accuracy and usefulness for diagnosing *H. pylori* infection in these patients.

An increasing body of evidence has reported *H. pylori* colonization in the esophageal mucosa of dyspeptic patients. Contreras *et al*[30] have further contributed to the field, with a study examining the presence of *H. pylori* in the gastroesophageal mucosa by by histology, fluorescence *in situ* hybridization (FISH) and PCR analysis of DNA (using genus- and species-specific PCR primers) extracted from gastric and esophageal biopsies of 82 symptomatic Venezuelan patients. *H. pylori* in the stomach was detected by PCR and FISH, respectively, in 61% and 90% of dyspeptic patients, and in the esophagus in 70% and 73% By combining the results of both methods, *H. pylori* was observed in the gastroesophageal mucosa in 86% of patients. These findings deserve specific attention and further elucidation.

Finally, the histology reporting of gastritis of the staging system OLGA (Operative Link on Gastritis Assessment) has also been re-examined, considering its interest in the prediction of the gastric cancer risk[31,32]***.*** Carrasco *et al*[33]reviewed the histology of the normal gastric mucosa, overviewing the role of *H. pylori* in the multistep cascade of GC. The role of the OLGA staging system in assessing the risk of GC wasemphasized; specifically, the epigenetic bases of chronic gastritis, mainly DNA methylation of the promoter region of E-cadherin in *H. pylori* -induced chronic gastritis and its reversion after *H. pylori* eradication. In addition, the authors discussed the finding of circulating cell-free DNA, offering the opportunity for noninvasive risk assessment of GC.

**Rapid Urease Test**

The RUT is based on the production of large amounts of urease enzyme by *H. pylori*, which splits the urea test reagent to form ammonia enabling its detection by a rapid indirect test. Many commercial RUT are available, including gel-based tests, paper-based tests and liquid-based tests, providing a result in 1-24 h, depending on the format of the test and the bacterial density in the biopsy specimen. Typically, the commercial RUT have specificities above 95%-100% but the sensitivity is slightly less, ranging from 85-95[34].

In comparison to histology and culture, urease tests are faster, cheaper and have comparable sensitivity and specificity, in normal clinical settings. The sensitivity can however decrease in patients with bleeding peptic ulcers (67%-85%) as well as in patients with partial gastrectomy (79%)[24,28,34,35]. Formalin contamination of forceps used to collect the biopsy may also be a cause contributing to reduced sensitivity[24,36].

An important conclusion of several studies is that enhancing the number of biopsy fragments and/or collecting from various regions of the stomach (antrum and body, from example), a higher sensibility of the RUT is achieved[37]. Moreover, it was recently shown that combining tissues prior to RUT increased the detection of *H. pylori*, in comparison to test separate specimens, and speed up the result since the reaction is faster[38].

**CULTURE**

Since the discovery of *H. pylori*, bacterial culture has been used as routine diagnostic test, being considered the gold standard. Currently, the Maastricht-4 Consensus Report recommends *H. pylori* culture for performing antibiotic susceptibility testing if primary resistance to clarithromycin is higher than 20% or after failure of second-line treatment[17].

Although its long use, culture test remains a challenge due to the fastidious nature of the bacterium, with particular growth requirements of medium and atmosphere. The most common used media include Brucella, Columbia Wilkins-Chalgren, brain-heart infusion or trypticase agar bases, supplemented with sheep or horse blood[39]. An alternative to blood is the supplementation of the agar base with -cyclodextrin or yolk emulsion[40,41].

The most recent advances on *H. pylori* culture concern growth medium composition, besides the usual serum or blood additives. A recent study showed that supplementation of media with cholesterol instead of serum was a valuable option for *H. pylori* growth[42]. Another original approach uses liquid culture medium for the rapid cultivation and subsequent antibiotics susceptibility testing of *H. pylori* directly from biopsy specimens, with a final detection step by enzyme linked immunosorbent assay (ELISA)[43].

Concerning the growth atmosphere, it is known that *H. pylori* is a capnophilic organism that requires an atmosphere CO2 enriched (varying from 5%-10%). In addition has been considered a microaerophile, but there is no general consensus about its specific O2 requirements[44]. A recent advance on this topic was brought by Park *et al*[45], that showed, unlike previous reports, that *H. pylori* may be a capnophilic aerobe whose growth is promoted by atmospheric oxygen levels in the presence of 10% CO2.

Typically, culture of *H. pylori* is performed on gastric biopsy samples, and since bacteria display an irregular distribution in the gastric mucosa, culture of more than one biopsy, from antrum and corpus, is sometimes mandatory, especially after antibiotic treatment. Another important issue is to bear in mind factors that may affect the outcome of *H. pylori* culture from endoscopic gastric mucosal specimens. Besides the issue concerning the bleeding peptic ulcers, for which culture has a lower sensitivity than in nonbleeding cases, other host-related factors such as high activity of gastritis, low bacterial load, drinking alcohol and the use of histamine H₂ receptor blockers have been recently described as the cause of failed *H. pylori* culture from gastric mucosa in the infected subjects[24,46].

Culturing from stools has been shown to be extremely difficult due to the complex nature of the sample regarding microbiota composition and shedding of unviable *H. pylori* cells, and this technique has been successful in the setting of rapid gastrointestinal tract transit[47]. In a recent study, the authors were able to culture *H. pylori* in nine and 12 rectal and ileal fluids, respectively, after polyethylene glycol (colyte) ingestion in 20 healthy adults with positive UBT[48]. Other studies have looked for the role of oral cavity as a reservoir of *H. pylori*. A recent work evaluated the occurrence of the organism in subgingival plaque and was able, by culture, to recover *H. pylori* in nine of 30 studied patients that were *H. pylori* positive with RUT and histopathologic examination. Thus, they conclude that detection of *H. pylori* in dental plaque of dyspeptic patients cannot be neglected and might represent a risk factor for recolonization of stomach after systemic eradication therapy[49]. The same conclusion comes from another study in which *H. pylori* was detected in subgingival dental plaque of children and their families, possibly acting as a "reservoir" contributing to the intra-familial spread[50].

**MOLECULAR METHODS**

Diagnostics tests rely more and more on molecular tests which can provide faster, more accurate and sensitive detection of the bacterium than conventional methods, with the possibility of extending to other purposes, such as detection of antibiotic resistance and virulence determinants, and bacterial quantification. Moreover, other biological samples than gastric biopsies can be used, obtained using less invasive methods, such as stool or oral cavity samples. Whatever the case, amplification of the nucleic acids by PCR is almost always present, either conventional PCR or, more and more, by real-time PCR.

*H. pylori*, like a few other bacteria, acquires resistance by mutation, which has enabled the development of numerous assays, in several formats, for detection of mutations leading to resistance, especially to macrolides and fluoroquinolones. For detection of *H. pylori* and resistances to fluoroquinolones and clarithromycin, there is a multiplex PCR followed by a hybridization and alkaline phosphatase reaction on a membrane strip (Genotype® HelicoDR kit), that uses as starting material biopsy specimen as well as culture material extracted from it. The performance of this test was evaluated, showing a high sensitivity and allowing detecting infection with multiple strains. The performance in detecting fluoroquinolone-resistance strains was however lower than culture, emphasizing the need to expand the broad of *gyrA* mutations included in the kit[51,52]. Several assays real-time PCR based, using either TaqMan or FRET (Fluorescence Resonance Energy Transfer) are available, as in-house assays or commercial kits, for clarithromycin resistance, performed on cultured strains, directly on biopsies[53-55] or in stool samples. This latter is particularly useful as non-invasive test in pediatric populations where a high prevalence of clarithromycin-resistant strains is suspected, as well as for tracking the emergence of clarithromycin resistance following eradication treatment[57-58].

Recently, a dual-priming oligonucleotide (DPO)-based multiplex PCR was developed to detect both *H. pylori* infection and the most common point mutations conferring resistance to clarithomycin, directly on gastric biopsy specimens. This assay showed to be fast, not requiring expensive instrumentation, and can thus be valuable in countries with high prevalence of clarithromycin resistance[59,60].

The detection of clarithromycin-resistance from formalin-fixed, paraffin-embedded gastric biopsies has also been described, and can be useful mostly before treatment when culture and susceptibility testing is not available, or to detect the primary resistance to clarithromycin in the case of failure of an empirical therapy based on this antibiotic. Real-time PCR assays as well as a peptide nucleic acid-fluorescence *in situ* hybridization (PNA-FISH) method have been lately described[61-63].

Another area of particular interest is the detection of virulence determinants, such as the *cagA* (cytotoxin-associated gene A) and the *vacA* (vacuolating cytotoxin) major toxins. Several studies showed that the risk of progression of gastric preneoplastic lesions is higher in patients infected with strains harboring the most virulent *cagA* and *vacA* genotypes than in patients infected with the least virulent strains. Therefore *H. pylori* genotyping may be useful for the identification of patients at high risk of progression of gastric preneoplastic lesions and who need more intensive surveillance[64]. Concerning *vacA*, a novel method for genotyping the vacA intermediate gene region was recently reported, using a novel primer combination allowing the amplification of smaller DNA fragments than the original PCR, that can therefore be applied to paraffin-embedded biopsies. Patients infected with *vacA* *i1* strains showed an increased risk for gastric atrophy and for gastric carcinoma, with odds ratios of 8.0 (95%CI: 2.3-27) and of 22 (95%CI: 7.9-63)[65].

CagA undergoes phosphorylation on tyrosines within Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs at the polymorphic C-terminus[66] and several studies suggest point for a role for the polymorphic CagA EPIYA-containing region in the pathogenicity of *H. pylori,* although have conflicting results have been reported[67,68]. The *in vivo* role of this region was recently emphasized in a study showing that infection with strains harboring two or more CagA EPIYA C motifs was associated with the presence of surface epithelial damage, and with atrophic gastritis and gastric carcinoma. Moreover, the presence of two or more CagA EPIYA C motifs increased the risk for atrophic gastritis from 7.3 (95%CI: 2.1–25) to 12 (*95*%CI: 2.5–58) and for gastric carcinoma from 17 (95%CI: 5.4–55) to 51 (95%CI: 13–198), when compared to one EPIYA C motif. Therefore, genotyping of *H. pylori* virulence determinants maybe a useful approach in defining severe gastric-disease risk.

Bacterial quantification can also be important for clinical management of the infection, for example for monitoring the treatment outcome or in particular settings such as upper gastrointestinal bleeding[69].

A recently developed real-time quantitative PCR assay based on *H. pylori* *ureC* (single copy gene) copy number measuring showed to be around 10 times more sensitive than the conventional PCR method. Moreover, the copy number of *ureC* was significantly increased when overall gastritis, bacterial density, chronic inflammation and intestinal metaplasia were present[70]. Nevertheless, further studies are necessary to determine the exact optimum cut-off point, making it possible to differentiate between asymptomatic colonization and infection with clinical repercussions for patients. These highly sensitive real-time quantitative PCR can have a large application on the study of environmental reservoirs as well[71,72].

By improving our knowledge of bacteria, at their molecular level, new strategies for treatment/prevention of bacterial-associated diseases as well as diagnostic tests can be developed. Proteomic approaches aiming at identifying gene products differentially expressed in association with a given pathology can give an important input towards understanding the pathways that are putatively associated with the respective disease, contributing therefore for identification of novel therapeutic or diagnostic targets.

Our current knowledge on the proteome of this organism was largely based on data obtained for the soluble proteome[73], membrane proteome[74,75] and secreted proteome[76] of the strain 26695, the first isolate to be sequenced. More recently, relevant contributions have made been through this approach, such as novel biomarkers for gastric cancer and for peptic ulcer disease[77,78].

**NONINVASIVE TESTS**

Although the reliability of both the 13C-UBT and a monoclonal ELISA stool test (HpSA) to diagnose *H. pylori* infection in very young children has been further confirmed, additional background information is yet warranted for epidemiological studies in infants and toddlers.

**UREA BREATH TESTS**

The 13 C-urea breath test (13C-UBT) is one of the most reliable tests for diagnosing *H. pylori* infection. It is a non-invasive, simple and safe test which provides excellent accuracy both for the initial diagnosis of *H*. *pylori* infection and for the confirmation of its eradication after treatment. The simplicity, good tolerance and economy of the citric acid test meal probably make its systematic use advisable. The UBT protocol may be performed with relatively low doses (< 100 mg) of urea: 75 mg or even 50 mg seem to be sufficient. With the most widely used protocol (with citric acid and 75 mg of urea), excellent accuracy is obtained when breath samples are collected as early as 10–15 min after urea ingestion. A unique and generally proposed cut-off level is not possible because it has to be adapted to different factors, such as the test meal, the dose and type of urea, or the pre-/post-treatment setting. As positive and negative UBT results tend to cluster outside of the range between 2 and 5, a change in cut-off value within this range would be expected to have little effect on clinical accuracy of the test[79,80]***.*** UBT is now marketed for use with a nondispersive, isotope-selective infrared spectroscope (NDIRS) or laser-assisted ratio analysis (LARA), reliable and valid alternatives to IRMS of potential interest for epidemiologic studies of children, for screening symptomatic children before endoscopy or assessment of treatment efficacy. These devices are far smaller and cheaper, and they allow for in-office, near-immediate reading of results. Validation studies to establish the cutoff value for this test were preliminarily performed in Japan[81] but novel data are needed[82,83].

The 13C-UBT in adults, has a high sensitivity (88%-95%) and specificity (95%-100%)[17]. However, the test has shown heterogeneity in accuracy in the pediatric population, especially in young children with values of sensitivity and specificity ranging from 75% to 100%, before and after treatment (using several protocols), despites being a simple and safe non-invasive test in children older than 6 years old[84]. Although several modifications have been proposed since the original description by Graham of the 13C-UBT to diagnose *H. pylori* infection[85], in children, performance criteria are not yet sufficiently established[86]. At the specific age group of younger children, accurate non-invasive tests for diagnosing *H. pylori* infection are strongly required, as they may avoid invasive and painful procedures, such as endoscopy and blood draw, additionally to overcoming the false negative results observed with gold standard tests (histology, culture, and RUT), where colonization of the stomach may be weak and patchy.

Potential explanations for UBT performance variability in children might include: (1) urease activity from the oral bacterial flora[87]; (2) differences in delta time (decrease in specificity if samples obtained at 15 min instead of 30 min); and (3) variability in cutoff values. The administration of 13C-urea in capsules to avoid activity of oral bacteria, though effective in adults, is not feasible in infants or toddlers[88]. Finally, the cutoff value (usually determined by the ROC curve) represents a crucial factor for accuracy of the test, where cutoff values low might increase sensitivity but reduce specificity, and *vice versa*[81]. Additionally, the individual’s CO2 production is known to be influenced by anthropometric characteristic as well as by age and sex (lower in young children with relativelylow weight and height)[89].

Leal *et al*[90] performed an informative systematic review and meta-analysis (31 articles and 135 studies from January 1998 to May 2009), aiming to evaluate the performance of the 13C-UBT diagnostic test for *H. pylori* infection in children. Selection criteria included studies with at least 30 children and reporting the comparison of 13C-UBT against a gold standard for *H. pylori* diagnosis (*H. pylori* culture, histologic examination, or RUT) were included for analysis. Children were stratified in subgroups of < 6 and ≥ 6 years of age. The 13C-UBT performance meta-analyses showed: (1) good accuracy in all ages combined [sensitivity 95.9%, specificity 95.7%, diagnostic odds ratio (DOR) 424.9]; (2) high accuracy in children > 6 years (sensitivity 96.6%, specificity 97.7%, DOR 1042.7); and (3) greater variability in accuracy estimates and a lower specificity, in children ≤ 6 years (sensitivity 95%, specificity 93.5%, DOR 224.8). The authors identified as potentially important sources of heterogeneity: (1) tracer dose; (2) pretest meal; and (3) cutoff value, observing that a unique tracer dose of 50 mg of 13C-urea shows greater accuracy than when it was adjusted to body weight (50–75 mg were used between studies). Accordingly, Mégraud[91] previously reported that reducing the dose from 75 to 45 mg in younger children resulted in improved specificity. Although citric acid has demonstrated good performance in adults, it is not well accepted by children, and apple, orange, or grape juice seems to be good alternatives. Finally, a cutoff value of 6.0 ‰ improved overall performance, in children younger than 6 years, as compared to a cutoff of 4.0 ‰ for children older than 6 years old.

Pacheco *et al*[92] have more recently contributed to the field at this age group, by evaluating the diagnostic accuracy for the detection of *H. pylori* infection of low dose C-UBT with early sampling at pediatric age (129 patients between the ages of 2.1 and 19 years old, median = 11.6 years) submitted to upper gastrointestinal endoscopy. The 13C-UBT was performed after a 4-hour fasting period with 4 points of collection: baseline (T0, at 10, 20 and 30 min) after ingestion of 25 mg C-urea diluted in 100 mL of apple juice; analysis of exhaled breath samples was performed with an isotope-selective infrared spectrometer. The sensitivity and specificity were similar at T10, T20 and T30 (94.7%/96.8%; 96.2%/96.1% and 96.2%/94.7%, respectively).

Recently also, Queiroz *et al*[93] investigated the agreement between the 13C-UBT and a monoclonal ELISA (HpSA) for detection of *H. pylori* antigen in stool in a prospective study enrolling 414 South-American infants (123 from Brazil and 291 from Peru) aged 6-30 months. Breath and stool samples were obtained at intervals of at least three-months. 13C-UBT and stool test results concurred with each other in 94.86% cases (kappa coefficient = 0.90, 95%CI: 87-92). In the *H. pylori*-positive group, DOB and OD values were positively correlated (*r* = 0.62, *P* < 0.001, suggesting that both 13C-UBT and stool monoclonal test are reliable to diagnose *H. pylori* infection in very young children.

Differently from pediatric studies where attention has been yet focused in methodological issues, in the adult studies, the validity and usefulness of UBT have increasingly been evaluated in a wide spectrum of specific clinic settings. Olafsson *et al*[94]evaluated 620 UBT in 595 subjects at a gastroenterology clinic. UBT was negative in 526 patients, but: (1) 45% patients were tested < 4 wk before end of treatment; and (2) 23% negative results occurred in patients recently treated. The authors emphasized the need of strict protocol adherence in clinical practice to a fully reliable UBT assessment. Velayos *et al*[95] aimed to investigate the accuracy of UBT performed immediately after emergency endoscopy in 74 patients with peptic ulcer bleeding by comparing the results with those of UBT performed after hospital discharge in a subset of 53 patients (gold standard). Although UBT carried out immediately after emergency endoscopy in peptic ulcer bleeding is an effective, safe, and easy-to-perform procedure, the relatively low sensitivity and specificity suggested the requirement of a subsequent control, in accordance with recommendations concerning peptic ulcer bleeding[96].

Few studies using UBT have yet been performed in patients submitted to partial gastrectomy, a specific group in which the identification of *H. pylori* infection is mostly relevant. Wardi *et al*[97] have evaluated the sensitivity and specificity of the continuous UBT (BreathID) in 76 post gastrectomized patients (older than 18 years) (lowering the gastric pH by the addition of citric acid), against a RUT and the histology as gold standards. *H. pylori* was positive in 14/76 (18.4%) patients when histology was considered as the gold standard method. The positive predictive value of the continuous UBT and the RUT was 0.64 and 0.35, respectively. The negative predictive value was high by both the methods, 0.92 and 0.95, respectively, supporting that BreathID might have some reliability to exclude *H. pylori* after partial gastrectomy.

**STOOL ANTIGEN TESTS**

The stool antigen test is a non invasion method for the detection of *H. pylori*, usually recommended when the UBT is not available[98]. Besides being a non-invasive test, the advantages of using this method include the unneeded requirement of expensive equipment and medical personnel, and the collection of the sample at home without a necessary visit to the hospital. This method is especially interesting for children access to a safe diagnosis and also for its low cost[99,100].

A meta-analysis revealed that the global sensitivity and specificity of stool antigen tests are 94% (95%CI: 93-95) and 97% (95%CI: 96-98), respectively[101]. A prospective study to evaluate the efficacy of a new EZ-STEP *H. pylori* polyclonal enzyme immunoassay (EIA) stool antigen test enrolled 555 patients undergoing routine checkups. For the optimal cut-off value (optical density 0.160) this test presented high level of sensitivity (93.1%), specificity (94.6%) and accuracy (93.8%)[99] .

There are two types of stool antigen tests used for *H. pylori* detection, the EIA and an assay based on immunochromatography. Two new stool test were recently developed[102]. These tests are the Testmate pylori antigen EIA, in which plastic 96-well EIA microtiter plates were coated with monoclonal antibody (Mab) 21G2[103], and the Testmate rapid pylori antigen, which is based in immunochromatography and is presented as a test strip. For the EIA test a drop of the suspended stool sample or a sample of the diluted bacterial antigen sample is mixed with the peroxidase-conjugated MAb 21G2, after proper incubation and washing the optical density is measured and considered positive if superior to 0.100. For the test strip, a drop of stool sample is applied in the specimen application of the test strip. When *H. pylori* antigens are present they form immune complexes with the red latex-labeled MAb 21Ge and migrate by capillarity until capture by the solid phase anti-mouse rabit polyclonal antibodies and form a visible red test line. A control line is also present. After application of these test to 111 stool samples, both these new tests provide 100% specificity, sensibility and accuracy[102], which is very promising. However, not all studies report this high values for sensitivity and specificity. This is the case of the report of Chehter *et al*[100] that analyzed the stools of 75 patients reveled and determined a lower sensitivity (87.2%) and specificity (44%); and of Iranikhah *et al* that, after analyzing the stools of 103 children, reach similar values for sensitivity (85%), but improved the specificity (83%)[104].

Recently, five different stool antigen tests were compared: the Premier Platinum HpSA Plus test is based on monoclonal EIA (Meridian Bioscience, Inc, Cincinnati, OH, United States); the Hp Ag test is based on monoclonal EIA (Dia.Pro Diagnostic Bioprobes Srl, Milano, Italy); the ImmunoCard STAT! HpSA test is based on monoclonal lateral flow chromatography (LFC) (Meridian Bioscience, Europe Srl Milano, Italy); the *H. pylori* fecal antigen test is based on monoclonal LFC (Vegal Farmaceutica, Madrid, Spain) and the one-step *H. pylori* antigen test is based on LFC with polyclonal antibodies (IHP-602, ACON Laboratories, Inc, San Diego, USA). Data comparison showed an uneven performance, favoring the Premier Platinum HpSA Plus test (sensitivity 92.2%; specificity 94.4%). The selection of the stool antigen assay is very important to achieve accurate results.

Stool antigen tests are also useful for detection of *H.* *pylori* in infected animal models, such as the C57BL/6 mice[105].

**Antibody -based tests**

Serology was one of the first methods used for diagnosis of *H. pylori* infection[106]. Currently, serology is recommended for initial screening, requiring further confirmation, by histology and/or culture, before treatment[107]. Detection of antibodies is useful for detection past or present exposure. In fact, a limitation of serology tests is the failure to distinguish between past and current *H. pylori* infection[99]. Moreover, the antibody levels to *H. pylori* are significantly heritable. Thus individual genetic differences of the human host contribute substantially to antibody levels to *H. pylori*[108].

Serological tests have several advantages, namely they are non-invasive and they do not produce false negative results in patients receiving treatment (proton pump inhibitors and antibiotics) or presenting acute bleeding[109].

Blood samples are used for serology testing, detecting anti-*H. pylori* antibodies (IgG), by ELISA. Recently the performance of 29 different serologic tests kits was compared, revealing a sensitivity from 55.6% to 100%, a specificity from 59.6% to 97.9 %, a positive predictive value from 69.8% and 100%, and negative predictive value from 68.3% and 100%[106]. According to the goal, screening, initial diagnosis, confirmation of another test, the most appropriate kit should be choose. Antibody-based tests for the detection of *H. pylori* are easy available but present high negative predictive value[110]. The heterogeneity of *H. pylori* strains has been well documented, with considerable variation in the prevalence of specific strains between isolates, specially from different geographic areas[111-113] and thus the success of the serology test depends on the use of antigens that are present in *H. pylori* strains from a given population. Moreover, kits developed using *H. pylori* strains from the west are not suitable for detecting *H. pylori* infection in the East[114]. The use of high-molecular-weight cell-associated antigens that are conserved in *H. pylori* strains allows overcoming this limitation[115]. Several *H. pylori* immunogenic proteins have been presented as candidates to detect the infection, such as FlidD protein[116]; multiple recombinant (CagA, VacA, GroEL, gGT, HcpC and UreA) proteins[116]; CagA[115] or Omp18[117].

Modifications to serology tests have been suggested, such as the automated immunoaffinity assay for *H. pylori* IgG detection using purified antigen of *H. pylori* immobilized on magnetic nanobeads, which is faster than ELISA and requires a smaller volume of serum[118]. The lateral flow immunoassays, an immunochromatographic assay, maintain the serological approach with the advantage of being fast, economic and require no additional equipment or experience[119].

Detection of gastrin and serum PG I/II ratio combined with *H. pylori* serology is useful for the prediction of gastric preneoplasric conditions[110]. PG I/II ratio decreases with the advancement of extensive atrophic gastritis, since PG I is produced by chief and mucous neck cells in the fundus glands, which are impaired in case of gastritis of the fundus; while PG II by the former cells and also by cardiac, pyloric and duodenal Brunner’s glands[120].

**DETECTION OF *H. PYLORI* IN OTHER SPECIMENS**

Other specimens have been evaluated to determine their usefulness to detect *H. pylori* infection. These include saliva[121,122], subgingival biofilm[123], dental plaque[124, gastric juice, gastroesophageal biopsies[125] and adenotonsillar tissue[126]. Contradictory results have been reported regarding *H. pylori* detection in adenotonsillar tissue, either favoring[127] or against[126] adenotonsillar tissue as an extra-gastric reservoir of *H. pylori*. The ability to detect *H. pylori* antibodies in saliva is lower than in blood-based serology. However, the use of molecular techniques for the detection of *H. pylori* infection in saliva or dental plaque may turn these specimens attractive since they are easier to collect[114]. The molecular techniques include PCR[122,123] and PCR-denaturing gradient gel electrophoresis (PCR-DGGE)[128]. Other techniques used to analyze these specimens are the RUT, immunohistochemistry and PNA-FISH[126].

The enterotest or string test was designed decades ago specially for children. String test consists of a gelatin capsule attached to a 90-140 cm long nylon string which unwinds during ingestion. Upon reaching the stomach, the gelatin capsule dissolves and the string absorbs gastric secretions. The extraction of the string occurs 30-180 min later and should avoid contact with teeth and tongue to prevent contamination. The string may be used for culture (sensitivity 65% and specificity 99%) or PCR (sensitivity 79% and specificity 99%) for *H. pylori* detection[129]).

**CONCLUSION**

Recent developments in both biopsy and non-biopsy based diagnostic methods for *H. pylori* infection, will further contribute to improve current clinical approach and management of *H.pylori*-associated diseases.

Predictably, in the future, standard and newer methods will evolve to improve the diagnostic yield of *H. pylori* infection detection in specific age groups (children versus adults) and clinical conditions, such as peptic ulcer bleeding, atrophic gastritis, post-gastrectomy status, as well as for wider application in epidemiological studies. The specific contribution of each method to the evolving strategies and algorithms for evaluation and management of *H. pylori* infection (test and treat) will persist of paramount relevance.

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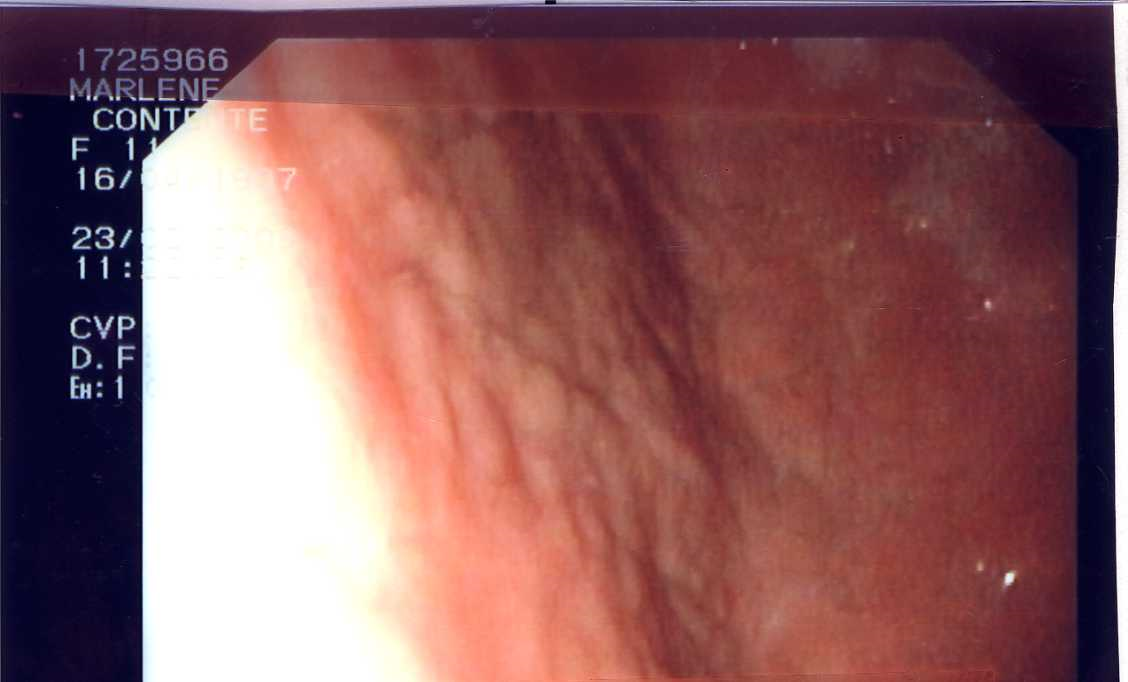
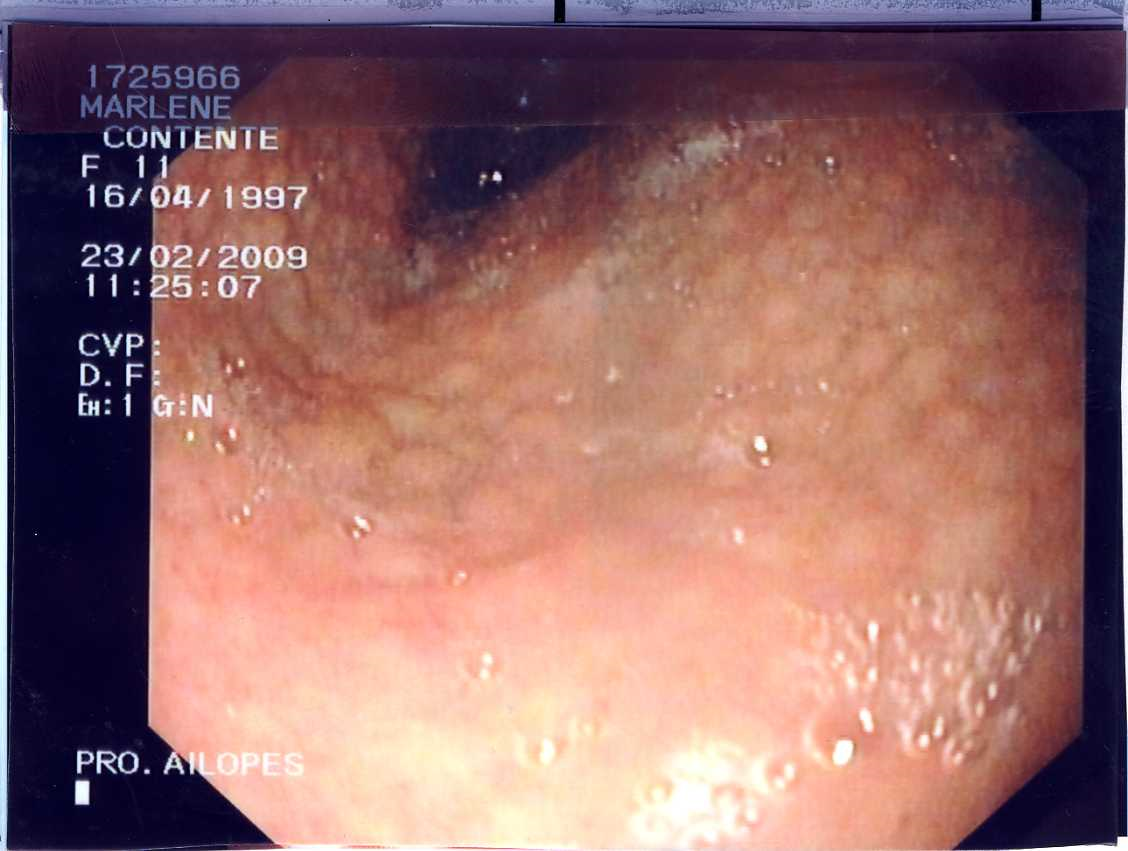
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**Figure 1 Endoscopic features of *Helicobacter pylori* infection (antral nodularity)**



**Table 1 Summary of diagnostic methods**

|  |  |  |  |
| --- | --- | --- | --- |
|  | Invasive / noninvasive | Reference method | Antibiotic resistance detection |
| Endoscopy | Invasive | Yes | No |
| Histology | Invasive | Yes | No |
| Rapid urease test | Invasive | No | No |
| Culture | Invasive | Yes | Yes |
| Molecular methods | Both | No | Yes |
| Serology | Noninvasive | No | No |
| Urea breath test | Noninvasive | No | No |
| Stool antigen test | Noninvasive | No | No |