Contingency nature of *Helicobacter bizzozeronii* oxygen-insensitive NAD(P)H-nitroreductase (HBZC1_00960) and its role in metronidazole resistance

Pradeep Kumar Kondadi, Claudia Pacini, Joana Revez, Marja-Liisa Hänninen and Mirko Rossi*

**Abstract**

Genomic analysis of a metronidazole resistant *H. bizzozeronii* strain revealed a frame length extension of the oxygen-insensitive NAD(P)H-nitroreductase HBZC1_00960 (RdxA), associated with the disruption of the C-terminal cysteine-containing conserved region (IACLXALGK). This was the result of the extension (from C₈ to C₉) of a simple sequence cytosine repeat (SSCR) located in the 3′ of the gene. A 3′ SSCR is also present in the *rdxA* homolog of *H. helmmani* sensu stricto, but not in *H. pylori*. We showed that in the majority of in vitro spontaneous *H. bizzozeronii* metronidazole resistant mutants, the extension of the 3′ SSCR of *rdxA* was the only mutation observed. In addition, we observed that *H. bizzozeronii* *ArdxA* mutant strain showed the same MIC value of metronidazole observed in the spontaneous mutants. These data indicate that loss of function mutations in *rdxA* and in particular the disruption of the conserved region IACLXALGK is associated with reduced susceptibility to metronidazole in *H. bizzozeronii*. Slipped-strand mispairing of the SSCR located in the 3′ of the *H. bizzozeronii rdxA* appears to be the main mechanism. We also observed that *H. bizzozeronii* acquires resistance to metronidazole at high mutation rate, and that serial passages in vitro without selection induced an increased level of susceptibility. In conclusion, contrary to what was previously described in *H. pylori*, the *H. bizzozeronii rdxA* appears to be a contingency gene which undergoes phase variation. The contingency nature of *rdxA* should be carefully considered when metronidazole is used in the treatment of *H. helmmani*-associated gastritis.

**Introduction**

The human-adapted pathogen *Helicobacter pylori* is one of the most common causes of bacterial infections worldwide, and it is recognized as an etiologic agent of chronic gastritis, peptic ulcers, gastric adenocarcinoma and MALT lymphoma [1]. Humans can also be sporadically infected by non-*H. pylori* gastric *Helicobacter* species, referred to as *H. helmmani* sensu lato, that are also able to cause gastritis [2]. *H. helmmani* sensu l.i. comprises very fastidious zoonotic *Helicobacter* species, including *H. bizzozeronii*, *H. felis*, *H. suis* and *H. helmmani* sensu stricto, which are all known to colonize the gastric mucosa of different animal species [2]. Although the absence of a simple laboratory test have lead to an underestimation of the infection rate, *H. helmmani* s.l. is consider to be a rare type of zoonosis, with prevalence ranging between 0.2% and 6.5% depending of the geographic region [2-5]. Due to the rarity of these infections and the peculiar growth requirements of *H. helmmani* s.l., which limits the isolation of pure cultures [2], very little is known about the prevalence of antibiotic resistance in these species [6-9]. Therefore, the optimal treatment regimen for these infections remains unclear, and conventional *H. pylori* eradication treatment is generally recommended [10,11]. The standard treatment for *H. pylori* appears to eradicate *H. helmmani* s.l. infection in most patients [3,5,10-13]. However, cases of failed treatment have been reported [7,14]. The peculiar
growth requirements of *H. heilmannii* s.l. have limited the application of molecular tools, affecting studies on the molecular mechanisms of antibiotic resistance in these species and hampering the adoption of new, specific strategies to treat patients after a failed treatment.

To better understand the molecular mechanisms of antibiotic resistance in *H. heilmannii* s.l., we investigated the potential reasons behind the failed treatment of a *H. bizzozeronii* infection in a 47-year-old woman suffering with chronic gastritis [7]. A few months after the diagnosis of *H. bizzozeronii*-associated gastritis, the patient was treated with a seven-day course of lansoprazole 30 mg twice daily, tetracycline 500 mg four times daily and metronidazole 400 mg three times daily. After the treatment, the patient’s symptoms became less severe and the patient started to gain weight. However, she continued to suffer from mild nausea associated with eating warm foods, and, after a few months, *H. bizzozeronii* was re-isolated from antrum samples obtained in a follow-up endoscopy [7]. The *H. bizzozeronii* obtained before the treatment was resistant to tetracycline, but a heterogeneous resistance profile for metronidazole was observed [15]. In fact, *H. bizzozeronii* CIII-1ORG (an isolate obtained from the corpus of the patient’s stomach before the treatment) showed an MIC of metronidazole equal to 32 μg/mL, but the MIC value for its derived clone CIII-1GEN, obtained by amplification of a single colony, was 4 μg/mL. These data indicated the simultaneous presence of metronidazole susceptible and resistant *H. bizzozeronii* variants before the treatment [15]. After treatment, the isolated *H. bizzozeronii* (antrum T1) was resistant to both drugs [15]. Metronidazole is considered a pro-drug whose activation requires intracellular reduction by anaerobic or microaerobic microorganisms; this results in the production of bacterial cytotoxic radicals [16,17]. In *H. pylori* the main causes of metronidazole resistance are mutations inactivating two nitroreductase genes: *rdxA* and *frxA* [16,17]. However, conflicting evidence correlating the oxygen-insensitive nitroreductase RdxA and/or the NAD(P)H flavin oxidoreductase FrxA with the resistant phenotype, indicate that the molecular basis of resistance in this species remains unclear [16,17].

A comparative genomic analysis of the *H. bizzozeronii* isolates obtained before and after the treatment showed that, among the five putative nitroreductases identified in the genome of *H. bizzozeronii* CIII-1GEN, only the oxygen-insensitive NAD(P)H-nitroreductase HBZC1_00960, showing 47% identity with *H. pylori* RdxA HP0954, was affected [15].

This study investigates the role of HBZC1_00960 (*H. bizzozeronii* RdxA homolog) in the molecular mechanisms of metronidazole resistance in *H. bizzozeronii*.

### Materials and methods

**Bacterial strains, growth conditions, DNA manipulations and PCR**

For this study, the human-derived *H. bizzozeronii* strain CIII-1GEN, which exhibited a metronidazole MIC of 4 μg/mL, was selected [7,15]. In addition, the canine-derived *H. bizzozeronii* CCUG 35545T strain (MIC = 8 μg/mL) [18] was used for the mutation analysis. *Helicobacter* spp. strains were cultured on HP agar plates (LabM Limited, Lancashire, UK) as previously described [19]. For electroporation, *H. bizzozeronii* strains were cultivated in liquid media constituted by Brain Heart Infusion (BHI, BD, Becton, Dickinson and Co., NJ, USA) containing 10% Fetal Bovine Serum (Gibco®, Invitrogen Carlsbad, CA, USA), Skirrow selective supplement (Oxoid Ltd., Cambridge, UK) and Vitox supplement (Oxoid) (BHI-FBv) at 37°C in a jar with microaerobic atmosphere supplemented with hydrogen. The *E. coli* TOPO10 strain (Invitrogen Corporation, Carlsbad, CA, USA) was cultivated on Luria-Bertani (LB) agar or broth supplemented with 100 μg/mL ampicillin or 10 μg/mL chloramphenicol when needed. The *H. bizzozeronii* genomic DNA was prepared as previously described [20]. PCRs were performed in 25 μL reactions using Phusion® High-Fidelity DNA Polymerase (Finnzymes, Oy, Espoo, Finland) and 25 pmol of primers (Table 1).

#### Antimicrobial susceptibility of *H. bizzozeronii*

The minimum inhibitory concentration (MIC) value of metronidazole (Sigma-Aldrich) was estimated using the agar dilution method. Briefly, HP agar plates supplemented with serial dilutions of the antibiotics were inoculated with 10 μL bacterial inoculums (corresponding to approximately 10^3 cfu). The inoculum was prepared by diluting 1:10 a 0.6-0.8 OD_600_ bacterial suspension obtained from 4 days’ culture on HP agar plates. The plates were incubated at 37°C in the microaerobic incubator. The MIC values were determined by three independent assays after 4 and 6 days of incubation. *H. bizzozeronii* CIII-1GEN (MIC = 4 μg/mL) was used as reference control [15]. The EUCAST 2012 clinical breakpoint of metronidazole (> 8 μg/mL) described for

### Table 1 Oligonucleotides used in this study

| Oligonucleotides | Sequence |
|------------------|----------|
| HBrdxAugFw-PstI  | ATTTGCGATGCAACCCCAAAACCCCTACCACCAGT |
| HBrdxAugRw-Xbal  | CATCTAGACCGCCAGATAATCCGCAATTGAGAGACCC |
| HBrdxAdwFw-KpnI  | ATGGTAGCGCTCCTCTACTGGAATTATCTGCGTGG |
| HBrdxAdwRw-EcoRI | ATGAAAGGTCTGTGCTTAAGCCACTGTACG |
| RdxAoutFw        | TTTAACACGCAGCCCAAGAGAGC |
| RdxAoutRw        | GCGACCAACGCGCAAGCCCAAGAC |
H. pylori was used to classify H. bizzozeronii as either resistant or susceptible [21].

Selection of spontaneous metronidazole-resistant H. bizzozeronii isogenic mutants

Spontaneous metronidazole-resistant H. bizzozeronii mutants were selected by a single passage in media containing four times the MIC of metronidazole. Briefly, a suspension containing approximately 10^6 CFU/mL of 3-day-grown H. bizzozeronii CIII-1^GEN was prepared in BHI-Fbv and cultured in biphasic medium (HP coupled with BHI-Fbv) for 36 h. Then, 100 µL was spread onto HP agar plates containing 16 µg/mL of metronidazole. After six to ten days of incubation, resistant colonies were picked up and transferred to a new HP plate containing 16 µg/mL of metronidazole before being frozen at -70°C in 10% glycerol for further use. From each mutant, HBZC1_00960 was amplified and sequenced. The experiment was repeated three times.

Construction of H. bizzozeronii rdxA::cat (ΔrdxA) isogenic mutants

Chromosomal inactivation of the HBZC1_00960 gene (H. bizzozeronii rdxA homolog) was performed by allelic exchange using the chloramphenicol resistance gene (cat), as previously described [22]. The cat gene was introduced in the same direction as the target gene using XbaI and KpnI restriction sites. The resultant plasmid, pCP5, was constructed and amplified in E. coli TOPO10 and used as a suicide plasmid in H. bizzozeronii. Mutants were obtained by electroporation as described for H. felis [23]. After electroporation, the bacteria were left to recover on HP agar plates for 48 h under microaerobic conditions. The mutant strains (H. bizzozeronii rdxA::cat) were selected on HP agar plates supplemented with chloramphenicol (10 mg/mL). The plates were incubated up to 10 days, and the site of recombination was verified by PCR.

Determination of mutation rate and mutation frequency by Luria-Delbrück fluctuation analysis

The mutation rate and frequency of H. bizzozeronii for metronidazole were calculated using Luria-Delbrück fluctuation analysis [24]. Briefly, from a three days’ culture, a suspension of approximately 10^6 H. bizzozeronii CIII-1^GEN per mL was prepared in BHI-Fbv broth and divided into twenty-four 0.5 mL aliquots. These aliquots were allowed to grow in biphasic medium (HP coupled with BHI-Fbv) for 36 h to obtain parallel, independent cultures. The number of resistant mutants that emerged in each culture was determined by plating an aliquot of the culture on HP agar plates containing 16 µg/mL metronidazole. The total number of cells (N_t) was determined by plating an appropriate dilution of three cultures on non-selective medium. Colonies on both selective and non-selective plates were counted after a maximum of 10 days of incubation. The frequency of resistant mutants was expressed as the mean number of resistant cells divided by the total number of viable cells per culture. For the calculation of the mutation rate, the most likely number of mutations per culture observed (m_{obs}) was first calculated from the distribution of numbers of resistant mutants in the independent cultures by the Ma-Sandri-Sarkar maximum-likelihood method [24] using the FALCOR web tool [25]. The effect of the sampling in the calculation of the most likely number of mutations was corrected for by applying the following equation: m_{act} = m_{obs} (z-1)/z^2, where z is the fraction of culture plated. Then, the mutation rate (μ) per cell division was calculated as: μ = m_{act} N_t, where N_t is the total cell number per culture [24].

Time-kill curve for metronidazole

H. bizzozeronii strain CIII-1^GEN and its derivate CIII-1^GEN ΔrdxA and CIII-1^GEN M11 (spontaneous metronidazole resistant mutant) were sequentially sub-cultivated. CIII-1^GEN was maintained on non-selective plates, CIII-1^GEN rdxA::cat was maintained in the presence of 16 µg/mL of metronidazole, and CIII-1^GEN M11 was maintained in both conditions. After 10, 12 and 15 passages, approximately 10^8 cells/mL of each H. bizzozeronii strain were suspended in BHI-Fbv with or without 32 µg/mL of metronidazole. After 16 h, the intracellular ATP levels were measured using BacTiter-GloTM (Promega). The experiment was performed in duplicate. The data were analyzed as percentage of relative light units (RLU) of the treated samples compared to the untreated ones. The statistical analysis was performed by applying One-way ANOVA analysis of variance followed by Tukey’s Multiple Comparison using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, California, USA).

Results

A multialignment of the C-terminal part of the amino acid sequence of the RdxA homologs of H. bizzozeronii CIII-1^GEN (HBZC1_00960) and of several other Helicobacter species with the predicted RdxA amino acid sequence of the resistant variant H. bizzozeronii Antrum T_1 [15] is shown in Figure 1. As compared to the isogenic strain CIII-1^GEN, the resistant H. bizzozeronii variant Antrum T_1 showed a frame length extension leading to the disruption of the C-terminal cysteine-containing conserved region IACLXALGK (amino acids from position 182 to position 190 of HBZC1_00960) in the RdxA homolog. The frame length extension was a result of the insertion of a single cysteine in a homopolymeric run
located in the C-terminal of *H. bizzozeronii* rdxA (from codon 178 to codon 180 of HBZC1_00960). A 3' simple sequence cytosine repeat (SSCR) was also present in the same position in the *rdxA* of the *H. heilmannii* s.s. type strain but not in other gastric *Helicobacter* species (Figure 1). To verify whether the instability of the 3' SSCR of *H. bizzozeronii* rdxA was associated with metronidazole resistance, the *rdxA* genes of 11 metronidazole-resistant *H. bizzozeronii* CIII-1GEN isogenic spontaneous mutants were sequenced. All of the isogenic mutants obtained in three independent experiments showed a MIC of 32 μg/mL (eight-fold more than the MIC described for the parental strain). In 10 out of 11 mutants, the extension of the SSCR (C8 to C9) in position 178–180 was the only modification observed in *rdxA*. However, in all of the replicates of the subsequent two experiments the number of mutant colonies was above 300, hampering the estimation of the mutation rate. Thus, in a fourth assay the fraction of plated volume (z) was decreased to 0.02, allowing the determination of a mutation rate of 1.74 × 10⁻⁵. The mutation frequencies were estimated to be 4.96 × 10⁻⁵ and 2.38 × 10⁻⁴ in the first and fourth experiments, respectively.

A luciferase-based bacterial cell viability assay was used to determine the bactericidal effect of metronidazole on *H. bizzozeronii* strain CIII-1GEN and its derivate CIII-1ΔrdxA and CIII-1M11 after sequential subculture. Time-kill curves were generated to measure the decrease in intracellular ATP after treatment with 1× the MIC of metronidazole (calculated for the mutant *H. bizzozeronii* CIII-1ΔrdxA). The results are shown in Figure 2, where the data are plotted as a percentage of relative light units (RLU) of the treated samples compared to the untreated ones. In order to minimize the effect on the phenotype of phase variation of loci other than *rdxA*, CIII-1M11, obtained by pooling several colonies of spontaneous mutants from different plates, was selected. The extension of the SSCR (C8 to C9) in the position 178–180 was the only modification observed in *rdxA* of CIII-1M11. After 16 h of exposure, the
spontaneous metronidazole-resistant isogenic mutant CIII-1GEN M11 maintained on non-selective plates survived similarly to the wild type but significantly less well than CIII-1GEN ΔrdxA. In contrast, when CIII-1GEN M11 was maintained on selective plates, the percentage of survival was not significantly different to any of the other conditions tested. However, in the absence of metronidazole, CIII-1 GEN M11 survived less well than the same strain maintained in medium containing the antibiotic. Therefore, although no statistical significance was found between the survival capability of CIII-1GEN M11 when sub-cultivated with or without metronidazole, it tends to become more susceptible in the absence of selection after a small number of in vitro passages.

### Discussion
In association with other antibiotics, metronidazole is largely used in the first-line treatment of *H. pylori* infections [26], and an increased incidence of resistant strains has been observed in the last few years [27], with current rates varying from 17% in Europe to 44% in America [28]. In contrast, almost no data are available concerning metronidazole resistance in *H. bizzozeronii* and other species of the *H. heilmannii* s.l. (such as *H. salomonis* and *H. felis*) [8]. The MICs of metronidazole for six *H. bizzozeronii* strains of animal origin have been estimated (by the agar dilution method) to range from 1 to 8 μg/mL [8], suggesting that this antibiotic could be efficiently applied to eradicate *H. bizzozeronii* infections. However, the analysis of isolates obtained from multiple biopsy samples from the same patient [7,15] revealed the simultaneous presence of metronidazole-susceptible and resistant variants [15]. These data suggest that the isogenic variation of *H. bizzozeronii* may lead to the accumulation of heteroresistant phenotypes for metronidazole, resulting in treatment failure [15].

Metronidazole resistance is a strong predictor of treatment failure for *Helicobacter* infections when the treatment contains metronidazole [26]. However, due to insufficient information about the genetic background of the resistant phenotype, non-invasive detection of metronidazole resistance is not yet feasible [16,26]. Several years of investigation have provided evidence that the main causes of metronidazole resistance in *H. pylori* are mutations that alter the correct function of the nitroreductases *rdxA* or *frxA* [16]. However, this resistance has recently been shown to involve more complex changes than the simple inactivation of *rdxA* or *frxA*, including intracellular redox potential [16] and global gene regulation [29,30]. To investigate possible changes in *H. bizzozeronii* after the acquisition of metronidazole resistance, a comparative genome analysis of susceptible and resistant isogenic strains has been performed [15]. Numerous single nucleotide polymorphisms (SNPs) and insertions or deletions (Indels) were detected in the metronidazole-resistant strain compared to the isogenic susceptible one, indicating that the modification of several

### Table 2 MIC values of metronidazole after 4 days of incubation for *H. bizzozeronii* strain CIII-1GEN, *H. bizzozeronii* CCUG 35545T, and corresponding mutants

| *H. bizzozeronii* strains | Cytosine stretch in 3′ of *rdxA* | Mutation | MIC value (μg/mL) |
|--------------------------|---------------------------------|----------|-------------------|
| CIII-1GEN                | C₈                               |          | 4                 |
| CIII-1GEN M1             | C₉                               |          | 32                |
| CIII-1GEN M2             | C₉                               |          | 32                |
| CIII-1GEN M3             | C₉                               |          | 32                |
| CIII-1GEN M4             | C₉                               |          | 32                |
| CIII-1GEN M5             | C₉                               |          | 32                |
| CIII-1GEN M6             | C₉                               |          | 32                |
| CIII-1GEN M7             | C₉                               |          | 32                |
| CIII-1GEN M8             | C₉                               |          | 32                |
| CIII-1GEN M9             | C₈                               | A₁₈S→     | 32                |
| CIII-1GEN M10            | C₉                               |          | 32                |
| CIII-1GEN M11            | C₉                               |          | 32                |
| CIII-1GEN C1             | C₉                               | *rdxA::cat* | 32            |
| CIII-1GEN C2             | C₉                               | *rdxA::cat* | 32                   |
| CCUG 35545T              | NS*                             |          | 8                 |
| CCUG 35545T S1           | NS                               | *rdxA::cat* | 64               |
| CCUG 35545T S2           | NS                               | *rdxA::cat* | 64               |

*The length of the 3′ SSCR and mutations in *rdxA* are indicated for each strain. NS*: not sequenced.

### Table 3 Fluctuation analysis for the calculation of the mutation frequencies and rates for *H. bizzozeronii* CIII-1GEN which becomes resistant to metronidazole

| Experiment | No. of cells per culture (Nt) | Fraction of culture plated (z) | Resistant bacteria mean | Mutation rate (μ) | Mutation frequency (f) |
|------------|-------------------------------|--------------------------------|------------------------|------------------|-----------------------|
| 1          | 2.18 × 10⁷                    | 0.08                           | 87                     | 4.78 × 10⁻⁵       | 4.96 × 10⁻⁵           |
| 2          | 1.07 × 10⁸                    | 0.08                           | > 300                  | ND               | ND                    |
| 3          | 1.40 × 10⁷                    | 0.08                           | > 300                  | ND               | ND                    |
| 4          | 1.30 × 10⁷                    | 0.02                           | 62                     | 1.74 × 10⁻⁵       | 2.38 × 10⁻⁴           |

There are 21 independent cultures for each experiment.
Figure 2 Killing effect of metronidazole (MTZ) after 16 h of exposure against *H. bizzozeronii* strains. The figure shows the time-kill curve for *H. bizzozeronii* strains CIII-1^GEN^, its derivative CIII-1^GEN^ ΔrdxA and CIII-1^GEN^ M11 maintained in the presence or absence of MTZ (marked with MTZ in the figure). Data are plotted as the percentage of intracellular ATP, shown as relative light units (RLU), of the treated samples compared to the untreated ones. Means statistically significant different from the wild type strain CIII-1^GEN^ are indicated with an asterisk, while means statistically significant different from the mutant strain CIII-1^GEN^ ΔrdxA are indicated with a hash tag (Turkey’s HSD, p < 0.05).

genes could participate in the resistant phenotype [15]. However, among the five nitroreductases identified in the *H. bizzozeronii* CIII-1^GEN^ genome [20], only the homolog of *H. pylori* rdxA was affected in the resistant strain, and its C-terminal cysteine-containing conserved region (IACLXALGK) was disrupted [15]. This mutation resulted from the extension (from C8 to C9) of a simple sequence cytosine repeat (SSCR) located in the 3’ region of the gene. A comparative analysis showed that a similar 3’ SSCR is also present in the same position in the *rdxA* homolog of *H. heilmannii* s.s. but not in *H. pylori*. In this study, we showed that the extension of the 3’ SSCR of *rdxA* was the only mutation detected in the majority of in vitro spontaneous *H. bizzozeronii* metronidazole resistant mutants. In addition, we observed that an *H. bizzozeronii* ΔrdxA mutant strain showed the same MIC value of metronidazole that was observed in the spontaneous mutants. These data indicate that loss of function mutations in *rdxA* and, in particular, the disruption of the conserved region IACLXALGK, are sufficient to produce clinically significant resistance to metronidazole in *H. bizzozeronii*. These findings extend our knowledge of the types of mutations that affect the functionality of *rdxA* in *Helicobacter* spp., and they support the idea that the C-terminal cysteine-containing conserved region plays a critical role in the ability of RdxA to catalyze nitroreduction [31]. Moreover, slipped-strand mispairing of the *rdxA* 3’ SSCR appears to be the most frequent mechanism leading to the inhibition of the *H. bizzozeronii* metronidazole-nitroreductase activity. Therefore, *H. bizzozeronii* rdxA represents the first example of a contingency gene being associated with metronidazole resistance.

Simple sequence repeats in *Helicobacter* and other bacterial genomes can mediate phase variation due to their high mutation rates and reversible mutations, providing a population-based mechanism for stochastic variation in expression of specific genes and rapid adaptation to environmental fluctuations [20,32]. In this study, we demonstrated that *H. bizzozeronii* acquires resistance to metronidazole at a mutation rate similar to that described for phase-variable genes in *Campylobacter jejuni* and other bacterial species [32]. In addition, we observed an increased level of susceptibility to metronidazole in a spontaneous mutant maintained in non-selective conditions after approximately 15 passages, indicating that the resistant phenotype is reversible. Based on these data, it is tempting to speculate that metronidazole resistance in *H. bizzozeronii* is a phase-variable phenotype due to the contingency nature of *rdxA*. However, several other unknown mechanisms could lead to metronidazole resistance in *H. bizzozeronii*, overcoming the stochastic variation. In addition, it is not clear how frequently the reversion of the mutation (C9 to C8) occurs. In fact, we recently detected that the larger fraction of the *H. bizzozeronii* population colonizing the stomach of a patient maintained the C9 allele in *rdxA* six months after the end of the therapy [15]. Although it is clear that disruption of the C-terminal cysteine conserved region of *rdxA* decreases the ability of *H. bizzozeronii* to catalyze the metronidazole nitroreduction, the effect of this mutation on NAD(P)H-oxidase activity is unknown. Therefore, it may be possible that the same mutation that provides resistance to metronidazole does not alter the physiological activity of *rdxA*, or paradoxically, induces an increased fitness of the bacterium. This could lead to the fixation of the mutation in the population, as observed in the patient [15].

In conclusion, *H. bizzozeronii*, and potentially other species of *H. heilmannii* s.l., easily acquired clinically significant resistance to metronidazole due to the high mutation rate of SSCR located in the 3’ region of *rdxA*. Although we observed in vitro reversibility of the phenotype, there is evidence that the mutation can be maintained in vivo even six months after the end of therapy. Therefore, the contingency nature of *rdxA* should be carefully considered when metronidazole is used in the treatment of *H. heilmannii*-associated gastritis in humans.

**Consent**

Written informed consent was obtained from the patient for the publication of this report and any accompanying images.
Abbreviations
BHI-FBv: Brain Heart Infusion containing 10% Fetal Bovine Serum, Skirrow selective supplement and Vitox supplement; LB: Luria-Bertani; MIC: Minimum inhibitory concentration; CFU: Colony forming unit; SSCR: Simple sequence cytosine repeat; RLU: Relative light unit; SNP: Single nucleotide polymorphism; Indel: Insertions or deletion.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
PKR performed the experiments and helped to draft the manuscript. CP constructed the tdsAc-tet mutants. JR participated in the design of the study and helped to draft the manuscript. MLH participated in the design of the study and helped to draft the manuscript. NR conceived and coordinated the study, designed the experiments and drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgements
This study was funded by Academy of Finland FiCO MiFoSa, no. 11411405, and of Academy of Finland Postdoctoral Fellowship no. 132940.

Received: 28 March 2013 Accepted: 18 June 2013

References
1. Suerbaum S, Michetti P: Helicobacter pylori infection. N Engl J Med 2002, 347:175–186.
2. Haesebrouck F, Pasmans F, Flahou B, Chiers K, Baele M, Meyns T, Decostere A: Gastric helicobacters in domestic animals and nonhuman primates and their significance for human health. Clin Microbiol Rev 2009, 22:202–223.
3. Iwanczak B, Biernat M, Iwanczak F, Grabińska J, Matulicevich K, Gosińska G: The clinical aspects of Helicobacter helimanni infection in children with dyspeptic symptoms. J Physiol Pharmacol 2012, 63:133–138.
4. Sykora J, Jejda V, Varvarovska J, Stozicky F, Gotttrand F, Siala K: Helicobacter helimanni related gastric ulcer in childhood. J Pediatr Gastroenterol Nutr 2003, 36:410–413.
5. Sykora J, Jejda V, Varvarovska J, Stozicky F, Siala K, Schwarz J: Helicobacter helimanni gastroduodenal disease and clinical aspects in children with dyspeptic symptoms. Acta Radiat 2004, 93:707–709.
6. Andersen LP, Baye K, Blom J, Holck S, Norgaard A, Elsborg L: Constructed the PKK performed the experiments and helped to draft the manuscript. CP read and approved the final manuscript.

10. Kato S, Ozawa K, Sekine H, Ohyashiki M, Shimosegawa T, Minoura T, Ilinuma K: Helicobacter helimanni infection in a child after successful eradication of Helicobacter pylori: case report and review of literature. J Gastroenterol 2005, 40:94–97.
11. Jothimani DK, Zanetto U, Owen RJ, Lawson AJ, Wilson PG: An unusual case of gastric erosions. Gut 2009, 58:1669–1708.
12. Siala K, Sykora J, Hes O, Varvarovska J, Pasko P: Helicobacter helimanni reinfection in a Helicobacter pylori negative adolescent: a 4-year follow-up. J Clin Gastroenterol 2007, 41:221–222.
13. Roehr MH, Hernandez-M, Yang S, Christensen TG, Moreau C, Wang YJ: Helicobacter helimanni gastritis in a young patient with a pet. Gastrointest Endosc 2012, 76:421–422.
14. Wupperhorst N, Von Loewenich F, Holmaier B, Vetter-Knoll M, Mohadjer S, Kist M: Culture of a gastric non-Helicobacter pylori Helicobacter from the stomach of a 14-year-old girl. Helicobacter 2013, 18:1–5.
15. Schott T, Kondadi PK, Hänninen ML, Rossi M: Microevolution of a zoonotic Helicobacter population colonizing the stomach of a human host before and after failed treatment. Genome Biol Evol 2012, 4:1310–1315.
16. Kaakoush NO, Asencio C, Megraud F, Mendz GL: A redox basis for metronidazole resistance in Helicobacter pylori. Antimicrob Agents Chemother 2009, 53:1884–1891.
17. Mendz GL, Megraud F: Is the molecular basis of metronidazole resistance in microaerophilic organisms understood? Trends Microbiol 2002, 10:370–375.
18. Hänninen ML, Happonen I, Saari S, Jalava K: Culture and characteristics of Helicobacter bizzozeronii, a new canine gastric Helicobacter sp. Int J Syst Evol Microbiol 1996, 46:160–166.
19. Kondadi PK, Rossi M, Tawkmeyer B, Schur MJ, Li U, Schott T, Paulin L, Auvinen P, Hänninen ML, Schweda EW: Identification and characterization of a lipopolysaccharide alpha2,3-sialyltransferase from the human pathogen Helicobacter bizzozeronii. J Bacteriol 2012, 194:2549–2550.
20. Schott T, Kondadi PK, Hänninen ML, Rossi M: Comparative genomics of Helicobacter pylori and the human-derived Helicobacter bizzozeronii CBII-1 strain reveal the molecular basis of the zoonotic nature of non-pylori gastric Helicobacter infections in humans. BMC Genomics 2011, 12:534.
21. European Committee on Antimicrobial Susceptibility Testing. Clinical breakpoints. http://www.eucast.org/clinical.breakpoints/.
22. Rossi M, Bolz C, Rever S, Javed S, El-Nagar N, Andelf F, Hyttinen H, Vuorela P, Gerhard M, Hänninen ML: Evidence for conserved function of gamma-glutamyltranspeptidase in Helicobacter genus. PLoS One 2012, 7:e30543.
23. Josenhans C, Ferrero RL, Labigne A, Suerbaum S: Cloning and allelic exchange mutagenesis of two flagellin genes of Helicobacter felis. Mol Microbiol 1999, 33:350–352.
24. Rosche WA, Foster PL: Determining mutation rates in bacterial populations. Methods 2000, 204–17.
25. Hall BM, Ma CK, Liang P, Singh KK: Fluctuation analysis CalculatOR: a web tool for the determination of mutation rate using Luria-Delbrück fluctuation analysis. Bioinformatics 2009, 25:1546–1565.
26. Rimbari E, Fischbach LA, Graham D: Optimal therapy for Helicobacter pylori infections. Nat Rev Gastroenterol Hepatol 2011, 8:879–88.
27. Kupcinckas L, Rasmussen L, Jonaitis L, Kupcinckas J, Miarcinski N, Tomarasovas U, Kupcinckas J, Miculeviciene J, Kadusevicius E, Berg D, Andersen LP: Evolution of Helicobacter pylori susceptibility to antibiotics during a 10-year period in Lithuania. APMIS 2013, 121:431–436.
28. Wu W, Yang Y, Sun G: Recent insights into antibiotic resistance in Helicobacter pylori eradication. Gastroenterol Res Pract 2012, 2012:e23183.
29. Tsubawa H, Suzuki H, Satoh K, Hirata K, Matsuzaki J, Saito Y, Suematsu M, Hibi T: Two amino acids mutation of ferric uptake regulator determines Helicobacter pylori resistance to metronidazole. Antioxid Redox Signal 2011, 14:5–23.
30. Choi SS, Chivers PT, Berg DE: Point mutations in Helicobacter pylori fur regulatory gene that alter resistance to metronidazole, a produg activated by chemical reduction. PLoS One 2011, 6:e18236.
31. Dolkovitchin IN, Goodman A, Hoffman PS: Characterization of the NAD(P)H oxidase and metronidazole reductase activities of the RdxA nitroreductase of Helicobacter pylori. FEBS J 2009, 276:3354–3364.
32. Bayliss CD, Bidmos FA, Anjum A, Manchev VT, Richards RL, Gasser JP, Wooldridge KG, Ketley JM, Barrow PA, Jones MA, Tretyakov MV: Phase variable genes of Campylobacter jejuni exhibit high mutation rates and specific mutational patterns but mutability is not the major determinant of population structure during host colonization. Nucleic Acids Res 2012, 40:5876–5889.

http://www.veterinaryresearch.org/content/44/1/56

Page 7 of 7

Cite this article as: Kondadi et al: Contingency nature of Helicobacter bizzozeronii oxygen-insensitive NAD(P)H-nitroreductase (HBZC1_00960) and its role in metronidazole resistance. Veterinary Research 2013 44:56.