Rapid screening of clarithromycin resistance in *Helicobacter pylori* by pyrosequencing

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Helicobacter pylori infections can be effectively treated with clarithromycin, a macrolide, in combination with other antibiotics, such as amoxicillin, tetracycline or metronidazole. The failure of *H. pylori* eradication is mainly associated with macrolide-resistant strains. Three point mutations (A2142G/C, A2143G, T2182C) in the peptidyltransferase region of domain V of the 23S rRNA have been described as being associated with clarithromycin resistance. Therefore, the determination of clarithromycin resistance by pyrosequencing was evaluated. *H. pylori* from 81 gastric biopsies was cultured and clarithromycin resistance was determined by Etest, as well as by pyrosequencing technology (PSQ 96 system; Biotage). The respective mutations were set in relation to the MIC measured in $\mu\text{g ml}^{-1}$ by Etest. In this study, point mutations in positions 2142 and 2143 were associated with clarithromycin resistance. Mutations in position 2182 did not contribute to clarithromycin resistance. In addition, from 22 out of the 81 biopsies, clarithromycin resistance was determined directly without culturing *H. pylori* to save additional time. Identical results were obtained as compared to resistance testing with pure *H. pylori* strains. All results obtained by pyrosequencing were evaluated by Sanger sequencing. The data show that pyrosequencing to detect point mutation is a fast and reliable method for determining clarithromycin resistance in *H. pylori*, and provides the same results as the Etest.

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INTRODUCTION

Helicobacter pylori is a microaerophilic, Gram-negative bacterium that colonizes the human gastric mucosa. To eradicate *H. pylori*, a specific triple therapy is recommended as a first-line approach using a proton pump inhibitor (e.g. esomeprazole) in addition to antibiotics, such as clarithromycin/metronidazole or clarithromycin/amoxicillin (Treiber *et al.*, 2005). Clarithromycin resistance is a major cause of treatment failure (Treiber *et al.*, 2005; Toracchio *et al.*, 2000; Fontana, 2003). Therefore, resistance testing should be considered before the first treatment.

H. pylori is a fastidious and slow-growing micro-organism. Therefore, standard cultures based on *in vitro* antibiotic susceptibility tests (agar dilution and Etest methods) are slow, and can take 7 to 14 days from initial receipt of the gastric specimen to reading and reporting the sensitivity results. Consequently molecular-based diagnostic assays for resistance detection offer an alternative approach for obtaining susceptibilities to antibiotics with higher speed and the possibility of a same-day result. Pyrosequencing has emerged as a new sequencing method. This technique is a widely applicable technology for the detailed characterization

of nucleic acids and has been explained in detail (Ronaghi, 2000, 2001; Ronaghi *et al.*, 1996, 1998, 1999). Pyrosequencing has the advantages of flexibility and parallel processing of the samples. Different studies have been performed to evaluate this specific method for detecting the antibiotic resistance of various micro-organism (Haanpera *et al.*, 2005; Sinclair *et al.*, 2003; Zhao *et al.*, 2005).

Genetic research and studies have revealed that clarithromycin resistance can be associated with distinct mutations in either one or both copies of the *H. pylori* 23S rRNA gene. Jiang *et al.* (1996) confirmed that the *H. pylori* genome carries just two copies of the 23S rRNA gene. Versalovic *et al.* (1996) showed that point mutations in two positions (an adenine to guanine transition at either position 2142 or position 2143, or an adenine to cytosine transversion at position 2142) in 23S rRNA domain V were associated with macrolide resistance. Point mutations at different additional sites have been described as occurring (Hultén *et al.*, 1997), although these mutations have never been subsequently reported. Furthermore, a T2182C mutation has been proposed to be associated with high levels of clarithromycin resistance by Kim *et al.* (2002).

In this study we concentrated on the rapid detection of the three above described point mutations, which are essentially associated with clarithromycin resistance. The aim of

Abbreviations: NPV, negative predictive value; PPV, positive predictive value; SNP, single nucleotide polymorphism.

this study was, therefore, to apply the pyrosequencing technique to DNA first extracted from cultured *H. pylori* strains, as well as directly from biopsy specimens, in order to obtain a result within a day of endoscopy.

METHODS

Gastric tissue. Gastric tissue was taken from 81 patients during gastric endoscopy examination (either from antrum or corpus). They were outpatients visiting the gastroenterology ambulance (outpatient clinic) or inpatients of the gastroenterology unit of the Otto-von-Guericke University Magdeburg. The outpatients were admitted into the ambulance for endoscopy by their general practitioner. A total of 16 samples were clinical isolates collected in a control study of patients included in a German surveillance study, the additional samples were from patients with chronic gastritis, peptic ulcer and gastric cancer. All patients were positive for the urea breath test and the antigen test in stool or urine. Immediately after endoscopy, the respective gastric biopsies were cultured on Pylori agar (bioMérieux). Plates were incubated for 3 to 7 days in a microaerobic atmosphere at 37 °C for primary culture. Every 2 to 3 days isolates were subcultured on the Pylori agar. Strains were identified as *H. pylori* by the use of standard criteria (Strobel *et al.*, 1998; Kist, 1991). The remaining gastric biopsies were stored at -80 °C until use, for example, in pyrosequencing.

Determination of MICs. MICs of clarithromycin were determined by Etest (AB Biodisk) on Iso-sensitest agar with 5 % (w/v) horse blood (Oxoid). The method was performed using the protocol of Heep *et al.* (2000), which is up to the standards of the DGHM (German Association for Hygiene and Microbiology) and the German reference centre for *H. pylori*. A sterile swab was dipped into the bacterial suspension equivalent to a McFarland standard of 2. After swabbing the entire plate surface with the inoculum, Etest strips impregnated with clarithromycin (concentration ranging from 0.016 to 256 µg ml⁻¹) were placed on the agar surface. After an incubation period of 48 to 72 h (37 °C, 98 % humidity in microaerobic atmosphere), the MICs of clarithromycin were determined. Isolates were classified to be resistant to clarithromycin if the MIC exceeded 1 µg ml⁻¹.

Isolation of genomic DNA. DNA from *H. pylori* isolates and from the biopsy samples was extracted using a QIAamp DNA mini kit (Qiagen).

Sanger sequencing of a part of the 23S rRNA gene. The primer pair DP1 and ZGE23 (Table 1) was used to amplify a 309 bp fragment of the peptidyltransferase region of the 23S rRNA, as described by Taylor *et al.* (1997). The purified PCR products were sequenced with an ABI PRISM BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. The PCR primers were used as sequencing primers. Sequencing was accomplished with an ABI PRISM 3100 genetic analyser (Applied Biosystems). The results were processed with Sequence Analysis Software (Applied Biosystems), and forward and reverse sequences were combined into a single consensus sequence with Assembler software (Applied Biosystems).

Pyrosequencing

PCR Amplification. Primers for PCR amplifications and pyrosequencing are listed in Table 1. The two primers cla1 and cla8, and the sequencing primers seq1 and seq2, were designed with the PSQ assay design program (Assay Design software version 1.0.6; Biotage) based on an alignment of the *H. pylori* sequence (U27270) that was obtained from the National Center for Biotechnology Information. All amplifications were performed in a final volume of

Table 1. List of primers used for PCR amplification and pyrosequencing

Name	Sequence (5'→3')	Position*
DP1†	ACGGCGGCCGTAAGTATA	2357–2374
ZGE23†	ACAGGCCAGTTAGCTA	2649–2664
cla1	ACGAGATGGGAGCTGTCTCAA	2434–2454
cla8	BIO-ATAAGAGCCAAAGCCCTTACTTC	2589–2611
seq1	ACCCGCGGCAAGACG	2126–2140
seq2	TTACTACAACTTAGCACTGC	2159–2178

BIO, Biotin.

*Numbering of the nucleotide position follows the system proposed by Taylor *et al.* (1997).

†Taylor *et al.* (1997).

50 µl containing both primers (cla1 and cla8) at 2.0 µM, 1x buffer, 800 µM dNTP, 8 mM MgCl₂, 1.25 U Qiagen *Taq* polymerase, 10 ng DNA-containing sample and HPLC-water to make up the volume. Conditions for cycling were 95 °C for 5 min, followed by 45 cycles of 95 °C for 15 s, 54 °C for 30 s and 72 °C for 15 s, and a final extension step at 72 °C for 5 min.

Single-stranded template preparation and pyrosequencing. Single-stranded DNA template preparation was performed using streptavidin-coated Sepharose beads (GE Healthcare, formerly Amersham Biosciences) and a vacuum prep tool (Pyrosequencing). All subsequent steps were performed according to the manufacturer's instructions. The templates were transferred to a 96-well plate containing annealing solution (20 mM tris-acetate, 2 mM magnesium acetate) and sequencing primers (seq1 and seq2) at 0.25 mM.

The PSQ96 plate was placed into the process chamber of a PSQ96MA instrument, and enzymes, substrates and nucleotides from the PyroGold reagent (Biotage) were dispensed. A charge-coupled device camera registered the light emitted from each incorporated nucleotide. The mutations A2142G and A2143G were analysed with the SQA (sequencing) mode (allows analysis of short sequences), while the mutation in position T2182C was analysed in SNP (single nucleotide polymorphism) mode (searches directly for given point mutations) of the PSA96MA software (version 2.1).

RESULTS

Determination of MICs by Etest

In this study 81 isolates of *H. pylori* were examined by Etest, and the clarithromycin MICs ranged from 0.016 to 256 µg ml⁻¹ (Table 2). Etest identified 21 (25.9 %) strains as clarithromycin resistant and 60 (74.1 %) strains as clarithromycin susceptible (Table 2).

Detection of point mutations in cultured *H. pylori* strains

Fig. 1 shows the DNA sequences, confirmed by Sanger sequencing and pyrosequencing, of wild-types *H. pylori*. Fig. 2 presents DNA sequences of *H. pylori* carrying the three mutations. Mutations were detected by pyrosequencing in 23 (28.4 %) of the respective isolates (Table 2). Nine

Table 2. Correlation between point mutations found in *H. pylori* isolates and MICs of clarithromycin

No. of strains*	Point mutations in 23S rRNA gene				No. of strains with the following MICs (µg ml ⁻¹) detected by Etest																			
	A2142G	A2142C	A2143G	T2182C	0.016	0.019	0.023	0.032	0.047	0.064	0.125	0.25	2	3	4	8	12	16	24	32	48	64	96	256
58	A	-	A	T	37	-	5	4	4	1	1	1	-	-	-	-	-	-	-	-	1	1	-	3
6	A	-	A	C	4	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	A	-	G	T	-	-	-	-	-	-	-	-	1	2	-	2	1	1	-	1	-	-	1	-
6	G	-	A	T	1	-	-	-	-	-	-	-	-	-	1	-	-	-	1	-	1	-	2	
1	G	-	A	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	
1	A/C	-	A	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
81	-	-	-	-	42	1	6	4	4	1	1	1	1	2	1	2	1	1	1	2	1	2	1	6

*No. of strains with the particular 23S rRNA genotype, as specified in columns 2–4, with the respective MICs showed in columns 5–24.

Fig. 1. Pyrosequencing and Sanger sequencing of the 2142, 2143 and 2182 positions of the 23S rRNA gene of a wild-type *H. pylori*. (a) Pyrosequencing pattern (sequencing mode) in comparison to direct Sanger sequencing of the 2142 and 2143 position. (b) Pyrosequencing pattern (SNP mode) in comparison to direct Sanger sequencing of the 2182 position.

strains carried the A2143G mutation and six the T2182C mutation without any other mutation. In six of the *H. pylori* strains just the A2142G mutation was detected. A heterozygous mutation, where only one copy of the 23S rRNA gene carried an A to C mutation in the position 2142, was found once. Finally, one strain carried two point mutations, the first in position 2142 (A→G) and the second in position 2182 (T→C) (Table 2). In 58 (71.6%) out of 81 clinical isolates, no mutations were detected in the specific positions. Sanger sequencing confirmed the results received by pyrosequencing (Fig. 1).

Detection of point mutations directly out of biopsies

In the next set of experiments, we chose 22 biopsies out of the total of 81 clinical samples used for *H. pylori* cultivation. DNA was extracted from the biopsies and

Fig. 2. Pyrosequencing and Sanger sequencing of the single point mutations at positions 2142, 2143 and 2182 of the 23S rRNA gene that are involved in clarithromycin resistance of *H. pylori*: (a) in position 2142 both alleles of the 23S rRNA gene show an A to G mutation, (b) in position 2142 one allele shows an A to C mutation, (c) in position 2143 both alleles of the 23S rRNA gene show an A to G mutation, (d) in position 2182 both alleles of the 23S rRNA gene show a T to C mutation.

pyrosequencing was applied with regard to clarithromycin resistance testing. All results derived directly from the clinical specimens without culturing *H. pylori* were identical to those derived from cultured *H. pylori*.

Association of MIC and point mutations

Out of 58 strains without SNP in the examined positions 53 were clarithromycin susceptible. The remaining five strains showed high MIC levels [$48 \mu\text{g ml}^{-1}$ ($n=1$); $64 \mu\text{g ml}^{-1}$ ($n=1$); $256 \mu\text{g ml}^{-1}$ ($n=3$)].

With regard to position 2142, eight strains showed the respective point mutations and among them seven were defined as clarithromycin resistant [MIC: $4 \mu\text{g ml}^{-1}$ ($n=1$); $24 \mu\text{g ml}^{-1}$ ($n=1$); $32 \mu\text{g ml}^{-1}$ ($n=1$); $64 \mu\text{g ml}^{-1}$ ($n=1$); $256 \mu\text{g ml}^{-1}$ ($n=2$)]. All nine strains with SNPs in position 2143 were clarithromycin resistant [MIC: $2 \mu\text{g ml}^{-1}$ ($n=1$); $3 \mu\text{g ml}^{-1}$ ($n=2$); $8 \mu\text{g ml}^{-1}$ ($n=2$); $12 \mu\text{g ml}^{-1}$ ($n=1$); $16 \mu\text{g ml}^{-1}$ ($n=1$); $32 \mu\text{g ml}^{-1}$ ($n=1$); $96 \mu\text{g ml}^{-1}$ ($n=1$)]. The mutation in position 2182 was detected in seven isolates. However, among them, six strains were clarithromycin susceptible and only one strain

showed clarithromycin resistance, MIC $256 \mu\text{g ml}^{-1}$. But in this particular strain an additional mutation in position 2142 was also found.

Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV)

Phenotypic resistance testing by agar dilution or Etest represents the gold standard. With the Etest used as the standard, pyrosequencing of the two point mutations at position 2142 and 2143 revealed a sensitivity/specificity of 75/98.3 % and a PPV/NPV of 93.75/92.2 %.

DISCUSSION

The first-line therapy against *H. pylori* is primarily based on the use of clarithromycin in combination with either metronidazol or amoxicillin. In this regard, resistance to clarithromycin is an important predictor of treatment failure. Resistance to clarithromycin is reported to be about 3 % in untreated patients and up to 50 % after treatment failure. Among the *H. pylori* isolates in this study, 25.9 %

showed resistance against clarithromycin as determined by Etest. To circumvent clarithromycin resistance, some clinicians favour the high-doses combination using a proton pump inhibitor plus amoxicillin. However, eradication rates are only between 70 and 80 %, and side effects such as diarrhoea are more severe. Yahav *et al.* (2006) evaluated the efficacy of susceptibility-guided versus empiric retreatment for *H. pylori* after treatment failure, and determined the prevalence of post-treatment antibiotic resistance. Their results showed that susceptibility-guided retreatment appears to be significantly more effective than empiric retreatment in eradicating *H. pylori* after at least one previous treatment failure. Comparable results have been found by Kobayashi *et al.* (2006). Therefore, to use the optimal therapy for patients, a rapid and reliable resistance testing against clarithromycin would be helpful.

The gold standard for identification and susceptibility testing of *H. pylori* is still culture based, including agar dilution and Etest. However, *H. pylori* is a slow-growing micro-organism, so all these methods are time consuming. The introduction of molecular methods for *H. pylori* antibiotic-resistance testing has been consequently facilitated by the fact that the mode of action of clarithromycin is well understood and resistance is due to the described point mutations in the peptidyltransferase region of domain V of the 23S rRNA gene. Therefore, molecular-based test methods were developed as an alternative approach. The basic assays were described 1996. Versalovic *et al.* (1996) first described the presence of the A2142G and A2143G mutations by PCR-RFLP. This method has been validated by direct sequencing as reported in many publications (Hultén *et al.*, 1997; Occhialini *et al.*, 1997; Szczebara *et al.*, 1997; Versalovic *et al.*, 1996). Alarcon *et al.* (2000), for instance, used a 3' mismatched PCR to detect the A2142C mutation as a further method. In addition, a DNA enzyme immunoassay (Alarcon *et al.*, 2000; Marais *et al.*, 1999; Pina *et al.*, 1998) was developed for directly screening gastric biopsies. The PCR line probe assay system (van Doorn *et al.*, 2001) is an alternative way for testing large number of samples. Other more complex microtitre plate-based systems, such as the PCR oligonucleotide ligation assay using labelled capture and reporter probes, and the preferential homoduplex formation assay using double labelled amplicons, have also been applied to the detection of mutations but have not been generally evaluated. A recent and conceptually unique approach to the rapid detection of mutations was provided by the LightCycler real-time PCR hybridization assay. The sequencing method developed in 2001 based on pyrosequencing was used for rapid detection and identification of *H. pylori* in gastric specimens, and showed the potential for clarithromycin resistance typing of *H. pylori*. In this regard, Hjalmarsson *et al.* (2004) used pyrosequencing as a single technology to determine species identity, clarithromycin susceptibility and virulence level in cultured *H. pylori* strains, as well as directly from biopsies. In their study only 5 *H. pylori* strains out of a total of 146 were clarithromycin resistant. Thus they were

not able to set the respective mutations in relation to the MIC measured in $\mu\text{g ml}^{-1}$ by Etest. Therefore, we extended their studies and developed assays for the detection of the respective point mutations (A2142G/C, A2143G, C2182T), which are, according to literature, associated with clarithromycin resistance. Our results were confirmed by Sanger sequencing, which is still the gold standard for mutation detection.

The relationship between the position of the mutation and phenotypic resistance against clarithromycin has been evaluated by different groups. Van Doorn *et al.* (2001) showed that the predominant mutations that are responsible for phenotypic clarithromycin resistance were A2143G and A2142G, the A2142C mutation was rare. Also strains containing a A2142T and A2143C were constructed and were clarithromycin resistant (Debets-Ossenkopp *et al.*, 1998, 1999). Owen (2002) indicated that the A2142G mutation alone is associated with MICs $>256 \mu\text{g ml}^{-1}$. Regarding the outcome from our study, we can confirm these results only partially. We confirmed in this study that the point mutations in positions 2142 and 2143 are associated with clarithromycin resistance in accordance with previous studies. However, strains with the A2142G mutation alone ($n=6$) expressed resistance at MICs of 4 ($n=1$), 24 ($n=1$), 64 ($n=1$) and 256 ($n=2$) or no resistance ($n=1$). Whereas three clarithromycin-resistant strains with MIC levels $>256 \mu\text{g ml}^{-1}$ had no mutations at positions 2142, 2143 and/or 2182. Further sequence analysis has to be done to detect additional mutations or resistance mechanisms associated with resistance to clarithromycin. Another controversial point is the stability of the point mutations leading to resistance. It is clear that if the mutations have a biological cost, they will not be maintained when selection pressure stops. Study groups claim that clarithromycin resistance is not stable over the long term (Xia *et al.*, 1996). However, it is unlikely that in our study the strains (MIC levels >256) lost their point mutations. The respective strains were retested by Etest when the discrepant results became obviously. In a Korean study the T2182C mutants showed a tendency to have higher MIC levels for clarithromycin than the A2143G mutants (Kim *et al.*, 2002). Khan *et al.* (2004) reported that a T2182C mutation in 23S rRNA is associated with clarithromycin resistance in *H. pylori* isolates obtained in Bangladesh. We could not confirm these findings. In our study a T2182C mutation by itself was not associated with clarithromycin resistance. Other mutations in the peptidyltransferase region of domain V of the 23S rRNA are also described (A2115G, G2141A, T2717C), but are rarely found (Hultén *et al.*, 1997). Fontana *et al.* (2002) found a T to C transition at position 2717, which conferred a low level of resistance, equivalent to the MIC for the isolates. Thus, further investigations are necessary to determine additional mutations, which are responsible for clarithromycin resistance by themselves or in combination and differ from already described point mutations in the literature.

Assuming that phenotypic resistance testing by agar dilution or Etest represents the gold standard, molecular

testing of the two point mutations at position 2142 and 2143 revealed a sensitivity/specificity of 75/98.3 %, and a PPV/NPV of 93.75/92.2 %. The results, derived from culturing *H. pylori* first, were compared to the results from the direct biopsy work up and showed identical findings. In summary, the pyrosequencing approach is a fast and reliable method that produces highly accurate results with regard to clarithromycin resistance as well as to other characteristics that are important for *H. pylori* therapy (Hjalmarsson *et al.*, 2004).

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