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ORIGINAL ARTICLE

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Adaptation of an in-house PCR for the detection of *Helicobacter pylori* and the mutations associated with macrolide resistance into ready-to-use PCR microwell strips

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Abstract

Background and Objectives: The present study describes the successful adaptation of an in-house Polymerase Chain Reaction (PCR) for *Helicobacter pylori* detection coupled with the main mutations associated with resistance to clarithromycin in ready-to-use PCR microwell strips.

Materials and Methods: These microwell strips can be used on LightCycler® 480, and are delivered with nine microliters of the reaction mixture dispensed into 8-well microwell strips. An extraction control PCR targeting the β-globin household gene is amplified in the same run as *H pylori* detection.

Results and Conclusion: These microwell strips can be stored at −20°C for 1 year and left at room temperature and in the light for up to 4 h with no impact on the PCR results. Microwell strips can also undergo a thaw and refreeze cycle without impacting the PCR results. These PCR microwell strips are available for purchase from Eurogentec.

KEYWORDS
clarithromycin resistance, *H pylori*, microwell strip, PCR

1 | INTRODUCTION

The PCR diagnosis of *Helicobacter pylori* infection allows a rapid, automated diagnosis of this infection and simultaneously detects the main mutations associated with resistance to clarithromycin located in *23S rDNA*.[1,2] Several commercial PCR formats exist, in particular, in the form of real-time PCR.[3] Some laboratories have developed in-house real-time PCRs.[4,5] Since 2003, the French National Reference Center for Campylobacters and Helicobacters (NRCCH) (Bordeaux, France) routinely uses a PCR in fluorescence resonance energy transfer format (FRET).[6] Our expertise in the field of diagnosis of *H pylori* infections means that we are regularly solicited to evaluate the performance of new real-time PCR kits.[3,7,8] Our in-house PCR has equivalent performances in terms of sensitivity and specificity compared with these kits.[3,7,8] It is however difficult to transfer from our laboratory to another, especially, if it does not properly control the entire analytical process, from DNA extraction to the preparation, in secure rooms, of the PCR reagents. The NRCCH is embarked on an accreditation process for its activities, notably with the desire to keep our in-house *H pylori* PCR automated, to simplify the analytical process, and to limit the risk of contamination.

Our aim was first to adapt our in-house *H pylori* PCR in ready-to-use microwell strips in order to facilitate the accreditation of our in-house PCR. Indeed, the validation and traceability of an in-house PCR is tedious. In order to facilitate the accreditation of our in-house *H pylori* real-time PCR, the NRCCH collaborated with the company Eurogentec to set up ready-to-use PCR microwell strips. The interest of these microwell strips is the insurance of a certificate

of cleanroom manufacturing and quality control, but this required adaptation of our in-house PCR. The second aim was to offer the possibility to interested biologists to use these microwell strips that are cheaper than commercialized PCRs.

Significance statement

Real-time PCR is one of the key diagnostic methods for the diagnosis of *Helicobacter pylori* infection. This study describes the adaptation of an in-house PCR for *H pylori* detection coupled with the main mutations associated with resistance to clarithromycin in ready-to-use PCR microwell strips. They can be purchased by any laboratory to the Eurogentec Company.

2 | MATERIALS AND METHODS

2.1 | DNA extraction on gastric biopsies

One hundred three gastric biopsies (47 *H pylori* negative and 56 *H pylori* positive (30 wild-type (WT), 11 A2142/43G mutation, 14 WT+A2142/43G mutation, 1 WT+A2142C mutation); *H pylori* status based on PCR and culture results), received at the NRCCH between 2018 and 2019, were digested and extracted using the MagNA Pure 96 DNA and Viral NA SV kit on the MagNA Pure 96 extractor (Roche Diagnostics), following the supplier's recommendations. These biopsies were from patients living in France, aged between 11 and 84 years old (average 46 years old), and with a sex ratio of 0.84.

Helicobacter pylori strains with known *23S rDNA* mutations: the WT strain (of reference CCUG 17874), mutated strains 4277, 4240, and 4538, from the NRCCH collection, with the mutation A2142G, A2143G, and A2142C, respectively. These DNAs, extracted with the same method as gastric biopsies, are used to determine the stability and sensitivity limit of the microwell strips.

2.2 | Design of PCR microwell strips

Our in-house PCR for the detection of *H pylori* in FRET format published in 2003,[6] required the preparation of a reaction mix and the use of capillaries onLightCycler® 2.0 (LC2.0) (Roche Diagnostics) for PCR. Eurogentec prepared for us the reaction mixture in a clean room while keeping the primers and probes for *H pylori* detection (Table 1) and at the same concentrations (0.4 and 0.2 μM, respectively) as originally published.[6] Among the few adaptations, the LightCycler® FastStart DNA Master HybProbe Master Mix (Roche Diagnostics) has been replaced by the Takyon ™ No ROX Probe 2X Master Mix dTTP master mix (Eurogentec), and the LC640 fluorophore of the emitter probe was also modified to ATTO647N, following step by step optimization on a limited number of samples in collaboration with Eurogentec. The microwell strips are in plastic, and the total volume is 9 μl in each well.

PCR amplification using the microwell strips on LightCycler® 480 (LC480) comprised an initial denaturation cycle at 95°C for 10 min, followed by 45 amplification cycles (with a temperature transition rate of 2.20°C/s) consisting of 95°C for 10 s, annealing at 60°C for 20 s, and extension at 72°C for 15 s. After amplification, a melting step is performed, consisting of 95°C for 30 s, cooling to 40°C for 50 s (with a temperature transition rate of 1.5°C/s), and finally a slow rise in the temperature to 85°C at a rate of 0.29°C/s with continuous acquisition of fluorescence decline.

The microwell strips are delivered to the NRCCH with nine microliters of the reaction mixture dispensed into eight-well microwell strips, closed with a hermetic cap. An extraction control PCR targeting of the β-globin household gene, already routinely used at the CNRCH (Table 1), was also adapted in microwell strips, in FRET format, in order to be amplified in the same run as *H pylori* detection. These microwell strips can be stored at −20°C for 1 year. Each DNA to be tested is added to the reaction well before amplification on LC480.

TABLE 1 Primers and probes used for 23S rDNA and *B*-globin PCR

	Primer sequences	Modifications	Final concentration in μM
H pylori—23S rDNA			
HpyS	AGGTTAAGAGGATGCGTCAGTC		0.4
HpyA	CGCATGATATTCCCATTAGCAGT		0.4
ANCHOR-FL	TGTAGTGGAGGTGAAAATTCCTCCTACCC	3′FAM	0.2
HpyRED	GGCAAGACGGAAAGACC	5′ATTO647N-3′P	0.2
Human—*B*-globin			
bGlob_fwd	TACGGCTGTCATCACTTAGAC		0.4
bGlob_rev	CTTCATCCACGTTCACCT		0.4
bGlob_donorP	CAAACAGACACCATGGTGCACCTGACTCCTGAGGA	3′FAM	0.2
bGlob_accepP	AAGTCTGCCGTTACTGCCCTGTGGGGCAA	5′ATTO647N-3′P	0.2

TABLE 2 Ct values for *23S rDNA* and B-globin for all positive *Helicobacter pylori* samples on LC2.0 and LC480

	23S rDNA				*B-globin*			
	Capillaries		Microwell strips		Capillaries		Microwell strips	
Sample	LC2.0	Ct	LC480	Ct	LC2.0	Ct	LC480	Ct
2018/0555	WT	24.7	WT	23.1	POS	19	POS	24.5
2018/1375	G+WT	25.8	G+WT	24.1	POS	15.6	POS	20.7
2018/1472	C+WT	26.6	C+WT	25	POS	17.3	POS	21.6
2018/1499	G+WT	28.8	G+WT	27.1	POS	17	POS	22.1
2018/1535	G+WT	33.9	G+WT	32.8	POS	20	POS	25.6
2018/1542	G+WT	21.3	G+WT	20.1	POS	18.1	POS	20.6
2018/1561	G+WT	25.4	G+WT	24	POS	17.2	POS	22.5
2018/1592	G+WT	22.9	G+WT	21.2	POS	17	POS	22.1
2018/1603	G+WT	22.1	G+WT	20.9	POS	18	POS	23.3
2018/1614	G+WT	25.8	G+WT	24	POS	17	POS	23.5
2018/1616	WT	33.6	WT	31.8	POS	17.3	POS	23.8
2018/1647	G+WT	27.4	G+WT	26	POS	15.9	POS	21.2
2018/1682	G+WT	29.1	G+WT	27.2	POS	19.5	POS	23.3
2018/1703	WT	31	WT	29.9	POS	15.7	POS	27.2
2019/0004	G+WT	25.1	G+WT	23.6	POS	20.7	POS	26
2019/0083	G+WT	32.3	G+WT	30.3	POS	17.3	POS	23.1
2019/0103	WT	17.7	WT	14.9	ND		ND	
2019/0106	WT	26.8	WT	24.1	POS	15.8	POS	21.1
2019/0108	WT	29.7	WT	25.6	POS	17.6	POS	22.7
2019/0113	G+WT	24.2	G+WT	22.8	POS	16.4	POS	22
2019/0142	G	32.7	G	31	POS	15.8	POS	21.2
2019/0149	G	24	G	22.6	POS	15.6	POS	21.1
2019/0157	WT	35.7	WT	33.1	POS	19.6	POS	25.1
2019/0171	WT	34	WT	33.2	POS	21.6	POS	27.3
2019/0176	WT	24.2	WT	23.6	POS	16.6	POS	22.1
2019/0177	WT	22	WT	21	POS	17.5	POS	22.5
2019/0178	WT	21.9	WT	20.5	POS	16.2	POS	21.9
2019/0179	WT	23.8	WT	22.9	POS	19.8	POS	25.5
2019/0180	G+WT	25.6	G+WT	24.9	POS	17	POS	22.5
2019/0182	WT	29.9	WT	28.6	POS	15.8	POS	21.1
2019/0217	WT	30.9	WT	29.4	POS	18.4	POS	23.9
2019/0220	neg	.	WT	35.9	POS	18.8	POS	24.5
P850	G	33.7	G	31.2	POS	18.6	POS	24.3
P857	WT	32.7	WT	30.3	POS	16	POS	21.8
P914	WT	33.8	WT	32	POS	17	POS	22.7
2019/229	WT	30.2	WT	28.7	POS	15.5	POS	21
2019/237	WT	22.8	WT	21.5	POS	16.6	POS	22
2019/246	WT	30.1	WT		POS	15.8	POS	21.5
2019/252	WT	23.7	WT	22.5	POS	17.9	POS	23.6
2019/259	WT	23.4	WT	22	POS	16.5	POS	22.1
2019/264	WT	27.3	WT	26	POS	17.8	POS	23.6
2019/266	G	28.1	G	27.1	POS	17.5	POS	23.3
2019/269	G	31	G	30.8	POS	16.8	POS	22.3

(Continues)

TABLE 2 (Continued)

	23S rDNA				*B-globin*			
	Capillaries		Microwell strips		Capillaries		Microwell strips	
Sample	LC2.0	Ct	LC480	Ct	LC2.0	Ct	LC480	Ct
2019/270	WT	30.7	WT	28.9	POS	21.5	POS	27.5
2019/271	WT	22.4	WT	31.4	POS	16.8	POS	22.7
2019/281	WT	23.9	WT	23.5	POS	17.8	POS	24
2019/282	G	25.5	G	25.7	POS	18.1	POS	24.1
2019/295	G	28.7	G	26.9	POS	19.1	POS	25.3
2019/301	G	27	G	25.2	POS	18.1	POS	23.8
2019/304	G	24.4	G	23.5	POS	18.2	POS	24.1
2019/313	WT	22.2	WT	20.9	POS	19.1	POS	24.5
2019/317	G	23.3	G	21.6	POS	33.9	POS	17.2
2019/324	WT	26.1	WT	24.3	POS	16.5	POS	21.2
2019/332	WT	20.7	WT	19.3	POS	17.7	POS	22.4
2019/336	G	27.6	G	25.1	POS	16.9	POS	21.7
2019/340	WT	29.9	WT	27.8	POS	17.7	POS	22.6

23rDNA genotypes are indicated: WT for wild-type, G for A2142/43G mutations.
The sample 2019/0103 is a strain: the Ct for B-globin cannot be reported.
Abbreviation: ND, not determined.

The results obtained on LC480 using the microwell strips were compared with those obtained on LC2.0 (gold standard).

2.3 | Ethics

All diagnostic methods were performed routinely. All patients were investigated in a hospital and private clinics setting, according to good clinical practices. In this routine process, the consent for the endoscopic procedure, and stool collection is always given in writing and kept in the patient's medical record. No informed consent for using human gastric DNAs or remaining stool samples were requested from the patients.

3 | RESULTS AND DISCUSSION

Comparison of PCR results obtained in capillaries and in microwell strips: Each DNA sample was tested on LC2.0 in capillaries, as previously described,[6] and on LC480 in Eurogentec microwell strips, the results were then compared. A 99% percentage overall agreement for the detection of *H pylori* was found (Table 3), 98.2% percentage positive agreement, and 100% percentage negative agreement. Using PCR microwell strip, one sample was positive (late detection with Ct = 35.9) while it was negative in capillaries. The positivity of this sample was confirmed by a commercial PCR using the Amplidiag® *H pylori* + ClariR (Mobidiag).[3,9] This discordant result could be explained by the change of fluorophore in PCR microwell strip which allowed a slight increase of PCR detection with a gain of 1.4 Ct on average (average of the Ct values obtained for the 55 positives samples with the two instruments: 27.1 for LC2.0 and 25.7 for LC480) (Table 2).

TABLE 3 Comparison of *Helicobacter pylori* detection on LC2.0 (capillaries) and LC480 (microwell strips)

	Capillaries 2.0	
	Positive	Negative
Microwell strips LC480		
Positive	55	0
Negative	1	47

The determination of the genotypes gave 100% agreement for WT, resistant of mix-population. *B*-globin PCR was positive in all 103 samples, with a mean Ct of 23.1, indicating a correct DNA extraction of the gastric biopsies. In conclusion, the two PCRs targeting *H pylori* and *B*-globin, can be performed in the same run, which allow to identify potential problem of DNA extraction or the presence of PCR inhibitors.

3.1 | Microwell strips stability

According to the manufacturer, upon receipt, the microwell strips are stored frozen at −20°C and are valid for 1 year. The stability of the microwell strips was nevertheless evaluated in particular their stability at room temperature and at 4°C before PCR. The acceptance criteria for the conditions tested were a bias <5% (% of variation = 100 × (mean delay − mean T0)/mean T0). To perform these tests, two DNAs (one extracted from a suspension prepared at 10^7

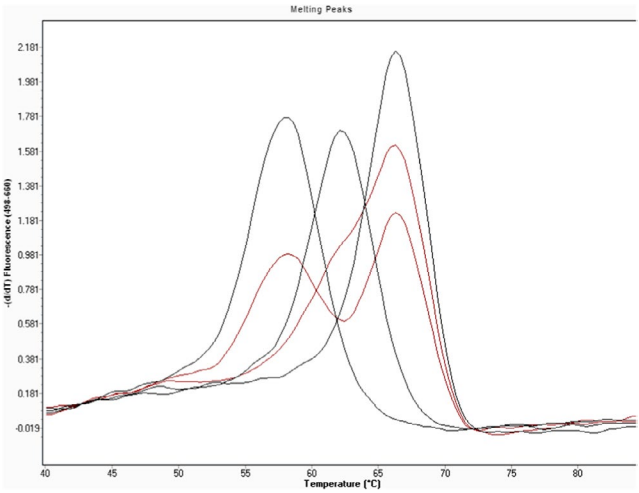

FIGURE 1 Melting peaks analysis of the *23S rRNA* gene obtained with LC480. Melting peaks for the wild-type (WT) and A2142C and A2142/43G mutations, and a mixture population of WT+A2142/43G or WT+A2142C are shown. Melting peaks with a Tm of approximately 65°C is obtained for WT genotype, 61°C for A2142C genotype, and 57°C for A2142/43G genotypes

H pylori/ml, and one at 10^4 *H pylori*/ml) from strains of each genotype (WT-*H pylori* CCUG 17874; and mutated *H pylori* strains 4277, 4240, and 4538, were used (Figure 1). Each DNA has been tested twice. The results of the stability tests are shown in Table 4, with the average of Ct obtained for the two DNA. The use of microwell strips that have been left at room temperature and in the light for up to 4 h did not impact the PCR results. It was not the case at *T* + 24 h (Table 4).

Microwell strips can also undergo a thaw and refreeze cycle without impacting the PCR results.

3.2 | Microwell strips sensitivity limit

The four *H pylori* strains with known *23S rDNA* mutations, CCUG 17874 (WT), 4277 (A2142G), 4240 (A2143G), and 4538 (A2142C), at 1.77×10^8, 3.8×10^8, 2.55×10^8, and 3.2×10^8 CFU/ml, respectively, were prepared in 10-fold dilutions in order to evaluate the sensitivity limit of the PCR on LC480. Each dilution was tested in triplicate (Table S1). Slope and efficiency for CCUG 17874 (WT), 4277 (A2142G), 4240 (A2143G), and 4538 (A2142C) were respectively −3.48 and 93.7%, −3.6 and 88%, −3.5 and 92.6%, and −4.06 and 76.2%. From 10^2 CFU/ml (dilution to 10^{-6}), no amplification was obtained (Figure S1). The detection limit was therefore determined at 1.10^3–4.10^3 CFU/ml.

4 | CONCLUSION

We successfully adapted our in-house *H pylori* PCR in ready-to-use microwell strips. This new PCR in microwell strips saves time, offers a better control of reagents, and will facilitate the accreditation of our in-house PCR. Since February 2019, *H pylori* PCR microwell strips are routinely used at the NRCCH. These microwell strips are also cheaper than commercialized PCR kits. The quality control purchased each year to the Quality Control for Molecular Diagnosis,

TABLE 4 Microwell strips stability tests

Tested conditions	Strain (genotype)	Average of Ct at T0	Average of Ct after test	% of variation
1. Microwell strips left at room temperature and in the light for 1 h 30 min	CCUG (WT)	27.5	27.7	0.85
	4277 (A2142G)	27.7	27.7	0.18
	4240 (A2143G)	26.6	26.8	0.88
	4538 (A2142C)	27.3	27.2	−0.43
2. Microwell strips remained at room temperature and in the light for 4 h	CCUG (WT)	27.5	27.4	−0.30
	4277 (A2142G)	27.7	27.4	−0.90
	4240 (A2143G)	26.6	26.3	−1.00
	4538 (A2142C)	27.3	26.8	−2.07
3. Microwell strips remained at room temperature and in the light for 24 h	CCUG (WT)	27.5	27.7	0.85
	4277 (A2142G)	27.7	27.5	−0.54
	4240 (A2143G)	26.6	29.2	9.59
	4538 (A2142C)	27.3	30.5	11.59
4. Deposit samples in microwell strips that have been thawed once, refrozen, and thawed again	CCUG (WT)	27.5	28.1	2.00
	4277 (A2142G)	27.7	27.7	0.3
	4240 (A2143G)	26.6	26.2	−1.63
	4538 (A2142C)	27.3	27.4	0.31

Helicobacter pylori CCUG 17874, 4277, 4240, and 4538: *H. pylori* strains from the NRCCH collection with known *23SrDNA* mutations used for the present study.

% of variation = 100 × ((mean delay − mean T0)/mean T0).

H pylori EQA program, are correct. Laboratories interested in this PCR can contact Eurogentec for manufacturing and ordering. Moreover, this technological evolution is adaptable to other home-made PCR formats.

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CONFLICT OF INTEREST

The authors declare no conflict of interest. Eurogentec (Liège, Belgique) collaborated with the NRCCH to design the microwell strips but was not involved in the data analysis.

AUTHORS' CONTRIBUTIONS

Philippe Lehours supervised the study. Lucie Bénéjat, Emilie Bessède, Francis Mégraud, and Philippe Lehours analyzed the data and drafted the paper. Lucie Bénéjat, Astrid Ducournau, and Chloé Domingues-Martins performed the experiments. All authors interpreted the data. All authors critically revised the manuscript for important intellectual content.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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