

## Quadruplex Real-Time PCR Assay Using Allele-Specific Scorpion Primers for Detection of Mutations Conferring Clarithromycin Resistance to *Helicobacter pylori*<sup>∇†</sup>

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**We developed a single-vessel multiplex real-time PCR assay that detects *Helicobacter pylori* infection and identified the four existing alleles of the 23S rRNA genes of *H. pylori*—the wild-type sequence and the three mutations conferring clarithromycin resistance—using allele-specific Scorpion primers directly on biopsy specimens. The Scorpion primers combine a primer and a probe in a single molecule and are able to distinguish single-nucleotide polymorphism. Fluorescent signals, produced when the probes are annealed, are read in four channels by a SmartCycler thermocycler. The assay was first applied successfully on 4 reference and 61 clinical strains. MICs of clarithromycin were determined by the Etest method. A perfect concordance was obtained between Etest and Scorpion PCR. Mixed populations were better detected by Scorpion PCR. We examined 259 biopsies from 229 patients by culture, PCR-restriction fragment length polymorphism (RFLP), and Scorpion PCR. One biopsy, positive for culture, exhibited inhibitors for both PCR-RFLP and Scorpion PCR. Twelve biopsies were positive for PCR-RFLP and Scorpion PCR but negative for culture with concordant determination of mutations in the 23S rRNA genes by the two PCR assays. Three biopsies were positive for Scorpion PCR only. Compared to culture, the sensitivity of Scorpion PCR was 98.3% and the specificity was 92.5%. The Scorpion PCR assay provides a highly accurate, rapid, and precise method for the detection and determination of mutations conferring clarithromycin resistance to *H. pylori*.**

*Helicobacter pylori* infection is one of the most common chronic bacterial infections in the world and has been established as a major cause of gastritis, peptic ulcer disease, and gastric cancer (5). Eradication therapy is recommended for patients with peptic ulcer disease, and recent guidelines extended indications of the eradication to mucosa-associated lymphoid tissue lymphoma, atrophic gastritis, first-degree relatives of gastric cancer patients, unexplained iron deficiency anemia, and chronic idiopathic thrombocytopenic purpura (13). Clarithromycin (Cla) is a key component of standard triple therapy used in the eradication of *H. pylori*. However, Cla resistance is increasing, reaching 20% in many countries (15). Cla resistance is the main risk factor for treatment failure: resistance reduces the efficacy of the first-line therapy by up to 70% (4, 10, 15). In countries where the prevalence of Cla resistance is high, Cla susceptibility tests should be performed to avoid Cla treatment in case of resistance. The threshold of the Cla resistance rate at which this antibiotic should not be used or a Cla susceptibility test should be performed is 15 to 20% (13).

The mechanism of resistance to Cla in *H. pylori* involves decreased binding of the antibiotic to the bacterial ribosome (18) and is due to point mutations within the 23S rRNA peptidyltransferase-encoding region. Three major point mutations

in two adjacent positions on the 23S rRNA genes are responsible for the Cla resistance in *H. pylori*. An adenine is replaced by a guanine or a cytosine residue in two different positions: A2143G, A2142G, and A2142C (18, 22, 25). Mutations A2143G and A2142G are the most frequently reported, whereas mutation A2142C is less common (24). Other rare mutations have also been identified, such as A2115G, G2141A, T2717C, and T2182C (2, 7, 11). The consequences for Cla resistance of these rare mutations are still under debate since A2115G, G2141A, and T2717C mutations have never been subsequently reported by other authors and since T2182C was also observed in Cla-susceptible isolates (2).

In routine practice, the detection of Cla resistance is mainly based on phenotypic methods performed after culture: agar diffusion for the disk diffusion and Etest or the agar dilution method, which is considered the reference (9, 16). These methods, however, are time-consuming and can take up to 2 weeks to be completed.

Detection of point mutations conferring resistance to Cla by molecular methods was developed to shorten the response delay. Several PCR-based assays that have been developed as alternatives to phenotypic methods for Cla susceptibility rely on the detection of resistance-associated point mutations, and include PCR-restriction fragment length polymorphism (RFLP) (6, 18, 20, 25), real-time PCR, fluorescent in situ hybridization (23), PCR line probe assays (24), 3'-mismatched reverse primer PCR (6), and double gradient-denaturing gradient gel electrophoresis. The molecular techniques correlate well with the conventional agar dilution, disk diffusion, and Etest for Cla.

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Several real-time PCR assays for Cla susceptibility determination of *H. pylori* have subsequently been published. Since 2001, seven studies using real-time PCR were performed on cultured strains or biopsy specimens to determine *H. pylori* susceptibility to Cla (3, 6, 8, 12, 14, 19, 21). All of them are two-step processes using LightCycler PCR for detection of *H. pylori* and are followed by a melting curve analysis by biprobe or HyProbe to detect resistance. Melting curve analysis is indirect and imprecise to determine accurately the different mutations. The occurrence of close melting temperatures ( $T_m$ s) between the two main mutations A2143G and A2142G rendered their differentiation difficult.

Scorpion primer technology allows the precise discrimination between different alleles of a target nucleotide in a single-step process (17, 26, 27). With Scorpion primers, sequence-specific priming and PCR product detection are achieved using a single oligonucleotide. The Scorpion primer maintains a stem-loop configuration in the unhybridized state. The fluorophore is attached to the 5' end and is quenched by a moiety coupled to the 3' end. The 3' portion of the stem also contains sequence that is complementary to the extension product of the primer. This sequence is linked to the 5' end of a specific primer via a nonamplifiable monomer. After extension of the Scorpion primer, the specific probe sequence is able to bind to its complement within the extended amplicon, thus opening up the hairpin loop. This prevents the fluorescence from being quenched, and a signal is observed. Scorpion primers have already been used for the detection of several pathogens (17, 27).

The aim of the present study was to develop a rapid and reliable single-step method to detect the wild type and the three most frequent Cla resistance-associated gene mutations in *H. pylori* directly on gastric biopsies, based on a real-time PCR assay using Scorpion primers with a SmartCycler thermocycler (Cepheid, Sunnyvale, CA).

The specificity and convenience of use of this Scorpion PCR assay format were compared with those of PCR-RFLP and conventional disk diffusion and Etest methods using a panel of 259 biopsy specimens from dyspeptic patients in Poitiers, France.

#### MATERIALS AND METHODS

**Bacterial strains and gastric biopsy specimens.** Four *H. pylori* strains—one reference strain (J99) with the wild-type phenotype/genotype (1) and three Cla-resistant strains (HP 825, HP 225, and HP 222) with mutations determined by sequencing of the 23S rRNA gene (mutations A2142C, A2142G, and A2143G, respectively)—were used as positive controls (20).

We examined a series of 259 preserved ( $-20^{\circ}\text{C}$ ) gastric biopsy specimens from 229 patients (199 patients with a single biopsy specimen, 17 patients with a pair of antrum and fundus concomitant biopsy specimens, 12 patients with two iterative biopsy specimens, and one with three iterative biopsy specimens) who underwent gastroduodenoscopy in Poitiers university hospital during 2005 and 2006.

The biopsy specimens (approximately 10 to 20 mg) were ground with a sterile scalpel in a petri dish. Half of this material was used for culture and half for DNA extraction.

Grinding material was spread on a Columbia agar plate supplemented with 5% (vol/vol) sheep blood and Skirrow antibiotic supplement (Oxoid, Basingstoke Hampshire, United Kingdom). The inoculated plates were placed at  $37^{\circ}\text{C}$  for 5 to 10 days under microaerobic conditions by using the GENbox Microaer generator (bioMérieux, Marcy l'Etoile, France) in an anaerobic jar. Identification of *H. pylori* was based on colony morphology; typical appearance on Gram staining; and positive urease, catalase, and oxidase activities.

DNA was isolated by using QIAamp DNA mini kit (Qiagen, Courtaboeuf,

France) according to the manufacturer's instructions. Lysis of biopsy specimens was performed on half of the grinding material in 180  $\mu\text{l}$  of ATL buffer (Qiagen, Courtaboeuf, France) for 1 h at  $56^{\circ}\text{C}$  and then  $95^{\circ}\text{C}$  for 10 min. The isolated DNA was eluted in 100  $\mu\text{l}$  of  $1\times$  TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and stored at  $-20^{\circ}\text{C}$  until use.

DNA extraction from cultured bacteria was performed on a 10- $\mu\text{l}$  loop of fresh culture suspended in 180  $\mu\text{l}$  ATL buffer, and the elution volume was 200  $\mu\text{l}$ .

**Determination of the Cla susceptibility by phenotypic methods.** Two phenotypic methods were used to determine the Cla susceptibility of the isolated strains: disk diffusion and Etest. Both methods were used according to a previously described protocol recommended by the French Study Group of *Helicobacter* (9). A bacterial suspension was prepared to a McFarland 3 turbidity standard (approximately  $10^9$  CFU/ml of *H. pylori*), spread with a sterile swab dipped into the inoculum suspension, and swabbed in three directions on a Mueller-Hinton agar plate supplemented with 10% horse blood.

A disk containing 15  $\mu\text{g}$  of erythromycin (Bio-Rad, Marnes-la-Coquette, France) and a Etest strip of Cla (AB Biodisk, Solna, Sweden) were applied to inoculated blood agar plates. Cla susceptibility was recorded after microaerobic incubation for 48 h at  $37^{\circ}\text{C}$ . A strain was considered resistant to Cla when the inhibition diameter for erythromycin was  $<17$  mm or the Cla MIC was  $\geq 1$  mg/liter.

Strains were stored at  $-80^{\circ}\text{C}$  in 3 ml meat-liver medium (bioMérieux, Marcy l'Etoile, France) added with 0.3 ml of glycerol.

**Detection of Cla resistance by PCR-RFLP analysis.** PCR-RFLP analysis was performed on all of the 259 biopsies. The enzymes BbsI and BsaI (New England Biolabs, Beverly, MA) were used in each case. We choose to study the two main mutations A2143G (detected by the BsaI enzyme) and A2142G (detected by the BbsI enzyme) because the mutation A2142C represents only 0% to 7% of the reported mutations (16, 24). We anticipated a direct sequencing if none of the aforementioned mutations was detected by PCR-RFLP while Scorpion PCR detected the A2142C mutation, which was the case in two of all tested biopsy specimens.

PCR-RFLP was performed as previously described (20). A 1,400-bp fragment of the 23S rRNA gene was amplified with primers 18 (5'-AGTCGGGACCTA AGGCGAG-3') and 21 (5'-TTCCCGCT6TAGATGCTTTCAG-3') (25).

PCR amplification of DNA was performed in a final volume of 50  $\mu\text{l}$  containing 10  $\mu\text{l}$  of biopsy genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01% gelatin, 1.5 mM  $\text{MgCl}_2$ , a 0.2 mM concentration of deoxynucleoside triphosphate mixture, a 1  $\mu\text{M}$  concentration of primers, and 2 U of *Taq* DNA polymerase (Sigma-Aldrich, Saint Quentin Fallavier, France). The cycling program was 1 cycle at  $94^{\circ}\text{C}$  for 10 min; 35 cycles of  $94^{\circ}\text{C}$  for 30 s;  $60^{\circ}\text{C}$  for 30 s;  $72^{\circ}\text{C}$  for 30 s; and a final elongation step at  $72^{\circ}\text{C}$  for 10 min.

The amplicon was digested with BsaI (New England Biolabs, Beverly, MA) for 1 h at  $50^{\circ}\text{C}$  and BbsI (New England Biolabs, Beverly, MA) for 1 h at  $37^{\circ}\text{C}$  to detect the restriction site occurring when the mutation was A to G at 2143 or 2142, respectively. The restriction products were analyzed by electrophoresis on a 2% agarose gel. BsaI cuts the PCR product of the wild-type sequence into two fragments of 1,000 and 400 bp and that of the A2143G sequence into three fragments of 700, 400, and 300 bp. BbsI cuts the PCR product into two fragments of 700 bp only when A2142G is present in the sequence.

**Determination of point mutation in the 23S rRNA gene of *H. pylori* by Scorpion PCR.** Primers were designed to target the location of the two adjacent adenines where mutations conferring Cla resistance occurred in *H. pylori* 23S rRNA (A2142 and A2143). The forward primer, 23SF2, a regular primer, was located 107 to 125 bases upstream of the adenines. Reverse Scorpion primers were designed to end on their 3' end with the complement of the mutation to detect, like 3'-mismatched reverse primers (6). The tails of the Scorpion primers were designed with the service of PrimerDesign, Ltd. (Southampton, United Kingdom) and synthesized by Atdbio (School of Chemistry, University of Southampton, United Kingdom). Primers and Scorpion primer sequences are shown in Table 1. They amplify a 140-bp fragment in the 23S rRNA genes.

PCR was performed in a final volume of 25  $\mu\text{l}$  with the Premix Ex *Taq* (TaKaRa, Shiga, Japan), 5  $\mu\text{l}$  of extracted DNA from biopsy specimen, or 1  $\mu\text{l}$  from culture, 0.1  $\mu\text{M}$  of oligonucleotide primer 23SF2, 0.14  $\mu\text{M}$  of 23SscA2142G, 0.18  $\mu\text{M}$  of 23SscA2143G, 0.1  $\mu\text{M}$  of 23SscA2142C, and 0.08  $\mu\text{M}$  of 23SscWT. Amplification was performed after a denaturation step ( $95^{\circ}\text{C}$  for 15 s) for 50 cycles of denaturation ( $95^{\circ}\text{C}$  for 15 s), annealing ( $55^{\circ}\text{C}$  for 30 s), and extension ( $72^{\circ}\text{C}$  for 20 s). The fluorescence reading for each sample was taken at the annealing step on four channels (6-carboxyfluorescein [FAM], Texas red, Cy3, and Cy5). Data analysis was performed with Cepheid software (Cepheid, Sunnyvale, CA). The cycle threshold ( $C_T$ ) value was calculated for each channel as the number of cycles at which the fluorescence exceeded the threshold limit,

TABLE 1. Scorpion primers and primer sequences

Oligonucleotide	Sequence
23SScWT	5'-FAM AAGGTAGGTGAAAATTCCTCC TACC BHQ1 HEG GGACCACGGGGTCT TT-3'
23SScA2142G	5'-Cy3 AAGGTAGGTGAAAATTCCTCC TACC BHQ1 HEG GGACCACGGGGTCT TC-3'
23SScA2143G	5'-Texas red AAGGTAGGTGAAAATTCCTCC CTACC BHQ2 HEG GGACCACGGGGTCT TC-3'
23SScA2142C	5'-Cy5 AAGGTAGGTGAAAATTCCTCC TACC BHQ2 HEG GGACCACGGGGTCT TG-3'
23SF2	5'-TGCGAAATTCCTTGTCGG-3'
BGLO1	5'-ACACAACCTGTGTGTTCACTAGC-3'
BGLO2	5'-CAACTTCATCCACGTTACCC-3'

which was set at the top of the second derivative fluorescence curve. They were expressed as fractional cycle numbers.

**Determination of the analytical sensitivity of the Scorpion PCR.** To test for the analytical sensitivity of the Scorpion PCR, DNAs extracted from numbered inocula from the four reference strains ( $3.5 \times 10^6$  CFU/ $\mu$ l of DNA for the J99 strain with the wild-type phenotype/genotype,  $1 \times 10^6$  CFU/ $\mu$ l of DNA for the Cla-resistant strain HP 222 (mutation A2143G),  $2.5 \times 10^6$  CFU/ $\mu$ l of DNA for the Cla-resistant strain HP 225 (mutation A2142G), and  $1.75 \times 10^6$  CFU/ $\mu$ l of DNA for the Cla-resistant strain HP 825 (mutation A2142C) were 10-fold diluted to  $10^{-7}$ . The seven 10-fold serial dilutions of each DNA were tested by Scorpion PCR.

Regarding the detection of multiple strains in the same biopsy sample, chromosomal DNA from the wild-type reference strain J99 and from a Cla-resistant reference strain harboring either the A2143G, A2142G, or A2142C mutation was mixed in different proportions: 50/50, 90/10, 95/5, and 99/1. The amounts of DNA of the three Cla-resistant strains at the 99/1 proportion corresponded to  $2.4 \times 10^3$ ,  $1.5 \times 10^3$ , or  $1.1 \times 10^3$  CFU, respectively. These mixtures were analyzed with the Scorpion PCR.

**Scorpion PCR in routine practice.** We performed a prospective study to assess the routine applicability of the Scorpion PCR in which 227 biopsy specimens were obtained over a 1-year period in 2007 from dyspeptic patients referred for endoscopy. Gastric biopsy specimens were processed as described above, except that the DNA extraction was performed with an automated extraction apparatus MagnaPur (Roche Diagnostic, Mannheim, Germany). The grinding material was placed in 380  $\mu$ l of lysis buffer (Roche Diagnostic, Mannheim, Germany); 20  $\mu$ l of proteinase K (Roche Diagnostic, Mannheim, Germany) was added before incubation at 65°C according to the manufacturer's instructions. The elution volume was 100  $\mu$ l. For these 227 biopsy specimens, culture and Scorpion PCR were performed.

A positive control of extraction was obtained by the detection of a 110-bp fragment of a human housekeeping gene coding for  $\beta$ -hemoglobin by real-time PCR using primers BGLO1 and BGLO2 (Table 1) and Sybr green detection. This control was performed for each biopsy specimen in a separate tube containing, in a final volume of 25  $\mu$ l with Premix Ex Taq (TaKaRa, Shiga, Japan), 5  $\mu$ l of extracted DNA from biopsy specimen, 0.25  $\mu$ M of oligonucleotide primers, and 0.5 $\times$  Sybr green 1 (Sigma Aldrich, Saint Quentin Fallavier, France). The cycling program was 1 cycle at 95°C for 10 s and 40 cycles of 95°C for 5 s, 55°C for 3 s, and 72°C for 10 s.

A positive control for Scorpion PCR was constituted by assembling the four extracted DNAs of four *H. pylori* strains: one reference strain (J99) with the wild-type phenotype/genotype (1) and three Cla-resistant strains (HP 825, HP 225, and HP 222) with mutations determined by sequencing of the 23S rRNA gene (mutations A2142C, A2142G, and A2143G, respectively). Two microliters of this mix was used as a positive control in each run of the Scorpion PCR assay.

## RESULTS

**Detection of *H. pylori* and Cla susceptibility testing by culture.** Among the 259 biopsy specimens tested in this study, culture was positive for 60 biopsy specimens and 61 strains

were isolated (one biopsy contained two strains: a susceptible and a resistant strain). Of these 61 strains, phenotypic methods for Cla susceptibility testing (Etest and disk) identified 37 Cla-susceptible strains and 24 Cla-resistant strains. The Etest and disk diffusion method were 100% concordant for the determination of the Cla susceptibility of the strains. Among the 229 patients tested in this study, 58 were detected as infected by *H. pylori* using culture (25%) and 24 were detected as infected with a resistant strain.

**Scorpion PCR on isolated strains.** Scorpion PCR was first performed on DNAs extracted from four reference strains as positive controls for the four possible alleles.

Scorpion PCR on DNA extracts of *H. pylori* control strains produced expected signals for the wild type and A2143G, A2142G, and A2142C mutants. We did not detect any cross-fluorescence during these experiments.

Using serial dilutions of DNA extracted from a standardized inoculum adjusted at  $10^7$  CFU/ml of these strains, we determined the minimal bacterial cell number allowing detection (Fig. 1, and see Fig. S3 to S5 in the supplemental material). The lowest pure *H. pylori* DNA concentration always delivering a positive result corresponded to 3.5, 1, 2.5, and 1.75 cells per  $\mu$ l of extracted DNA for the wild type and A2143G, A2142G, and A2142C mutants, respectively. At the detection limit,  $C_T$  values varied between 43 and 45.

To confirm that the Scorpion PCR could detect the simultaneous presence of different genotypes, the technique was carried out by using mixtures in various proportions of DNA from the wild-type and A2143G, A2142G, or A2142C mutant strains (from 50 to 1%). For all three mutations, the Scorpion PCR was able to detect the mutant DNA until reaching concentration as low as 5% (see Fig. S6 in the supplemental material).

DNA extracted from the 61 strains isolated in this study was tested by Scorpion PCR. Of the 37 strains found as Cla susceptible by the phenotypic methods, the susceptible allele was confirmed by Scorpion PCR in all cases. Of the 24 strains found as Cla resistant by phenotypic methods, Scorpion PCR detected the A2143G allele in 15 cases, the A2142G allele in 6 cases, a mixture of wild-type and A2142C alleles in two cases (isolated from the same patient), and a mixture of the wild type and A2143G alleles in one case.

All strains detected as resistant by phenotypic methods were detected as resistant by Scorpion PCR. Mixed populations were detected by Scorpion PCR, whereas they were not detected by culture and Etest in three cases. In these three cases, the susceptible population was detected by Scorpion PCR but was missed by phenotypic methods.

**PCR-RFLP on biopsy specimens.** Of the 259 biopsy specimens tested, PCR-RFLP was positive for 70 biopsy specimens (Table 2). Of the 60 biopsies positive for culture, PCR-RFLP was positive for 58 biopsies. For these two discrepancies, only a few colonies were grown on the culture plates, indicating a very small amount of bacterial cells present in these biopsies. Twenty-one of 24 resistant strains detected by phenotypic methods were detected by PCR-RFLP and were distributed as follows: 16 A2143G genotypes and 5 A2142G genotypes. Because the restriction enzymes used in this study are not able to detect the A2142C genotype, two resistant strains detected as A2142C genotypes by the Scorpion PCR were not detected by

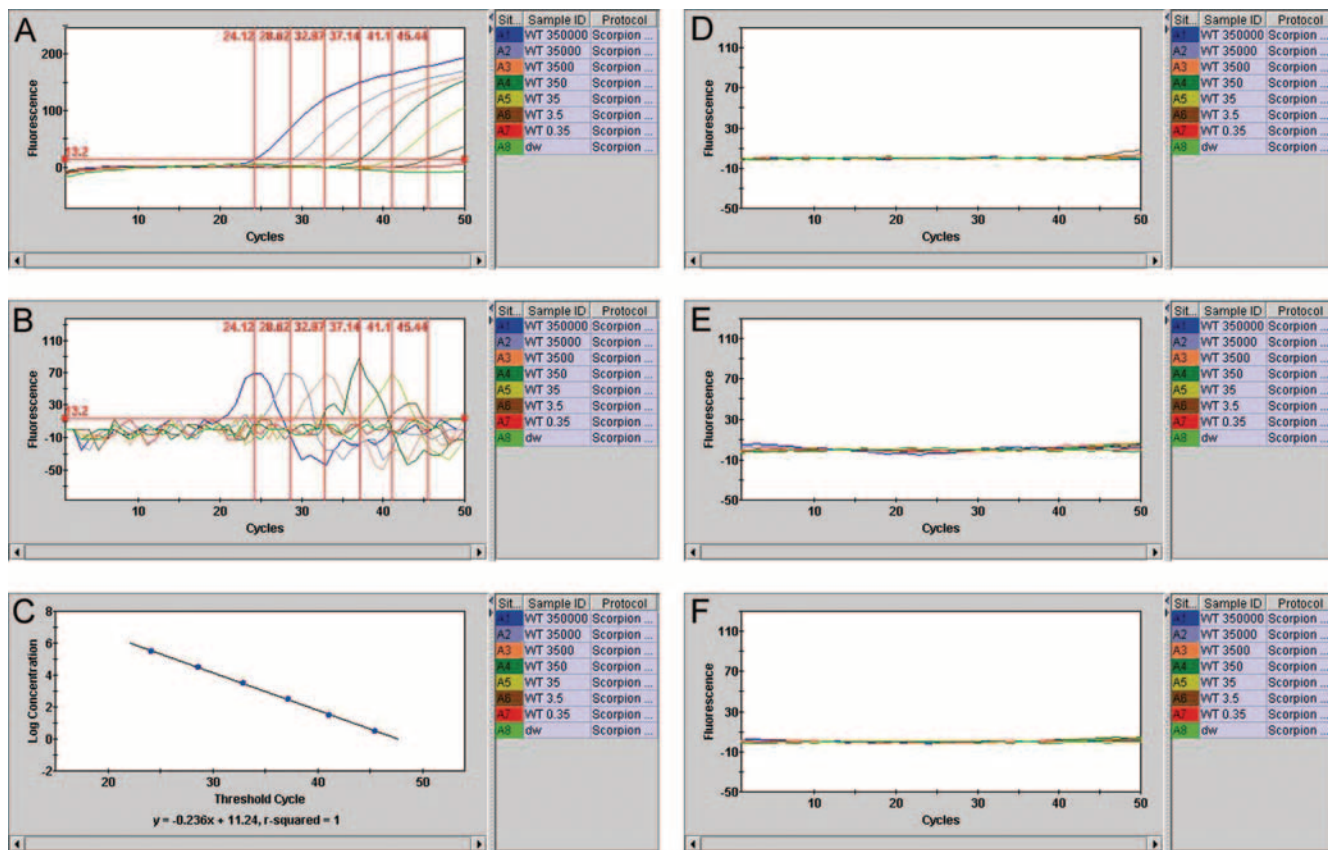


FIG. 1. Example of a sensitivity assay performed on the SmartCycler thermocycler with 10-fold serial dilution of the genomic DNA purified from strain J99. (A) The fluorescence signal was from FAM dye of the 23SScWT-specific Scorpion primer. (B) Second derivative curves of fluorescence for FAM dye. (C) Standard curve plotting log concentrations versus  $C_T$ s, calculated linear fit equation, and correlation coefficient. (D) Fluorescence curves for Texas red. (E) Fluorescence curves for Cy3 dye. (F) Fluorescence curves for Cy5 dye.

PCR-RFLP. The sequencing of the amplified fragment of the 23S rRNA gene confirmed the A2142C genotype for these two strains. A case of a mixed population with a susceptible strain associated with a resistant strain, detected by culture with only three colonies in the inhibition area of the Etest, was detected as susceptible only by the PCR-RFLP method. The PCR-RFLP missed the resistant strain. Twelve biopsies were positive for PCR-RFLP but negative for culture: 7 were wild type, 4 were of the A2143G genotype, and 1 was a mixture (wild type and A2142G genotype).

**Scorpion PCR of biopsy specimens: determination of the cutoff for the  $C_T$  values.** The detection of fluorescence was realized in the four channels of the SmartCycler thermocycler during 50 cycles of amplification. Among the 259 biopsy specimens, a detection of fluorescence was positive in one of the four channels in 119 cases. Sixteen biopsy specimens displayed two  $C_T$  values for two different genotypes.  $C_T$  values ranged from 27.4 to 49.6. Since experiments on DNA extracted from reference strains indicated a limit of detection of 1 to 3 CFU at 43 to 45 cycles, detection of fluorescence over 45 cycles might be false positive. Then to determine the  $C_T$  cutoff that distinguish positive from negative Scorpion PCR assays, we analyzed the distribution of  $C_T$  values among culture-positive or -negative and PCR-RFLP-positive or -negative biopsy specimens (Fig. 2).

The bimodal distribution of  $C_T$  values allowed us to define an

optimal cutoff at 40 cycles. This cutoff is confirmed by the fact that all but one of the  $C_T$  values obtained from biopsy specimens positive for culture were lower than 40 and that the 12 biopsy specimens that were positive for PCR-RFLP exhibit  $C_T$  values lower than 40 cycles. Thus, a Scorpion PCR result was considered positive if the  $C_T$  value was lower than 40 cycles.

**Performances of the three methods for detection of *H. pylori* in gastric biopsy specimens.** All biopsy specimens with a positive culture were also confirmed by Scorpion PCR with  $C_T$ s lower than 40, with the exception of one biopsy specimen with a  $C_T$  of 42.7. Only a few colonies were obtained on culture plate from this biopsy specimen: the direct stain on the biopsy smear was negative, and the urease test was too. The DNA extracted from the cultured strain was positive for Scorpion PCR, with a wild-type (susceptible) sequence detected. The PCR-RFLP was negative too for this biopsy specimen, since no amplification product was obtained. A search for PCR inhibitors was positive for this biopsy specimen, since the  $C_T$  of a quantified amount of DNA extracted from a cultured strain exhibits five  $C_T$ s smaller than expected. This biopsy was collected from a patient with a mucosa-associated lymphoid tissue lymphoma who underwent a new biopsy 3 months later providing a positive Scorpion PCR.

The mean  $C_T$  for positive results (<40) was 34.25. By comparison to  $C_T$ s obtained with serial dilutions of a bacterial

TABLE 2. Results obtained by culture, PCR-RFLP, and Scorpion PCR from 259 biopsy specimens

Outcome and no. of specimens (total)	Result by <sup>a</sup> :		
	Culture and Etest	PCR-RFLP	Scorpion PCR
Concordance of all 3 methods (238 [92%])			
184	Negative	Negative	Negative
34	S	S	S
15	R	A2143G	A2143G
5	R	A2142G	A2142G
Mixtures not detected by culture and by PCR-RFLP (3)			
2	R	A2142C <sup>b</sup>	A2142C + S
1	R	A2143G	A2143G + S
Default of PCR-RFLP (2)			
1	S	Negative	S
1	R + S	S	A2142G + S
Default of PCR methods (1)			
1	S	Negative <sup>c</sup>	Negative <sup>c</sup>
Default of culture (12)			
7	Negative	S	S
4	Negative	A2143G	A2143G
1	Negative	A2142G + S	A2142G + S
Detection only by Scorpion PCR (3)			
3	Negative	Negative	S

<sup>a</sup> S, susceptible; R, resistant.

<sup>b</sup> Detected by sequencing.

<sup>c</sup> Negative because of the presence of PCR inhibitors.

suspension of a CFU-numbered suspension, the estimated mean number of bacterial cells that contain biopsies is 10,000.

Twelve biopsy specimens were positive by Scorpion PCR and PCR-RFLP but negative by culture. Among them, two biopsies took 7 days to reach the lab. One was sampled only 1 month after eradication treatment, and one was positive for microscopic examination of a smear prepared from the biopsy specimen but never grew. One was positive for urease activity but never grew. All of the biopsies that were positive by PCR-RFLP were positive by Scorpion PCR. Moreover, there was a perfect concordance of the two PCR-based techniques (PCR-RFLP and Scorpion PCR) for the determination of the mutations conferring *Cla* resistance.

Three biopsies were found to be negative by culture and by PCR-RFLP but positive by Scorpion PCR. Two of these three biopsies were obtained 2 months after eradication treatment. The last one was obtained from an atrophic gastric mucosa of a 93-year-old man.

The remaining 184 biopsy specimens were negative by culture, PCR-RFLP, and Scorpion PCR.

When the culture was regarded as the "gold standard," the

sensitivity and the specificity of Scorpion PCR were 98.3% and 92.5%, respectively. Those of PCR-RFLP were 97% and 94%, respectively.

When the gold standard was defined by a positive culture and/or a positive PCR-RFLP, the sensitivity and specificity of Scorpion PCR were 98.6% and 98.4%, respectively.

**Cla susceptibility testing by Scorpion PCR and PCR-RFLP on biopsy specimens.** *Cla* susceptibility test results are presented on Table 2.

Among the 60 biopsy specimens positive for culture, 24 (40%) harbored a *Cla*-resistant strain. Of these 24 biopsy specimens, both PCR-RFLP and Scorpion PCR detected the A2143G genotype in 16 biopsy specimens (a mixture of an A2143G genotype and a wild-type genotype was detected by both PCR-RFLP and Scorpion PCR but not by phenotypic methods in one of these 16 biopsy specimens), the A2142G genotype in 6 biopsies (one of them with a mixture of a A2142G genotype not detected by PCR-RFLP and a wild genotype) and the A2142C genotype in 2 biopsies (from the same patient). As expected, the A2142C genotype was not detected by PCR-RFLP because we did not use a specific restriction enzyme for its detection, but this genotype was confirmed by sequencing the PCR product.

Of the 24 biopsy specimens with a resistant strain isolated by culture, the resistant genotype was confirmed by Scorpion PCR and PCR-RFLP in all 24 cases, except for one biopsy specimen containing a mixture of resistant and susceptible strains in which the PCR-RFLP did not detect the resistant genotype. In this biopsy specimen, the resistant population consisted of only three colonies in the inhibition area on the Etest.

Mixed populations were detected by Scorpion PCR that were not detected by culture and Etest in three cases. Thus, in 3 of 24 cases with a resistant strain, as shown by the Etest, Scorpion PCR results suggest the presence of both wild-type and mutant genotypes. These cases and the single case in which PCR-RFLP failed to detect the resistant genotype were further investigated. *Cla* susceptibility testing of the corresponding isolates was repeated by Etest. Although numerous colonies showed *Cla* MICs of >1 mg/liter, an inhibition ellipse indicating the presence of the susceptible population could also be observed. Bacteria were harvested both within and outside the inhibition ellipse, the DNA

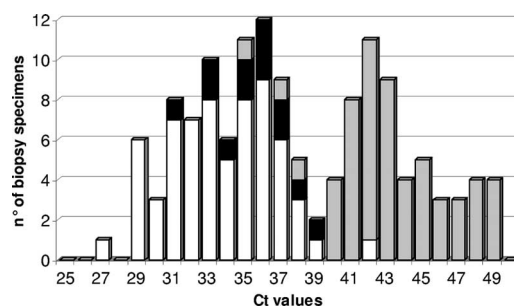


FIG. 2. Distribution of  $C_T$  values determined by Scorpion PCR according to culture and PCR-RFLP results among the 119 biopsy specimens displaying a  $C_T$  value during the 50 cycles of the assay (16 biopsy specimens displayed two  $C_T$  values for two different genotypes). White bars, biopsy specimens positive for culture; black bars, biopsy specimens positive for PCR-RFLP but negative for culture; gray bars, biopsy specimens negative for culture and PCR-RFLP.

was extracted, and Scorpion PCR was performed. Within the inhibition ellipse, Scorpion PCR revealed the A2143G mutant in two cases and the A2142C mutant for the third case, whereas outside the ellipse, isolated single colonies showed either the susceptible or mutant genotype.

The 12 biopsy specimens that were negative by culture and positive by both PCR-RFLP and Scorpion PCR presented concordant genotypes.

Finally, the two genotypic methods agreed in 254 cases (98%), and among them, the culture gave concordant results in 238 cases (92%).

**Scorpion PCR in routine practice.** Since that study, we have used this technique in routine practice. We perform DNA extraction with an automatic extractor (MagNApure Compact; Roche), we use extraction and inhibitor controls by detection of the  $\beta$ -globin gene in biopsy specimens and with distilled water used as a negative control. Two hundred twenty-eight biopsy specimens from 214 patients have been tested. Thirty patients were positive for culture: among them, 10 (33%) harbored a Cla-resistant strain. In only one biopsy specimen was a mix of a resistant strain and a susceptible strain detected by Etest. The Scorpion PCR detected all of the culture-positive patients. Mixed populations with susceptible and resistant strains in the same biopsy specimen were detected in seven patients by Scorpion PCR (including the one detected by Etest). The  $C_T$ s obtained by the detection of the  $\beta$ -globin gene ranged from 19.86 to 22.67 (mean, 21.56).

## DISCUSSION

We have developed an easy-to-use, real-time PCR technique based on the amplification of a fragment of the 23S rRNA gene with Scorpion primers allowing for specific detection directly from gastric biopsy specimens of the four possible alleles of this gene: the wild type, susceptible to Cla, and the three mutations conferring resistance to Cla. The four fluorescence results are readable on a SmartCycler thermocycler.

The determination of the  $C_T$  cutoff to distinguish positive from negative Scorpion PCR assays was based on several results. The distribution of  $C_T$  values presented on Fig. 2 shows a clearly bimodal distribution with two peaks and a valley at 40 cycles. Moreover, the  $C_T$  values of all but one of the culture-positive biopsy specimens were lower than 40 cycles, and among the 15  $C_T$  values lower than 40 obtained for culture-negative biopsy specimens, 12 were confirmed by PCR-RFLP and 7 present clinical arguments for a false-positive culture. All of these results together allowed us to propose a  $C_T$  cutoff of 40 cycles. Thus, biopsy specimens with  $C_T$  values lower than 40 were considered as positive by the Scorpion PCR. Considering this cutoff value, we decided to shorten the number of cycles run for the Scorpion PCRs from 50 to 45 cycles, in this way shortening the assay to 1 h 8 min.

The sensitivity and specificity of the Scorpion PCR are equivalent to those reported for real-time PCR assays and better than those of PCR-RFLP. The use of a culture result as a gold standard to evaluate performances of the Scorpion PCR has certainly shortened the specificity of the test. Histological analysis of the biopsy specimens of our study was done in different centers with different techniques and read by four different physicians, prohibiting the use of the histological re-

sults to determine positive cases. Only a few patients benefit from an indirect diagnostic test (serology or breath test). The performances of our Scorpion PCR assay could, in fact, be higher than those reported here if these indirect methods are taken as references.

The sensitivity and specificity of Scorpion PCR compared to culture obtained in our study (98.3% and 92.5%, respectively) are comparable to those reported in the literature: Lascols et al. reported sensitivity and specificity of 98.3% and 96%, respectively, Oleastro et al. reported results of 98.4% and 94.1%, respectively, Schabereiter-Gurtner et al. reported results of 100% and 94%, and Chisholm et al. reported results of 93% and 99% (3, 12, 19, 21).

The relatively low specificity observed in our study is probably due to false-negative culture. Indeed, among the 15 biopsy specimens positive by Scorpion PCR and negative by culture, 12 were positive by PCR-RFLP with concordant results for detection of mutations conferring resistance to Cla. Furthermore, the transport of two of them was delayed for 7 days, with probable death or a viability decrease of the microorganisms present in the specimen but persistence of the DNA, which was detected by both PCR methods. One specimen was collected only 1 month after eradication treatment, too early to be tested, with a very low density of viable bacteria or persistence of dead bacteria. Two others displayed arguments for *H. pylori* infection with urease activity or the presence of spiral gram-negative bacteria on a smear prepared from the biopsy specimen. The three biopsy specimens positive only by Scorpion PCR also displayed an indication of *H. pylori* infection. Two specimens were collected 2 months after eradication treatment. Among them, one patient's specimen was positive by culture of a new biopsy 12 months later, confirming treatment failure and the possible persistence of a small number of bacteria at the time of the biopsy. The second specimen was collected from a 93-year-old man with gastric atrophy and under circumstances in which the density of *H. pylori* infection is often too low to allow isolation of bacteria in culture.

Susceptibility testing by Scorpion PCR could be performed for all culture-positive biopsy specimens, except one due to PCR inhibitors. In comparison with phenotypic Cla resistance measurement by the Etest, the detection of resistance mutations by Scorpion PCR revealed a perfect concordance since all of the resistance detected by Etest was detected by Scorpion PCR. The only discrepancies concerned mixtures of susceptible and resistant cells, which were better detected by Scorpion PCR than by Etest in three cases. It might be underlined that the Scorpion PCR was able to distinguish a mixture not detected by PCR-RFLP in four cases.

Our tests used to determine the analytical sensitivity of the Scorpion PCR confirm these observations. The Scorpion PCR allows detection of the two strains in a mixture in which the lowest proportion of one allele was 5% (95/5). This result is better than those published for real-time PCR assays using LightCycler (10%) (6, 14, 19).

Mixed populations containing a resistant clone associated with a susceptible clone are difficult to detect by PCR-RFLP. The resistant clone is often well detected because the mutation conferring resistance produces a restriction site that results in a new band on the electrophoresis profile. However, the uncut fragment corresponding to the susceptible clone is usually not taken into consideration. The uncut fragment is indeed more

often attributed to an incomplete restriction than to a mixed population. Even for pure resistant strains, a band corresponding to the uncut fragment of DNA is sometimes still present on the electrophoresis profile due to partial restriction. Thus, a mixed population containing a susceptible clone associated with a resistant clone is detected as a single resistant strain.

The results we obtained for several biopsy specimens that require 7 days to be processed by the laboratory with a positive Scorpion PCR and negative culture highlight the advantage of PCR techniques that do not require viable bacteria. The transport conditions are thus not as critical as they are for culture, and shipments are cheaper.

The cost of the reagent necessary for the Scorpion PCR is acceptable: \$9.86, including extraction for a single reaction detecting the four different alleles. This price is \$1.26 more expensive than detection by culture (\$1.70) and determination of Cla susceptibility by Etest (\$6.90) (12).

Our Scorpion PCR technique is rapid and simple. It can be performed in 1 h 8 min for 45 cycles. Since DNA extraction of biopsy specimens takes 1 h, the total detection and Cla susceptibility testing could be performed in less than 3 h. Since that study, we have used this technique in routine practice. We perform DNA extraction with an automatic extractor (MAGNApure Compact; Roche), we use extraction and inhibitor controls by detection of the  $\beta$ -globin gene in biopsy specimens and a negative control. Two hundred twenty-eight biopsy specimens have been tested with perfect concordance with culture and Etest, except for minor discrepancies concerning mixed populations. The control using detection of the human  $\beta$ -globin gene did not detect any PCR inhibition.

In conclusion, we have developed a Scorpion PCR assay that permits accurate, fast, and cost-effective detection of *H. pylori* directly from gastric biopsy specimens as well as detection of the mutations that confer Cla resistance. This PCR technique is a good candidate for automated real-time PCR methods allowing simple and rapid detection of *H. pylori* and its resistance to Cla by clinical laboratories which do not practice *H. pylori* culture.

#### REFERENCES

- Alm, R. A., L. S. Ling, D. T. Moir, B. L. King, E. D. Brown, P. C. Doig, D. R. Smith, B. Noonan, B. C. Guild, B. L. DeJonge, G. Carmel, P. J. Tummino, A. Caruso, M. Uria-Nackelsen, D. M. Mills, C. Ives, R. Gibson, D. Merberg, S. D. Mills, Q. Jiang, D. E. Taylor, G. F. Vovis, and T. J. Trust. 1999. Genomic sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. *Nature* **397**:176–180.
- Burucoa, C., C. Landron, M. Garnier, J. L. Fauchère, R. Khan, and M. Rahman. 2005. T2182C mutation is not associated with clarithromycin resistance in *Helicobacter pylori*. *Antimicrob. Agents Chemother.* **49**:868–870. (Letter.)
- Chisholm, S. A., R. Owen, E. L. Teare, and S. Saverymuttu. 2001. PCR-based diagnosis of *Helicobacter pylori* infection and real-time determination of clarithromycin resistance directly from human gastric biopsy samples. *J. Clin. Microbiol.* **39**:1217–1220.
- Dore, M. P., G. Leandro, G. Realdi, A. R. Sepulveda, and D. Y. Graham. 2000. Effect of pre-treatment antibiotic resistance to metronidazole and clarithromycin on outcome of *Helicobacter pylori* therapy: a meta-analytical approach. *Dig. Dis. Sci.* **45**:68–76.
- Dunn, B. E., H. Cohen, and M. J. Blaser. 1997. *Helicobacter pylori*. *Clin. Microbiol. Rev.* **10**:720–741.
- Elviss, N. C., A. J. Lawson, and R. J. Owen. 2004. Application of 3'-mismatched reverse primer PCR compared with real-time PCR and PCR-RFLP for the rapid detection of 23S rDNA mutations associated with clarithromycin resistance in *Helicobacter pylori*. *Int. J. Antimicrob. Agents* **23**:349–355.
- Fontana, C., M. Favaro, S. Minelli, A. A. Criscuolo, A. Pietroiusti, A. Galante, and C. Favalli. 2002. New site of modification of 23S rRNA associated with clarithromycin resistance of *Helicobacter pylori* clinical isolates. *Antimicrob. Agents Chemother.* **46**:3765–3769.
- Gibson, J. R., N. A. Saunders, B. Burke, and R. J. Owen. 1999. Novel method for rapid determination of clarithromycin sensitivity in *Helicobacter pylori*. *J. Clin. Microbiol.* **37**:3746–3748.
- Grignon, B., J. Tankovic, F. Megraud, Y. Glupczynski, M. O. Husson, M. C. Conroy, J. P. Emond, J. Loudergue, J. Raymond, and J. L. Fauchère. 2002. Validation of diffusion methods for macrolide susceptibility testing of *Helicobacter pylori*. *Microb. Drug Resist.* **8**:61–66.
- Houben, M. H., D. van de Beek, E. F. Hensen, A. J. Craen, E. A. Rauws, and G. N. Tytgat. 1999. A systematic review of *Helicobacter pylori* eradication therapy: the impact of antimicrobial resistance on eradication rate. *Aliment. Pharmacol. Ther.* **13**:1047–1055.
- Hultén, K., A. Gibreel, O. Sköld, and L. Engstrand. 1997. Macrolide resistance in *Helicobacter pylori*: mechanism and stability in strains from clarithromycin-treated patients. *Antimicrob. Agents Chemother.* **41**:2550–2553.
- Lascols, C., D. Lamarque, J.-M. Costa, C. Copie-Bergman, J.-M. Le Glaunec, L. Deforges, C.-J. Soussy, J.-C. Petit, J.-C. Delchier, and J. Tankovic. 2003. Fast and accurate quantitative detection of *Helicobacter pylori* and identification of clarithromycin resistance mutations in *H. pylori* isolates from gastric biopsy specimens by real-time PCR. *J. Clin. Microbiol.* **41**:4573–4577.
- Malferteiner, P., F. Megraud, C. O'Morain, F. Bazzoli, E. El-Omar, D. Graham, R. Hunt, T. Rokkas, N. Vakil, and E. J. Kuipers. 14 December 2006, posting date. Current concepts in the management of *Helicobacter pylori* infection. The Maastricht III Consensus Report. *Gut*. doi:10.1136/gut.2006.101634.
- Matsumura, M., Y. Hikiba, K. Ogura, G. Togo, I. Tsukuda, K. Ushikawa, Y. Shiratori, and M. Omata. 2001. Rapid detection of mutations in the 23S rRNA gene of *Helicobacter pylori* that confers resistance to clarithromycin treatment to the bacterium. *J. Clin. Microbiol.* **39**:691–695.
- Megraud, F. 2004. *H. pylori* antibiotic resistance: prevalence, importance and advantages in testing. *Gut* **53**:1374–1384.
- Megraud, F., and P. Lehours. 2007. *Helicobacter pylori* detection and antimicrobial susceptibility testing. *Clin. Microbiol. Rev.* **20**:280–322.
- Ng, C. T., C. A. Gilchrist, A. Lane, S. Roy, R. Haque, and E. R. Houpt. 2005. Multiplex real-time PCR assay using Scorpion probes and DNA capture for genotype-specific detection of *Giardia lamblia* on fecal samples. *J. Clin. Microbiol.* **43**:1256–1260.
- Ochialiini, A., M. Urdaci, F. Doucet-Populaire, C. M. Bébéar, H. Lamouliatte, and F. Mégraud. 1997. Macrolide resistance in *Helicobacter pylori*: rapid detection of point mutations and assays of macrolide binding to ribosomes. *Antimicrob. Agents Chemother.* **41**:2724–2728.
- Oleastro, M., A. Ménard, A. Santos, H. Lamouliatte, L. Monteiro, P. Barthélémy, and F. Mégraud. 2003. Real-time PCR assay for rapid and accurate detection of point mutations conferring resistance to clarithromycin in *Helicobacter pylori*. *J. Clin. Microbiol.* **41**:397–402.
- Raymond, J., C. Burucoa, O. Pietrini, M. Bergeret, A. Decoster, A. Wann, C. Dupont, and N. Kalach. 2007. Prevalence and coexistence of different mutations conferring clarithromycin resistance in *Helicobacter pylori* strains isolated from French children. *Helicobacter* **12**:157–163.
- Schabereiter-Gurtner, C., A. M. Hirschl, B. Dragosics, P. Hufnagl, S. Puz, Z. Kovách, M. Rotter, and A. Makristathis. 2004. Novel real-time PCR assay for detection of *Helicobacter pylori* infection and simultaneous clarithromycin susceptibility testing of stool and biopsy specimens. *J. Clin. Microbiol.* **42**:4512–4518.
- Taylor, D. E., Z. Ge, D. Purych, T. Lo, and K. Hiratsuka. 1997. Cloning and sequence analysis of two copies of a 23S rRNA gene from *Helicobacter pylori* and association of clarithromycin resistance with 23S rRNA mutations. *Antimicrob. Agents Chemother.* **41**:2621–2628.
- Trebesius, K., K. Panthel, S. Strobel, K. Vogt, G. Faller, T. Kirchner, M. Kist, J. Heesemann, and R. Haas. 2000. Rapid and specific detection of *Helicobacter pylori* macrolide resistance in gastric tissue by fluorescent *in situ* hybridisation. *Gut* **46**:608–614.
- van Doorn, L.-J., Y. Glupczynski, J. G. Kusters, F. Mégraud, P. Midolo, N. Maggi-Solcà, D. M. M. Queiroz, N. Nouhan, E. Stet, and W. G. V. Quint. 2001. Accurate prediction of macrolide resistance in *Helicobacter pylori* by a PCR line probe assay for detection of mutations in the 23S rRNA gene: multicenter validation study. *Antimicrob. Agents Chemother.* **45**:1500–1504.
- Versalovic, J., D. Shortridge, K. Kibler, M. V. Griffy, J. Beyer, R. K. Flamm, S. K. Tanaka, D. Y. Graham, and M. F. Go. 1996. Mutations in 23S rRNA are associated with clarithromycin resistance in *Helicobacter pylori*. *Antimicrob. Agents Chemother.* **40**:477–480.
- Whitcombe, D., J. Theaker, S. P. Guy, T. Brown, and S. Little. 1999. Detection of PCR products using self-probing amplicons and fluorescence. *Nat. Biotechnol.* **17**:804–807.
- Xia, Q. F., S. X. Xu, D. S. Wang, Y. A. Wen, X. Qin, S. Y. Qian, Z. L. Zhan, H. M. Wang, Y. Z. Lin, and Z. G. Tu. 12 January 2007, posting date. Development of a novel quantitative real-time assay using duplex scorpion primer for detection of *Chlamydia trachomatis*. *Exp. Mol. Pathol.* doi:10.1016/j.yexmp.2006.11.005.