Identification and molecular characterization of triple- and quadruple-resistant *Helicobacter pylori* clinical isolates in Germany

Nicole Wueppenhorst¹, Hans-Peter Stueger², Manfred Kist¹† and Erik Glocker¹*†

¹National Reference Centre for Helicobacter pylori, Department of Microbiology and Hygiene, Institute of Medical Microbiology and Hygiene, University Hospital Freiburg, Hermann-Herder-Str. 11, 79104 Freiburg, Germany; ²Institute of Biostatistics, Austrian Agency for Health and Food Safety, Graz, Austria

Received 12 August 2008; returned 29 September 2008; revised 14 November 2008; accepted 3 January 2009

**Objectives:** The aim of this study was to estimate the frequency of triple- and quadruple-resistant *Helicobacter pylori* isolated in Germany, to characterize those isolates molecular genetically and to identify risk factors for the development of multiresistance.

**Methods:** Antimicrobial susceptibility to metronidazole, clarithromycin, amoxicillin, tetracycline, ciprofloxacin/levofloxacin and rifampicin in 1118 clinical isolates obtained between July 2006 and December 2007 was tested by the Etest® method. For patients harbouring triple- or quadruple-resistant strains (n = 169), data on prior eradication therapies and underlying diseases were collected and evaluated. A select number of quadruple- and triple-resistant strains were examined for resistance-mediating mutations in their 23S rRNA, 16S rRNA, *gyrA* and *rpoB* genes, respectively.

**Results:** From 1118 clinical isolates, 13.4% (n = 150) showed phenotypic resistance to metronidazole, clarithromycin, amoxicillin, tetracycline, ciprofloxacin/levofloxacin and rifampicin in 1118 clinical isolates obtained between July 2006 and December 2007 was tested by the Etest® method. For patients harbouring triple- or quadruple-resistant strains (n = 169), data on prior eradication therapies and underlying diseases were collected and evaluated. A select number of quadruple- and triple-resistant strains were examined for resistance-mediating mutations in their 23S rRNA, 16S rRNA, *gyrA* and *rpoB* genes, respectively.

**Conclusions:** We show that more than 15% of *H. pylori* strains isolated from routine samples in the German National Reference Centre are resistant to three or more antimicrobials and identified prior unsuccessful eradication therapies as a key factor for the development of multiresistance. Our data emphasize the need for further comprehensive surveillance studies monitoring the role of treatment regimens in antimicrobial resistance in *H. pylori*.

Keywords: multiresistance, mutations, *gyrA*, 23S rRNA, *rpoB*, 16S rRNA, treatment failure

**Introduction**

*Helicobacter pylori* infection is chronic in nature, causes gastritis and may result in complications such as peptic ulcer disease, gastric cancer and mucosa-associated lymphoid tissue lymphoma.¹⁻⁴ In line with the Maastricht III Consensus Report, the German National Reference Centre (NRC) for *H. pylori* recommends an empirical standard first-line regimen consisting of clarithromycin and amoxicillin or metronidazole combined with a proton pump inhibitor (PPI) to eradicate the bacteria in previously untreated patients.⁵

Treatment failures due to drug-resistant *H. pylori* strains have become an increasing clinical problem and prompt gastroenterologists and attending physicians to administer alternative antimicrobial strategies. A large number of studies favour the use of quinolone- or rifabutin-based therapies, which were shown to be highly efficacious and well-tolerated.⁶⁻⁹

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**Triple- and quadruple-resistant *H. pylori* in Germany**

*H. pylori* may acquire resistance to those rescue antibiotics during eradication therapies or treatments of unrelated bacterial infections, thereby becoming multiresistant and difficult to eradicate.\textsuperscript{10–14} Several studies have pointed to the alarming increase in infections, thereby becoming multiresistant and difficult to eradicate during eradication therapies or treatments of unrelated bacterial infections.\textsuperscript{13–18} and we recently reported the emergence of the first quadruple-resistant *H. pylori* clinical isolates in Western Europe.\textsuperscript{13} Those data underline the need for studies such as ResiNet, a German surveillance study that aims to collect prospective and systematic data on the development of antimicrobial resistance.\textsuperscript{19}

In order to update data on multiresistance in *H. pylori*, we tested the antimicrobial susceptibility of 1118 clinical routine isolates obtained between July 2006 and December 2007, collected data on patients’ previous eradication therapies and genotyped a select number of isolates characterized by resistance to three or more antimicrobials.

**Materials and methods**

**Gastric tissue samples, culture of bacteria and antimicrobial susceptibility testing**

Between July 2006 and December 2007, 1118 *H. pylori* strains were isolated from gastric biopsies sent to our diagnostic laboratory by a total of 280 centres from all over Germany: 39.7% (n = 444) of the gastric specimens were received from centres in the South, 37.7% (n = 421) from the West, 16.5% (n = 184) from the North and 6.2% (n = 69) from the East of Germany. Samples were taken in hospital-based (n = 223; 19.9%) and community-based (n = 705; 63.1%) endoscopy centres or sent by other microbiological laboratories (n = 190; 17%).

Isolates were derived from patients suffering from gastritis (n = 602; 53.8%), peptic ulcer disease (n = 96, 8.6%) or other diseases (n = 11; 1%) for 409 patients (36.6%), information on underlying diseases was not available. Of the 1113 patients for whom gender was known, 65% (n = 724) were female and 35% (n = 389) were male. Prior eradication therapies were reported for 746 patients (66.7%) with information on the antimicrobials used given for 652 patients. Of the patients, 5.4% (n = 60) had never been treated before, and for 27.9% (n = 312) data on earlier antimicrobial eradication treatments were lacking. The majority of pre-treatments included PPI in combination with amoxicillin and clarithromycin (n = 285 of 652; 43.7%), PPI with amoxicillin, clarithromycin and metronidazole (n = 228; 35%), PPI with metronidazole and clarithromycin (n = 43; 6.6%) or PPI with amoxicillin and metronidazole (n = 22; 3.4%), respectively.

Primary *H. pylori* cultures were grown on Columbia-agar-based culture medium containing 10 vol% washed human erythrocytes and 10 vol% heat inactivated horse serum under microaerobic conditions at 37 °C for 44–70 h. Grown bacteria were identified as *H. pylori* by typical morphology, biochemical reactions and Gram staining.\textsuperscript{20}

Antimicrobial susceptibility to amoxicillin, metronidazole, clarithromycin, ciprofloxacin/levofloxacin, tetracycline and rifampicin was tested by using the Etest\textsuperscript{®} method (AB Biodisk, Sweden). In brief, Iso-Sensitest agar plates (Oxoid, Germany) were flooded with a bacterial suspension adjusted to a turbidity equivalent to that of a 2–3 McFarland standard. After removing the supernatants and drying the plates for 15–20 min at 37 °C, Etest\textsuperscript{®} strips were placed in the centre of the dried plates. After incubation at 37 °C for 48–74 h in a microaerobic atmosphere, MICs were read according to the manufacturer’s instructions. In accordance with earlier recommendations, we applied the following resistance breakpoints: metronidazole, 8 μg/mL; clarithromycin, 1 mg/L; ciprofloxacin/levofloxacin, 1 mg/L; amoxicillin, 1 mg/L; tetracycline, 1 mg/L; and rifampicin, 4 mg/L.\textsuperscript{13,21,22}

**DNA extraction from culture and screening for mutations conferring antimicrobial resistance**

DNA of all quadruple-resistant *H. pylori* strains was isolated using the QIAmp DNA Mini Kit (Qiagen, Germany). Mutations in the 23S rRNA and 16S rRNA genes conferring resistance to clarithromycin and tetracycline were detected by real-time PCR as described previously.\textsuperscript{23,24} The method included the amplification of short 16S and 23S rRNA gene fragments of *H. pylori* and the simultaneous detection of the PCR product with fluorescence-labelled hybridization probes followed by a melting curve analysis of the hybridization probes. In brief, PCRs were accomplished in 20 μL volumes in glass capillaries (Roche Diagnostics, Germany) by using a LightCycler instrument (Roche Diagnostics) and primers and hybridization probes as described previously.\textsuperscript{23,25} After amplification, the PCRs were denatured at 95 °C for 0 s and cooled down to 30 °C, where they were held at that temperature for 30 s. Samples were then slowly heated up to 90 °C with continuous acquisition of the decline in fluorescence. Melting curves were plotted automatically and analysed with LightCycler software, thereby enabling us to distinguish between wild-type and mutant *H. pylori*.

The detection of mutations mediating resistance to rifampicin and quinolones was carried out by amplification and sequencing of the rpoB and gyrA gene fragments as described previously.\textsuperscript{13,14} In brief, a 569 bp fragment of the gyrA gene was amplified by using the primers 321fw (5'-ATATTGTAAAGGTGGAGTTGAT-3') and 600rv (5'-ATGCCTAAGGCTTAGATT-3'), and a 490 bp fragment of the rpoB gene was amplified by using the primers 1369fw (5'-AGGCCACATTGGGCAATCGTG-3') and 1867rv (5'-TAGCGGTCAAAATACGTCAC-3') (all primers were delivered by Apara Bioscience GmbH, Germany). Cycling conditions consisted of an initial denaturation at 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s, followed by a final extension at 72 °C for 7 min. PCR amplicons were examined by applying 5 μL on a 1.2% agarose gel (Peqlab, Germany) and then purified using the QIAquick PCR Purification Kit (Qiagen).

Purified PCR products were sequenced with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1 (Applied Biosystems, Germany) using the PCR primers as sequencing primers. Sequencing was accomplished with an ABI 3130 Genetic Analyzer (Applied Biosystems), and the obtained results were analysed with Sequencing Analysis software and Sequencher\textsuperscript{®} software (GeneCodes, USA).

**Statistical analysis**

A χ\textsuperscript{2} test was used to test for statistical significance at an error level of 5%. The 95% confidence intervals were constructed by a Bayesian approach by using a non-informative prior function.

**Results**

**Frequency of triple- and quadruple-resistant isolates and impact of prior eradication therapies on multiresistance**

In a total of 1118 *H. pylori* isolates examined between July 2006 and December 2007, we detected 14.4% (n = 161) that were triple-resistant: 13.4% (n = 150) showed resistance to metronidazole, clarithromycin, and quinolones; 0.9% (n = 10) showed...
resistance to metronidazole, clarithromycin and rifampicin; and <0.1% (n=1) showed resistance to clarithromycin, quinolones and rifampicin. In eight patients, we detected quadruple-resistant isolates with resistances to metronidazole, clarithromycin, quinolones and rifampicin (n=7; 0.6%) or tetracycline (n=1; <0.1%), respectively (Figure 1). None of the isolates included in our study was amoxicillin-resistant. Of the 169 patients carrying triple- or quadruple-resistant strains: 71% (n=120) were female and 29% (n=49) were male; 55% (n=93) suffered from gastritis and 8.3% (n=14) from peptic ulcer disease; and in 62 patients (36.7%) data on the underlying disease were not available. Our statistical analysis excluded any significant association between the evolution of multiresistant *H. pylori* and gender or underlying gastrointestinal disease (α-error 0.05). We did not observe any significant differences in resistance rates when comparing isolates from North, South, West and East Germany (data not shown).

Prior eradication treatments were documented for 746 patients including 132 patients carrying triple- or quadruple-resistant isolates, with detailed information on the antimicrobials used in previous eradications given for 120 patients (Table 1); 60 patients have never been treated before. Since triple- and quadruple-resistant strains were nearly exclusively isolated from pre-treated patients (17.6%; 95% confidence interval: 15.1%–20.6%), unsuccessful antimicrobial eradication significantly proved to be a major risk factor for the development of triple or quadruple resistance. In contrast, only a single isolate resistant to metronidazole, clarithromycin and quinolones was derived from an untreated patient (1.7%; 95% confidence interval: 0.4%–8.8%) (Figure 2).

**Molecular genetic analysis of multiresistant clinical isolates**

All isolates with quadruple resistance to metronidazole, clarithromycin, quinolones and rifampicin (MR-1 to MR-7) revealed mutations in their 23S rRNA genes at nucleotide 2142 or 2143 and mutations at codon 87 or 91 in gyrA conferring resistance to clarithromycin and quinolones, respectively (Table 2). Six of the isolates showed high-level resistance to rifampicin (>32 mg/L) and harboured mutations at triplets 525, 530 and 540 in their *rpoB* genes (Table 2); one isolate with moderate rifampicin resistance characterized by an MIC of 12 mg/L (MR-7) did not exhibit any mutation in the examined *rpoB* gene fragments (Table 2).
One quadruple-resistant strain (MR-8) with combined resistance to metronidazole, clarithromycin, quinolones and tetracycline showed resistance-associated mutations in the 23S rRNA (A2143G) and gyrA (N87K) genes; the phenotypic resistance to tetracycline was confirmed by the detection of a double nucleotide exchange at the critical positions 926–928 in the 16S rRNA genes rrnA/B from AGA to ATC (Table 2).

Next, we examined a select number of triple-resistant strains \((n=7)\) characterized by resistance to rifampicin for resistance-associated mutations. Except for one, all isolates (MR-9 to MR-14) were resistant to metronidazole and clarithromycin as confirmed by the detection of mutations in the 23S rRNA genes. Four of these strains (MR-9 to MR-12) harboured additional mutations in their rpoB genes at codon 525, 530 or 540 consistent with high-level (>32 mg/L) resistance to rifampicin, while two isolates (MR-13 and MR-14) with moderate resistance to rifampicin (≤8 mg/L) did not reveal any mutations in rpoB. One isolate (MR-15) that was clarithromycin-, quinolone- and rifampicin-resistant carried corresponding mutations in the 23S gene (A2143G), in GyrA (D91N) and RpoB (L525P) (Table 2).

### Discussion

Drug resistance represents a major issue in the management of \(H.\ pylori\) infections and may prompt gastroenterologists and attending physicians to prescribe reserve antimicrobials such as quinolones or rifabutin. However, the widespread use of quinolones in refractory \(H.\ pylori\) infections or in unrelated bacterial infections was shown to result in a sharp rise in resistance to quinolones between 2001 and 2005,\(^\text{14–16}\) and in 2007 the emergence of rifabutin-resistant strains including eight quadruple-resistant strains in Germany was reported.\(^\text{13}\)

We show that more than 15.1% of \(H.\ pylori\) strains isolated in our routine diagnostic laboratory between July 2006 and December 2007 are resistant to more than two antimicrobials; 13.4% exhibited triple resistance to metronidazole, clarithromycin and quinolones, thereby supporting earlier observations of a remarkable increase in strains with that pattern of resistance.\(^\text{14}\)

In contrast, prevalence of resistance to four antimicrobials is still low, since we detected only eight isolates exhibiting...
resistances to metronidazole, clarithromycin, quinolones and rifampicin or tetracycline during the 18 month period of observation. The presented data are not representative for the situation in the community though, since more than two-thirds of the patients included in our study had undergone one or more prior unsuccessful eradication therapies and hence are ‘difficult to treat’ patients.

In all quadruple-resistant and in a select number of triple-resistant isolates, we confirmed the observed phenotype by the detection of well-known resistance-conferring mutations in the 23S rRNA, gyrA and 16S rRNA genes. The detection of rpoB mutations in rifampicin-resistant strains at codons 530 and 540 indicated cross-resistance to rifabutin. Mutations at triplet 525, only found in vitro so far, are known to mediate high-level resistance to rifampicin, but their role in conferring cross-resistance remains to be clarified by further studies. In contrast, an A1589T transition resulting in a D530V amino acid exchange was shown to confer resistance to rifampicin and cross-resistance to rifabutin. In three isolates with moderate rifampicin resistance (MIC ≤ 8 mg/L), we could not detect any mutations in rpoB, thereby largely excluding cross-resistance to rifabutin. However, we cannot completely rule out other additional mutations outside of the sequenced fragments or as yet unknown mechanisms of rifampicin resistance.

Except for one, all triple- and quadruple-resistant strains were isolated from patients after one or multiple treatment failures, thereby stressing that prior unsuccessful eradication attempts appear to be a critical factor for the development of multi-resistance. Resistance to clarithromycin, quinolones and rifampicin/rifabutin is due to single point mutations and in vitro experiments showed that acquisition of resistance to those antimicrobials is a rapid one-step event. Early reports on failures of monotherapies with ofloxacin or ciprofloxacin and the well-known association of clarithromycin consumption and resistance demonstrate the ease with which resistance to those antimicrobials may develop.

This may be a possible explanation for the high rate of quinolone- and clarithromycin resistance in H. pylori, since both antimicrobials are frequently used in the treatment of both H. pylori eradication therapies and unrelated bacterial infections such as infections of the urinary tract or the airways.

In contrast, the resistance rate to rifampicin/rifabutin is still low, most likely due to the as yet restricted use on inpatients and reservation for mycobacterial infections, severe infections due to Staphylococcus aureus or life-threatening diseases such as Legionnaires’ disease.

The use of prior antimicrobial susceptibility testing and its impact on eradication success is still controversial. Pre-treatment testing was shown to be cost-saving and associated with better eradication rates, but other studies question the use of antimicrobial susceptibility testing before first- or second-line therapies. The German NRC for H. pylori has proved that antimicrobial resistance in H. pylori is closely associated with prior unsuccessful eradication therapies and recommends susceptibility testing both after the first therapy failure and in multi-morbid patients with high consumption of antimicrobials.

We have to curb the expectation that prior susceptibility testing guarantees a successful therapy, but it allows an antibiogram-adapted therapy and avoids the unnecessary administration of inefficient antimicrobials, thereby contributing to a reduction of costs and maybe less resistance to reserve antimicrobials.

To what extent bacterial factors contribute to the proneness of H. pylori to develop multiresistance still remains to be elusive. Previous studies have demonstrated that the presence of cytotoxin-associated gene A (encoding CagA) results in more severe inflammation but higher eradication rates. In addition, host factors such as distinct polymorphisms in cytochrome P450 2C19 or interleukin-1β were shown to be associated with lower eradication rates, thereby reflecting the complex impact of the bacterial and human genetic background on treatment failures. By using advanced technologies such as microarrays or gene expression assays, future studies may discover further genetic parameters affecting successful eradication and, in a broader sense, persistence of bacteria and possible development of antimicrobial resistance.

In conclusion, our data show that multiresistance in H. pylori is a serious problem. The majority of eradication therapies are based on positive serologies, urea breath tests or stool antigen ELISAs, and only little microbiological examination is performed. Antimicrobial susceptibility testing after the first treatment failure may prevent the development of multiresistant H. pylori and help reduce costs due to ineffective eradication.

We emphasize the need for continuous surveillance studies across Europe monitoring antimicrobial resistance to identify further risk factors for the development of resistance and to work out evidence-based recommendations for diagnostic procedures and antimicrobial therapies in untreated or pre-treated patients.

Acknowledgements

We thank Christine Ganter, Beate Hobmaier, Christine Melzl and Marianne Vetter-Knoll for excellent technical assistance.

Funding

This work was supported by the Robert-Koch-Institut by a grant to M. Kist (1369-239) of the German Ministry of Health.

Transparency declarations

None to declare.

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