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Characterisation of the genes encoding resistance to metronidazole (*rdxA* and *frxA*) and clarithromycin (the 23S-rRNA genes) in South African isolates of *Helicobacter pylori*

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Helicobacter pylori has been incriminated in human diseases, such as peptic ulcer, gastritis and gastric malignancy. Although modern triple-drug regimens are usually highly effective in the treatment of *H. pylori* infection, the emergence of resistance to two of the most used antibiotics, metronidazole (Mtz) and clarithromycin (Cla), is a serious and increasing problem. Truncations in the *rdxA* and *frxA* genes of *H. pylori* are thought to be associated with Mtz resistance whereas mutations in the pathogen's 23S-ribosomal-RNA (23S-rRNA) genes are associated with Cla resistance. In a recent study, PCR and sequence analysis of the *rdxA*, *frxA* and 23S-rRNA genes were used to explore the genetic basis of resistance to Mtz and Cla in *H. pylori*. When 200 isolates of *H. pylori* from the Eastern Cape province of South Africa were tested for antibiotic susceptibility, almost all (95.5%) were found resistant to Mtz and 20.0% were found resistant to Cla. Only the Mtz-resistant isolates showed *rdxA* and *frxA* truncation. Two point mutations were detected in the 23S-rRNA genes of the Cla-resistant isolates. Many significant changes (resulting in 13 amino-acid substitutions in nine loci and truncated proteins in 14 loci) were observed in the *rdxA* genes of the Mtz-resistant isolates, and it appears that, compared with the rarer changes detected in *frxA*, such mutations may contribute more significantly to the high prevalence of Mtz resistance. To guide empiric treatment, the genotypes and antibiotic susceptibility of *H. pylori* in the Eastern Cape province of South Africa need to be monitored regularly.

Helicobacter pylori is a gastric pathogen that infects >50% of the world's population, the major cause of several gastro–duodenal pathologies in infected patients (Tankovic *et al.*, 2000; Cover and Blanke, 2005), and an early risk factor for gastric cancer (Matsuhisa *et al.*, 2003). The large number of individuals infected, the tenacity of *H. pylori* infection and the associated morbidity make effective treatment regimens extremely important. Eradication of the organism usually leads to a feeling of general well-being among the treated patients (Sepulvedo and Coelho, 2002). The triple therapy that is generally recommended for eradicating *H. pylori* consists of a proton-pump inhibitor, clarithromycin (Cla) and either metronidazole (Mtz) or amoxicillin (Kalach *et al.*, 2001). Unfortunately, *H. pylori* has acquired resistance to many classes of antibiotics (Kwon *et al.*, 2001; Ahmad *et al.*, 2009) and such resistance is now a primary cause of treatment failure, especially in Africa (Asrat *et al.*, 2004; Ndip *et al.*, 2008).

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Although Mtz has been the cornerstone of many triple-drug regimens for the eradication of *H. pylori*, resistance to this drug can now be detected in 80%–90% of *H. pylori* isolates from many developing countries (Asrat *et al.*, 2004; Ndip *et al.*, 2008) and in 5%–50% of such isolates (depending on geographical region and patient group) from Western Europe (Alarcón *et al.*, 1999; Ables *et al.*, 2007). Such resistance clearly decreases the effectiveness of Mtz-containing anti-*H. pylori* therapies, which tend to be popular because they are relatively inexpensive (Kim *et al.*, 2009).

If patients infected with Mtz-susceptible *H. pylori* are treated with the drug, reduction of the nitro moiety of the Mtz produces highly reactive compounds that cause DNA strand breakage, helix destabilization, helix unwinding and, ultimately, death of the bacterial cells (Kwon *et al.*, 2001; Kim *et al.*, 2009). Acquisition of Mtz resistance by *H. pylori* is highly associated with mutational inactivation of the bacterium's *rdxA* gene, which encodes an oxygen-insensitive NADPH nitroreductase (Tankovic *et al.*, 2000). Inactivation of *frxA*, which encodes NAD(P)H-flavin oxidoreductase, may also contribute to the Mtz-resistant phenotype, either alone or in association with *rdxA* (Jeong *et al.*, 2000; Kwon *et al.*, 2001; Llanes *et al.*, 2010). Although gene sequencing has provided some support for the idea that *frxA* and/or *rdxA* play a role in resistance to Mtz, frameshift mutations in *frxA* have been found to occur with similar frequencies in Mtz-sensitive and -resistant isolates (Chisholm and Owen, 2004). According to Jeong *et al.* (2000), most Mtz resistance in *H. pylori* depends on *rdxA* inactivation (although mutations in *frxA* can enhance such resistance), and genes conferring Mtz resistance without *rdxA* inactivation are either rare or non-existent in *H. pylori* populations.

Clarithromycin resistance is also becoming increasingly prevalent in *H. pylori* (Lee *et al.*, 2005). Most (>90%) of the macrolide resistance seen in *H. pylori* appears to be mediated by a transition (A→G) mutation at position 2142 or 2143 in one or both of the bacterium's two 23S-rRNA genes (Matsuoka *et al.*, 1999). Other mutations in these 23S-rRNA genes, such as A→C, A→T and G→T, have also been detected (Doorn *et al.*, 2001; Kim *et al.*, 2002).

About 50%–60% of the people living in South Africa are thought to be infected with *H. pylori* (Tanih *et al.*, 2010*a*). In the country's Eastern Cape province, the resistance of *H. pylori* to Mtz is a common problem (Tanih *et al.*, 2010*b*). Resistance to Mtz and/or Cla is clinically significant because it increases the risk of treatment failure (Mégraud, 1997). The main aim of the present study was to employ PCR and sequence analysis of the genes that are believed to be involved in Mtz resistance (*rdxA* and *frxA*) and Cla resistance (the 23S-rRNA genes) to determine if specific mutations in these genes were responsible for the drug resistance seen in *H. pylori* isolates from Eastern Cape province. It was hoped that the results would improve prognosis and empiric treatment.

MATERIALS AND METHODS

Study Population and Ethics

The *H. pylori* isolates used in this study were isolated from patients from Eastern Cape province who were suffering from gastric-related morbidities and, when the isolates were collected, had no recorded history or memory of treatment with Cla or Mtz. After informed consent was obtained, gastric biopsies were collected by a resident gastro-enterologist.

The study protocol was approved by the institutional review board of the University of Fort Hare and the Eastern Cape Department of Health (protocol number EcDoH-Res 0002).

Bacteriology

Helicobacter pylori was isolated from the gastric biopsies by following standard microbiological procedures (Ndip *et al.*, 2008).

Briefly, biopsies were homogenized, under aseptic conditions, in sterile brain–heart-infusion (BHI) broth (Oxoid, Basingstoke, U.K.) supplemented with cysteine (0.2 g/litre) and glycerol (20%, v/v). A loopful of this homogenate was then plated on freshly prepared Columbia agar base (Oxoid) containing 7% (v/v) sheep's blood (Oxoid) and Skirrow's supplement (Oxoid; two vials/litre, giving 5 mg trimethoprim, 10 mg vancomycin, 5 mg cefsulodin and 5 mg amphotericin/litre). All plates were incubated at 37°C for 3–5 days under micro-aerophilic conditions (5%–6% O_2, 10% CO_2, 80%–85% N_2) produced using the Anaerocult® P reagent (Merck, Darmstadt, Germany). Isolates were identified based on colony morphology and positive results in oxidase, urease and catalase tests. A reference strain of *H. pylori* (NCTC 11638) was included as a positive control. Isolates identified as *H. pylori* were suspended in 20% (v/v) glycerol and stored at −80°C, for use in future experiments.

Testing Antibiotic Susceptibility

Susceptibility testing was carried out by the disk-diffusion (Kirby–Bauer) technique (Tanih *et al.*, 2010*b*), using disks (Mast Group, Bootle, U.K.) pre-dosed with Cla (15 μg) or Mtz (5 μg) on plates of BHI agar (Oxoid) containing 7% (v/v) horse blood and *Helicobacter pylori* selective supplement (Oxoid; two vials/litre). Reference strains of *H. pylori* (NCTC 11638 and J99) were included in all of the experiments.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC of Mtz and Cla were determined, for each isolate, using the agar-dilution method (Osato, 2000). After incubation, the MIC value was read as the lowest concentration of the antibiotic that inhibited all visible bacterial growth. An isolate was considered Mtz-resistant if it had an MIC of >8 μg Mtz/ml and Cla-resistant if it had an MIC of >1.0 μg Cla/ml (Osato, 2000; Ndip *et al.*, 2008).

Molecular Characterisation

DNA EXTRACTION

The QIAamp DNA kit (QIAGEN, Hilden, Germany) was used, according to the manufacturer's recommendations, to extract the DNA from cell pellets produced from 14 of the test isolates found Mtz-resistant, three of the isolates found Mtz-susceptible and three of the test isolates found Cla-resistant. The extracted DNA was stored at −20°C until analysis.

PCR-BASED AMPLIFICATION

PCR-based analysis of the targeted genes was performed using Thermo-stat *Taq* DNA polymerase (ABgene, Epsom, U.K.) and the reaction buffer provided by the enzyme's manufacturer. Each 50-μl reaction mixture contained 5 μl DNA in 1 × PCR buffer containing 3 mM $MgCl_2$, 0.2 mM of each deoxynucleotide (Abgene), 62.5 U Thermo-stat *Taq* DNA polymerase and either 0.5 μM (*rdxA* and 23S-rRNA genes) or 0.2 μM (*frxA*) of each of the two relevant oligonucleotide primers (see Table 1). The thermal cycler (MJ Research, Waltham, MA) was set to give an initial denaturation of target DNA at 95°C for 15 min and then 35 cycles of denaturation at 94°C for 1 min, primer annealing at 56°C for 1 min and extension at 72°C for 1 min before a final extension step, at 72°C for 5 min. As a negative control (used in each PCR run), the template DNA was replaced with ultrapure water (Sigma–Aldrich, Gillingham, U.K.).

The amplicons produced (5 μl) were separated by electrophoresis in 2% (w/v) high-resolution agarose gel, using Tris–acetate–EDTA buffer and staining with ethidium bromide (0.5 μg/ml). The bands were visualized under ultraviolet light and photographed.

MUTATIONAL ANALYSIS

Mutations and genetic diversity in the genes of interest were explored by sequencing the PCR products. Each sample of amplified DNA (20 μl) was first cleaned by

mixing with 2 µl shrimp alkaline phosphatase (Promega, Madison, WI) and 2 µl Exonuclease solution (Promega). This mixture was then incubated for 30 min at 37°C, 15 min for 72°C, and then 5 min at 8°C. Sequencing was carried out using version 3.1 of the Big Dye® Terminator DNA sequencing kit (Applied Biosystems, Carlsbad, CA) and a mixture of 2 µl chromosomal DNA, 0.25 µl primer (10 pmol/µl), 2 µl Big Dye buffer and 2 µl Big Dye. The thermal cycler used was set to give 30 cycles, each of denaturation at 96°C for 10 s, annealing at 50°C for 20 s, and extension at 60°C for 4 min. The cycling was followed first by dye-terminator removal, using the Agencourt® CleanSeq® system (Agencourt Bioscience, Beverly, MA), and then by sequencing on the ABI 3130xl automated sequencer (Applied Biosystems). The sequences were edited, aligned and analysed using BioEdit (www.mbio.ncsu.edu/bioedit/bioedit.html), Clustalw2 (www.ebi.ac.uk/Tools/msa/clustalw2/) and the DNAMAN software package (Lynnon, Pointe-Claire, Canada).

RESULTS

Patient Characteristics

Overall, 254 patients (90 males and 164 females) were enrolled in a larger study on *H. pylori* (Tanih *et al.*, 2010*c*) of which this investigation forms part. Their mean (S.D.) age was 44.5 (15.7) years (range= 5–93 years). Most (83%) were older than 35 years.

Susceptibility Testing and MIC Determination

Of the 200 isolates of *H. pylori* (from the 254 patients) that were subjected to tests of antibiotic susceptibility, 95.5% were found Mtz -resistant and 20.0% Cla-resistant. The isolates showed MIC of 1–256 µg/ml for Mtz and 0.125–256 µg/ml for Cla.

PCR Amplification of 23S-rRNA, *frxA* and *rdxA Genes*

As expected, PCR based on the primers for the 23S-rRNA, *frxA* and *rdxA* produced amplicons of 307, 445 (Fig. 1) and 427 (Fig. 2) bp, respectively.

Characterisation of *rdxA* and *frxA* Genes of the Mtz-sensitive and Mtz-resistant Isolates

rdxA

Although a Mtz-sensitive isolate (237C) exhibited a Glu(27)Val amino-acid substitution (i.e. a missense mutation) in its *rdxA* gene that was not seen in the Mtz-resistant isolates, the other two Mtz-susceptible isolates checked by sequencing (238A and 238C) remained unchanged for their *rdxA* genes (Table 1). Of the 14 Mtz-resistant isolates investigated by sequencing, two (279C and 305C) had missense mutations, nine (243A, 243C, 265C, 266A, 268C, 293A, 294A, 308A and 308C) showed a total of 13 amino-acid substitutions, and seven (243A, 243C, 266A, 268C, 279A, 293C and 296C) had nonsense mutations; 243A, 243CA, 266A and 268C therefore

TABLE 1. *The primers used for the PCR-based amplification of sequences from the* rdxA, frxA *and 23S-rRNA genes of* Helicobacter pylori

Gene	Primer sequences	Amplicon size (bp)	Reference
rdxA	5′-GTTAGGGATTTTATTGTAATG-3′ 3′-ACGCCAAGCATTTGAGCAAA-5′	427	Kwon *et al.* (2001)
frxA	5′-TCTCAAGCGGAAAAATCCGG-3′ 3′-AATTTTTGATGATTTGAGCG-5′	445	Kwon *et al.* (2001)
23S-rRNA	5′-ACGGCGGCCGTAACTATA-3′ 5′-ACAGGCCAGTTAGCTA-3′	307	Wang *et al.* (2001)

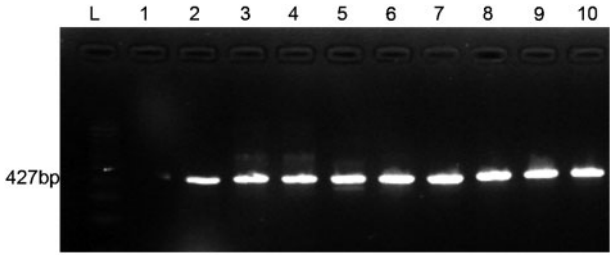

FIG. 1. The results of the electrophoresis of the amplicons produced in PCR targeting the *rdxA* gene of *Helicobacter pylori*. The lanes contained a molecular-weight 'ladder' (L), the products from a negative-control reaction (lane 1) and samples produced from test isolates of *H. pylori* (lanes 2–10).

showed both nonsense mutations and amino-acid substitutions (Table 2). The changes seen in the Mtz-resistant isolates resulted in an *rdxA* protein truncated at position 16, 22, 27, 32, 56, 60, 71, 78, 97, 106, 111, 113, 115 or 121.

frxA

In almost all of the isolates investigated by sequencing (including all of the three Mtz-susceptible isolates), the open reading frame for the *frxA* gene was disrupted by a deletion of a nucleotide at position 54 or 98 (Table 2). In one Mtz-resistant isolate (293C), such disruption was caused by a nucleotide insertion at position 224 (Table 2). These deletion/insertion events led to the occurrence of a stop codon at positions corresponding to amino acids 39, 72 or 84. Some point mutations were also observed, in five of the Mtz-resistant isolates (Table 2).

Detection of Mutation in the 23S-rRNA Gene, by Sequencing

Mutations of the 23S-rRNA genes of three isolates showing high levels of resistance to Cla (with MIC no higher than 256 μg/ml) were investigated by sequencing. All three of the isolates showed a mutation at position A2142G, and one also showed an A2143G mutation.

DISCUSSION

The present results add to the description of Mtz and Cla resistance and MIC values reported, for the same *H. pylori* isolates, by Tanih *et al.* (2010*b*). Resistance to Mtz is currently the most common type of resistance found in *H. pylori* and is, along with other types of antibiotic resistance, a major cause of elimination failure (Marais *et al.*, 2003; Nahar *et al.*, 2004; Tanih *et al.*,

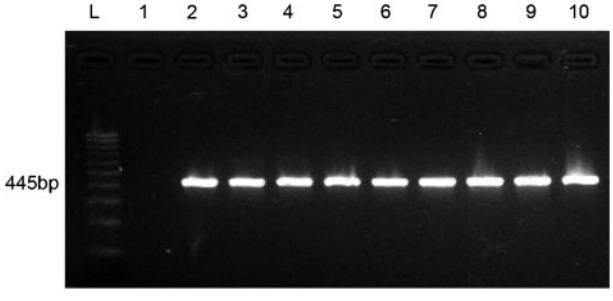

FIG. 2. The results of the electrophoresis of the amplicons produced in PCR targeting the *frxA* gene of *Helicobacter pylori*. The lanes contained a molecular-weight 'ladder' (L), the products from a negative-control reaction (lane 1) and samples produced from test isolates of *H. pylori* (lanes 2–10).

TABLE 2. *Detection of mutations in the* rdxA *and* frxA *genes in 17* Helicobacter pylori *isolates, only three of which (237C, 238A and 238C) were considered metronidazole-susceptible*

Isolate	Minimum inhibitory concentration of metronidazole (μg/ml)	Changes in *rdxA*		Changes in *frxA*	
		Nucleotide (nt) sequence	Amino-acid sequence	Nucleotide sequence	Amino-acid sequence
237C	1	–	Glu(27)Val	Frameshift (nt deletion at position 54)	Val(44)Gly, Tyr(60)Phe
238A	8	–	–	Frameshift (nt deletion at position 54)	Stop codon at position 39
238C	1	–	–	Frameshift (nt deletion at position 54)	Stop codon at position 39
243A	256	–	Leu(71)Phe, Gln(113)stop codon	Frameshift (nt deletion at position 54)	Stop codon at position 39
243C	256	–	Pro(106)Ser, Ser(111)Leu, Arg(16)stop codon	Frameshift (nt deletion at position 54)	Stop codon at position 84
265C	256	–	Arg(16)Cys, Met(56)Val	Frameshift (nt deletion at position 98)	Stop codon at position 39, His(6)Leu, Ser(7)Phe
266A	64	–	Ser(111)Leu, Arg(56)stop codon	–	Stop codon at position 39, Thr (110)Ser, Glu(169)Lys
268C	256	–	Arg(16)His, Thr(16)Ala, Glu(60)stop codon	Frameshift (nt deletion at position 54)	Thr(26)Glu
279A	128	–	Leu(121)stop codon	Frameshift (nt deletion at position 54)	Stop codon at position 39
279C	64	Missense [deletion of three nt (80–82)]	–	Frameshift (nt deletion at position 54)	Stop codon at position 39
293A	128	–	Glu(27)Gln	Frameshift (nt deletion at position 54)	Glu(199)stop codon
293C	128	–	Glu(60)stop codon	Frameshift (nt insertion at position 224)	Stop codon at position 72
294A	256	–	Val(32)Ala	Frameshift (nt deletion at position 54)	Stop codon at position 39
296C	256	–	Glu(78)stop codon	Frameshift (nt deletion at position 54)	Stop codon at position 39
305C	64	Missense [deletion of four nt (80–83)]	–	Frameshift (nt deletion at position 54)	Stop codon at position 72
308A	64	–	Asn(22)Ser	–	Ala(112)stop codon
308C	128	–	His(97)Tyr, Arg(115)Ile	Frameshift (nt deletion at position 54)	Glu(120)Lys, Met(126)Phe, Thr(26)Glu

2010*b*). In South Africa, the high prevalence of resistance to Mtz in *H. pylori* might be due to the drug's frequent use against intestinal parasites and in the treatment of gynaecological disorders; among patients attending the primary healthcare system in South Africa, Mtz is one of the most widely used antibiotics (Tanih *et al.*, 2010*b*). Given that patients who admitted taking Mtz for their current gastric morbidity were excluded from the present study, it is unlikely (but not impossible) that any of the tested isolates came from patients who had failed Mtz treatment (i.e. patients who were particularly likely to be infected with Mtz-resistant *H. pylori*).

Mtz resistance in *H. pylori* has previously been associated with inactivation of *rdxA* and/or *frxA* (Jeong *et al.*, 2000; Tankovic *et al.*, 2000; Kwon *et al.*, 2001). Both of these genes can be inactivated without leading to the death of the pathogen (Kwon *et al.*, 2001) and mutations in them appear to be quite common, allowing the selection of resistance during the clinical use of Mtz (Kwon *et al.*, 2001; Jeong *et al.*, 2000; Tanih *et al.*, 2010*b*). The Glu(27)Val mutation detected, in the present study, in a Mtz-susceptible isolate, was not observed in any of the Mtz-resistant isolates and therefore cannot be required for resistance to occur. Some of the mutations detected in the *rdxA* genes of the Mtz-resistant isolates that were investigated, by sequencing, in the present study — Arg(115)Ile, Arg(16)His, Ser(111)Leu and Arg(16)Cys — have also been described previously (Kwon *et al.*, 2001; Wang *et al.*, 2001; Yang *et al.*, 2004). The other mutations detected, either in the *rdxA* genes [Thr(16)Ala, Glu(27)Gln, Val(32)Ala, Asn(22)Ser, His(97)Tyr, Met (56)Val, Ser(111)Leu, Leu(71)Phe, Pro (106)Ser, Gln(113)stop codon, Arg(16)stop codon, Arg(56)stop codon, Glu(60)stop codon, Leu(121)stop codon, and Glu(78) stop codon] or *frxA* genes [Val(44)Gly, Tyr(60)Phe, His(6)Leu, Ser(7)Phe, Thr (110) Ser, Glu(169)Lys, Thr(26)Glu, Glu(120)Lys, Met(126)Phe, Thr(26)Glu, Ala(112)stop codon, and Glu(199)stop codon] of the Mtz-resistant isolates have not been previously described in the literature. The phenotypic changes resulting from any of these mutations may include antibiotic resistance (Jeong *et al.*, 2000).

It has been suggested that inactivation of *frxA* cannot cause Mtz resistance in the absence of mutations in *rdxA* (Kwon *et al.*, 2001; Yang *et al.*, 2004). In the present study, however, not only did some of the Mtz-resistant isolates have no mutations in their *frxA* genes but also the mutations seen in the *frxA* genes of many of the Mtz-resistant isolates were identical to those seen in the Mtz-susceptible isolates (Table 2). It therefore seems unlikely that the detected *frxA* mutations contributed to the observed Mtz resistance. The present results are in accordance with the notion that Mtz susceptibility is dependent on the level of nitroreductase activity produced by *rdxA* (Kwon *et al.*, 2001).

Resistance to Cla can considerably reduce the success of the standard triple therapies used against *H. pylori* infection, as Cla is generally used in such treatments (Duck *et al.*, 2004; Ahmad *et al.*, 2009). The prevalence of Cla resistance among *H. pylori* isolates has been found to vary from 1% to 29%, the value depending on the location of the study site and tending to increase with the amount the drug is used locally, many respiratory-tract infections being treated with the drug (Kato *et al.*, 2002). The resistance of *H. pylori* to Cla has been associated with an adenosine-to-guanosine (A-to-G) substitution within the peptidyltransferase-encoding region of the pathogen's 23S-rRNA genes (Versalovic *et al.*, 1996). Mégraud (2004) stated that most Cla-resistant isolates of *H. pylori* show the A2142G mutation seen in the present study and/or the A2143C mutation, although, previously, Kim *et al.* (2002) had described A2143G as the mutation that was most frequently detected in Cla-resistant *H. pylori*. In the present study, A2142G appeared more common than A2143G but

only three Cla-resistant isolates were investigated for mutations in their 23S-rRNA genes. The A2142G mutation detected, in the present study, in all three Cla-resistant isolates might be responsible for the high level of Cla resistance seen in *H. pylori* from the present study area (Tanih *et al.*, 2010*b*). Both of the 23S-rRNA mutations detected in the present study (A2142G and A2143G) have, however, already been associated with resistance to Cla (Versalovic *et al.*, 1996; Matsuoka *et al.*, 1999; Mégraud, 2004; Ahmad *et al.*, 2009).

It is hoped that the present results help guide clinicians in the study area to choose effective (Mtz-free) regimens for the treatment of *H. pylori* infection.

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