Real-time PCR detection and frequency of 16S rDNA mutations associated with resistance and reduced susceptibility to tetracycline in Helicobacter pylori from England and Wales

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Received 4 May 2005; accepted 20 May 2005

Objectives: To investigate the occurrence of 16S rDNA mutations associated with resistance or reduced susceptibility to tetracycline in Helicobacter pylori isolated in England and Wales, and to develop a real-time PCR assay to detect these DNA polymorphisms from culture and gastric biopsies.

Methods: Tetracycline susceptibility was determined by disc diffusion. The MIC of isolates with reduced susceptibility was determined by Etest and agar dilution methods. The 16S rDNA of these isolates was sequenced and resistance-associated mutations identified. A LightCycler assay developed to detect these mutations was applied to DNA extracted from culture and gastric biopsies.

Results: From 1006 isolates of H. pylori examined, 18 showed reduced susceptibility to tetracycline. Of these, three were resistant (≥4 mg/L). Mutations in 16S rDNA were detected in 10 of the reduced susceptibility isolates: one double base mutation of A926T/A928C, one A926C, one A928C; and seven A926G. The first two polymorphisms were novel and had not been reported from clinical isolates previously. The LightCycler assay identified each of the 10 isolates with 16S rDNA mutations, but did not detect polymorphisms in 100 tetracycline-susceptible H. pylori isolates. The assay correctly determined the tetracycline susceptibility of H. pylori in 20 gastric biopsy samples.

Conclusions: Mutations in 16S rDNA were detected in H. pylori isolated in England and Wales with reduced susceptibility to tetracycline, but resistance to this antibiotic was uncommon. We show molecular-based susceptibility testing for tetracycline is possible direct from biopsy material.

Keywords: antimicrobial resistance mechanisms, LightCycler, DNA polymorphisms

Introduction

Tetracycline is used globally as a key component of eradication regimens for Helicobacter pylori, the bacterium associated with peptic ulcer disease. In Europe, ‘quadruple therapy’ of tetracycline, metronidazole, bismuth citrate and a proton pump inhibitor is employed as a second-line after failure of initial ‘triple therapy’ treatment (typically amoxicillin or metronidazole together with clarithromycin, and a proton pump inhibitor). Worldwide, tetracycline resistance in H. pylori remains relatively uncommon. However, evidence has emerged of increasing resistance in regions where tetracycline is more widely used in H. pylori therapy. For example, reported resistance levels were 5.3% in Korea,2 58.8% in China,3 3.3% in Bulgaria4 and 9% in Brazil.5

In H. pylori, tetracycline resistance is caused by mutations in 16S rDNA corresponding to the helix 31 region of the 16S rRNA molecule, an area that contains the key binding point of the tetracycline molecule to the bacterial ribosome.6 A triple mutation, AGA926–928TTC, has been shown to cause high-level tetracycline resistance (≥256 mg/L),6,7 whilst other single or double base mutations amongst these same three bases are associated with lower level resistance.8,9

Previous studies have described tetracycline-resistant H. pylori with 16S rDNA mutations from Australia, The Netherlands, India, El Salvador, Lithuania and Brazil.5–8 Recent surveys of H. pylori
isolation of H. pylori from gastric biopsies. Isolates were stored on beads at –80°C and sequenced strain 20-456 was obtained using the QIAmp DNA mini kit (Qiagen Ltd., Crawley, West Sussex, UK). The isolates from these biopsies were all tetracycline-susceptible.

### Materials and methods

#### Bacterial strains and cultivation

A total of 1006 isolates of H. pylori were isolated from laboratories and hospitals in England (predominantly London and Chelmsford) and Wales (predominantly Bangor) between June 2000 and May 2004. Isolates were obtained from gastric biopsy material and from culture with standard laboratory procedures as described previously, and from cultures sent for antimicrobial susceptibility testing. All isolates were cultured on Columbia agar base (Oxoid Ltd., Basingstoke, UK) with 10% (v/v) defibrinated horse blood (E & O Laboratories Ltd., Basingstoke, UK), referred to as CBA. All plates were incubated at 37°C under microaerobic conditions (4% O2, 5% H2, 5% CO2 and 86% N2) in a MACS-VA500 Microaerophilic Workstation (Don Whitley Scientific Ltd., Shipley, UK). The genome sequenced strain H. pylori 26695 (NCTC 12455) was used as a control. Isolates were stored on beads at −80°C (Pro-Lab Diagnostics, Neston, UK).

#### Antibiotic susceptibility

All H. pylori isolates were tested for susceptibility to tetracycline by disc diffusion. In the absence of published guidelines on the interpretation of disc zone sizes for H. pylori, a zone of inhibition of ≤30 mm diameter with 2 μg tetracycline disc was defined as showing ‘reduced susceptibility’. The tetracycline MIC of isolates with reduced susceptibility was determined by both the Etest and agar dilution methods to allow comparison with previous studies. Isolates with Etest MIC ≥4 mg/L were defined as tetracycline-resistant.

For susceptibility testing, growth from a 48 h culture was suspended in maximum recovery diluent (Oxoid) to a density equivalent to a McFarland 3 standard (~9.0 × 10^8 cfu/mL) and inoculated onto a pre-dried CBA plate. A 2 μg tetracycline disc (Oxoid) or a tetracycline Etest strip (AB Biodisk, Solna, Sweden) was placed on the surface. Plates were incubated and results were recorded after 48 h. MIC determination by agar dilution was determined as described by Trieber and Taylor except that CBA was used in place of brain heart infusion–yeast extract agar.

Susceptibilities for antimicrobials were determined by Etest with the following resistance breakpoints: metronidazole, 8 mg/L; clarithromycin, 2 mg/L; amoxicillin, 2 mg/L; tetracycline, 4 mg/L.

#### DNA extraction from culture and gastric biopsies

Genomic DNA was extracted using the MagNA Pure LC total nucleic acid extraction kit (Roche Diagnostics Ltd., Lewes, East Sussex, UK) from the 18 isolates with reduced susceptibility to tetracycline and from 100 tetracycline-susceptible H. pylori isolates. This latter group consisted of 50 isolates with tetracycline disc diffusion zone sizes of between 31 and 39 mm, and 50 with zone sizes ≥40 mm.

DNA from 20 H. pylori-positive gastric biopsy samples was extracted using the QIAamp DNA mini kit (Qiagen Ltd., Crawley, West Sussex, UK). The isolates from these biopsies were all tetracycline-susceptible.

### Sequence analysis

The 16S rDNA sequences of isolates with resistance or reduced susceptibility to tetracycline were determined. H. pylori 16S rDNA was amplified and sequenced using primers described by Dewhirst et al. DNA sequencing was done using the CEQ dye terminator cycle sequencing quick start kit run on a Beckman CEQ 8000 (Beckman Coulter Inc., Fullerton, CA, USA). Sequence alignments and comparisons were performed using the BioEdit software.

### Real-time PCR assay

The assay contained two PCR primers (MWG-Biotech AG, Ebersberg, Germany): TefF (5′ CGG TCG CAA GAT TAA AAC 3′) and TefR (5′ GCC GAT TCT CTC AAT GTC 3′) designed to amplify a 118 base pair fragment of 16S rDNA, together with a probe, Tensensor (5′ LCRed705-GCA TGT GGT TTA ATT CGA AGA TAC AC- phos 3′), labelled with the fluorophore LC Red 705 (TIB Molbiol, Berlin, Germany), which was complementary to the tetracycline-susceptible allele. Samples were processed using a LightCycler-FastStart DNA Master SYBR Green I kit (Roche) with 5 pmol of each primer and 2.5 pmol of probe. The assay was run on a Roche LightCycler using the following cycling conditions: 95°C (ramp rate, 20°C/s, held for 10 s); 50 cycles of amplification at 95°C (ramp rate, 20°C/s); 50°C (ramp rate, 20°C/s); 55°C (ramp rate, 3°C/s), and 72°C (ramp rate, 20°C/s, held for 10 s; acquisition mode, single). This was followed by a single probe melting cycle: 95°C (ramp rate, 20°C/s), 45°C (ramp rate, 20°C/s), and 95°C (ramp rate, 0.2°C/s; acquisition mode, continuous).

BLASTn analysis of the TefF and TefR primers suggests that, in combination, they should be specific for H. pylori in human gastric biopsy specimens. However, they might also amplify 16S rDNA from closely related Helicobacter species found in animals such as Helicobacter cetorum (dolphins) and Helicobacter acinonychis (cheetahs).

The LightCycler assay was applied to DNA extracted from all 18 isolates with resistance or reduced susceptibility to tetracycline, 100 tetracycline-susceptible H. pylori isolates and 20 H. pylori-positive gastric biopsies.

### Natural transformation of H. pylori

Transformation of H. pylori 26695 was done as described by Gerrits et al. using 16S rDNA amplicons produced by the F24 and F25 universal primers and the TefF and TefR primers (designed for the real-time PCR assay) from selected isolates with reduced susceptibility to tetracycline.

### Results

For the 1006 H. pylori isolates from England and Wales tested for tetracycline susceptibility by disc diffusion, the maximum zone of inhibition recorded was 60 mm, minimum 0 mm, mean 44 mm, and mode 48 mm. A total of 18 isolates showed reduced susceptibility with zones of inhibition of ≤30 mm diameter. Of these, three isolates were resistant to tetracycline by the criterion of King: Etest MIC ≥4 mg/L (Table 1). One of the resistant isolates had an MIC of >256 mg/L, whereas the other two had an MIC of 4 mg/L, just within the definition of resistance. Tetracycline MICs were also determined by the agar dilution method to allow comparison with
other studies. All MIC and susceptibility results are summarized in Table 2.

Sequence analysis showed that 10 of the 18 reduced susceptibility isolates had 16S rDNA mutations in the region associated with tetracycline resistance (Table 2). Seven showed an A to G mutation at base 926 (A926G), one an A to C mutation at base 926 (A926C), one an A to C mutation at base 928 (A928C) and one a two base polymorphism of A to T at 926 and A to C at 928 (A926T, A928C).

The LightCycler assay produced a peak at a melting temperature (T_m) of ~68°C for all wild-type (AGA) sequences. Samples with 16S rDNA mutations in the helix 31 region produced peaks at lower T_m, characteristic for each mutation. The A926C mutations had a T_m of ~66°C, the A926G mutation a T_m of ~64°C, the A926G mutation a T_m of ~63°C and the A926T/A928C mutation a T_m of ~62°C. A control isolate of H. pylori with the triple AGA926–928TTC mutation (not part of the study) was also tested. It produced a T_m of ~61°C (Figure 1). The LightCycler assay was applied to DNA extracted from 100 tetracycline-susceptible H. pylori isolates and 20 H. pylori-positive gastric biopsies. In each case, no tetracycline resistance-associated 16S rDNA mutations were detected and the tetracycline susceptibility of the infecting H. pylori was correctly predicted.

The control strain 26695 was transformed with amplicons from the isolates with the novel A926C and A926T/A928C 16S rDNA mutations. The resulting 26695-A926C transformants had an Etest MIC of 1.5 mg/L (8 mg/L by agar dilution), identical with that of the donor isolate H3615. However, the 26695-A926T/A928C transformants had an Etest MIC of 2.0 mg/L (8 mg/L by agar dilution), which was significantly lower than the donor isolate H4534 Etest MIC of >256 mg/L, but higher than the recipient strain 26695 Etest MIC of 0.38 mg/L.

PCR amplicons generated with the F24 and F25 universal 16S rDNA primers from the eight reduced susceptibility isolates without mutations were also used in transformation experiments with strain 26695. No transformants with reduced susceptibility to tetracycline were produced.

Discussion

This study shows that tetracycline resistance amongst H. pylori from England and Wales is uncommon, occurring in just 0.3% of the isolates examined. Over the 4 year period of the investigation, there was no evidence of an increasing trend in either resistance or reduced susceptibility to tetracycline. This is consistent with previous UK-based studies. However, comparison between studies is hampered by the lack of standardized methodology for determining tetracycline susceptibility and of guidelines for the interpretive reading of resistance.

There was no evidence of resistance among any of the isolates to more than two of the antibiotics typically used in H. pylori eradication therapy in UK (Table 2). However, four out of 18 (22%) isolates with reduced susceptibility to tetracycline showed dual resistance to metronidazole and clarithromycin (two of which were from the same patient), whereas in a previous study dual resistance occurred in only 4% of isolates. Interestingly, the three tetracycline-resistant isolates were not from treatment failure cases, they were not dual resistant and one was resistant only to tetracycline. Detailed patient information was not available and it was not possible to establish if reduced susceptibility or resistance could have arisen due to induction of mutations in a previously sensitive strain during treatment with tetracycline for H. pylori eradication or for an unrelated infection.

Ten of the 18 isolates with reduced susceptibility to tetracycline had mutations in the 16S rDNA coding for the helix 31 region of 16S rRNA. The A926G mutation was found in seven isolates with Etest MICs that ranged between 0.75 and 4 mg/L (2 to 16 mg/L by agar dilution), two of which were scored as resistant by the criterion of King. The A926G mutation had previously been reported...
amongst two El Salvadorian isolates with an agar dilution method MIC of 2 mg/L. The A928C mutation was found in one isolate with an Etest MIC of 1 mg/L (4 mg/L by agar dilution). This mutation had been reported previously in a Lithuanian isolate with an agar dilution method MIC of 2 mg/L. However, the A926C mutation present in one isolate with an Etest MIC of 1.5 mg/L was novel as it had not been reported previously. To confirm that the mutation was responsible for the high tetracycline MIC, 16S rDNA from this isolate was used to transform strain 26695 (Etest MIC 0.38 mg/L). The resultant transformant had an Etest MIC of 1.5 mg/L identical with that of the donor isolate. The A926T/A928C mutation occurred in one isolate with an Etest MIC of >256 mg/L. This mutation had not been found before in a naturally occurring strain, but was created artificially by site-directed mutagenesis with an Etest MIC of 2 mg/L. A transformant created using 26695 and 16S rDNA from H4534, the isolate with the A926T/A928C mutation, had an MIC of 2.0 mg/L. This was higher than the MIC of the parent 26695 and lower than that of the donor isolate, but similar to that reported in the artificially created strain.

The variable MIC of isolates with the A926G mutation and the discrepancy between the MICs of isolate H4534 and transformants with the A926T/A928C mutation suggest that factors other than 16S rDNA polymorphism contribute to resistance and reduced susceptibility to tetracycline. In the closely related enteropathogen Campylobacter jejuni, tetracycline resistance can occur by acquisition of DNA encoding the ribosomal protection protein Tet(O) or may be due to efflux pump activity. However, all of the reduced susceptibility H. pylori were negative for tet(O) by PCR assay and tetracycline MIC was not affected by pretreatment with carbonyl cyanide m-chlorophenylhydrazone (CCCP), an efflux pump inhibitor (data not shown). However, it remains possible that efflux mechanisms unaffected by CCCP are involved.

LightCycler-based assays have been used to identify 23S rDNA mutations associated with clarithromycin resistance in H. pylori both from cultured isolates and biopsies. In this study a LightCycler assay successfully distinguished four distinct 16S rDNA mutations associated with resistance and reduced susceptibility to tetracycline from isolates with the wild-type allele. The assay also correctly detected the presence of the H. pylori isolated from clinical material containing tetracycline-resistant H. pylori was not available.

Tetracycline resistance has been associated with 16S rDNA mutations other than those found in this study. These include AG2926–927GT and A926G/A928C mutations associated with low-level resistance in clinical isolates and other mutations have been created in vitro. It is unlikely that the LightCycler assay would have sufficient resolution to reliably identify all of these reported mutations. However, any polymorphism in the region of the probe would result in a lower $T_m$ and thus be distinguishable from the wild-type allele. Possibly additional probes could be designed for future assays to identify specific 16S rDNA mutations. A possible shortcoming of the LightCycler assay is reduced sensitivity of detection of resistance-associated mutations in mixed populations with wild-type alleles. Previous studies have shown that detection of resistance-associated alleles present at less than 10% of a mixed population is unreliable. It remains unclear how such mixed populations respond to the antimicrobial therapies typically used in H. pylori eradication.

In contrast to the well-documented single nucleotide mutations in 23S rDNA associated with clarithromycin resistance, our data show the relationship between mutation and resistance is not as clear-cut in the case of 16S rDNA mutations and resistance to tetracycline. Nevertheless, 16S rDNA mutations are useful markers for tetracycline resistance based on the evidence of their presence in the three tetracycline-resistant isolates, 10 of the 15 isolates with highest Etest MICs in the survey of 1006 isolates, and absence from 100 tetracycline-susceptible isolates with disc diffusion zone sizes of >30 mm diameter.

In summary, novel and previously reported 16S rDNA mutations associated with reduced susceptibility to tetracycline are
present in isolates of *H. pylori* from England and Wales. However, tetracycline resistance is uncommon at present. The 16S rDNA polymorphisms are excellent targets for real-time PCR detection assays that allow ‘culture-free’ susceptibility testing direct from biopsies. This approach could be especially useful in the management of patients with a history of treatment failure as it allows the direct and rapid analysis of clinical material containing sub-lethally damaged *H. pylori* that might be difficult to culture.

**Acknowledgements**

We thank the hospitals and laboratories that submitted gastric biopsies and isolates to our Unit in the course of this study.

**References**

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