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Original article

Efficacy of a novel, multiplex, real-time PCR kit for the detection of *Helicobacter pylori* and clarithromycin resistance

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ABSTRACT

Background: *Helicobacter pylori* is a pathogen involved in the etiopathogenesis of gastroduodenal infections and some gastric malignancies. Accurate diagnosis and rapid detection of antibiotic resistance are crucial for effective treatment. **Methods:** We investigated the efficacy of a novel, multiplex, real-time PCR (qPCR) kit (DiaRD-HPykl, Diagen, Turkey) to detect *H. pylori* on 181 gastric biopsy specimens and simultaneously search clarithromycin resistance. qPCR results obtained from 49 fresh and 132 paraffinized gastric biopsy specimens were compared with histopathologic examination (HPE). **Results:** qPCR was positive in 89.2% and 74.2% of the fresh and paraffinized samples with HPE positive result, respectively. These values were 41.7% and 5.7% for HPE negative samples. The overall agreement between HPE and qPCR was 80.1% and the Kappa coefficient of agreement was 0.551. In fresh tissues, the actual agreement was 81.6% and the Kappa value was 0.489, while in paraffinized tissues, these values were 79.6% and 0.566, respectively. Clarithromycin resistance was detected in 12.5% of 112 samples, which were *H. pylori* positive by qPCR. **Conclusion:** The DiaRD-Hpykl qPCR kit, which is resulted in two hours, can be used in combination with HPE or alone as an alternative test for rapid diagnosis and accurate management of treatment.

Introduction

Helicobacter pylori is a spiral-shaped, gram-negative bacterium that infects more than 40% of the world's population of all ages. It was classified as a Group 1 carcinogen by the world health organization in 1994. *H. pylori* can lead to a wide range of clinicopathological outcomes ranging from permanent colonization of the gastric mucosa to chronic gastritis, gastric and duodenal ulcers, gastric adenocarcinoma, and gastric lymphoma [1-3].

Different conventional methods have been used in the diagnosis of *H. pylori* related infections. Some of these are invasive methods such as histopathological examination (HPE), rapid urease test (RUT), fluorescence in situ hybridization (FISH), and culture of gastric biopsy specimens. The others such as stool antigen detection (SAT), urea breath test

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(UBT) and antibody determination in serum are non-invasive. The non-invasive methods do not provide information on susceptibility to antimicrobials and the most used UBT and SAT have a sensitivity and specificity of 85-95% [4,5]. RUT and UBT may give false negative results due to the absence of urease activity in the coccoid form of *H. pylori* and false positive results in the presence of *Proteus*, *Yersinia*, *Klebsiella* and *pseudomonas* [6,7]. Serological tests have only epidemiologic value. The invasive tests require tissue samples obtained from the stomach by endoscopic biopsy. The most important limitation of biopsy specimens is the low sensitivity due to only a small portion of the gastric mucosa examined [4,5]. The widely accepted gold standard for the diagnosis of *H. pylori* is cultivation of the bacterium from a gastric biopsy specimen, which is a difficult and time-consuming procedure with limited sensitivity. There are also publications that recommend HPE as the gold standard [8,9]. Espghan/NASPGHAN (European/North American society of pediatric gastroenterology-hepatology and nutrition) guidelines stipulate that RUT or PCR positivity must be demonstrated in addition to culture or HPE positivity for the definitive diagnosis of *H. pylori* infection in children **[10].**

Molecular methods have also been developed for investigation of *H. pylori* in invasive and non-invasive samples. Although various genes are targeted in *H. pylori* research by PCR, 16S rRNA gene is commonly preferred to show the presence of bacteria and 23S rRNA gene is preferred to determine clarithromycin resistance. PCR-based tests are superior to many conventional tests, especially in patients with gastric bleeding, with a sensitivity and specificity of up to 95% [11-13]. PCR is recommended in pediatric patients on PPI treatment with achlorhydria, intestinal metaplasia and bleeding gastric ulcers, as HPE may miss *H. pylori* in this group [14]. There are also some studies suggesting that PCR-based molecular tests can be used as the gold standard test in certain cases [15,16]. Although these methods can give rapid and highly accurate results, they have not been sufficiently standardized and widely used yet [17].

The ideal approach in the treatment of *H. pylori* infections requiring multiple and long-term drug regimens is to determine the treatment protocol under antibiogram guidance. However, this is very difficult due to the need for biopsy and the low sensitivity of culture. As a matter of fact, treatment protocols commonly consist of empirical combinations. Clarithromycin is a first-line drug in these protocols and should be included in the treatment if the bacteria are susceptible [18]. On the other hand, the Maastricht/Florence consensus reports (VIth report, 2021), considering the rising resistance rates, stipulated that antibiograms should be performed for this drug in regions having resistance rates higher than 15% [19].

In this study, we sought to determine the efficacy of a novel, commercial multiplex qPCR kit (DiaRD-HPykl, Diagen Biyoteknoloji, Ankara, Turkey) in detecting *H. pylori* and its clarithromycin resistance in paraffinized and fresh gastric biopsy specimens obtained from the pediatric patients followed in a tertiary city hospital with a 3810-bed capacity.

MATERIALS AND METHODS

Patients and biopsy samples

This study was performed in line with the principles of the declaration of Helsinki. Approval was granted by the Ethics Committee of the Ankara Yıldırım Beyazıt University Medical Faculty (Date: 07.04.2021/ No:36). Parents of all patients signed informed consent forms for using the anonymised clinical samples in the study.

A total of 181 biopsy samples, including 132 paraffinized tissue and 49 fresh tissue samples, were included in the study. Paraffinized tissue samples of 132 patients aged <18 years who admitted to the pediatric gastroenterology (PGE) outpatient clinic with dyspepsia between 2019 and 2021 and underwent gastric biopsy were included in the study. To prevent cross-contamination, 10-µm thick tissue sections were taken from the paraffin blocks by changing the microtome blade between blocks and 5 sections of each sample were added to sterile microcentrifuge tubes. Fresh tissue samples of 49 patients in the same age group who were admitted between April and June 2022 and underwent gastric biopsy were included in the study. One part of each fresh sample was sent to the pathology laboratory for HPE, another part was transferred into sterile microcentrifuge tubes containing 2 ml saline solution (NaCl 0.9%). HPE was performed by the same pathologist and results were evaluated according to the Sydney classification [20]. Information including age, gender, complaints at admission and upper gastrointestinal endoscopy findings were obtained from hospital records.

Deparaffinization and DNA isolation

Deparaffinization and DNA isolation were performed using GeneMATRIX Tissue and bacterial

DNA Purification kit (EURx, Poland) according to the manufacturer's instructions (https://eurx.com.pl/docs/manuals/en/e3551.pdf/ last accessed: 11.05.2023). Briefly; 1 ml xylene was added onto the paraffinized tissue sections in microcentrifuge tube. It was thoroughly vortexed and incubated at room temperature for 15 min. Then the tube was centrifuged at 11,000 x g for 3 min and the supernatant was removed. One milliliter ethanol (96-100%) was added on the pellet and vortexed. The supernatant was then removed by centrifugation at 11,000 x g for 3 min. Ethanol treatment was performed twice to remove residual xylene. The tubes were incubated at 37°C until the ethanol evaporated. Bacterial DNA isolation from deparaffinized and the fresh tissues was performed using the GeneMATRIX kit according to the manufacturer's instructions.

A multiplex qPCR analyses

DiaRD-HPykl kit (Diagen Biyoteknoloji, Ankara, Turkey), a multiplex qPCR kit, was used to investigate *H. pylori* 16S rDNA in the samples and the presence of mutations in the 23S rDNA V region leading to clarithromycin resistance. The procedures were performed according to the manufacturer's recommendations. The kit included a 2XMasterMix , positive and negative controls, and primer-probe mix. The primer-probe mix consisted of 1) a primer pair specific for 16S rDNA of *H. pylori* plus TaqMan probe, 2) a primer pair plus molecular beacon probe to investigate the presence of A2143G, A2142G and A2142C mutations for clarithromycin resistance 3) and a primer pair plus TaqMan probe specific for beta-actin gene as an internal control. The working principle of the kit is as follows: In *H. pylori* positive and clarithromycin-sensitive samples, fluorescent signal should be obtained with all three probes. In *H. pylori* positive but clarithromycin-resistant samples, fluorescent signal should be obtained with *H. pylori* and internal control probes, while the probe designed for the "wild" type-specific sequence in 23S rDNA should not give fluorescence. Amplifications were performed in a total volume of 20 µL containing 10 µL 2XMasterMix, 5 µL primer-probe mix, 5µL DNA sample. After an initial denaturation at 95°C for 15 min, 50 cycles of amplification were performed in a thermal cycler (Rotor Gene, Germany), including denaturation at 95 °C for 15 s, annealing and elongation at 62 °C for 60 s. The results were evaluated according to the criteria given in the kit package insert (www.diagen.com.tr).

Statistical analyses

The prevalence of *H. pylori* in the Turkish pediatric population is reported to be approximately 50% [21]. Accordingly, the sample size for the study was determined as 47, which would meet the requirements of at least 70% (>50%) sensitivity (/selectivity), <0.05 type I error and at least 80% power. All analyses were performed in the R programming language v4.0.[22] The "epiR" R package was used for sample size, diagnostic accuracy criteria, and 95% confidence interval (CI) calculations.[23] The significance of the difference between HPE and qPCR test results in samples divided into two groups as paraffinized and fresh tissues was evaluated by McNemar test. The agreement between the results of the two methods was interpreted by calculating the kappa coefficient according to the following criteria: <0.2 insignificant/slight agreement, 0.2 - 0.4 low agreement, 0.4 - 0.6 moderate agreement, 0.6 - 0.8 significant agreement, >0.8 almost perfect agreement. Sensitivity (D), specificity (S), positive predictive value (PPV), negative predictive value (NPV) and correct classification rate (CCR) were calculated with 95% Wilson's CI. A lower limit of CI greater than 50.0 indicated that the relevant coefficient was statistically significantly higher than 50% based on the binomial test. Statistical significance level $p<0.05$ was accepted.

RESULTS

Demographic data of the patients were summarized in Table 1. The clinical diagnoses of the patients were antral gastritis, erosive gastritis, pangastritis, chronic gastritis, duodenitis, reflux esophagitis and/or peptic ulcer. Histopathological diagnoses included *H. pylori* gastritis (22.4%), chronic active gastritis (73.4%), and chronic gastritis (40.8%).

HPE reported that 12 (25%) of the 49 fresh samples were *H. pylori* positive and the remaining 37 (75%) were negative. DiaRD-HPykl qPCR kit was positive in 89.2% of fresh samples with HPE (+) and 42% of fresh samples with HPE (-). These rates were 74.2% and 5.7% in paraffinized samples, respectively. The results obtained by DiaRD-HPykl qPCR kit in paraffinized, and fresh tissues were given in Figure 1 and Tables 2-4.

Considering the total of 181 samples, 80.1% agreement was observed between HPE and DiaRD-HPykl qPCR kit results (Table 3). The *Kappa* coefficient of agreement, which included the probability of prediction in addition to the true agreement, was 0.551, indicating a moderate level of agreement between the two methods. The true

agreement and *Kappa* values were 81.6% and 0.489 for fresh tissues and 79.6% and 0.566 for paraffinized tissues, respectively. In fresh tissues, there was no statistically significant difference in sensitivity between the qPCR and HPE ($p>0.05$). In paraffinized tissues, HPE was significantly more sensitive than qPCR ($p<0.001$). HPE was also significantly more sensitive when fresh and paraffinized samples were evaluated together ($p<0.001$).

Clarithromycin resistance was detected in 14 of 112 samples (12.5%; CI: 6.38 - 18.63) which were found to be *H. pylori* positive by DiaRD-HPykl qPCR kit.

Table 1. Demographic data of the patients.

	All tissues		Paraffinizzed tissues		Fresh tissues	
	HPE (+)	HPE (-)	HPE (+)	HPE (-)	HPE (+)	HPE (-)
Gender						
Male	63	24	50	18	13	6
Female	71	23	47	17	24	6
Age (year)[1]	13.16±4.35 15 (1 – 18)	12.30±4.17 13 (2 – 18)	12.85±4.32 14 (1 – 18)	11.46±3.83 11 (3 – 17)	13.97±4.39 16 (1 – 18)	14.75±4.29 16 (2 – 18)

HPE(+): Histopatological examination result positive, HPE(-): Histopatological examination result negative . [1]*Mean (± SD), median (min-max)*

Table 2. HPE and qPCR results of fresh tissue samples.

		HPE			
		Positive	Negative	Total	%
qPCR	Positive	33	5	38	77.6
	Negative	4	7	11	22.4
	Total	37	12	49	100

HPE: Histopatological examination. Aggreement per cent: 81.63 %, Kappa (standard error): 0.489 (0.147)

Table 3. HPE and qPCR results of paraffinizzed tissue samples.

		HPE			
		Positive	**Negative**	**Total**	**%**
qPCR	**Positive**	**72**	**2**	**74**	**56**
	Negative	**25**	**33**	**58**	**44**
	Total	**97**	**35**	**132**	**100**

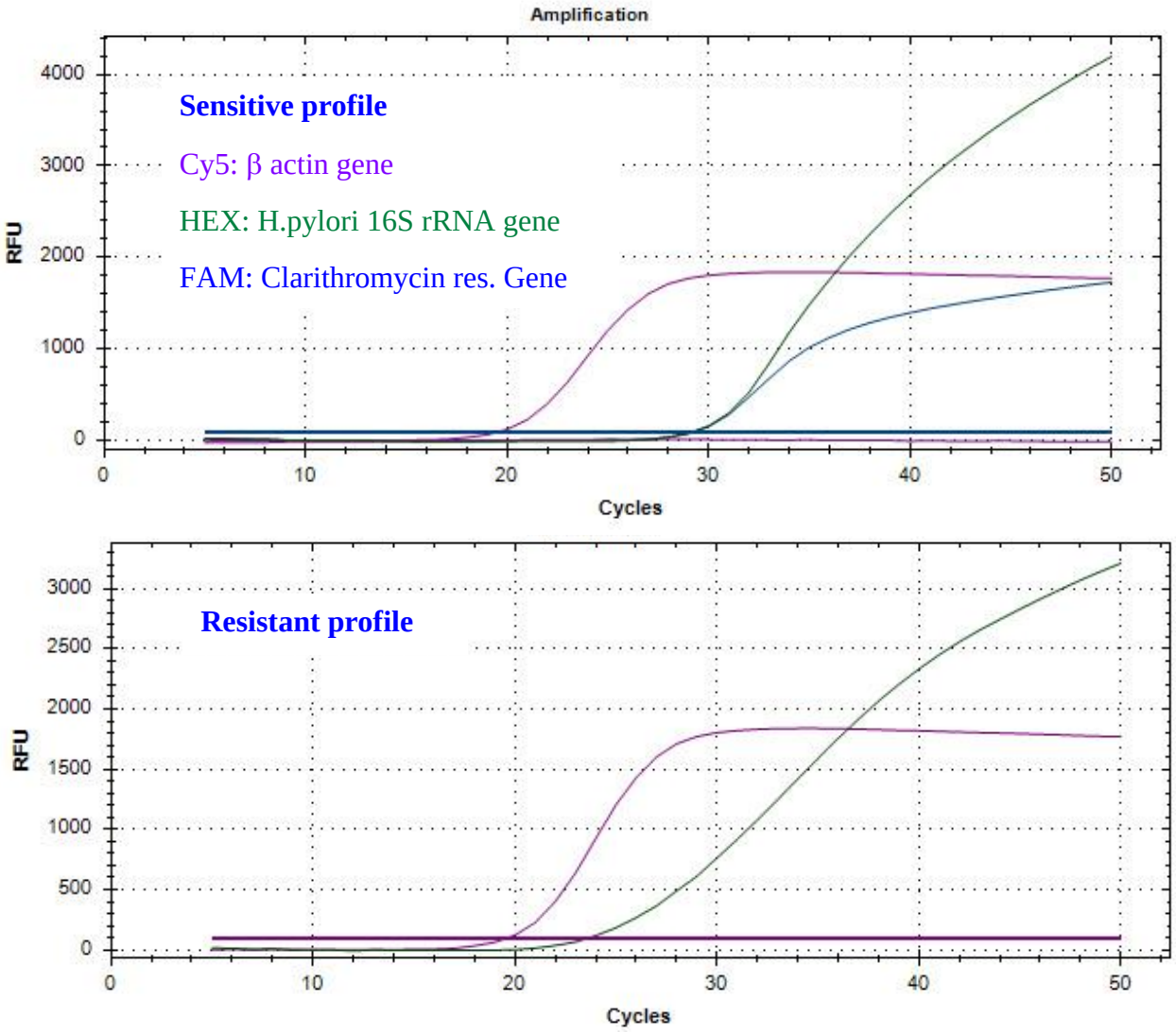
HPE: Histopatological examination. Aggreement per cent: 79.55 %, Kappa (standard error): 0.566 (0.069)

Table 4. Accuracy criteria and 95% CI values of qPCR according to HPE[1].

	Sensitivity (%) (LL – UL)	Specificity (%) (LL – UL)	PPV (%) (LL – UL)	NPV (%) (LL – UL)	CCR (%) (LL – UL)	□ (SE)[2]
All tissue samples	78.36 (70.65; 84.49)	85.11 (72.31; 92.59)	93.75 (88.90; 96.65)	57.97 (50.41; 65.19)	80.11 (73.40; 85.51)	0.551 (0.064)
Paraffinized tissues	74.23 (64.72; 81.89)	94.29 (81.39; 98.42)	97.30 (92.40; 99.20)	56.90 (47.99; 65.39)	79.55 (71.46; 85.87)	0.566 (0.069)
Fresh tissues	89.19 (75.29; 95.71)	58.33 (31.95; 80.67)	86.84 (73.46; 94.34)	63.64 (48.62; 76.52)	81.63 (67.50; 90.76)	0.489 (0.147)

HPE: Histopathological examination, CCR: Correct classification rate, □: Kappa coefficient, CI: Confidence interval, LL: Lower limit of 95% CI, UL: Upper limit of 95% CI, NPV: Negative predictive value , PPV: Positive predictive value, SE: Standard error.
[1]*The p-value from the McNemar test was >0.999 for fresh tissues and <0.001 for deparaffinized tissues and total tissues.*
[2]*Standard error, p<0.05, all kappa coefficients were significantly higher than zero.*

Figure 1. Three-channel screenshots in qPCR. Top, *H. pylori* (+), clarithromycin-sensitive; bottom, *H. pylori* (+), clarithromycin-resitant sample.



Discussion

Although there are many approaches for the diagnosis of *H. pylori* infection, still there is no certain gold standard for accurate diagnosis and detection drug resistance. Each of the available approaches has different advantages and limitations [7,10,14].

In several studies conducted to detect *H. pylori* in gastric biopsy, gastric fluid, or feces of patients with dyspepsia, PCR demonstrated diagnostic ability equal to or higher than the HPE with a sensitivity rate of over 90%, and 100% sensitivity in detecting antibiotic resistance [5]. Bazin et al.reported that HPE was less sensitive than molecular tests, and Silva et al [16,24]. reported that PCR was approximately 1.6 times more sensitive than HPE. In a study conducted in Poland in 2022, the sensitivity and specificity of PCR-based methodology were 95.3% and 92.6%, respectively, and the proportion of samples in which PCR and HPE were both positive was 88.6% [14]. In another study, the sensitivities of qPCR and HPE for detecting the presence of *H. pylori* in paraffinized tissues were found to be 95.6% and 69.9%, respectively [25]. In these and many similar studies, a moderate or high concordance between HPE and molecular test results in the detection of *H. pylori* in pediatric patients was reported [14].

In our study, a moderate level of agreement was observed between qPCR kit and HPE in fresh and paraffinized samples. It is noteworthy that the sensitivity of the qPCR kit was lower in paraffinized specimens compared to fresh specimens. In agreement with our data, a study conducted in Brazil found that the efficiency of *H. pylori* detection by PCR was higher in fresh tissues; this was explained by the fact that paraffinized specimens contain less tissue, inhibition of the reaction due to paraffin and xylol in extraction procedures, and/or low quality and degradation of DNA [26]. The negative qPCR results detected in 10.8% of HPE-positive fresh tissue samples in our study might be due to the fact that only one sample was evaluated in qPCR while two biopsy materials were examined simultaneously in HPE. As it was indicated previously, two or more biopsy specimens taken from the antrum and corpus can improve sensitivity of the qPCR techniques [4,5]. On the other hand, as in agreement with some previous studies showing PCR superior to HPE .[15,16,24], we found that qPCR were positive in 5 (42%) of 12 fresh and 2 (5.7%) of 33 paraffinized specimens having negative results with HPE.

When the HPE results is accepted as a gold standard, sensitivity, specificity, PPV, and NPV of DiaRD-HPykl qPCR kit were calculated as 74 %, 94%, 97 %, and 57% on paraffinized tissues and 89%, 58%, 87%, and 64% on fresh tissues, respectively. Correct classification rates were also calculated around 80% for all, fresh, and paraffinized sample groups separately. Although the specificity and PPV seem to be high in paraffinized tissues; specificity and NPV seem to be low in fresh tissues these results may be misleading due to limitation of the ability of HPE as a referee test/gold standard. As it was mentioned above, HPE used as a reference test in this study may give false positive results in the presence of *Helicobacter* species other than *H. pylori* or *Campylobacter jejuni* in the samples and false negative in the presence of intestinal metaplasia or a small amount of *H. pylori* in coccoid form. Therefor positive results obtained by qPCR in HPE-negative samples may in fact be positive, in which case the percentages mentioned will change in favor of qPCR.

Although inter-regional variability is observed, *H. pylori* is becoming increasingly resistant to different antibacterial drugs, especially clarithromycin, metronidazole, and quinolones, and therefor empirical *H. pylori* eradication therapies often fail. Local surveillance studies to determine resistance profiles are necessary to select regionally appropriate eradication regimens [4,5]. Clarithromycin is still the key antibiotic of choice in the first line of *H. pylori* eradication [18,19].

In a meta-analysis including 178 studies from sixty-five countries, primary and secondary resistance rates to clarithromycin among the *H. pylori* isolates were reported as ≥15% [27]. A 24-center study from eighteen European countries found 21.4% resistance to clarithromycin [28], while an analysis including 176 studies from 24 countries in the Asia-Pacific region reported the rate as 17% [29]. Clarithromycin resistance rate was reported as 20-50% in China, 8-31% in Korea, and 38.5% in Japan [5]. The clarithromycin resistance was reported as 26.7% in Turkey in 2023 [30].

The Maastricht V/Florence Consensus Report recommends that if a standard clarithromycin-based protocol is to be used for first-line treatment, clarithromycin susceptibility testing should be performed by a standard culture-based method (antibiogram) or by a molecular test directly on a gastric biopsy specimen [19]. In recent years, there has been a shift from culture-based phenotypic tests to

molecular microbiological methods. Clarithromycin resistance, which is mostly due to point mutations at codon A2143G, A2142G and A2142C in the 23S rRNA V region that led to a conformational change in the peptidyl transferase, can be easily detected by molecular methods [31]. Since these methods can be applied directly to biopsy material and stool samples, they provide advantages in obtaining faster results and increasing eradication success by planning the treatment correctly. PCR based approaches for the detection of clarithromycin resistance are more sensitive than culture. [19,31]

In our study, using the DiaRD-HPykl qPCR kit that searches mutations at codon A2143G, A2142G and A2142C in the 23S rRNA, the clarithromycin resistance was detected in 14 (12.5%) of a total of 112 samples with positive qPCR results from pediatric patients. Although this rate is below the threshold value of 15% for antibiogram requirement, it closes to the rate (9.50%) reported by Çagan-Appak et al. [32] and it is below the current rate reported as >20% for pediatric patients in our country[33]. Considering the location of our hospital in the country and province and the diversity of the patient population admitted, it cannot be said that this is an exceptional situation specific to the region/population. Although a possible explanation for the low rate of macrolide exposure in the pediatric patient group included in our study may come to mind, we do not have concrete data on this. Another possible explanation is clarithromycin heteroresistance, which might be present in the isolates and not detected by qPCR, leading to false negative results. Quite different rates of clarithromycin heteroresistance have been reported from various countries. A recent metanalysis found an average heteroresistance rate of 6.8%, ranging from 1% to 24%.[34]. Since the probe in the DiaRD-HPykl qPCR kit was designed for the "wild" type-specific sequence in 23S rDNA in the sample, it might lead to the omission of resistant DNAs that may coexist with wild type DNA in the population.

In conclusion, the DiaRD-HPykl qPCR kit has statistically moderate compatibility with HPE in terms of detecting *H. pylori* in gastric biopsy specimens, especially in fresh specimens. It gives results within two hours after the sample reach to laboratory. Considering these features, it is an advantageous test option for early planning and management of treatment. It can be used in combination with HPE or as an alternative to HPE. It can be said that the positive results produced by this kit in the determination of clarithromycin resistance can also be trusted, but negative results should be evaluated carefully - considering the high average resistance rate in the country - and the 12.5% rate found in our study needs to be confirmed by larger scale studies.

Statements and declarations

Ethics committee approval

This study was performed in line with the principles of the declaration of Helsinki. Approval was granted by the Ethics Committee of the Ankara Yıldırım Beyazıt University Medical Faculty (Date: 07.04.2021/ No:36). Parents of all patients signed informed consent forms for using the anonimysed clinical samples in the study.

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Availability of data and materials

All data within the article or available on request from the authors.

Conflict of interest

The authors have no conflict of interest regarding this study.

Author contributions

All authors contributed to the study conception and design. Material preparation, sample and data collections were performed by Ahmet Murat YAVAŞ, Ziya Cibali AÇIKGÖZ, Rıza DURMAZ, Şamil HIZLI, Fatma YILDIRIM and Fatih DURAN. Experimental analyses were performed by Ahmet Murat YAVAŞ, Ziya Cibali AÇIKGÖZ and Rıza DURMAZ. Statistical analyses were performed by Pervin DEMİR. The first draft of the manuscript was written by Ahmet Murat YAVAŞ and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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