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Chronic gastritis as an indication for reflex molecular testing in histologically negative gastric biopsies: detection of *Helicobacter pylori* infection and assessment of clarithromycin susceptibility in formalin-fixed, paraffin-embedded tissues

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Background: Histopathological examination of formalin-fixed, paraffin-embedded (FFPE) gastric biopsies is a gold-standard method for diagnosing *Helicobacter pylori* (*H. pylori*) infection. However, infections may remain undetected histologically due to focal bacterial distribution at low bacterial densities. Furthermore, antimicrobial susceptibility testing is not routinely integrated into pathology workflows, despite recommendations in guidelines for susceptibility-guided eradication therapy. We aimed to define histopathological indications for reflex molecular testing in *H. pylori*-negative gastric biopsies and to evaluate an FFPE-optimized real-time quantitative PCR (qPCR) assay for simultaneous detection of *H. pylori* and assessment of clarithromycin susceptibility.

Materials and Methods: A novel short-amplicon primer pair (HPJ; 122 bp) was designed for dual hybridization probe-based qPCR and compared with a conventional assay (HPY; 267 bp) and fluorescence *in situ* hybridization (FISH). We analyzed 108 histologically *H. pylori*-positive and 101 negative FFPE gastric biopsies from patients with chronic gastritis, along with 100 consecutive biopsies without current or previous chronic gastritis.

Results: HPJ-qPCR detected *H. pylori* in 102/108 histologically positive cases and 25/101 histologically negative cases with current or previous chronic gastritis, significantly outperforming HPY-qPCR (89/108 and 12/101, respectively; $p < 0.0001$). In contrast, only 3/100 histologically negative cases without chronic

gastritis were PCR-positive. HPJ-qPCR showed concordance with FISH for clarithromycin susceptibility assessment and outperformed HPY-qPCR in FFPE tissues. Histologically negative but HPJ-qPCR-positive cases showed a trend to display rates of chronic gastritis and activity intermediate between double-negative and double-positive subgroups ($p < 0.001$).

Conclusion: Histological evidence of chronic gastritis is a key indicator for reflex qPCR testing in *H. pylori*-negative gastric biopsies. Beyond detecting histologically undiagnosed infection, HPJ-qPCR simultaneously determines clarithromycin susceptibility, providing clinically relevant information for targeted eradication therapy.

KEYWORDS

clarithromycin resistance, FFPE tissue, FRET-qPCR, *Helicobacter pylori*, molecular diagnostics

Introduction

Helicobacter pylori (*H. pylori*) bacterium is a human pathogen, chronically infecting more than 40% of the global population [1]. While the infection is often asymptomatic, it can cause chronic atrophic gastritis, peptic ulcer disease, gastric adenocarcinoma, and B-cell mucosa-associated lymphoid tissue (MALT) lymphoma [2]. The infection is lifelong without treatment, and the eradication therapy became more challenging due to the increasing resistance to antibiotics, particularly to clarithromycin (Cla), the most effective antibiotic against this bacterium [3–5]. Clarithromycin resistance (Cla-res) is caused by specific point mutations in the 23S rRNA of *H. pylori* [4]. The emergence of Cla-res has significantly reduced the success rate of conventional triple therapy (clarithromycin combined with levofloxacin, amoxicillin or metronidazole and proton pump inhibitor), which, in the absence of new antibiotics and widely available susceptibility testing, has led to a general decline in the first-line use of clarithromycin [6].

Diagnostic methods for *H. pylori* include both invasive (biopsy-based) and non-invasive (urea breath test, serology tests, stool tests) approaches [7–9]. Histopathological examination of tissue slides from formalin-fixed, paraffin-embedded (FFPE) biopsies with stains such as hematoxylin and eosin (H&E) and Giemsa is still the standard method, but immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) are superior for detecting *H. pylori* [10]. However, the FISH technique is primarily used to detect the Cla-susceptibility of the pathogen rather than the bacterium itself [8, 11].

Several variants of PCR-based diagnostics have also been developed to detect *H. pylori* and its Cla-susceptibility status from gastric biopsies [8, 12–17]. It can be performed on gastric tissue either from fresh, unfixed biopsy samples taken specifically for the microbiological diagnosis of *H. pylori*, or from FFPE specimens used in routine histopathological diagnostics. However, FFPE materials have several disadvantages in terms of the preservation of nucleic acids, including single- and double-stranded breaks, which prevents amplification of longer DNA fragments with PCR-based techniques. Therefore, primer pairs amplifying the shortest possible target sequences are recommended for FFPE tissues [18–20]. However, number of PCR tests capable of simultaneously detecting *H. pylori* and determining its Cla-susceptibility status from FFPE samples that meet these requirements is very limited, and to our knowledge, none of the currently available methods use dual hybridization probe FRET (Förster resonance energy transfer)-based real-time quantitative PCR (qPCR) with a short amplicon (see Supplementary Table S1). Accordingly, our first goal was to develop a new, short amplicon-producing primer pair based on the dual hybridization probe qPCR test of the French National Reference Center for Campylobacters & Helicobacters [17].

The significance of diagnosing not only *H. pylori* infection, but its antibiotic susceptibility from FFPE tissues that susceptibility testing is required by the Maastricht VI/Florence Consensus Report prior to the use of clarithromycin-based regimens in populations with high resistance rates [2, 5]. However, this requirement is largely unknown in pathology practice, as histopathology guidelines do not address it. Therefore, as our second objective, we aim to highlight the importance of PCR-based *H. pylori* diagnostics in identifying cases of *H. pylori* infection negative by routine histopathological diagnostic methods, as well as to promote targeted antimicrobial treatment for *H. pylori*-infected patients.

Materials and methods

Patient selection, clinical data, and ethics

Gastric biopsy or resection cases, with presence of histological abnormalities (e.g., chronic mucosal inflammation, intestinal metaplasia, atrophy) suggestive of *H. pylori* infection–either in the specific case or in the patient's medical history–were retrospectively selected from the institutional electronic archive of the Department of Pathology, Forensic and Insurance Medicine, Semmelweis University, Budapest, Hungary. A total of 115 histologically *H. pylori*-positive and 115 histologically *H. pylori*-negative cases were randomly selected from the period 2011–2023 but taking into account a balanced representation of the clarithromycin susceptibility/resistance status among positive cases. The primary inclusion criteria included the availability of FFPE tissue blocks and complete clinical, histopathological and *H. pylori* Cla-susceptibility FISH documentation. Cases with insufficient tissue (gastric mucosa) in the block were excluded. After this pre-selection, 209 patients was finally included: 108 *H. pylori*-positive by initial stainings (Giemsa/IHC/FISH), and 101 negative (Figure 1). These patients living in Hungary, aged between 3 and 91 years old (average 57 years old), and with a sex ratio of 0.71 (87M/122F). As a control group, qPCR testing

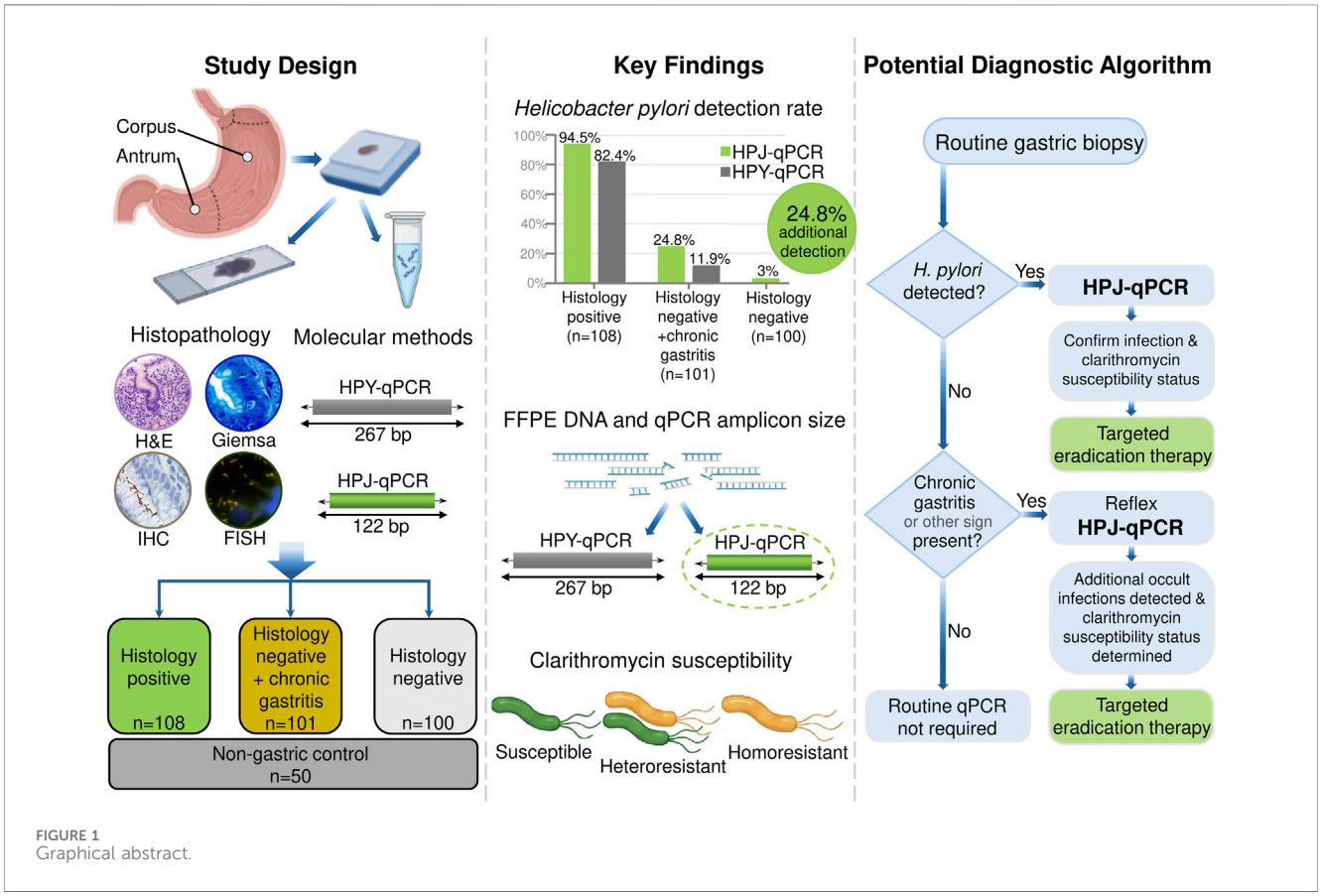

FIGURE 1
Graphical abstract.

was also performed on 100 consecutive FFPE gastric biopsy samples from 2025 that had tested negative for *H. pylori* by immunohistochemistry, and no tissue abnormalities suggestive of *H. pylori* infection were observed. As a negative tissue control group, 50 non-gastric FFPE tissue blocks (including 13-13 liver adenocarcinoma metastasis and prostate tissue samples as well as 12-12 endometrial and bladder tissue samples) were also examined by qPCR. The sections of all tissue blocks from the cases included in the study, as well as the special stains, immunohistochemical slides, and FISH images of them, were reviewed centrally by two expert pathologists (É.K., G.L.). The detailed patient data are available in the Supplementary Table S2.

From the French National Reference Center for Campylobacters and Helicobacters (NRCCH, Hospital University Center, Bordeaux, France), 99 consecutive cases with request for *H. pylori* PCR testing on non-fixed tissue samples were enrolled in the study. These gastric biopsies were obtained in 2023 from patients living in France, aged between 1 and 82 years old (average 44 years old), and with a sex ratio of 0.65 (39M/60F). Detailed patient information is shown in the Supplementary Table S3.

The study protocol followed the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethical Committee of Semmelweis University, Budapest (#277/2021). Under current Hungarian law, retrospective studies do not generally require the informed consent of patients. Accordingly, the Committee waived the informed consent procedure for the study. The Ethics Committee of the Bordeaux University Hospital deemed it unnecessary to issue ethical approval and obtain informed consent from patients whose gastric samples were submitted to the National Reference Centre for Campylobacters & Helicobacters, Bordeaux, France (NRCCH), as these are used in accordance with the scientific missions assigned to the NRCCH by Santé Publique France (https://www.santepubliquefrance.fr) by recognizing the NRCCH as a national reference centre in the Official Journal of the French Republic.

Routine histological work-up and assessment of formalin-fixed, paraffin-embedded tissue samples

Gastric biopsy specimens were obtained during upper gastrointestinal tract endoscopy procedures at referring clinical centers. Mapping biopsies were taken from both the antral and corpus compartments of the stomach, and samples were fixed in 10% buffered formalin for 12–36 h prior to paraffin embedding. Sections of 2–4 μm thickness were prepared and stained with H&E and Alcian Blue–Periodic Acid-Schiff for routine histological evaluation. Gastric mucosal alterations were assessed according to the updated Sydney system, including grading of inflammation, atrophy, intestinal metaplasia, and activity.

Detection of *H. pylori* positivity from the formalin-fixed, paraffin-embedded tissue samples

The presence of *H. pylori* was determined using modified Giemsa staining and *Helicobacter*-specific immunohistochemistry.

IHC was performed on Ventana BenchMark automated staining system (Ventana Medical Systems Inc., Tucson, AZ, United States) with B0471 polyclonal rabbit anti-*H. pylori* primary antibody (Dako, Glostrup, Denmark), using a dilution of 1:350 for 32 min at 42 °C, followed by detection using the UltraView DAB Detection Kit, and hematoxylin counterstaining. Bacterial density was assessed semi-quantitatively according to the updated Sydney system [Mild (1+): low density, scattered distribution; Moderate (2+): medium density, small-medium-sized groups of bacteria; Severe (3+): high density, large groups or fields of bacteria].

Fluorescence *in situ* hybridization (FISH) for detection of clarithromycin susceptibility

The clarithromycin susceptibility/resistance of *H. pylori* infections was routinely determined according to previously published protocols [10, 11, 21, 22]. Briefly, 4 μm thick FFPE tissue sections were deparaffinized and treated by microwave at 400 W for 20 min in Vector Antigen Unmasking Solution (Cat. No. H-3300, Vector Laboratories, Burlingame, CA, United States). The tissue sections were hybridized for 90 min at 46 °C with the DNA probe mix containing species-specific FITC-labeled (green) Hpy-1 probe targeting the 16S-rRNA of *H. pylori*, as well as three resistance-specific probes targeting clarithromycin resistance-specific point mutations of 23S rRNA (ClaR1 [A2142-3G], ClaR2 [A2143-4G], and ClaR3 [A2142-3C], each labeled with orange-red fluorochrome Cy3). The slides were washed 2 × 15 min at 46 °C in Wash Buffer and mounted using Vectashield with DAPI counterstaining (Cat. No. H-1200, Vector Laboratories). Fluorescent signals were acquired using a Leica DMRXA epifluorescence microscope (Leica Microsystems, Wetzlar, Germany) equipped with DAPI, Spectrum Green, and Spectrum Orange filters (Vysis, Downers Grove, United States), Leica DFC365 FX camera, and Leica CW4000 FISH analysis software. Results were interpreted based on fluorescence patterns: susceptible bacteria showed only green signal (Hpy-1), whereas resistant bacteria appeared yellow in merged images due to overlapping green (Hpy-1) and red (ClaR1–3) signals. Heteroresistance was defined by the coexistence of green-only and yellow bacteria within the same sample or at different biopsy sites. Bacterial density was graded semi-quantitatively according to the updated Sydney system as mild (1+), moderate (2+), or high (3+) (Figure 2).

DNA extraction from gastric biopsy samples

FFPE tissue samples: The bacterial and human genomic DNA was extracted from of each FFPE tissue specimen using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) with additional pretreatment to tissue protocol. Three 5 μm sections were vortexed with 1 mL of xylene for 10 s, and after centrifugation (2 min at 13000 RPM) the supernatants were removed. Tissue pellets were vortexed with 1 mL of 96% ethanol; after centrifugation (2 min at 13000 RPM), the supernatants were discarded, and the pellets were air-dried to evaporate the remaining ethanol. Lysis was carried out at 56 °C for 1 h in the presence of 180 μL Buffer ATL and 20 μL Proteinase K, followed by incubation at 90 °C for 1 h. After this step, the original tissue protocol was applied. In three *H. pylori*-positive cases, DNA was isolated from a histologically *H. pylori*-negative block to assess whether qPCR could detect bacteria present in other gastric areas (Supplementary Table S2).

Non-fixed tissue samples: Gastric biopsy specimens were transported from the referring clinical centers in Portagerm pylori transport medium (bioMérieux SA, Marcy-l'Etoile, France) to the NRCCH. Upon receipt, the biopsies were ground in 500 μL nutrient broth with a tissue homogenizer (UltraTurax; LaboModerne, Paris, France), according to the routine diagnostic protocol. A portion of the suspension was treated with 20 μL proteinase K (Roche Diagnostica, Meylan, France) in 180 μL ATL Qiagen buffer at 56 °C for 3 h, from which 200 μL was further processed for DNA extraction using the MagNA Pure 96 DNA and Viral NA SV kit on the MagNA Pure 96 extractor (Roche Diagnostics GmbH, Mannheim, Germany), following the supplier's recommendations.

Real-time qPCR

A new primer pair (HPJ) was designed using Array Designer (Premier Biosoft International, San Francisco, United States) (HPJ-S: TGTCTCAACCAGAGATTCAG, HPJ-A: CGCATGATATTC CCATTAGC) targeting a 122 bp sequence of the *Helicobacter pylori* 23S rRNA gene (GenBank: U27270) within a 267 bp amplicon defined by HPY primer pair (HPYS: AGGTTAAGA GGATGCGTCAGTC, HPYA: CGCATGATATTCCCATTAGCA GT), including the positions of FRET probes from the previously published FRET-qPCR reaction (ANCHOR-FL: TGTAGTGGA GGTGAAAATTCCTCCTACCC with 3′FAM, HPYRED: GGCAAGACGGAAAGACC with 5′ATTO647N-3′P) [13, 17]. PCR reactions were performed using a LightCycler® 480 II/96 system (Roche Diagnostics Ltd., Rotkreuz, Switzerland) with software version 1.5.0 SP4. The reaction mix was prepared under clean-room conditions, adjusting the concentration of primers and probes as described previously (0.4 μM and 0.2 μM, respectively) using Takyon™ No ROX Probe 2X Master Mix dTTP (Kaneka Eurogentec S.A., 4102 Seraing, Belgium) [17]. The reactions were carried out in plastic microwell strips with a final volume of 10 μL per well (9 μL mix plus 1 μL DNA sample). Extraction control PCR targeting the β-globin housekeeping gene was also performed on all DNA samples using bGlob primer pair (bGlob_fwd: TACGGCTGT CATCACTTAGAC, bGlob_rev: CTTCATCCACGT-TCACCT) and FRET probe-based detection (bGlob_donorP: CAAACAGAC ACCATGGTGCACCTGACTCCTGAGGA with 3′FAM, bGlob_ accepP: AAGTCTGCCGTTACTGCCCTGTGGGGCAA with 5′ATTO647N-3′P), with the same Takyon™ Master Mix and reaction conditions as described above.

The qPCR conditions for the HPJ primer pair (HPJ-qPCR) were optimized based on the previously established HPY-based qPCR protocol (HPY-qPCR). Reactions began with a 10-min denaturation at 95 °C, followed by 45 cycles of 10 s at 95 °C, 20 s annealing at 58 °C for HPY/bGlob or 57 °C for HPJ, and 15 s extension at 72 °C (with a temperature transition rate of 2.20 °C/s). A melting curve was then generated: 95 °C for 3 min (FFPE) or 30 s (non-fixed), cooling to 40 °C for 50 s, and a gradual rise to 85 °C at 0.29 °C/s with continuous fluorescence acquisition (2 acq./s).

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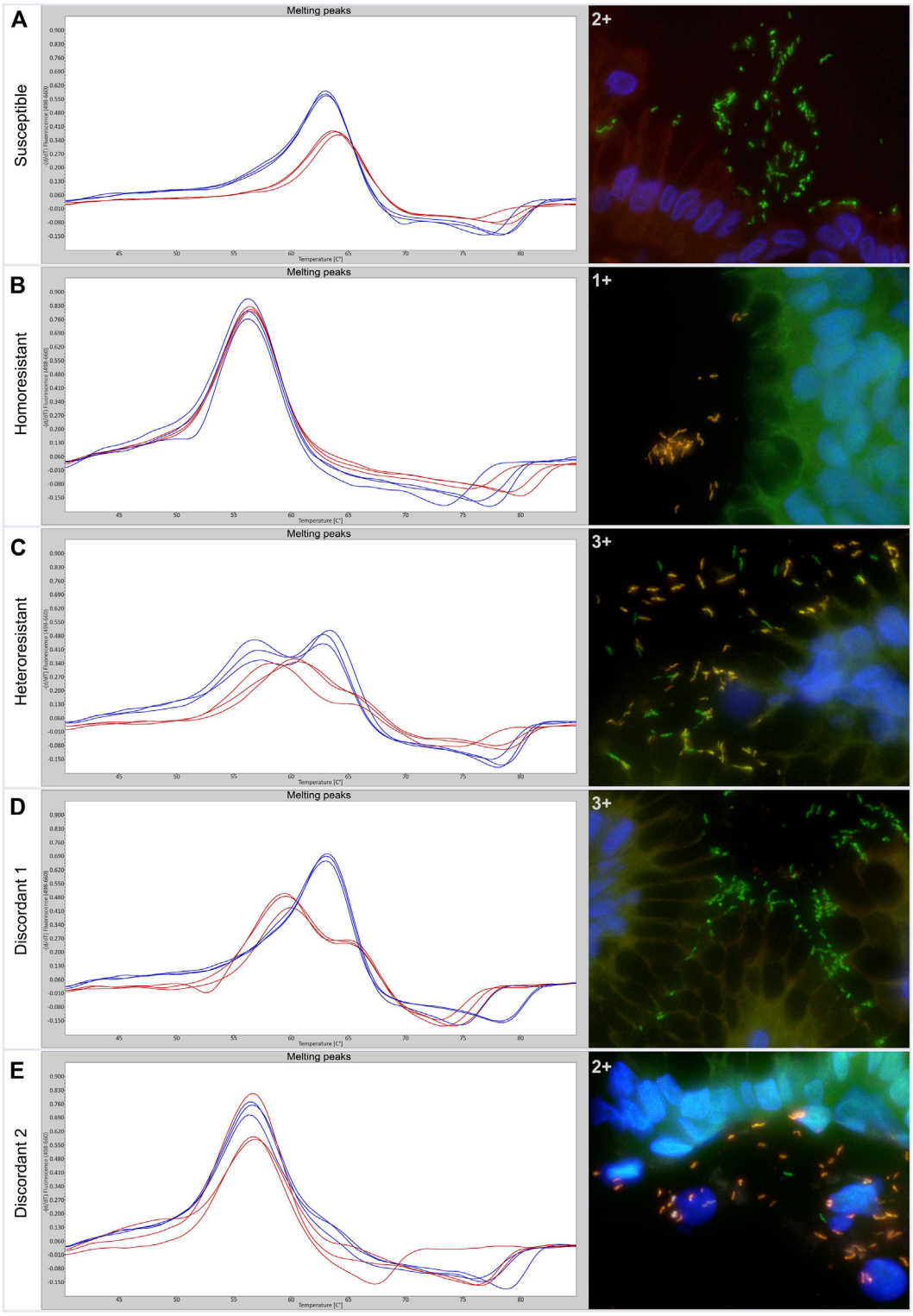

FIGURE 2
Detection of *Helicobacter pylori* and its clarithromycin susceptibility status by qPCR and FISH. Melting curves are displayed on the left (HPY-qPCR in red and HPJ-qPCR in blue) while FISH images presented on the right. The melting curve peaks of the clarithromycin-susceptible *H. pylori* population were detected at 64 °C–65 °C with HPY-qPCR and at 63 °C–64 °C with HPJ primer pair. In cases carrying the A2142-3G clarithromycin resistance mutation, melting curve peaks were detected at 56 °C–57 °C with both primer sets, while in the presence of the A2142C resistance mutation, they were detected at 59 °C–61 °C. Clarithromycin-susceptible case by all the three methods **(A)** Clarithromycin-homoresistant case by all the three methods **(B)** Clarithromycin-heteroresistant case by all the three methods **(C)** A discordant case found to be clarithromycin-susceptible by HPJ-qPCR and FISH but diagnosed as heteroresistant by HPY-qPCR **(D)** A discordant case in which clarithromycin-heteroresistant *H. pylori* population with less than five percent susceptible bacteria was found by FISH but diagnosed as homoresistant by both HPY-qPCR and HPJ-qPCR **(E)**. Original magnification of FISH images is 630x, bacterial density is indicated as 1+ (mild), 2+ (moderate), or 3+ (high) in the left-upper corner of the image.

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TABLE 1 *Helicobacter pylori* detection rates by staining methods (IHC/FISH) as well as by HPJ-qPCR and HPY-qPCR.

A)	*H. pylori* positive by staining	*H. pylori* negative by staining		Non-gastric tissue samples
		With histology suggestive of *H. pylori* infection	Without histology suggestive of *H. pylori* infection	
H. pylori positive by HPJ qPCR	102/108	25/101	3/100	0/50
H. pylori negative by HPJ qPCR	6/108	76/101	97/100	50/50
Positivity rate	94.5%	24.8%	3%	0%

B)		*Helicobacter pylori* detection by staining techniques (IHC/FISH)		*p*
		Positive	Negative	
HPJ-qPCR	Positive	102	25	0.0018
	Negative	6	76	
HPY-qPCR	Positive	89	12	0.2810
	Negative	19	89	
	Total	108	101	

Interpretation of technical replicates and melting-curve analysis

All qPCR reaction was performed in triplicate. The interpretation of the results was based on both the characteristics of the amplification curve and the analysis of the melting curve. A sample was considered *H. pylori*-positive if the characteristic amplification and the corresponding melting peak were observed in at least two of the three technical replicates. Samples that yielded a positive result only in one replicate were assessed on a case-by-case basis. Reactions exhibiting atypical amplification characteristics or melting peaks outside the predefined temperature range were considered non-specific and classified as negative. If even a single replicate produced a characteristic amplification curve and an expected melting peak, we carried out further technical replicates. A reproducible detection during the repeat tests was required for a definitive positive result. Samples that failed to be confirmed were classified as negative.

Melting-curve interpretation

The melting curves were interpreted blindly in accordance with the principles originally described by [13] Wild-type strains susceptible to clarithromycin exhibited melting peaks at approximately 63 °C–65 °C, whilst the A2142G/A2143G mutations resulted in peaks at approximately 56 °C–57 °C, and the A2142C mutations resulted in peaks at approximately 59 °C–61 °C. Samples that exhibited only the wild-type peak were classified as susceptible to clarithromycin. Samples exhibiting only mutation-associated peaks were classified as homoresistant to clarithromycin. The simultaneous presence of susceptible and resistant melting peaks was interpreted as heteroresistance.

Investigated parameters and statistical analysis

All analyses were performed in R (v4.0.2). McNemar's test was used to compare the results of FISH, HPY-qPCR and HPJ-qPCR within the same (sub)population, with Holm–Bonferroni correction applied to control for multiple dependent comparisons. This approach was used to evaluate diagnostic performance across clinical and histological categories and different bacterial loads. The performance of the three detection methods across susceptible, homoresistant, and heteroresistant subgroups was assessed with the Stuart–Maxwell test, moreover Cohen's kappa was calculated to stratify the agreement level between the different diagnostic methods. Differences in clinical symptoms, diagnoses, and histopathological findings among three different subgroups were analyzed using 3 × 2 Fisher's exact test, and Cochran-Armitage test for trend. P values were two-tailed, with significance defined as $p < 0.05$.

Results

In the 209 cases of the FFPE cohort suggestive of *H. pylori* infection, HPJ-qPCR detected *H. pylori* in 102/108 histologically positive and 25/101 histologically negative cases, outperforming HPY-qPCR (89/108 and 12/101, respectively; $p < 0.0001$) (Table 1). Of the 25 IHC/FISH-negative but HPJ-qPCR-positive cases, 11 had received PPI and 3 received antimicrobial therapy shortly before sampling. HPJ-qPCR detected *H. pylori* significantly more frequently than IHC/FISH stainings ($p = 0.0018$), HPY-qPCR showed no significantly better detection performance than the staining-based methods ($p = 0.2810$) (Supplementary Tables S3, S4).

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TABLE 2 Clarithromycin susceptibility results by the three different methods (A); McNemar p-values of clarithromycin susceptibility results by the three different methods (B).

A) FISH results of the 108 histologically *H. pylori* positive cases by clarithromycin -susceptibility/-resistance status →	Susceptible n = 42	Heteroresitant n = 33	Homoresistant n = 33	Susceptible n = 42	Heteroresitant n = 33	Homoresistant n = 33
qPCR results for the two different primer pairs	HPJ			HPY		
Clarithromycin-susceptible	34	4	0	13	1	0
Clarithromycin-heteroresistant	5	19	2	20	13	0
Clarithromycin-homoresistant	1	8	29	4	15	23
Negative	2	2	2	5	4	10

B) Detection methods		*p*
FISH ↔ HPJ-qPCR	Clarithromycin-susceptible/clarithromycin-resistant	0.3438
FISH ↔ HPY-qPCR		<0.0001
HPJ-qPCR ↔ HPY-qPCR		<0.0001
FISH ↔ HPJ-qPCR	Clarithromycin-homoresistant/clarithromycin-heteroresistant	0.0391
FISH ↔ HPY-qPCR		0.0004
HPJ-qPCR ↔ HPY-qPCR		0.0259

Clarithromycin susceptibility testing results are given in Table 2. Stuart-Maxwell test showed significant differences between the three methods in detecting clarithromycin-susceptible, homoresistant, and heteroresistant cases. Using the McNemar test, no significant difference was found between the FISH technique and HPJ-qPCR in identifying clarithromycin-susceptible and clarithromycin-resistant cases ($p = 0.3438$), while the agreement between the two methods was 81.34% (83/102), with a Cohen's κ of 0.850 (95% CI: 0.764–0.916). HPY-qPCR classified significantly more cases as Cla-resistant (FISH↔HPY-qPCR $p < 0.0001$, HPJ↔HPY $p < 0.0001$), as it detected Cla-heteroresistance in 17 (55%) of the 31 cases classified as clarithromycin susceptible by the other two methods. Accordingly, the greatest difference between the methods was observed in the classification of cases as homo- and heteroresistant, with the smallest discordance between FISH and HPJ-qPCR (FISH↔HPJ-qPCR $p = 0.0391$, FISH↔HPY-qPCR $p = 0.0004$, HPJ↔HPY $p = 0.0259$). In the three different subgroups of clarithromycin-susceptibility FISH and HPY-qPCR agreement was 56.18% (50/89) with a Cohen's κ of 0.587 (95% CI: 0.460–0.702), HPJ-qPCR and HPY-qPCR agreement was 61.22% (60/98) with a Cohen's κ of 0.680 (95% CI: 0.572–0.771).

At the NRCCH, the original DNA isolates from 16 representative FFPE cases (three Cla-susceptible, three heteroresistant, four homoresistant, and three *H. pylori*-negative with all methods in the DPFIM) were also tested with HPY-qPCR. In these cases, identical results were obtained at the NRCCH. In two further samples, HPY-qPCR detected Cla-heteroresistance in DPFIM, while FISH and HPJ-qPCR confirmed Cla-susceptible status. In one case, despite heteroresistant FISH and HPJ-qPCR results, HPY-qPCR detected homoresistance (Supplementary Table S2). In these three discordant cases, the NRCCH team confirmed the FISH and HPJ-qPCR results of the DPFIM by an optimized HPY-qPCR setup. The Ct values showed an advantage in detection performance of HPJ-qPCR over HPY-qPCR with an average of 2.96 cycle (range: 0.55–7.85) on FFPE samples (Supplementary Table S2).

The two qPCRs were also tested on a cohort of 99 unfixed routine diagnostic gastric biopsies in the NRCCH, yielding identical *H. pylori* detection and clarithromycin susceptibility status results with both primer pairs. The advantage in detection performance of HPY-qPCR over HPJ-qPCR was subtle (~half a cycle Ct, range: −3.8–4.6) on unfixed samples (Supplementary Table S3).

We examined the diagnostic performance of the three methods (FISH, HPY-qPCR, HPJ-qPCR) according to the patient's clinical symptoms, endoscopic and histopathological diagnoses, previous gastrointestinal diseases, and eradication status in the 209 cases of the FFPE cohort suggestive of *H. pylori* infection (Figure 3; Supplementary Table S5). The three different tests showed similar trends in *H. pylori* detection rates and clarithromycin susceptibility status among these symptom and diagnosis groups, with characteristic inter-test differences: the greater *H. pylori* detection ability of the HPJ-qPCR, while HPY-qPCR performed worse even compared to the IHC/FISH staining-based *H. pylori* detection (with the exception of intestinal metaplasia). Moreover, similarly to the total cohort, the HPY-qPCR categorized higher proportion of cases as heteroresistant instead of Cla-susceptible (Figure 3). We investigated this in more detail in subgroups of histopathological diagnoses with sufficient sample sizes, namely, in the chronic gastritis and activity cases (Supplementary Table S4). HPJ-qPCR detected significantly

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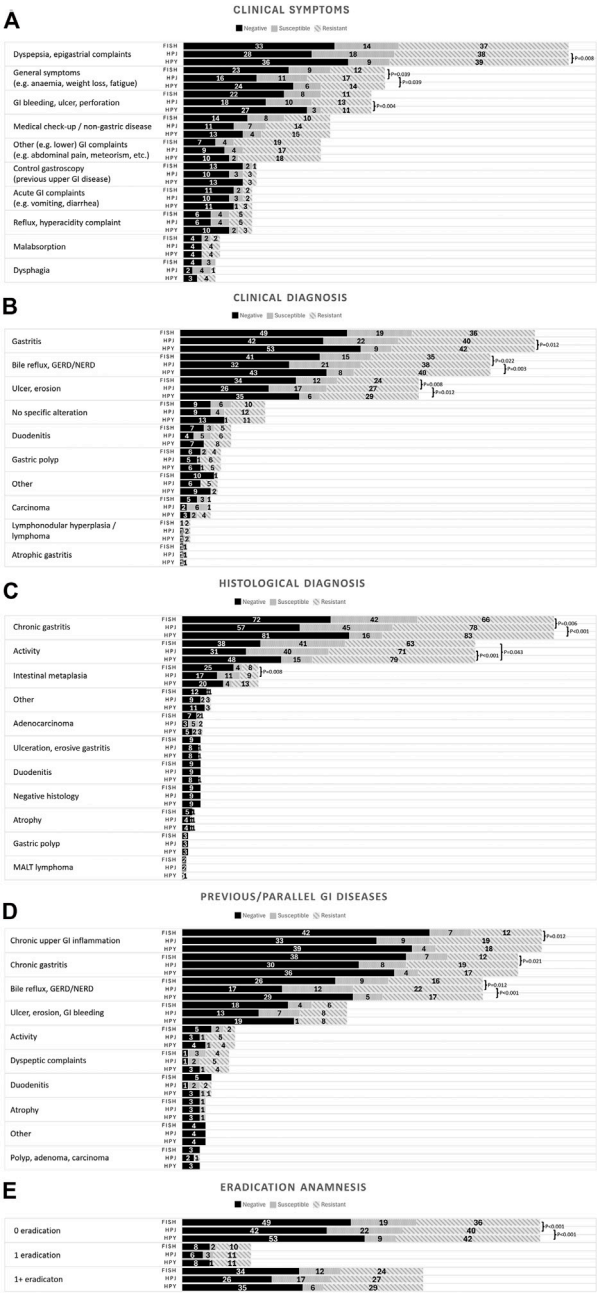

FIGURE 3
Comparison of the detection rates of *Helicobacter pylori* infection and its clarithromycin susceptibility between the three methods (FISH, HPY-qPCR, HPJ-qPCR) according to the patient's clinical symptoms **(A)**, endoscopically diagnosed alterations (clinical diagnosis) **(B)**, histopathological diagnosis **(C)**, previous/parallel gastrointestinal diseases **(D)**, and eradication history **(E)**. (GI, gastrointestinal; GERD, Gastroesophageal Reflux Disease; NERD, Non-Erosive Reflux Disease).

more *H. pylori* infections in cases of chronic gastritis than both staining-based approach and HPY-qPCR (FISH↔HPJ-qPCR p = 0.0059, HPY-qPCR↔HPJ-qPCR p < 0.0001, FISH↔HPY-qPCR p = 0.1360). HPJ-qPCR detected Cla-susceptibility status in a manner similar to FISH (FISH↔HPJ-qPCR p = 0.3438) in chronic gastritis, while HPY-qPCR performed significantly worse (FISH↔HPY-qPCR p < 0.0001; HPJ-qPCR↔HPY-qPCR p < 0.0001). On the other hand, in cases showing inflammatory activity, no significant difference was found between the diagnostic performance of FISH and HPJ-qPCR (p = 0.1671 in *H. pylori* and p = 0.5078 in Cla-susceptibility detection), while HPY-qPCR showed significantly poorer results in detecting both *H. pylori* (FISH↔HPY-qPCR p = 0.0433; HPJ-qPCR↔HPY-qPCR p < 0.0001) and Cla-susceptibility status (FISH↔HPY-qPCR p < 0.0001; HPJ-qPCR↔HPY-qPCR p < 0.0001).

In histologically negative but HPJ-qPCR-positive cases, the occurrence of chronic gastritis, as well as inflammatory activity, was intermediate between the rates in the double-negative and double-positive subgroups (Table 3). This was proved to be a statistically significant trend by Cochran-Armitage test (p < 0.0001). Of the 100 cases with no histological or anamnestic findings suggestive of *H. pylori* infection, three tested positive for *H. pylori*, including one susceptible and two homoresistant cases (Supplementary Table S2). Dyspeptic and general symptoms, as well as endoscopically diagnosed reflux disease (bile, GERD/NERD) and gastritis were less frequent in this cohort (p = 0.0003, 0.0215, 0.0136, 0.0119, respectively), while only dyspepsia was less frequent among the histologically negative cases suggestive of *H. pylori* infection compared to the staining positive group (p = 0.0351) (Supplementary Table S2B). All samples from the negative tissue control group were negative by HPJ-qPCR (Supplementary Table S6).

Discussion

Although clarithromycin is one of the most effective antibiotics against susceptible *H. pylori* infections, its use is not recommended in populations with high (≥15%) resistance rates without prior susceptibility testing [2]. Bacterial culture and resistance testing requires dedicated sampling and processing, as does non-fixed tissue-based microbiological PCR diagnostics, although both culture and PCR can be performed on the same sample if required [17]. On the other hand, the detection of *H. pylori* is still based on histopathological diagnosis in most of the cases where tissue sampling is performed, as, clinicians usually do not take separate samples for microbiological testing. However, FFPE tissue blocks of routine pathology diagnostics can also be used for DNA isolation for PCR without separate microbiological sampling and logistics chain. This is important because the Maastricht VI/Florence Consensus Report on the management of *H. pylori* infection recommends antibiotic susceptibility testing [2]. However, this is not known to the majority of pathologists, as the guidelines for pathological processing do not address this issue. Therefore, this is an unmet need not only in Europe but also worldwide, which in most cases makes targeted antibiotic eradication of *H. pylori* unrealistic. As a result, clarithromycin-based triple therapy, which would be the best option for the majority of patients, even in populations with high (≥15%) resistance rates, has been widely declined, despite the fact that typically 60%–85% of the infected individuals in these areas still carry susceptible *H. pylori* infections [5]. Therefore, there is a need to develop susceptibility testing methods that are widely available for the pathology departments, require little human resources, and are inexpensive. The qPCR method is ideal in this respect, as it can rapidly detect the infection and its clarithromycin susceptibility/resistance simultaneously.

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TABLE 3 The presence of clinical symptoms and diagnosed diseases in cases suspected of having *H. pylori* infection (based on the presence of histological abnormalities like chronic mucosal inflammation, intestinal metaplasia, atrophy, either in the specific case or in the patient's medical history).

Cases suspected of having *H. pylori* infection	Alterations, diseases	209 cases suggestive of *H. pylori* infection	FISH and HPJ-qPCR negative (76 cases)	FISH and HPJ-qPCR positive (102 cases)	FISH negative/ HPJ-qPCR positive (25 cases)
Clinical symptoms and diagnoses (actual and anamnestic)	GI bleeding, peptic ulcer, erosion	103/209 49.3%	31/76 40.8%	54/102 52.9%	13/25 52.0%
	Gastroesophageal and/or bile reflux	106/209 50.7%	34/76 44.7%	54/102 52.9%	16/25 64.0%
	Dyspeptic, epigastrial complaints	87/209 41.6%	24/76 31.6%	50/102 49.0%	9/25 36.0%
	Gastritis	116/209 55.5%	39/76 51.3%	60/102 58.8%	13/25 52.0%
Histology (of the actual slide)	Chronic gastritis	180/209 86.1%	51/76 67.1%	102/102 100.0%	21/25 84.0%
			$p < 0.0001$		
	Activity	142/209 67.9%	25/76 32.9%	98/102 96.1%	13/25 52.0%
			$p < 0.0001$		

The subgroup of FISH-positive/HPJ-qPCR-negative cases (n = 6) in which *H. pylori* DNA could not be detected by HPJ-qPCR due to preanalytical reasons are not listed in a separate column. The p-values are displaying the results of the Cochran-Armitage trend test showing a significantly increasing trend across the FISH & HPJ-qPCR negative, the FISH negative / HPJ-qPCR positive and the FISH & HPJ-qPCR positive subgroups.

Due to the patchy mucosal distribution of *H. pylori* bacteria, histological staining techniques (e.g., H&E, Giemsa, immunohistochemistry and FISH) may not necessarily be able to detect *H. pylori* infection, as the sampling site itself could be a bacteria-free area [10]. In contrast, PCR can detect *H. pylori* bacterial DNA. In our study, HPJ-qPCR was able to detect infection in one of the three cases where DNA was isolated from a histologically *H. pylori*-negative tissue block of an *H. pylori*-positive case. Kiss et al. reported that *H. pylori* was detected by PCR testing in 146 of 341 histologically negative samples showing inflammation activity and pattern suggestive of *Helicobacter* gastritis (43.1%) [14]. Ramírez-Lázaro et al. used the highly sensitive droplet digital PCR (ddPCR) method and identified *H. pylori* infection in 36% of patients with dyspeptic symptoms who were negative in conventional diagnostic tests (histology, rapid urease test, urea breath test) [16]. We observed a 25% PCR positivity rate in histologically *H. pylori*-negative biopsy samples showing chronic gastritis. This underscores the importance of molecular detection, but also highlights the added value of short-amplicon HPJ-qPCR in FFPE samples, as HPY-qPCR was only able to detect about half of these. On the other hand, of the 108 histologically *H. pylori* positive cases, HPJ-qPCR confirmed infection in 102 samples, while HPY-qPCR demonstrated the presence of the bacterium in only 89 samples.

Our results were also verified at the NRCCH in Bordeaux in selected cases, and HPJ-qPCR, with an average cycle threshold of 2.96, also demonstrated better detection performance than HPY-qPCR. In addition, HPJ-qPCR and HPY-qPCR were tested on 99 unfixed biopsy cases of NRCCH, showing identical *H. pylori* and Cla-susceptibility status detection results. This also confirms that the DNA-degrading effects of formalin fixation and paraffin embedding must be taken into account. Sub-optimal conditions during handling, transport, fixation, embedding and storage of tissue samples may also further reduce nucleic acid integrity [23–25]. Fragmented DNA and formalin cross-links impair qPCR performance with longer amplicons, therefore primer pairs resulting in amplicons longer than 300 base pairs (bp) should be avoided; target sequences of 75–150 bp are generally recommended for FFPE tissues [20, 25–27]. This was confirmed by the sensitivity advantage of HPJ-qPCR (122 bp amplicon) compared to HPY-qPCR with a 267 bp target sequence. Moreover, preanalytical damages do not necessarily affect protein (IHC), ribosomal RNA (FISH), and DNA molecules (PCR) uniformly, or even bacterial and human nucleic acids equally [27]. This may explain the cases that are IHC-positive but PCR-negative, and also suggest that the qPCR is recommended primarily for the further investigation of negative cases rather than for completely replacing *Helicobacter* staining techniques on FFPE tissue sections.

In terms of clarithromycin susceptibility testing performance, HPJ-qPCR and FISH identified susceptible and resistant cases at similar rates in the FFPE cohort. In the classification of homo- and heteroresistant cases, significant differences were observed between all three methods, with the smallest discrepancy between FISH and HPJ-qPCR (Table 2). The discrepancy between FISH and HPJ-qPCR can be explained by the fact that the sensitivity limit of qPCR generally does not allow the detection of Cla-susceptible or -resistant *H. pylori* subpopulations below ~10% in cases diagnosed as heteroresistant by FISH. FISH is capable of detecting heteroresistant infections containing mixed populations of Cla-susceptible and -resistant bacteria with a sensitivity that significantly exceeds the variant allele frequency (VAF) detection capabilities of standard PCR methods, as even a single bacterium with different color and thus susceptibility/resistance status can be identified (as demonstrated in Figure 2E) [8, 11]. In general,

however, HPJ-qPCR outperformed the FISH and HPY-qPCR methods in detecting bacteria and also showed near-perfect agreement with FISH in determining susceptibility status. HPY-qPCR performed poorly in this regard, misdiagnosing a subset of Cla-susceptible cases as heteroresistant. This is probably due to suboptimal amplification of poor-quality, fragmented DNA with a longer amplicon, producing an additional melting-curve peak that may be indistinguishable from the double-peak curves of heteroresistant cases.

We demonstrated significant differences in the prevalence of *H. pylori* among subgroups in which infection was not detected by staining techniques: cases exhibiting no features suggestive of *H. pylori* infection showed only 3% positivity rate, whereas 25% of cases with characteristic alterations of *H. pylori* infection (whether currently present or in the medical history) tested positive using the HPJ-qPCR. Accordingly, in these cases we demonstrated that the rates of currently present chronic gastritis and inflammatory activity were higher than in double-negative cases and were intermediate between these and the double-positive subgroups, showing a stistically significant trend. This suggests the presence of *H. pylori*, but with a lower bacterial load, when not only inflammatory activity but also the occurrence of other histological changes may be less frequent (as we confirmed this correlation in one of our previous studies) [10], but still considerably higher than in truly negative cases. The reduced bacterial load in 14 of the 25 IHC/FISH-negative but HPJ-qPCR-positive cases can be explained by PPI or antimicrobial therapy administered shortly before sampling, which has an *H. pylori*-reducing effect (see in Supplementary Table S2). The Furthermore, none of the negative tissue control group cases tested positive, which also supports that the infections detected by qPCR are not false positives.

Given this, it raises serious questions about what strategy should be pursued in cases where staining techniques have yielded negative results. As indicated by the relatively few but existing IHC-positive, PCR-negative cases, there is no definitive diagnostic method; every technique has its own limitations. Under inadequate preanalytical conditions, DNA degradation can lead to false-negative PCR results. Conversely, infection may be missed by staining if no viable bacteria are present, whereas PCR can still detect bacterial DNA. Therefore, as noted above, we recommend qPCR for staining-negative cases. Since testing all histologically negative samples may not be cost-effective (depending on the healthcare system), pathologists should be guided toward cases most likely to benefit from further investigation. Based on our results, this is most warranted in cases of gastric tissue samples showing chronic gastritis and/or intestinal metaplasia, atrophy. During that three-month period (Jan–Mar 2025), from which we collected consecutive cases with no histological or anamnestic findings suggestive of the *H. pylori* infection, a total of 590 adult gastric samples underwent *H. pylori* diagnostics using staining methods at our institute. Of these, *H. pylori* was detected in 183 cases (31%), all of which presented with chronic gastritis. An additional 407 cases (69%) were *H. pylori*-negative; among these, chronic gastritis and/or intestinal metaplasia, atrophy were identified in 187 cases (46%). Although *H. pylori* infection is closely associated with chronic gastritis, chronic inflammation with characteristics typical of *H. pylori* gastritis is frequently observed in other conditions as well [28]. We found a 25% *H. pylori* positivity rate in cases exhibiting specific histological alterations despite negative *H. pylori* staining, and others have reported rates as high as ~40% [14, 16]. This justifies further qPCR testing for *H. pylori* in this group. However, in the other half of the negative cases, where histological changes characteristic of *H. pylori* are not observed, our extremely low (3%) PCR-positivity rate suggests that routine *H. pylori* qPCR testing is not necessarily required. Rather, this may be a clinical decision, depending, for example, on whether there are symptoms that persist or recur despite treatment.

Moreover, as we pointed out in the introduction, it is recommended to not only diagnose *H. pylori* infection, but also determine its clarithromycin susceptibility for the targeted antimicrobial eradication therapy. However, this requirement–which is based on gastroenterological and microbiological considerations of the Maastricht VI/Florence Consensus Report–is not followed in the practice of pathology departments, as pathology guidelines do not address it. Therefore, it is essential to emphasize that, in the absence of separate microbiological sampling, performing the susceptibility testing required for targeted antibiotic-based eradication is the responsibility of molecular pathology. To this end, the adaptation of inexpensive, high-throughput, highly sensitive genetic tests that show high concordance with phenotypic susceptibility tests to FFPE specimens is of particular importance. One of the most promising candidates for this is the qPCR technique and this was the reason why we developed our HPJ-qPCR test. However, further studies involving a large number of patients are needed to precisely validate the clinical diagnostic accuracy of the HPJ-qPCR.

As with the majority of studies, this study also has several limitations that should be acknowledged. First, its retrospective design inherently limits the ability to control for confounding variables and introduces potential biases related to sample selection and data availability. This is particularly true because the main objective was to test the different methods in all possible scenarios of routine diagnostics, so we had to maintain a balanced representation of *H. pylori*-positive and -negative cases, as well as within the positive ones, the balance between clarithromycin-susceptible, homo- and heteroresistant status. Although the overall cohort size was relatively large, sequential subgrouping based on clinical, histological, and molecular parameters resulted in low event numbers in certain subgroups, reducing statistical power and limiting the interpretability of some comparisons. Furthermore, the presence of bacterial DNA can be detected using the PCR technique, even in the absence of viable bacteria. Finally, although the newly developed HPJ-qPCR method showed excellent performance in detecting *H. pylori* and determining its clarithromycin susceptibility in FFPE samples, its performance needs to be validated in further studies with larger patient cohorts.

Conclusion

One of the gold-standard methods for diagnosing *H. pylori* infection remains the histopathological examination of gastric biopsies. However, pathology departments could contribute not only to the diagnosis of infection but also to susceptibility-guided eradication therapy, provided that molecular testing becomes more widely integrated into routine diagnostic workflows. In the absence of dedicated microbiological sampling, FFPE tissue blocks represent a unique opportunity to obtain both diagnostic and therapeutic

information from the same specimen. The FFPE-optimized HPJ-qPCR developed in this study proved superior for detecting *H. pylori* in fragmented DNA and showed high concordance with FISH in determining clarithromycin susceptibility. By simultaneously identifying infection and clarithromycin susceptibility status, it provides clinically relevant information required for targeted antimicrobial therapy. We propose a simple pathology-based diagnostic strategy: gastric biopsies positive for *H. pylori* by routine histology should undergo molecular testing to determine clarithromycin susceptibility, whereas histologically negative biopsies should be selected for reflex qPCR primarily when chronic gastritis, intestinal metaplasia, atrophy, or other characteristic *H. pylori*-associated lesions are present. Such an approach may facilitate both the detection of infections missed by routine histopathological methods and the implementation of susceptibility-guided eradication therapy.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Ethical Committee of Semmelweis University, Budapest, Ethics Committee of the Bordeaux University Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because based on the current Hungarian law for scientific research, contacting the patients in order to have their informed consent is not required for the retrospective studies. Ethics Committee of the Bordeaux University Hospital deemed it unnecessary to issue ethical approval and obtain informed consent from patients whose gastric samples were submitted to the National Reference Centre for Campylobacters & Helicobacters, Bordeaux, France (NRCCH), as these are used in accordance with the scientific missions assigned to the NRCCH by Santé Publique France (https://www.santepubliquefrance.fr) by recognizing the NRCCH as a national reference centre in the Official Journal of the French Republic.

Author contributions

GL, PL, and ÉK performed study concept and design; AKz, AB, LB, ÁJ, GL, and PL performed development of methodology; AKz, AB, LB, ÁJ, and EB performed experiments; AKz, AB, LB, ÁJ, BZ, IS, IK, EB, GB, VP, TV, ÉK, and GL provided acquisition, analysis and interpretation of data; AB, ÉK, and GL performed statistical analysis; AS, AKs, LG, and PL provided technical and material support and oversaw the study. AKz, AB, GL, and PL drafted the manuscript. All authors interpreted the data, critically read and revised the manuscript in terms of important intellectual content and approved the final paper.

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Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.por-journal.com/articles/10.3389/pore.2026.1612654/full#supplementary-material

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