Non-invasive molecular tests for *H. pylori*

Recently developed molecular methods for the non-invasive detection of *H. pylori* infection and antibiotic resistance offer a potentially rapid and sensitive alternative to current endoscopic tests.

The gastric pathogen *Helicobacter pylori* infects the stomachs of half of the world’s population. The prevalence of *H. pylori* infection varies globally but increases with older age and with lower socioeconomic status. The higher prevalence in older age groups is thought to reflect poorer childhood living conditions in the past. Most infected individuals do not develop clinically significant complications. However, infection can progress to a number of gastrointestinal disorders, including peptic ulcers, gastric cancer and gastric mucosa-associated lymphoid tissue lymphoma. The World Health Organization’s International Agency for Research on Cancer has classified *H. pylori* as a definite (group 1) carcinogen. Disease risk in infected individuals varies greatly in different populations and is associated with both host and bacterial factors.

The majority of *H. pylori*-infected patients are managed using a ‘test-and-treat’ strategy and diagnosed by non-invasive methods, including the urea breath test, serologic tests and the stool antigen test. The urea breath test involves ingesting $^{13}$C-labelled urea. In *H. pylori*-infected individuals, the *H. pylori* enzyme urease converts the $^{13}$C-labelled urea to labelled carbon dioxide detectable in a breath sample. The stool antigen test detects *H. pylori* antigens in stool samples, while serologic tests detect IgG antibodies to *H. pylori* in blood.

Invasive testing by means of endoscopy is recommended in those with a family history of gastric cancer, older patients (>45 years in Europe) with dyspepsia or those with accompanying alarm symptoms such as weight loss, iron deficient anaemia, persistent vomiting, gastrointestinal bleeding or an abdominal mass. Diagnosis of *H. pylori* following endoscopy is by means of a rapid urease test (RUT) for Campylobacter-like organisms, histological examination of stained biopsy specimens and/or by culture.

Treatment is recommended in symptomatic patients and those at risk of gastric cancer. However, successful eradication of *H. pylori* has become a significant challenge in recent years.
The standard first-line triple therapy for *H. pylori* infection, comprising a proton pump inhibitor (PPI) and the antibiotics clarithromycin and amoxicillin for seven to 14 days, has been widely used worldwide. Amoxicillin is replaced with metronidazole in penicillin-allergic patients. Unfortunately, suboptimal eradication rates for first-line triple therapy have been reported in many countries. Levofloxacin-based triple therapy is recommended as a second-line treatment option and subsequent rescue therapies should be guided by antimicrobial susceptibility testing. The main factors contributing to treatment failure are poor patient compliance and the emergence of antibiotic resistant strains of *H. pylori*. The most recent survey of antimicrobial resistance for *H. pylori* in Europe reported primary resistance rates of 17.5%, 14.1% and 34.9% for clarithromycin, levofloxacin and metronidazole respectively in *H. pylori* treatment-naive patients. Combined resistance to clarithromycin and metronidazole was detected in 7.8% of cases. Of note, the rates of antibiotic resistance varied greatly according to geographic region within Europe; the resistance rate for clarithromycin was <10% in Northern European countries, while most countries in the rest of Europe (except Spain and Germany) had a resistance rate of >15%. Overall, primary clarithromycin resistance had almost doubled since the previous European survey. As clarithromycin resistance decreases the efficacy of first-line triple therapy by up to 70%, European guidelines recommend that clarithromycin-containing triple therapy should be abandoned in regions where the resistance rate is higher than 15–20% and alternative treatment regimens, such as bismuth or non-bismuth quadruple therapy, have been suggested.

Antimicrobial susceptibility testing
Given that the recommended first-line therapy should be guided by the local prevalence of clarithromycin resistance and third-line and subsequent treatment regimens should be guided by antimicrobial susceptibility testing, methods for detecting antibiotic resistance are of great interest. The current gold standard method for *H. pylori* antimicrobial susceptibility testing recommended by the European Helicobacter and Microbiota Study Group (EHMSG) is culture and determination of the minimum inhibitory concentration of antibiotic required to inhibit bacterial growth using the Etest method (Biomerieux, Basingstoke, UK). The European Committee on Antimicrobial Susceptibility Testing (EUCAST) have published clinical breakpoints for *H. pylori* antibiotic resistance based on this method. However, this approach is not without limitations. Firstly, *H. pylori* is isolated from gastric biopsy samples taken from patients during endoscopy, thus this method is only available for individuals undergoing invasive testing for *H. pylori*. As such, findings on the prevalence of antibiotic resistance based solely on this patient cohort may not reflect the true rates of resistance in a given population. Additionally, concurrent use of PPIs inhibits the growth of *H. pylori* and reduces the chance of successful culture. For this reason, endoscopy patients should avoid taking PPIs for at least two weeks prior to endoscopy. Furthermore, *H. pylori* is a fastidious organism and culture is time-consuming with very specific growth requirements. Indeed, culture sensitivity values as low as 55–73% have been reported. As a result culture and antimicrobial susceptibility testing is not routinely performed in the majority of hospitals.

Molecular testing for *H. pylori* offers an attractive and faster alternative to culture and has enabled the identification of *H. pylori* DNA directly from gastric biopsy samples and isolated culture material. Molecular methods targeting genes related to antibacterial resistance are suitable to both detect the presence of the infection and provide information regarding antimicrobial resistance. Recent studies on the diagnosis of *H. pylori* infection in gastric biopsies have concluded that molecular detection of *H. pylori* is more precise than routine culture, histology or the rapid urease test alone and enables the detection of low bacterial loads, which is important given that patients on PPIs have reduced *H. pylori* cell numbers. Moreover these tools are accurate in detecting the coexistence of *H. pylori* strains susceptible and resistant to the same antibiotic within the same patient sample.

European guidelines have suggested that if standard susceptibility testing is not possible, molecular tests may be used to detect *H. pylori* and clarithromycin resistance directly on gastric biopsies. Single point mutations within the *H. pylori* trl gene encoding the 23S ribosomal subunit result in clarithromycin resistance, with three major mutations described; A2146C, A2146G and A2147G (Genbank Accession number NC_009195). To date, these mutations have primarily been detected with polymerase chain reaction (PCR)-based molecular methods using bacterial culture samples or gastric biopsies. Several molecular testing kits are commercially available for the molecular detection of clarithromycin resistance, including the MutaREAL Helicobacter *Hp* assay kit (Immunodagnostik, Bensheim, Germany), the ClariRes real-time PCR assay (Ingentix, Vienna Austria), and the Seeplex ClaR-H. pylori ACE detection system (Seegene, Eschborn, Germany) and the GenoType HelicoDR assay (Hain Lifescience, Nehren, Germany). Accumulating evidence has demonstrated that the presence of mutations detected by molecular tests correlates well with culture-based susceptibility testing.

Non-invasive molecular tests for *H. pylori* and clarithromycin resistance
In terms of patient comfort, reduction of risks associated with endoscopy and economic factors, non-invasive tests are preferred over endoscopy. Not only would extending molecular-based methods to non-invasive *H. pylori* diagnosis greatly enhance our ability to more accurately assess the prevalence of antibiotic resistance, it would also provide clinicians with the option of tailoring therapy based on antimicrobial susceptibility data to an increased number of patients. *H. pylori* DNA has been detected in various clinical specimens, including blood, stool samples and oral cavity specimens such as inflamed dental pulp and dental plaque, providing an exciting opportunity for the diagnosis of *H. pylori* infection and antimicrobial susceptibility testing through non-invasive procedures. In terms of sensitivity and specificity, data using stool samples have shown the most promise for the molecular detection of clarithromycin resistance-mediating mutations to...
date. As with all molecular-based assays for H. pylori detection and antimicrobial susceptibility testing, factors such as cost, local expertise in molecular diagnosis, and the availability of specialised equipment should be considered. It must also be kept in mind that most of the molecular tests available do not detect resistance based on uncommon genetic mechanisms. Some rare mutations within the rrl gene that mediate clarithromycin resistance have been described. Additionally, DNA mutations that lie outside the rrl gene or other possible mechanisms of clarithromycin resistance (for example, efflux pumps) must be considered. As with all DNA-based assays, the amino acid sequence is not directly analysed. Therefore, it is possible that detection of silent DNA mutations that do not lead to phenotypic resistance could result in a false-positive result.

A number of factors should be further considered when performing molecular analysis using stool samples. Firstly, stool samples contain many compounds that can degrade DNA and inhibit downstream analysis. Therefore, reliable sample collection protocols should be established. Commercially available stool DNA extraction kits are available, such as the QIAamp Stool DNA mini kit (Qiagen, Hilden, Germany) and the PSP Spin Stool DNA plus Kit (Invitek, Hayward, CA, USA). Stools also harbour a rich diversity of other commensal bacteria, thus false-negative and false-positive results represent a potential problem and rigorous experimental controls should be put in place. Importantly, further studies are required to determine the correlation between molecular test results determined using stool samples with antimicrobial susceptibility test data obtained using gastric biopsy specimens from the same patient. Moreover, the impact of genetic resistance detected using stool samples on treatment outcome will provide key insight into the long-term applicability of these methods for the routine detection of antibiotic resistance by non-invasive methods in the clinic.

References