

Pyrosequencing assay to rapidly detect clarithromycin resistance mutations in Canadian *Helicobacter pylori* isolates

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BACKGROUND: Mutations at positions 2142 or 2143 in the two-copy 23S ribosomal RNA gene of *Helicobacter pylori* are highly predictive of in vitro clarithromycin resistance and failure of clarithromycin-containing treatment regimens.

OBJECTIVE: To design an assay to rapidly detect these mutations using rapid polymerase chain reaction and pyrosequencing, a novel method of 'sequencing by synthesis', and to test this assay with a collection of Canadian *H pylori* isolates.

METHODS: Forty-two *H pylori* isolates (24 clarithromycin-resistant, 18 clarithromycin-susceptible) were studied. A target region in the 23S gene was rapidly amplified and sequenced by pyrosequencing.

RESULTS: Mutations at one of the two positions studied were present in 20 of the 24 (83%) clarithromycin-resistant isolates; 13 had double-copy A2143G mutations, four had double-copy A2142G mutations and three had single-copy A2143G mutations. There were no mutations in 17 of the 18 (94%) susceptible isolates. A single-copy A2142G mutation was detected in one susceptible isolate.

CONCLUSIONS: The pyrosequencing assay developed was able to detect and differentiate mutations at positions 2142 and 2143 in either one or both copies of the *H pylori* 23S ribosomal RNA gene. Further study is needed to determine whether this pyrosequencing assay can be used to determine *H pylori* susceptibility to clarithromycin from clinical specimens such as stools or gastric biopsies.

Key Words: Antibiotic; Clarithromycin; *H pylori*, PCR; Pyrosequencing; Resistance

Clarithromycin is a component of first-line therapy regimens for *Helicobacter pylori* infections. However, the prevalence of pretreatment resistance to this antibiotic appears to be increasing, potentially due to the widespread use of macrolide therapy for respiratory tract infections. Resistance rates vary widely by region, from 0% to 50% worldwide (1).

This in vivo resistance is clinically important because it has been shown to greatly increase the risk of treatment failure when clarithromycin-containing regimens are used (2,3). However, despite the clinical importance and increasing prevalence of clarithromycin resistance in *H pylori* strains, susceptibility is not generally determined before treatment. This is partially due to the fact that conventional clarithromycin antimicrobial susceptibility testing for *H pylori* by agar dilution or E test (AB Biodisk, Sweden) is a relatively slow process that takes 72 h to perform.

Pyroséquençage en vue de la détection rapide de mutations causant une résistance à la clarithromycine dans des isolats d'*Helicobacter pylori* obtenus au Canada

CONTEXTE : La présence de mutations aux positions 2142 et 2143 dans le gène de l'ARN ribosomique 23S à double copie d'*Helicobacter pylori* est fortement prédictive d'une résistance *in vitro* à la clarithromycine et de l'échec des traitements composés de clarithromycine.

BUTS : L'étude visait à concevoir une épreuve pouvant détecter rapidement la présence de ces mutations, à l'aide de l'amplification en chaîne par polymérase et du pyroséquençage, nouvelle technique de « séquençage de synthèse », et à vérifier son efficacité dans une série d'isolats d'*H. pylori* obtenus au Canada.

MÉTHODE : Quarante-deux isolats d'*H. pylori* (24 résistants à la clarithromycine, 18 sensibles à la clarithromycine) ont été examinés. Une région cible dans le gène 23S a été rapidement amplifiée et séquencée par pyroséquençage.

RÉSULTATS : La présence de mutations à l'une ou l'autre des deux positions a été relevée dans 20 isolats résistants à la clarithromycine sur 24 (83 %) : 13 étaient porteurs de la mutation A2143G en deux copies; 4, de la mutation A2142G en deux copies; et 3, de la mutation A2143G en une copie. Il n'y avait pas de mutation dans 17 isolats sensibles sur 18 (94 %). Une seule copie de la mutation A2142G a été détectée dans un isolat sensible.

CONCLUSIONS : Le pyroséquençage mis au point a pu détecter et distinguer des mutations aux positions 2142 et 2143 dans l'une ou l'autre des copies, ou les deux copies, du gène de l'ARN ribosomique 23S d'*H. pylori*. Il faudrait toutefois pousser la recherche pour savoir si cette technique de pyroséquençage peut détecter la sensibilité d'*H. pylori* à la clarithromycine dans des échantillons cliniques comme les selles ou des prélèvements biopsiques.

Clarithromycin acts by binding to the 23S ribosomal RNA (rRNA) gene, thereby blocking protein synthesis. Resistance to this agent is most commonly caused by point mutations at positions 2142 or 2143 in either one or both copies of the 23S rRNA gene, which then leads to altered macrolide binding (1). Because of the difficulties involved in performing traditional susceptibility testing with bacterial cultures, and because resistance is highly associated with mutations in these two adjacent positions, rapid genotypic susceptibility determination using molecular diagnostics has become an attractive and feasible option.

Rapid detection and sequencing of mutations in the 23S gene from DNA extracted from *H pylori* cultures may potentially lead to improved antibiotic selection for patients infected with *H pylori*, and also provide epidemiological information

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regarding the types of mutation(s) present in a population. DNA sequencing is considered the 'gold standard' for mutation detection, but is rarely used in the clinical microbiology laboratory setting due to issues of time, cost and lack of familiarity with techniques.

Recently, pyrosequencing, a rapid method of sequencing relatively short DNA targets, has been used in a number of microbiological applications (4-8). This is a novel method of sequencing that potentially has advantages over conventional sequencing when only a short target sequence is required because it can be less costly and more rapid.

Pyrosequencing is a polymerase chain reaction (PCR)-based method of DNA sequencing, in which primers must be biotin labelled at the 5' end for capturing the PCR product with streptavidin-coated beads. Once captured, single-stranded DNA is prepared by denaturation with sodium hydroxide. A pyrosequencing primer is then added. This primer anneals to the single-stranded DNA bound to the beads. DNA synthesis then begins at the 3' end of the primer and proceeds in a manner in which the addition of a nucleotide to the DNA strand results in the generation of light via a series of enzymatic reactions. This is known as 'sequencing by synthesis'.

On incorporation of a deoxynucleotide, the primer is extended by DNA polymerase and an inorganic phosphate (PPi) is released. ATP sulfurylase is then used to convert the PPi to ATP. The reaction also contains the substrate luciferin and a luciferase enzyme that uses the ATP to produce light. This is a stoichiometric reaction, and the amount of light produced is proportional to the number of PPi molecules produced and hence the number of incorporated nucleotides. Any unincorporated nucleotide is degraded with apyrase and the next nucleotide is then added, and the series of reactions is repeated.

Nucleotides are added at a rate of approximately 1 nucleotide/min. Following the addition of each nucleotide, the light emitted from each individual reaction well is detected by a camera and visualized in real time on a computer monitor. Software then analyzes the peak heights and determines the sequence. When more than one copy of a gene is present, as in the case of *H pylori* 23S rRNA genes, pyrosequencing has been shown to be able to clearly determine the number of copies of each variant present (9).

In Canada, the mutations responsible for clarithromycin resistance in *H pylori* strains are not well described; consequently, we decided to develop a pyrosequencing assay to provide this information. A previous publication (10) described the pyrosequencing of a small number of clarithromycin-resistant *H pylori* strains. We designed new PCR primers for a short 23S target region in *H pylori* to allow for a rapid PCR protocol, and used these with the published sequencing primer. To increase assay speed, a primer designed to bind directly adjacent to position 2142 of the *H pylori* 23S gene was used, thereby expediting the sequencing of only the minimum essential number of bases. We then tested a collection of Canadian *H pylori* isolates to investigate which mutations were most prevalent.

METHODS

Forty-two *H pylori* isolates were studied, the majority of which were from patients in Nova Scotia. Clarithromycin susceptibility of these isolates was determined using an E-test method (11)

that included 24 clarithromycin-resistant and 18 clarithromycin-susceptible *H pylori* strains. A clarithromycin-susceptible *H pylori* strain (American Type Culture Collection reference 43504) was used as a control.

DNA was extracted from isolated colonies of *H pylori* in pure culture using the PrepMan Ultra reagent (Applied BioSystems, USA) according to the manufacturer's protocol. The DNA extracts were stored frozen at -80°C until PCR was performed. DNA concentrations were estimated by measuring optical densities. DNA extracts were diluted with water and a DNA concentration of 50 $\mu\text{g}/\mu\text{L}$ was used in the PCR reaction.

Primers were designed using the Primer 3 program (<http://frodo.wi.mit.edu/primer3>) to amplify a short 23S target region that included the binding site for the previously described pyrosequencing primer and positions 2142 and 2143. A short amplicon was chosen to allow for efficient rapid-cycle PCR, and for the sequencing of only the two target positions of interest.

A 56 bp fragment of the 23S gene was amplified in the PCR reaction using commercially synthesized primers (Integrated DNA Technologies Inc, USA): the forward primer HpF-GAGGTGAAAATTCCCTCCTACCC and the reverse primer HpR Biotin-AGTAAAGGTCCACGGGGTCT.

The total volume for each PCR reaction was 10 μL , which included 500 $\mu\text{g}/\text{mL}$ of bovine serum albumin, 2 mmol/L magnesium chloride, 200 $\mu\text{mol}/\text{L}$ of each deoxynucleoside triphosphate, 0.5 $\mu\text{mol}/\text{L}$ of each primer, 50 $\mu\text{g}/\mu\text{L}$ of extracted bacterial DNA, 0.4 units of KlenTaq polymerase (AB Peptides Inc, USA), 88 ng of TaqStart antibody (ClonTech, USA) and 1 \times LCGreen Plus+ (Idaho Technology, USA).

The rapid PCR protocol consisted of 45 cycles of 94 $^{\circ}\text{C}$ denaturation, 58 $^{\circ}\text{C}$ annealing and 72 $^{\circ}\text{C}$ extension, with a programmed transition rate of 9.9 $^{\circ}\text{C}/\text{s}$ with no holds. After PCR, samples were transferred to a high-resolution melting instrument (HR-1, Idaho Technology, USA) for melting curve analysis. The derivative curve of the measured fluorescence plotted against temperature was examined to determine whether amplification was successful.

The pyrosequencing assay was optimized using the PSQ96MA system (Biotage, Sweden). Reagents were mixed together in preparation for the sequencing reaction according to the pyrosequencing sample preparation protocol. A forward pyrosequencing reaction was performed using the sequencing primer 5'-GCGGCAAGACGG-3', at a concentration of 0.5 $\mu\text{mol}/\text{L}$.

RESULTS

All of the 42 *H pylori* isolates were successfully amplified and sequenced using the procedures described. Representative pyrograms for clarithromycin-resistant and -susceptible strains are presented in Figure 1. Sequencing results and clarithromycin minimum inhibitory concentration (MIC) range and susceptibility status for the isolates are shown in Table 1.

Mutations at one of the two positions studied were present in 20 of 24 (83%) clarithromycin-resistant isolates. Seventeen isolates had identical mutations in both copies of the 23S gene: 13 with double adenine (A) to guanine (G) (A2143G) mutations and four with double A2142G mutations. Three resistant isolates had a mutation in only one copy of the 23S gene (all were A2143A/G). Thus, an A to G mutation in either one or both copies of the 23S gene at position 2143 was present in 16 of 24 (66%) resistant isolates.

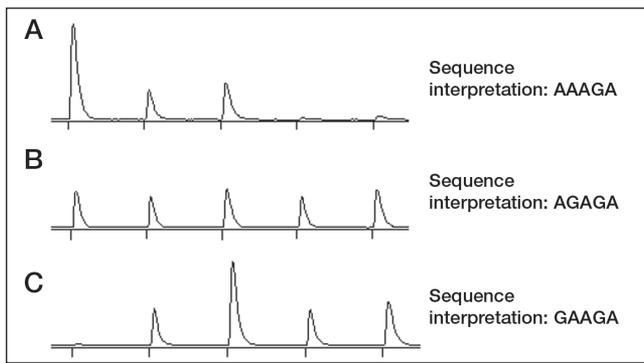


Figure 1 Pyrosequencing pyrograms of the point mutations at positions 2142 and 2143 of the *Helicobacter pylori* 23S ribosomal RNA gene associated with clarithromycin resistance. Adenine (A) bases were dispensed followed by guanine bases in a sequential manner to create distinct pyrograms. The order of bases added is shown on the x-axis and light intensity is on the y-axis. The height of the base peaks is proportional to the number of bases of that type that occur at any given base position. **A** Clarithromycin-susceptible strain showing A at positions 2142 and 2143. **B** At position 2143, both alleles of the 23S rRNA gene show an A to guanine (G) mutation (ie, A2143G). **C** At position 2142, both alleles of the 23S rRNA gene show an A to G mutation

There were no mutations in 17 of 18 (94%) susceptible isolates. A single-copy mutation was detected in one susceptible isolate (A2142A/G).

Procedure times were short. Rapid-cycle PCR and amplicon detection were completed in approximately 30 min. Pyrosequencing procedures were completed in approximately 20 min.

DISCUSSION

The pyrosequencing assay developed in the present study was able to detect and differentiate mutations at positions 2142 and 2143 in either one or both copies of the *H pylori* 23S rRNA gene. A high proportion of resistant isolates had mutations in the assay target region. As reviewed by Gerrits et al (2), the A2143G mutation is linked to intermediate-level clarithromycin resistance (MIC of 2 µg/mL to 64 µg/mL), and the A2142G mutation is associated with high-level clarithromycin resistance (MIC greater than 64 µg/mL). Although the number of strains tested was small, this association appears to exist with our isolates. The MIC range was 2 µg/mL to more than 256 µg/mL for our 13 dual-copy A2143G isolates, with a MIC required to inhibit the growth of 50% of organisms (MIC₅₀) of 16 µg/mL. For the four A2142G mutants, the MIC range was 32 µg/mL to more than 256 µg/mL, with a MIC₅₀ of greater than 256 µg/mL.

Clarithromycin resistance not due to mutations at positions 2142 or 2143 was seen in 17% of the isolates. However, because this was primarily a feasibility study to determine whether a pyrosequencing assay could be developed and used successfully for genotypic analysis of *H pylori*, rather than an epidemiological study, the proportion of resistance due to mutations at positions 2142 and 2143 of the 23S gene in Canadian *H pylori* isolates cannot be accurately estimated from our data. In large studies, resistance due to these two mutations typically accounts for more than 90% of clarithromycin resistance. A prospective Canadian study to establish the pretreatment prevalence of

TABLE 1
Pyrosequencing results and clarithromycin susceptibilities for Canadian *Helicobacter pylori* isolates

23S gene nucleotide position		Isolates, n	Clarithromycin susceptibility	Clarithromycin MIC range, µg/mL	23S gene copies with mutation at position 2142 or 2143, n
2142	2143				
A	A	17	S	<1	0
A/G	A	1	S	<1	1
A	G	13	R	2 to >256	2
G	A	4	R	32 to >256	2
A	A/G	3	R	4 to 16	1
A	A	4	R	2 to >256	0

A Adenine; G Guanine; MIC Minimum inhibitory concentration; R Resistant; S Susceptible

clarithromycin resistance in *H pylori* is warranted and could help determine whether current empirical treatment regimens need to be modified.

Further investigation is also needed to determine the mechanism of resistance of the four resistant isolates in our collection that lacked mutations at positions 2142 or 2143. Mutations at other 23S rRNA positions are most probably responsible (1,2), and these mutations could possibly be investigated with additional pyrosequencing assays or by conventional sequencing.

Development of pyrosequencing assays for the detection of mutations in *H pylori* that lead to resistance to certain other antibiotics should also be possible. For example, high-level tetracycline resistance in *H pylori* is mediated by a triple base-pair substitution AGA926-928→TTC in the 16S rRNA gene, and resistance to fluoroquinolones is caused by point mutations in the DNA gyrase-encoding gene *gyrA* at amino acid positions 87, 88, 91 and 97 (2).

Additional studies are needed to determine whether the PCR/pyrosequencing method can be applied after the direct amplification of DNA from clinical specimens such as stools and gastric biopsies, as is performed with other nonculture-based molecular methods such as PCR with fluorescent probe detection of clarithromycin resistance (12,13). Although pyrosequencing requires an additional step after PCR, it does provide gold standard results because the sequence itself is obtained. This is not the case with fluorescent PCR methods, in which the possibility of false-positive or false-negative results exist.

Zhou et al (14) performed a cost comparison as part of a study examining resistance mutations in malaria parasites. They demonstrated pyrosequencing to be less expensive and more rapid than conventional sequencing. If assays are designed in which valuable clinical information can be obtained from short DNA sequences, as in the present study, pyrosequencing may gain wider acceptance for use in clinical microbiology laboratories.

NOTE: This work was presented as an abstract at the Association of Medical Microbiology and Infectious Disease Canada and Canadian Association for Clinical Microbiology and Infectious Diseases Annual Conference, Halifax, Nova Scotia March 14-18, 2007.

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