The 16S rRNA gene is one of the most extensively used genes for molecular bacterial identification or genotypic studies, and several methods for study using this gene are described, including metagenomics studies. Accordingly, Gantuya et al. discussed the advantages of 16S rRNA sequencing in the diagnosis of *Helicobacter pylori*, by performing 16S rRNA sequencing on gastric biopsies and calculated cutoffs for operational taxonomic units (OTUs) and relative abundance (RA) to define positive results using ROC curves. *H. pylori* was defined as positive based on histology/immunohistochemistry, culture, rapid urease test, or serology results, and as negative by negative results from all these tests. Compared with the conventional methods, in a group of 161 patients (122 positive), the sensitivity and specificity for 16S rRNA sequencing were 94.3% and 82.1% (OTUs) or 93.4% and 82.1% (RA), respectively. When combining the validated values, the concordance rate was higher (91.1%). In addition, 16S rRNA sequencing detected nine additional positive

**Keywords:** Antibiotic resistance, Epidemiology, Molecular diagnostic methods, Next Generation Sequencing.
cases (5.6%) compared with routine diagnostic methods\(^2\). Nevertheless, the clinical value of these additional cases must be interpreted with caution, as it might correspond to free DNA resulting from bacterial lysis and not viable bacteria.

The newly developed CRISPR-Cas12 technology enables the detection of pathogens without amplification steps, reducing the processing time. A lateral flow biosensor based on CRISPR-Cas12a to detect *H. pylori* in stool samples was developed. The limit of detection was five copies/μl, and the method was specific for *H. pylori*. Overall, it proved to be a rapid, simple, and inexpensive method for the detection and screening of *H. pylori*, although the comparison with gold standard tests is still lacking\(^3\).

For the detection of *H. pylori* and macrolide resistance associated mutations, an in-house real-time PCR was adapted into ready-to-use PCR microwell strips, allowing time saving, better control of reagents and facilitating the accreditation of the assay\(^4\).

For a noninvasive *H. pylori* diagnosis and antimicrobial susceptibility testing (AST), Rolon et al\(^5\) developed a real-time PCR assay, based on TaqMan probes, targeting the 23S rRNA gene, for simultaneous detection of the bacterium and clarithromycin resistance determinants, directly from stools. The extraction method was optimised to enhance inhibitor removal and maximise DNA extraction from stools. Using an *H. pylori* stool antigen test (SAT) as reference, the assay was validated in 524 samples (sensitivity 89%, specificity 97%) and Sanger sequencing to confirm genotypic susceptibility led to 100% agreement. Due to its noninvasive nature, the assay can be used when endoscopy is not required for guiding treatment choices at the time of diagnosis by providing baseline clarithromycin resistance status\(^5\).

Two new multiplex real-time PCR assays were developed for simultaneous detection of *H. pylori*, as well as clarithromycin and levofloxacin resistance, offering an additional advantage particularly in regions with high rates of primary resistance. A multiplex quantitative PCR (qPCR) was developed by Binmaeil et al\(^6\), with primers and TaqMan probes being designed for *ureA* gene for *H. pylori*, and 23S rRNA and *gyrA* genes for clarithromycin and levofloxacin resistance, respectively, and validated in gastric biopsy samples. An optimal cut-off value was set up (≥0.5 copies number/100 human cells at the Cq≤30 value). In addition, the positive detection rate for qPCR assay was significantly higher than conventional methods (23.8% vs 14.7%, P<0.0001), being able to detect *H. pylori* at low concentration in gastric biopsies. For both 23S rRNA and *gyrA* mutations, the sensitivity and specificity of qPCR were 100% and ≥99%, respectively, when compared with E-test. In addition, the multiplex qPCR was able to detect mixed genotypes with a low proportion of the mutant DNA (1%; 99/1 ratio). The assay includes the more recently described GyrA N87I mutation conferring resistance to fluoroquinolones, not included in many commercial genotyping tests, but lacks the D91G substitution, which may result in false negative results for levofloxacin resistance, depending on the distribution of the mutations in different populations\(^6,7\).

The second assay was described by Zhao et al\(^8\) and is based on TaqMan-MGB probe multiplex real-time PCR. Using a two-tube approach, the PCR detects *H. pylori* (16S rRNA), the two N87 and the three D91 mutations (*gyrA*) conferring levofloxacin resistance, and the three clarithromycin resistance mutations (23S rRNA), including also an internal control targeting human DNA. The assay was compared with standard phenotypical methods and Sanger sequencing, in a group of 697 gastric mucosal samples, showing 100% sensitivity for *H. pylori*, levofloxacin, clarithromycin and mixed resistance to both antibiotics. The specificity was 100% for both levofloxacin and mixed resistance, while it was 97.9% for *H. pylori* and 95.6% for clarithromycin resistance\(^8\).

Finally, a simple and fast method for detection of *cagA*-positive *H. pylori* was developed and evaluated, based on a duplex recombinase aided amplification (RAA) combined with lateral flow dipstick (LFD) (Duplex RAA-LFD). The RAA is a novel isothermal nucleic acid amplification method, not requiring automated thermocyclers. The detection step takes only 25 min with visual detection limits of 1.2x10^2 CFU/mL and 10 pg. The diagnostic performance of the assay was tested on 56 clinical isolates, showing 96% sensibility and 100% specificity. To further assess its usefulness and point-of-care application, the assay will be evaluated in stools, gastric juice, formalin-fixed and paraffin-embedded (FFPE) tissue and saliva\(^9\).
NOVEL NON-MOLECULAR METHODS

A technique using quantum dots-based immunofluorescence histochemistry (QDs-IHC) to detect *H. pylori* in FFPE gastric biopsies was compared to conventional immunohistochemistry (IHC). QDs are a new type of fluorescent label, widely used in many biological and biomedical imaging applications. A retrospective study using 203 FFPE from patients with known *H. pylori* status (112 *H. pylori* positive and 91 negative) was conducted to compare the sensitivity and specificity of QDs-IHC and conventional IHC. Using the same mouse anti-HP monoclonal antibody, QDs-IHC had higher sensitivity (99.11% and 97.32%, respectively), its performance being superior to conventional IHC in detecting *H. pylori* in FFPE tissues, especially in those with low bacterial content, while both techniques had a specificity of 100%.

Rapid urease tests (RUTs) are still used in the management of *H. pylori* infection in daily practice. A colorimetric RUT using a novel and natural pH indicator, the anthocyanins from red cabbage, was developed. This natural compound has several advantages compared to synthetic organic indicators, including very low cost, ease of preparation, biocompatibility, wide availability, high stability and production of a wide colour spectrum. This assay could efficiently detect *H. pylori* in liquid, on filter paper and in agar, being able to detect bacterial loads in the range of 1-10 CFU/mL. Besides a colourimetric detection by the naked eye, it is possible to quantitatively monitor colour changes using a smartphone application. Optimisation of the assay to be used directly on gastric biopsies is the next step to increase the applicability of this test.

Faecal samples were used to evaluate the usefulness of faecal immunochemical tests (FIT) to detect *H. pylori* stool antigen. The test showed high accuracy and sensitivity for diagnosis of *H. pylori* and it was rated as the most convenient among several non-invasive *H. pylori* tests, based on accuracy and patient preferences.

*H. pylori* infection causes oxidative stress, chronic inflammation and DNA damage. Activated neutrophils are the main source of reactive oxygen species (ROS) production in the *H. pylori*-infected stomach, and *H. pylori* itself also produces ROS. Therefore, ROS detection may be an indirect strategy to evaluate *H. pylori* infection and gastric cancer. Accordingly, Jain et al. performed a comparative study of various methods available for the measurement of ROS released during *H. pylori* infection, by evaluating their pros and cons. More recently, several biosensor devices, based on microfluids and cell-based and affinity-based biosensors, are being developed for detection of ROS, presenting higher sensitivity and ease of use.

Advances in endoscopic imaging technologies improve mucosal visualisation and enhance fine structural and microvascular details. Three studies addressed the use of high-quality endoscopic imaging for a more accurate diagnosis of *H. pylori* gastritis and pre-cancerous conditions, using narrow-band imaging endoscopy with or without magnification. In particular, Glover et al. also evaluated the value of regular arrangement of collecting venules within the distal body as a marker for *H. pylori* naive status and developed an algorithm for classification of *H. pylori* status based on endoscopic predictive features.

Serological tests can be useful in particular settings with limited access to diagnostic tools and where specific genetic background is a reality. Two studies developed serological assays using local *H. pylori* strains for preparing antigens instead of foreign strains. In one study, an in-house ELISA (HpAfr-ELISA) was developed using whole-cell antigens prepared with strains belonging to the main African genetic *H. pylori* populations (*hpAfrica1* and *hpNEAfrica*). The diagnostic accuracy of the HpAfr-ELISA was evaluated in an unmatched case-control study, using a set of samples which had been tested with invasive conventional tests, collected from dyspeptic patients from two African countries. An optimal cut-off-value was defined at 20.2 U/mL, for which the assay achieved a sensitivity of 97.6% and specificity of 90.5%. The overall results indicate that the HpAfr-ELISA assay is a useful tool for accurately assessing the *H. pylori* infection in an African setting.

In the other study, a latex aggregation turbidity assay (latex) and a conventional ELISA (E-plate) were developed, both containing Japanese *H. pylori* protein lysates as antigens. The assays were then tested using sera from seven Asian countries. The *H. pylori* in-
Infection status and atrophy status were determined by culture and histology. Overall, the assays displayed different levels of accuracy and parameters, showing better adaptation to some of the studied populations than to others, which might be associated with different genetic backgrounds of \textit{H. pylori} circulating strains or even to diverse composition of the gastric microbiota in these different populations. With the appropriate adjustments to increase the accuracy in particular populations, authors suggest that the quick and high-throughput characteristics of the automated latex system might benefit regional central hospitals and allow for mass screening of local populations\textsuperscript{19}.

\textbf{DIAGNOSIS ACROSS THE GLOBE: WHO IS REPORTING?}

Lin et al\textsuperscript{20} presented the global incidence rate of gastric cancer worldwide and modelled the prediction of the incidence rates for the next decade. The incidence rate between 1988 and 2012 diminished in all world regions, with a world age standardized incidence rate decrease of 56.4\%. This study points out that towards 2030 the incidence rates of gastric cancer will tend to decrease in most countries, yet exceptions will occur. Thus, a rise in incidence is expected in India (from 10 to 20 per 100,000), Ecuador (from 20 to >80 per 100,000), and Lithuania (from 20 to 55 per 100,000). Of notice, some European countries face a small increase or stable trend: Italy, Lithuania, Malta, the Netherlands, Norway, Poland, Slovakia, Slovenia, Spain, Switzerland, and the United Kingdom. The reduction in the prevalence rate of \textit{H. pylori} explains the globally decreasing tendency in the gastric cancer incidence rate.

A consensus for \textit{H. pylori} infection control in China proposed a family-based \textit{H. pylori} infection control and management, to follow the transmission between family members\textsuperscript{21}.

Several studies evaluated the \textit{H. pylori} prevalence, estimated at 46.8\% in Iranian dyspeptic patients (evaluated by RUT)\textsuperscript{22}, and at 63.0\% among therapy-naive patients in Peru (determined by histopathology)\textsuperscript{23}. In Benin, a cohort of 150 patients subjected to a carbon-14-urea breath test (C14-UBT) allowed the detection of \textit{H. pylori} infection in 34.7\%\textsuperscript{24}. In Vietnam the prevalence of \textit{H. pylori} was as high as 83.5\% (determined by RUT and/or C14-UBT in a sample of 1,272 patients from 482 households)\textsuperscript{25}. For Turkey, using culture and PCR, the prevalence of \textit{H. pylori} in 422 adult dyspeptic patients was 75.6\%\textsuperscript{26}.

In Tanzania, using immunohistochemistry, 170 tissue blocks of gastric biopsies from patients with different gastric pathologies were retrospectively analysed, revealing 37.1\% prevalence of \textit{H. pylori}\textsuperscript{27}. In Italy, another retrospective study addressed the prevalence of \textit{H. pylori} in 1,512 histological samples, showing a global prevalence of 16.8\%. However, for the patients born in South America and Africa the prevalence was significantly higher, confirming the unbalanced rate of \textit{H. pylori} worldwide\textsuperscript{28}. Nath et al\textsuperscript{29} showed that the prevalence of \textit{H. pylori} is decreasing in Bangladesh and, based on SAT performed on 360 dyspeptic patients, the prevalence was 37.2\%. Thus, collecting results reported across the globe, despite using distinct methodologies, allows one to gain a perspective of the current prevalence of \textit{H. pylori} infection.

Other studies addressed the proportion of antibiotic resistance among \textit{H. pylori} isolated strains. A retrospective observational study on clarithromycin resistance involving 4,744 \textit{H. pylori} infected Hungarian patients showed a 5.5\% primary clarithromycin resistance and 30.5\% for secondary resistance. This study inferred \textit{H. pylori} infection and clarithromycin resistance using immunohistochemistry and fluorescence \textit{in situ} hybridization on fixed gastric tissue samples. A mathematical model including the infection status, distinction between clarithromycin homo or heteroresistance, and medication history (use of clarithromycin for eradication or other purposes and macrolide-naive) predicted that primary clarithromycin resistance was 98.7\% due to transmission of resistant bacteria, while the remaining 1.3\% corresponded to spontaneous mutations. Moreover, there was an age-dependent preponderance of females with secondary clarithromycin resistance\textsuperscript{30}. Further studies should confirm the percentages presented, since the detection of clarithromycin resistance may have been underestimated, considering that no antibiogram was done and that the emergence
of resistance after mutation without the selective pressure of the antibiotic may be difficult to detect even if present. In Turkey, the resistance rates determined by antibiogram for 133 isolates revealed a resistance rate to metronidazole, levofloxacin, clarithromycin, and rifampicin of 62%, 36%, 19%, and 12%, respectively. In another study from Vietnam involving 308 patients with *H. pylori* infection, the amoxicillin MIC was performed by E-test on a subset of 101 isolates, of which 25.7% were resistant. Here, seven amino acid changes in the PBP1A protein were associated with resistance, including the substitutions F366L, S414R, V469M, F473V, D479E, and the insertions E/N and S/A/G595–596. In the study of Bilgili et al., using gastric biopsies from Peru, clarithromycin and quinolone resistance determined by real-time PCR were 34.0% (33/97) and 68.3% (56/82), respectively. The *H. pylori* resistome was accessed in a set of 1,040 worldwide sequences. Resistome analysis revealed the occurrence of common point mutations which were previously found to correlate with phenotypic antibiotic resistance, evidencing the potential of using next generation sequencing (NGS) data to determine the resistance profile.

**COMPARISON OF DETECTION METHODS**

An important aspect for clinical microbiology methods is the existence of external quality control assessment. An external quality control performed in China involved 130 participating laboratories, 93 of which passed the quality threshold (75.6%).

The success of *H. pylori* isolation relies on the quality of the starting material itself, the bacterial load in the sample, transport conditions, and time taken to start the processing. Accordingly, Brenan et al. showed that detection of *H. pylori* increases when biopsies from the antrum and corpus are used in combination for *H. pylori* culture, as opposed to processing antrum biopsies only. Also, an increasing bacterial load improved the sensitivity of culture and histologic methods, revealing that a reduced bacterial load may lead to a false negative diagnosis.

Choosing the best method for *H. pylori* detection may be difficult given the multiple alternatives available. Thus, the comparison of method performance appears to be good source of information, which helps ascertaining and continuous efforts to find the best model to fit each laboratory. Several studies conducted comparison studies, focusing from the biopsy collection itself, to the biopsy processing in the laboratory. Concerning *H. pylori* detection methods during upper gastrointestinal endoscopy, the exclusive use of the RUT with biopsies has not been recommended as a stand-alone method, but rather in combination with histological examination. Likewise, serology does not constitute a reliable diagnostic method for current *H. pylori* infection. The usefulness of using EndoFaster®, a device that analyses the gastric juice determining the ammonium concentration (reaction product of urease activity) during endoscopic procedure, was assessed, pointing to a sensitivity of detection between 73% and 87%, and a specificity between 84% and 86%.

The use of molecular diagnosis tests for *H. pylori* is especially gaining adepts for detecting antimicrobial resistance, which is crucial for treatment success and avoidance of the rise of antibiotic resistance bacteria. Regarding cost-effectiveness, PCR based methods present high sensitivity and are cost-effective. In another study, the cost-effectiveness of *H. pylori* management useful for determining the value of intervention was assessed for the UK, revealing that the most cost-effective methods were UBT and faecal antigen test.

**EVALUATION OF DIAGNOSTIC KITS**

The new generation of ELISA test GastroPanel® (biomarker panel of pepsinogen I, pepsinogen II, gastrin-17, Hp IgG antibody) was evaluated on 522 patients referred for gastroscopy at a university hospital in Finland, presenting a sensitivity of 95.0% and a specificity of 97.5%, using biopsy-confirmed *H. pylori* as the reference.
For *H. pylori* detection by SAT, polyclonal antibody-based *H. pylori* Stool Antigen Lateral Flow Immunochromatography assay (HpSA-LFIA - Rightsign®) was compared to the monoclonal antibody-based ELISA kit (FORESIGHT®)\textsuperscript{46}, which was used as reference since a meta-analysis suggested that monoclonal antibody-based SAT performed better\textsuperscript{47}. The HpSA-LFIA showed good sensitivity but poor specificity, thus an additional confirmatory test was recommended in the case of positive test. The discrepancy of performance of HpSA-LFIA across different studies was discussed\textsuperscript{46} and could appear as a consequence of the well-known geographic genetic variability of *H. pylori*\textsuperscript{48}.

Concerning molecular diagnosis of *H. pylori* in gastric biopsies, the RIDA\textsuperscript{®}GENE Helicobacter pylori assay, which is a quantitative PCR assay for detecting both *H. pylori* and 23S rRNA gene mutations (associated with clarithromycin resistance) in gastric biopsies, was tested on 436 patients referred for gastroscopy. In comparison to culture, the sensitivity and specificity were 100% and 99%, respectively\textsuperscript{49}.

We are beginning to see NGS methods for *H. pylori* diagnosis, allowing the detection of *H. pylori*, characterisation of virulence factors and antibiotic resistance that can ultimately lead to higher levels of personalised treatment and management of *H. pylori*-associated infections\textsuperscript{50,51}. In this regard, Hulten et al\textsuperscript{52} compared targeted NGS (PyloriAR NGS) for the genes 23S rRNA (clarithromycin), gyrA (fluoroquinolones), 16S rRNA (tetracycline), \textit{pbp}1 (amoxicillin), \textit{rpo}B (rifabutin) and \textit{rdx}A (metronidazole) with culture-based *H. pylori* AST, using agar dilution, in paired clinical isolates. NGS could determine resistance for all tested antibiotics reliably, except for metronidazole and amoxicillin whose resistance mechanisms are more complex and not yet totally understood.

**CONCLUSIONS**

There was a considerable number of reports concerning the prevalence of *H. pylori* and the antibiotic resistance rates found in different countries, evidencing a global tendency of a decline in the former and an increase in the latter. We are reaching a paradigm change where it is expected to find a smaller number of *H. pylori* positive patients that are harder to treat. This points to the major importance of detecting antibiotic resistance before treatment prescription.

Concerning diagnostic methods based on antigen or anti-*H. pylori* antibody detection, the importance of using reference regional antigens appears to be of paramount importance due to the genetic variability of *H. pylori*. Moving towards molecular diagnosis, more studies are using and recommending the approach, which is still very much based on several types of PCR. A few studies have started to rely on NGS applications for diagnosis and antibiotic resistance determination, and it is expected that many more studies will appear in the future.

**Conflict of Interest**

The authors declare no conflict of interest.

**Acknowledgment**

None.

**Author’s Contribution**

F.F.V. and M.O. conceptualized the study, and wrote, reviewed and edited the manuscript.

**Funding**

F.F.V. is funded by Fundação para a Ciência e a Tecnologia through an Assistant Researcher grant CEEC-IND/03023/2017, and project grants (PTDC/BTM-SAL/28978/2017 and PTDC/BTM-TEC/3238/2020).

**ORCID ID**

Filipa Vale: 0000-0003-4635-0105, Mónica Oleastro: 0000-0001-6360-2576.
REFERENCES


