**Introduction**

Resistance of *Helicobacter pylori* to either clarithromycin or metronidazole (Mtz) has been associated with therapeutic failure and reduced eradication rates with multi-agent treatment regimens. In The Netherlands, reductions in treatment efficacy with triple agent therapies approached 50% when Mtz-resistant isolates were present. Reductions in efficacy were also noted when clarithromycin-resistant strains were present in individual patients. Multiple nitroreductases are expressed by *H. pylori* and probably contribute to the reductive activation of Mtz. In protozoan and bacterial pathogens susceptible to nitroimidazole compounds, reduction of the nitro group is considered essential for reductive activation and subsequent hydroxylamino intermediate formation. Hydroxylamino intermediates are likely to mediate chromosomal DNA strand breakage with resultant cytotoxicity. Null mutations in *rdxA*, encoding an oxygen-insensitive (type I) NADPH nitroreductase, confer Mtz resistance on *H. pylori*. Goodwin et al. demonstrated that insertional inactivation of *rdxA* in *H. pylori* resulted in a Mtz-resistant phenotype by preventing reduction of Mtz. In this study, *rdxA* mutations in *H. pylori* isolates from the USA were examined and compared with mutations reported recently from other geographical regions.

**Materials and methods**

**Determination of Mtz MIC**

*H. pylori* isolates were cultivated from gastric biopsies isolated by oesophagastroduodenal endoscopy at the Veterans’ Affairs Medical Center in Houston, TX, USA. MIC values for *H. pylori* isolates were determined by two-fold agar dilution. Plates were prepared using Mueller–Hinton (MH) agar as the basal medium supplemented with 5% aged sheep blood (2 weeks old). Serial concentrations ranging from 0.015 to 256 mg/L were achieved by addition of...
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Mtz (Sigma Chemical Co., St Louis, MO, USA) prepared in sterile distilled water and added to 5% sheep blood–MH basal medium. Fresh H. pylori cultures (2–3 days old) were suspended in saline at an optical density (OD₆₀₀) between 0.38 and 0.4. Using a Steers-type replicating device (Cathra, St Paul, TN, USA), 1–5 μL of adjusted inocula were delivered to the agar plates. Plates were incubated in microaerobic jars with CampyPak Plus sachets (Becton Dickinson BBL, Cockeysville, MD, USA) at 37°C for 3 days, without anaerobic pre-incubation. Mtz-resistant H. pylori ATCC 43504 (NCTC 11637) was used as control. H. pylori ATCC 43504 was evaluated by agar dilution antimicrobial susceptibility testing and found to possess an MIC >256 mg/L. A breakpoint MIC (8 mg/L) was defined in accordance with published studies.4

Amplification and sequencing of rdxA alleles

Genomic DNA was isolated from H. pylori isolates by phenol/chloroform extraction and ethanol precipitation. Genomic DNA specimens were suspended in TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0).

Primers complementary to regions flanking the 630 bp coding sequence of rdxA were used to amplify an 882 bp product. PCRs (50 μL final volume) included 0.5 U Taq DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN, USA), 1 × reaction buffer supplemented with 2.2 mM MgCl₂ (Roche Molecular Biochemicals), 5 ng bovine serum albumin (Roche Molecular Biochemicals), 50 nmol dNTP (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and 100 pmol each primer RdxA-19 (5’-GCC-AGATAGCCAAATGGGGG, forward) and RdxA-900R (5’-GAAAACGCTTTGAAAAACACCCC, reverse).

PCR was performed in a DNA Thermal Cycler 480 (Applied Biosystems, Foster City, CA, USA) with the following conditions: an initial denaturation (94°C, 6 min), followed by 35 cycles of denaturation (94°C, 30 s), annealing (48°C, 1 min) and extension (72°C, 3 min), and a final extension (72°C, 10 min). Amplification products were separated in 1% agarose by electrophoresis, visualized with 0.5 μg/mL ethidium bromide and purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech).

RdxA amplicons were sequenced in the ABI Prism 310 Genetic Analyzer (Applied Biosystems) using ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit chemistry (Applied Biosystems) according to the manufacturer’s recommendations. An internal sequencing primer, RdxA-255 (5’-AATCGCTGAATCGCCAGGC) was used to determine and verify sequence data. Sequences obtained were aligned and translated by Megalign sequence analysis software (LaserGene, DNASTar, Madison, WI, USA). Newly obtained rdxA sequences were compared with published rdxA data of Mtz-susceptible H. pylori strain 500.²

Results

Clinical isolates of H. pylori were obtained from 17 random, unpaired gastric biopsy specimens. MICs of eight Mtz-susceptible H. pylori isolates (Mtz MIC range: 0.25–1.0 mg/L) and nine Mtz-resistant isolates (Mtz MIC range: 16–256 mg/L) were determined by agar dilution antimicrobial susceptibility testing (AST). Median MICs were 0.5 mg/L for Mtz-susceptible isolates and 64 mg/L for Mtz-resistant isolates.

DNA sequence analyses of rdxA alleles from Mtz-susceptible H. pylori isolates revealed intact reading frames (i.e. lacking nonsense mutations). Pairwise alignment of susceptible isolates against a reference Mtz-susceptible H. pylori strain 500 (Hp 500), using the Martinez–Needleman–Wunsch method, yielded rdxA allelic identities ranging from 95.5 to 97%. Nucleotide substitutions varied in number (19–29), with two to eight resultant amino acid substitutions. Peptide similarity indices ranged from 95.8 to 99.1 using the Lipman–Pearson method.

In contrast, most Mtz-resistant H. pylori isolates contained nonsense mutations (seven of nine) in rdxA, resulting in expression of putatively truncated nitroreductase, RdxA (Table). Insertion or deletion (indel) events were identified in six of seven rdxA alleles with nonsense mutations. Four of six Mtz-resistant H. pylori isolates contained single nucleotide insertions in rdxA, whereas two isolates contained poly-nucleotide deletion mutations. These indel mutations resulted in translational frameshifts, creating premature stop codons at downstream positions. Premature termination codons in the six frameshift mutants were distributed between codon positions 32 and 176 in the 209 amino acid RdxA polypeptide (Figure). One of the nine Mtz-resistant isolates contained a transition mutation, C→T, at nucleotide position 148. This substitution mutation created a premature termination codon at that site, probably resulting in a truncated RdxA polypeptide containing 49 amino acids.

Two Mtz-resistant isolates lacked nonsense mutations. Missense mutations and resultant amino acid substitutions may alter the function of RdxA and preclude reductive activation of Mtz. A pairwise alignment of the two missense mutants, B1/18 and 52/16, yielded identities of 95.3 and 96.2%, respectively, when compared with Hp 500. Interestingly, the A80T mutation has been found only in Mtz-resistant H. pylori isolates and was present in both resistant isolates lacking nonsense mutations (Table). The Table summarizes mutations found in rdxA alleles of Mtz-resistant isolates.

Discussion

In this study, North American H. pylori isolates resistant to Mtz contained mutations yielding truncated RdxA polypeptides. Diminished nitroreductase activity inhibits
Metronidazole resistance in *Helicobacter pylori*

Table. Sequence analysis of rdxA from Mtz-resistant *H. pylori* isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>MIC (mg/L Mtz)</th>
<th>Codon substitutions</th>
<th>Nature of mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>51/053</td>
<td>64.0</td>
<td>E32stop</td>
<td>frameshift due to polynucleotide deletion (–4 nt at 63–66)</td>
</tr>
<tr>
<td>B1/18</td>
<td>128.0</td>
<td>R16H, E31T, V68A, A80T, Q197K, V204F</td>
<td>multiple missense mutations</td>
</tr>
<tr>
<td>B0/014</td>
<td>128.0</td>
<td>Q50stop</td>
<td>nonsense mutation (C→T at 148)</td>
</tr>
<tr>
<td>B6/012</td>
<td>32.0</td>
<td>R112stop</td>
<td>frameshift due to nucleotide addition (+1G at 332)</td>
</tr>
<tr>
<td>52/16</td>
<td>64.0</td>
<td>L62V, A80T, S88P, R90K, A118S</td>
<td>multiple missense mutations</td>
</tr>
<tr>
<td>A2/010</td>
<td>16.0</td>
<td>S92stop</td>
<td>frameshift due to nucleotide addition (+1G at 240)</td>
</tr>
<tr>
<td>B0/17</td>
<td>256.0</td>
<td>R176stop</td>
<td>frameshift due to polynucleotide deletion (–9 nt at 112–120)</td>
</tr>
<tr>
<td>57/18</td>
<td>32.0</td>
<td>M154stop</td>
<td>frameshift due to nucleotide additions (+1T at 430; +1G at 467)</td>
</tr>
<tr>
<td>2723</td>
<td>64.0</td>
<td>S158stop</td>
<td>frameshift due to nucleotide addition (+1T at 459)</td>
</tr>
</tbody>
</table>

*aPairwise alignment with rdxA of *H. pylori* strain 500 (Goodwin et al.)*.  
*bProtein sequence analysis using Lipman–Pearson alignment method*.  
*cAmino acid substitutions found only in Mtz-resistant isolates*.  

Figure. Locations of mutations within rdxA. A schematic diagram depicts the location of premature translation termination (STOP) codons (indicated by dark arrows) within the rdxA coding sequences of *Helicobacter pylori* isolates presented in this study. Unfilled, box-shaped arrows depict sites of point mutations present in Mtz-resistant *H. pylori* isolates.

reductive activation of Mtz, rendering nitroimidazole agents ineffective in *H. pylori* eradication regimens. The *rdxA* mutations in resistant isolates included the addition of individual nucleotides or polynucleotide deletions. These results are consistent with recently published studies of European and North African *H. pylori* isolates documenting the presence of indel mutations, yielding truncated, presumably inactive, RdxA.5,6

Mtz is inactive in its parent form and requires reductive activation by RdxA or other nitroreductases in *H. pylori* to exert antimicrobial activity. In contrast to macrolide resistance in *H. pylori* and associated drug target alteration of 23S ribosomal RNA,7 frameshift and point mutations in *rdxA* result in truncated nitroreductase and diminish the ability of *H. pylori* to activate Mtz. In addition to frameshift mutations caused by insertion or deletion events, substitution mutations in important structural or catalytic domains of *rdxA* may lead to functional inactivation of nitroreductase. Nucleotide analyses showed that in Mtz-susceptible isolates, transition substitutions were most frequently found in *rdxA* alleles, as expected (140 of 166 nucleotide substitutions, data not shown). Sequence divergence in susceptible isolates ranged from 3 to 4.5% and is consistent with previously reported (c. 5%) in allelic differences *rdxA*.2 Presumably, these nucleotide polymorphisms do not result in functionally important amino acid substitutions.
Functionally important point mutations in rdxA are proposed in this study. Point mutations found only in resistant isolates are described in the Table. The A80T mutation (G→A transition event), resulting in an alanine to threonine residue substitution (A80T) is intriguing due to its presence in both resistant strains (B1/18 and 52/16) lacking nonsense mutations. This amino acid is conserved in an E. coli nitroreductase, nfsB, and lies adjacent to the mini-IS605 insertion in H. pylori NCTC 11637 (ATCC 43504). Mutations in nfsB may decrease reductive activation of nitrofuran compounds (e.g. furazolidone, nitrofurazone) leading to nitrofuran resistance in E. coli. As with Mtz, nitrofurans require reduction of nitro moieties to cause DNA breakage. We propose that this amino acid lies in a functionally important domain and structural alteration of this region inactivates RdxA. The identical mutation (A80T) has been reported in a resistant North African isolate, and substitution mutations at nearby codon positions 79 and 87 have recently been reported in resistant isolates from France.

Metronidazole and related nitroimidazole compounds (e.g. tinidazole) represent important agents in H. pylori eradication regimens. The prevalence of resistant isolates in patients varies depending on the geographical region in question, but has reached alarming proportions in both developed and developing nations. This study is the first report describing the mutational mechanisms of Mtz resistance in H. pylori from the USA and corroborates the findings of previous studies. The panoply of mutations found in rdxA is consistent with the high level of genetic diversity of H. pylori. Mutations in rdxA and other nitroreductases may explain molecular mechanisms of nitroimidazole drug action and resistance and may facilitate the development of novel antimicrobial agents for future use in patients.

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