Enzymic colorimetry-based DNA chip: a rapid and accurate assay for detecting mutations for clarithromycin resistance in the 23S rRNA gene of *Helicobacter pylori*

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Macrolide drugs, such as clarithromycin (CAM), are a key component of many combination therapies used to eradicate *Helicobacter pylori*. However, resistance to CAM is increasing in *H. pylori* and is becoming a serious problem in *H. pylori* eradication therapy. CAM resistance in *H. pylori* is mostly due to point mutations (A2142G/C, A2143G) in the peptidyltransferase-encoding region of the 23S rRNA gene. In this study an enzymic colorimetry-based DNA chip was developed to analyse single-nucleotide polymorphisms of the 23S rRNA gene to determine the prevalence of mutations in CAM-related resistance in *H. pylori*-positive patients. The results of the colorimetric DNA chip were confirmed by direct DNA sequencing. In 63 samples, the incidence of the A2143G mutation was 17.46 % (11/63). The results of the colorimetric DNA chip were concordant with DNA sequencing in 96.83 % of results (61/63). The colorimetric DNA chip could detect wild-type and mutant signals at every site, even at a DNA concentration of 1.53×10^2 copies μl^{-1} . Thus, the colorimetric DNA chip is a reliable assay for rapid and accurate detection of mutations in the 23S rRNA gene of *H. pylori* that lead to CAM-related resistance, directly from gastric tissues.

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INTRODUCTION

Helicobacter pylori is a Gram-negative bacterium involved in digestive diseases such as peptic ulcers, gastritis and mucosa-associated lymphoid tissue, and is a risk factor in the development of gastric cancer (Dunn *et al.*, 1997). Eradication of *H. pylori* infection can cure peptic ulcer disease and reduce the risk of gastric adenocarcinoma and mucosa-associated lymphoid tissue (Sung *et al.*, 1995). Indeed, macrolide drugs such as clarithromycin (CAM) form a key component of most treatment recommendations in eradicating *H. pylori* (Treiber *et al.*, 2005). In recent years, however, the increasing use of CAM has resulted in the development of resistance. It is now widely accepted that resistance to CAM is the main reason for failure in *H. pylori* eradication therapy (Sezgin *et al.*, 2008; Treiber *et al.*, 2005).

Many laboratories use agar disc diffusion or an Etest for the determination of macrolide resistance after isolation of the

pathogen from gastric biopsies. Both methods require further serial subcultivation for several days and neither identifies the type of point mutation present in the strain (Rüssmann *et al.*, 2001). Recently, numerous molecular techniques have been developed to detect point mutations in the 23S rRNA gene such as fluorescent *in situ* hybridization, PCR-RFLP, DNA sequencing and an oligonucleotide line probe assay (Kim *et al.*, 2002; Rüssmann *et al.*, 2001; Sezgin *et al.*, 2008; Van Doorn *et al.*, 2001). However, these are also time-consuming and laborious methods. Here, we have established an enzymic colorimetry-based DNA chip for the detection of point mutations in the 23S rRNA gene of *H. pylori* that are associated with resistance to CAM. We also evaluated the sensitivity and specificity of this method by comparison with DNA sequencing.

METHODS

Sample collection and DNA extraction. Gastric tissues were obtained from 63 patients during gastric endoscopy at the Affiliated

Abbreviations: CAM, clarithromycin; NC, negative control; PC, positive control; SNP, single-nucleotide polymorphism.

Hospital of Nantong University and The People's Hospital of Dongtai City, PR China, from October 2007 to January 2008. All patients were enrolled randomly. Primary and secondary drug resistance were not classified. The mean age of the patients was 48.7 years (range 19–62 years). They had erosive gastritis (17 patients, 26.98 %), gastro-duodenal ulcers (38 patients, 60.32 %), atrophic gastritis (5 patients, 7.94 %) or gastric cancer (3 patients, 4.76 %). One piece of biopsy specimen was obtained from the patient for the purpose of genomic DNA extraction, whilst another was obtained from the same patient for cultivation. All patients were infected with *H. pylori*, confirmed by a rapid urease test (Livzon Group Reagent Factory) and by a [^{14}C]urea breath test (GMS Pharmaceutical). DNA was extracted from each bacterial and biopsy specimen with a DNA extraction kit (TaKaRa) according to the manufacturer's protocol. The isolated DNA was stored at $-20\text{ }^{\circ}\text{C}$.

Culture conditions and antibiotic susceptibility test. Biopsy specimens were cultured on Columbia agar with 10 % sheep blood and Dent antibiotic supplement (Oxoid) at $37\text{ }^{\circ}\text{C}$ for 3–5 days under microaerobic conditions (5 % O_2 , 10 % CO_2 , 85 % N_2). Identification of *H. pylori* was based on colony morphology, microscopy, and positive urease, catalase and oxidase activities. The MICs of *H. pylori* to CAM were evaluated with Etest strips (AB Biodisk). Strains were classified as resistant to CAM when the MIC was $>1\text{ }\mu\text{g ml}^{-1}$.

Amplification of the 23S rRNA gene of *H. pylori* by PCR. Primers used for PCR amplification (GenBank accession no. U27270) were an *H. pylori* forward primer (HPFP: 5'-ATGAATGGCGTAACGA-GATGGGAGC-3', nt 2050–2074) and reverse primer (HPRP: 5'-CCAGTCAAACTACCCACCAAGCATT-3', nt 2335–2311). The amplification product was 285 bp. The HPRP primer was 5' labelled with digoxigenin. PCR amplification was in a 50 μl volume containing 50 ng genomic DNA, $1\times$ PCR buffer, 1.5 mmol MgCl_2 , 0.2 μmol each primer, 1.0 U *Taq* polymerase and 200 μmol each dNTP. Amplification was carried out in a DNA thermal cycler (PTC-200; Bio-Rad). Cycling parameters were 5 min of denaturation at $94\text{ }^{\circ}\text{C}$; 30 cycles of 30 s at $94\text{ }^{\circ}\text{C}$, 30 s at $53\text{ }^{\circ}\text{C}$ and 30 s at $72\text{ }^{\circ}\text{C}$; and a final elongation step at $72\text{ }^{\circ}\text{C}$ for 10 min. Amplification products were routinely analysed by electrophoresis in 2.0 % agarose gels, which were stained with ethidium bromide, and then stored at $4\text{ }^{\circ}\text{C}$.

Design of probes. A total of 13 oligonucleotide probes were designed using Primer 5.0 software from the conservative regions of

the 23S rRNA gene of *H. pylori* according to GenBank accession no. U27270. The sequences of the probes were analysed with a BLAST search. The probe lengths were between 15 and 18 nt, and the 5' end of each probe was modified by adding poly(T) and was amino-modified for immobilization. The positive-control (PC) probe was designed to be complementary to HPRP to evaluate the quality of the oligonucleotide chip fabrication and hybridization. The negative-control (NC) probe used had two base mismatches compared with the sequence of the PC probe. The probes sequences are shown in Table 1.

Mutant construction by site-directed mutagenesis. To verify the specificity of the probe, five mutant clones were constructed by site-directed mutagenesis with a QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. The mutants were cloned in vector pGEM-T (Promega) and the clones were analysed to confirm the mutagenic sequences. The concentrations of recombinant plasmids were detected using a nucleic acid and protein analyser (U-0080D; Hitachi). Recombinant plasmids were diluted to 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 and 10 copies μl^{-1} . PCR was performed with different plasmid copy numbers and the PCR products were used for hybridization. The primers used for mutagenesis are presented in Table 2.

Production of the chip. The oligonucleotide probe spotting mixture contained 10 μl probe dilution (30 pmol μl^{-1}) and 10 μl spotting solution (750 mM NaCl, 75 mM sodium acetate, 5 % glycerol, 1 % Ficoll and 0.1 % SDS). The spotting mixture was spotted onto silylated glass using a Pixsys 7500 microarrayer (Cartesian). The same oligonucleotide probe was spotted sequentially four times in a row to examine the reproducibility of probe hybridization. The printed slides were left for at least 24 h at $25\text{ }^{\circ}\text{C}$, so that the probes could dry onto the slide surface. Probes were spotted onto the glass slides as shown in Fig. 1.

Hybridization and scanning. A 1 μl aliquot of digoxigenin-labelled unpurified PCR product was mixed with 10 μl hybridization solution (750 mM NaCl, 75 mM sodium acetate, 0.2 % SDS, 1 mg fragmented salmon sperm DNA ml^{-1} , 2.5 % formamide). The mixture was denatured for 5 min at $95\text{ }^{\circ}\text{C}$, chilled on ice for 5 min, and 10 μl was applied to the oligonucleotide probe area and covered with a coverslip. Hybridization was performed at $37\text{ }^{\circ}\text{C}$ for 30 min in a hybridization chamber. After hybridization, the hybridized slide was

Table 1. Probes for detection of the SNPs in the 23S rRNA gene from *H. pylori*

Bases associated with the wild-type/mutant type to be detected are indicated in bold.

Probe	WT/mutant detected	Probe sequence (5'→3')
P1	2142, 2143 (WT)	(NH ₂)T ₁₀ AGACGGA AA GACCCC
P2	A2142G	(NH ₂)T ₁₀ CAAGACGG G AAGACC
P3	A2142C	(NH ₂)T ₁₀ AGACGGCAAGACCCC
P4	A2142G, A2143G	(NH ₂)T ₁₀ CAAGACGG GG GAGACC
P5	A2142G, A2143C	(NH ₂)T ₁₀ CAAGACGG GC CAGACC
P6	A2143G	(NH ₂)T ₁₀ AAGACGGAGAGACCCC
P7	A2143C	(NH ₂)T ₁₀ AAGACGGACAGACCCC
P8	A2142C, A2143G	(NH ₂)T ₁₀ CAAGACGG CG GAGACC
P9	2224 (WT)	(NH ₂)T ₁₀ AAGTAAGG CT TTGGCT
P10	A2142C, A2143C	(NH ₂)T ₁₀ CAAGACGG CC CAGACC
P11	G2224A	(NH ₂)T ₁₀ AAGTAAAG CT TTGGCT
P12	PC	(NH ₂)T ₁₀ AATGGCGTAACGAGATGG
P13	NC	(NH ₂)T ₁₀ AATGGCGTGGCGAGATGG

WT, Wild-type.

Table 2. Primers for site-directed mutagenesis

Point mutations in the 23S rRNA gene sequence are indicated in bold.

Genotype	Clone	Primer name	Sequence (5'→3')
A2142G	HC23S-1	GAF	AAGACGG G AAGACCCCGTGGGA
		GAR	GGGGTCTTCCCGTCTTGCCG
A2142C	HC23S-2	CAF	AAGACGG C AAGACCCCGTGGGA
		CAR	GGGTCTTGCCCGTCTTGCCG
A2143G	HC23S-3	AGF	AAGACGG A GAGACCCCGTGGGA
		AGR	GGGGTCT C TCCGTCTTGCCG
A2143C	HC23S-4	ACF	AAGACGG A CAGACCCCGTGGGA
		ACR	GGGTCT G TCCGTCTTGCCG
G2224A	HC23S-5	AAF	TGAAGTAAAGGCTTTGGCTCT
		AAR	AAAGCCTTTACTTCAAAGC

washed sequentially in washing solution A (150 mM NaCl, 15 mM sodium acetate, 0.2 % SDS, pH 7.5) for 10 min (60–100 r.p.m. on a shaker) followed by solution B (150 mM NaCl, 150 mM toxic acid, 0.3 % Tween 20, pH 7.5) for 1 min. The hybridized slide was dried with absorbent paper, 20 µl 1:2500-diluted alkaline phosphatase-conjugated anti-digoxigenin (Roche) was added and the slide was incubated at 30 °C for 30 min. A 50 µl aliquot of detection buffer (0.1 M Tris/HCl (pH 9.5), 0.1 M NaCl) was added to the hybridization area for 1 min and then the hybridized slide was washed in washing solution A for 1 min. A piece of nylon membrane (~1 cm²) was soaked in 1:50-diluted substrate (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium; Roche) solution for 10 s and placed over the hybridization area away from light at room temperature for 30 min. For the final step, the nylon membrane was washed in ddH₂O and dried at room temperature. The nylon membrane was scanned with an EPSON Perfection 1640SU scanner or examined with a magnifying lens.

Sequence analysis. To confirm the results of the DNA chip, all PCR products were sequenced. DNA sequencing was performed by Shanghai Bioasia Biotechnology with an ABI Prism 3730 genetic analyzer (Applied Biosystems).

Fig. 1. Diagram of the probe layout on the DNA chip. C1–6, PC; A1, A2142G mutation; B1, wild-type nt 2142/2143; A2, A2142G/A2143G mutation; B2, A2142C mutation; A3, A2143G mutation; B3, A2142G/A2143C mutation; A4, A2142C/A2143G mutation; B4, A2143C mutation; A5, A2142C/A2143C mutation; B5, wild-type nt 2224; A6, G2224A mutation; B6, NC. Each probe in columns A and B was spotted sequentially four times in a row to examine the reproducibility of probe hybridization.

RESULTS AND DISCUSSION

Single-nucleotide polymorphism (SNP) genotyping using a DNA chip

The mechanism of CAM resistance in *H. pylori* is mostly due to point mutations in the peptidyltransferase-encoding region of the 23S rRNA gene. CAM inhibits protein synthesis by binding to the peptidyltransferase loop of 23S rRNA. Mutations A2142G/C and A2143G are the most commonly associated with macrolide resistance in natural *H. pylori* strains (Kato *et al.*, 2002; Kim *et al.*, 2004; Mégraud, 2004; Van Doorn *et al.*, 2001). Recently, a new point mutation, G2224A, was found to be related to CAM resistance in *H. pylori* isolates in north-east China (Hao *et al.*, 2004). Thus, probes were designed and a DNA chip was produced to detect the SNPs at these three sites (nt 2142, 2143 and 2224). In this study, only one mutation of the 23S rRNA gene, A2143G, was observed in gastric tissue biopsy specimens and strains. The prevalence of this mutation was 17.46 %, higher than that reported by Liu *et al.* (2008), who found that that primary prevalence of the A2143G mutation was 10–14 % in a Chinese population without a history of CAM therapy (Liu *et al.*, 2008). It may be relevant that we did not distinguish between primary and secondary drug resistance. We did not find the A2142G/C, A2143C or G2224A mutation in any of our samples. The absence of these mutation types in east China may be due to geographical differences. The genotype distributions in our samples are shown in Table 3.

MICs of *H. pylori* strains with the A2143G mutation

In East Asia, studies from Japan and Korea showed that more than 90 % of CAM-resistant strains of *H. pylori* had the A2143G mutation (Kato *et al.*, 2002; Kim *et al.*, 2002; Maeda *et al.*, 2000). A study from China (Pan *et al.*, 2002) showed the A2143G mutation present in 100 % of CAM-resistant *H. pylori*. We also determined MIC values by Etest. A total of 10 *H. pylori* strains were isolated successfully from 11 gastric biopsy specimens (*H. pylori*

Table 3. SNP results of the *H. pylori* 23S rRNA gene from 63 gastric tissue biopsy specimens using the DNA chip

Nucleotide	Genotype			
	A	G	C	T
2142	63/63 (100 %)	0	0	0
2143	52/63 (82.54 %)	11/63 (17.46 %)	0	0
2224	0	63/63 (100 %)	0	0

could not be isolated from T23) with the A2143G mutation. The CAM MIC values of these ten *H. pylori* strains ranged from 4 to 256 µg ml⁻¹. All of the A2143G mutations in the CAM-resistant strains were confirmed in our study by sequencing.

Comparison of the DNA chip and DNA sequencing

To evaluate the specificity of the probes, we compared the results of our DNA chip with DNA sequencing using plasmids constructed by site-directed mutagenesis. Amplification products of clones HC23S-1, HC23S-2, HC23S-3, HC23S-4 and HC23S-5 (Table 2) were hybridized to the probes and the results were consistent with the respective probe locations (data not shown). The results of the DNA chip and DNA sequencing were almost identical. Out of 63 gastric tissue samples (T1–T63), the results of the DNA chip were concordant with DNA sequencing in 96.83 % (61/63). DNA sequencing of two samples, T8 and T40, was unsuccessful, probably because the DNA concentration of these samples was lower than the other samples, although the DNA chip was able to detect whether

these two gastric tissue samples had a mutation. One reason for this discrepancy is that the microarray-based method may be less affected than direct sequencing by the fragmentation of DNA caused by processing of the tissue. Another reason is that it is possible that the microarray-based method can detect smaller amounts of mutant, whilst direct DNA sequencing requires higher levels (Taylor *et al.*, 1997). We also compared the information about the point mutations in the 23S rRNA gene of the ten CAM-resistant *H. pylori* strains with the corresponding biopsy specimens using the DNA chip. The results showed a complete concordance between the biopsy specimens and the *H. pylori* strains. The results of DNA chip and DNA sequencing are shown in Fig. 2.

Sensitivity and reproducibility of the DNA chip

To evaluate the sensitivity of hybridization with the DNA chip, recombinant plasmids were diluted to 10⁷, 10⁶, 10⁵, 10⁴, 10³, 10² and 10 copies µl⁻¹. The sensitivity of this assay was 1.53 × 10² copies of *H. pylori* DNA µl⁻¹ (data not shown). Thus, based on this result, the microarray-based method would not be a suitable approach if the amount of template is less than 100 copies of the 23S rRNA gene. We randomly selected five clinical samples and tested each sample five times using our colorimetric enzyme assay. The results of this assay were concordant, indicating that the assay had good reproducibility (data not shown).

Evaluation of the quality of the DNA chip and optimization of probes

To evaluate the quality of chip production and hybridization, the PC probe used in this study was designed to be

Fig. 2. Comparison of results from sequencing analysis and the DNA chip. (a, c) Sequence analysis (a) of the A2143G mutant and wild-type nt 2224, which yielded the same results as the DNA chip (c). (b, d) Sequencing analysis (b) of the wild-type nt 2142, 2143 and 2224, which yielded the same results as the DNA chip (d).

complementary to HPRP. We designed the NC probe by substituting two base mismatches compared with the sequence of HPRP. The NC probe was thus used to evaluate whether non-specific hybridization occurred. In order to increase the hybridization efficiency of the assay, the lengths of the probes and spacer arms and the hybridization conditions were optimized. A probe length of 15–18 nt with a poly(T)₁₀ spacer was found to be optimal.

The prevalence of *H. pylori* infection, which is higher in developing countries and low socio-economic populations (Bener *et al.*, 2002; Strnad *et al.*, 2002), is about one-half of the world's population (Dunn *et al.*, 1997). It is widely accepted that all patients with gastric or duodenal ulcers and *H. pylori* infection should be treated with antimicrobial agents, as eradication of the bacteria cures peptic ulcer disease and efficiently prevents relapse (Drumm *et al.*, 2000). However, patients in unsuccessful eradication groups have been found to be infected with strains of *H. pylori* that become more resistant to CAM on exposure to the drug (Kobayashi *et al.*, 2006). The prevalence of CAM resistance is increasing, which may be due to the more extensive use of macrolides (Buzás *et al.*, 2007). In China, the resistance rates of *H. pylori* to CAM in Beijing were 10.0 % in 1999–2000 and 18.3 % in 2001–2002 (Cheng & Hu, 2005). Thus, prediction of CAM resistance plays a key role before treatment in the eradication of *H. pylori*. Antibiotic susceptibility tests have been considered the 'gold standard' for confirmation of the resistance of *H. pylori*. However, isolation of *H. pylori* from gastric biopsies is affected by the biopsy preparation, culture conditions and the medium used. It has been reported that isolation rates of *H. pylori* from infected individuals vary from 23.5 to 97 % (Fresnadillo Martínez *et al.*, 1997; Heep *et al.*, 1999). In the present study, the isolation rate of *H. pylori* from 63 gastric biopsy specimens was 79.37 % (50/63). *H. pylori* was not isolated successfully from 13 gastric biopsy specimens, although the DNA extracted from the corresponding biopsy specimen could be used for detecting the mutant using the DNA chip. To detect mutants associated with antibiotic resistance and identify the mutation types, many molecular methods have been used. However, these methods can generally detect only one specific marker per reaction (Chizhikov *et al.*, 2002). Compared with these methods, an oligonucleotide chip-based method is less time-consuming and is highly sensitive for the detection of point mutations (Xing *et al.*, 2005).

In conclusion, our method detected point mutations in the 23S rRNA gene of *H. pylori* accurately and rapidly (<5 h), and we could process many samples simultaneously. Thereby, our colorimetric DNA chip for detecting the resistance gene in *H. pylori* is highly specific, has a high throughput and is also technically feasible for clinical application. Compared with a fluorescence microarray, the colorimetry chip has a similar sensitivity and the results are easy to interpret (Mao *et al.*, 2006). In addition, the laser scanner and Cy3/Cy5-labelled dUTPs required for fluor-

escence microarray are expensive, limiting this method for general clinical application. A colorimetric DNA chip does not require expensive instruments such as a laser scanner. Therefore, because of its lower cost and simplicity, a colorimetric DNA chip is technically feasible for clinical application, especially for small- and medium-sized hospitals in developing countries.

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