Assessment of Helicobacter pylori clarithromycin resistance mutations in archival gastric biopsy samples

Alex Soltermann, Aurel Pevere, Sonja Schmidt, Franz Eigenmann, Rudolf Güller, Kurt B. Weber, Jürg Meier, Peter Eichenberger, Paul Komminoth

a Institute of Clinical Pathology, University Hospital Zürich, Switzerland
b Institute of Pathology, Cantonal Hospital Baden, Switzerland
c Division of Gastroenterology, Department of Internal Medicine, Cantonal Hospital Baden, Switzerland

Aim: First, to assess the clarithromycin resistance (ClaR) rate 1) in patients with persistent Helicobacter pylori (H. pylori) infection after eradication, 2) in patients with untreated infection and 3) in patients with successful status post eradication. Second, to evaluate the techniques sequencing and line probe hybridisation INNO-LipA for resolution of uniform and mixed populations in archival gastric biopsy samples.

Methods: The genomic 2142/43 23s rRNA mutations of the 50S ribosomal subunit conferring ClaR were detected by PCR-based assays.

Results: A total of 130 patients were investigated. Out of 21 patients of a first series with persistent infection after eradication, 19 (90%; CI (95%): 67–99%) exhibited point mutations at position 2142/43. In the second series of untreated patients, primary resistance was observed in 8 out of 93 patients (9%; CI: 4–16%). In a third series of 16 successfully eradicated patients, pure wild type populations (WT; for loci 2142/43) without any minimal mutated part were found (resistance rate 0%; CI: 0–21%). Further, in all 24 biopsies with uniform mutated and in 8 of 11 biopsies with mixed populations the two molecular biological methods yielded concordant results (100%; CI: 86–100% and 73%; CI: 39–94%, respectively).

Conclusion: In the Baden region of Switzerland, most clarithromycin resistant H. pylori strains harbour mutations at position 2142/43. The primary resistance rate is below 10%. Mixed populations, even with minor mutated part, cannot become successfully eradicated. The two investigated techniques are equally valid for resolution of uniform mutated or mixed H. pylori populations in archival biopsy material.

Key words: Helicobacter; resistance; clarithromycin; 23s rRNA; gastric biopsy; histology

Introduction

The Gram negative bacteria Helicobacter pylori colonize the human stomach with prevalence rates from 25% in western to over 90% in developing countries. Persistent infection is associated with chronic gastritis and peptic ulcer disease. After prolonged infection some patients will develop gastric cancer or non-Hodgkin lymphoma. H. pylori are eradicated by a triple or quadruple therapy regimen, containing a proton-pump inhibitor (PPI) and antibiotics, mainly clarithromycin and amoxicillin.

Multiple resistances of bacteria to antibiotic drugs are an increasingly encountered problem [1]. In the setting of H. pylori gastritis, the resistance against the macrolide component clarithromycin is particularly important, since this drug appears to be effective against endocyotosed intracellular bacteria [2] and is therefore considered essential for a successful treatment. The drug binds to the bacterial 50S ribosomal subunit and confers a translation block (3). Clarithromycin resistance (ClaR) of H. pylori is caused by point mutations of the genomic 23s rRNA, the main component of the 50S subunit, mostly at position 2142/43 (A2142 to G/C/T; A2143 to G/C) in the peptidytransferase region of the V domain, thereby preventing drug binding [4]. ClaR is increasing due to widespread use of macrolides for other diseases in the western world.

We aimed (i) for assessing the rate of H. pylori genomic 2142/43 23s rRNA point mutations in patients with persistent infection (failed eradication),
with untreated infection and with successful status post eradication and (ii) for evaluating the two molecular biological methods sequencing and line probe hybridisation for detection of uniform mutated and mixed mutated/wild type (WT) populations.

Material and methods

Patients

One hundred and forty-one patients were enrolled in the period of March 2002 till July 2003 and 130 of them were analysed. The first (AS) and last author (PK) diagnosed during this time range 12 patients with persistent H. pylori infection after clarithromycin containing eradication. This number was supplemented to 22 by equal cases from the 3 other staff pathologists of the institute (first series). Second, AS/PK diagnosed in the same time range 98 other patients with untreated H. pylori gastritis. Seventy-eight of the 98 patients were newly diagnosed by a first endoscopy, twenty patients had one or more previous H. pylori-positive biopsies in our institute, but did not receive an eradication therapy (second series). Third, AS/PK diagnosed in the same time range successful status post eradication in another 21 patients (third series). Eleven patients were then removed from further analysis because of unclear therapy (6 patients), not amplifiable DNA, not interpretable sequence (3 patients), concomitant celiac disease (1 patient) or lambliasis (1 patient). Patients with histologically documented gastro-oesophageal reflux disease (37 patients), ulcer (28) and partial gastrectomy (1 patient) remained. Biopsies and clinical data were obtained from 9 gastroenterologists who constitute the regional gastro-entero-logical service. Clinical data were also obtained from general practitioners. A total of 181 gastric biopsies (177 antrum, 4 corpus) were analysed. In patients with failed eradication, the pre-eradication biopsies were analysed additionally when available. In the 2 patients with failed eradication and WT strain, both pre- and post-eradication biopsies of antrum and corpus were investigated. Clarithromycin was included in all eradication regimens. Further components were the antibiotics amoxicillin, metronidazol and ornidazol as well as different proton pump inhibitors (PPI).

Controls

As positive controls we used the H. pylori strains V15–17469 (Cla–, wild type), V15–37 400 (Cla–, A2143G) and V15–4434 (Cla–, A2143G), kindly provided by Dr. R. Zbinden, Institute of Microbiology, University of Zürich. In brief, biopsy specimens were spread over the surface of Wilkins-Chalgren agar, supplemented with human blood and antibiotics (vancomycin, cefsulodin, trimethoprim and actidione). Plates were incubated for 4 days at 37 °C in a microaerophilic atmosphere and H. pylori was identified by typical morphology in a Gram stain and positivity for catalase, oxidase and urease. Susceptibility testing was performed by inoculation of sheep blood agar plates with H. pylori isolates followed by application of E-test stripes (AB Biodisk, Solna, Sweden). A breakpoint of >1 µg/ml was used for clarithromycin resistance. Ileum mucosa, a vas deferens specimen and blood DNA from healthy individuals served as negative controls.

DNA extraction

Samples were fixed in buffered formaldehyde and processed by paraffin-embedding and H&E-staining. Additionally, a Giemsa–C and alcian-blue/periodic acid schiff (AB-PAS) stained sections were produced. From the paraffin blocs fifty µm (5 × 10) sections were cut on a microtome and dewaxed in a microtube with 500 µl of xylene at 50° for 15 min followed by washes with 100% and 70% ethanol. The microtome was cleaned and the blade changed after each bloc. The pellets were digested with proteinase K (1.75 mg/ml) in 100 µl digestion buffer (100 mM Tris pH 8.0, 5 mM EDTA, 0.5% NP40, 0.5% Tween-20) at 54° for 24 hrs, followed by enzyme inactivation at 94° for 10 min. An aliquot of the extraction digest was used for PCR.

Polymerase chain reaction, restriction digest and sequencing

PCR was performed on a 6700 cycler (Applera Biosciences) with two sets of primers: First was the primer pair CRF-4/CRR-1 (forward primer 5'-AGTGGAGGTT-GAAATTCC-3', reverse primer 5'-TAAGAGCCAAAG-GCCCTTAC-3') with a product length of 135 bp (5). Second was the primer mix from the line probe reverse hybridisation clarithromycin resistance kit (INNO-LiPA, Innogenetics, Nethelands [6]). A standard PCR reaction of 50 µl and 45 cycles, using 1 µl of digestion extract was run with 30 sec denaturation at 94°, 30 sec annealing at 55° for the first and 62° for the second primer pair and 30 sec of extension at 72°. Products were analysed by gel electrophoresis after restriction digest with Mbo2 (A2142G) and BstI (A2143G). For sequencing, a second PCR with labelled dNTP's was performed as described [7].

Single strand conformational polymorphism analysis (SSCP) and single base pair mismatch hybridisation

SSCP was performed as described [8]. The line probe kit assay was performed according to the manufacturer's recommendations in a closed water bath by hybridisation of the PCR product at 50° for one hour, followed by a stringent wash at 50° for 30 min and visualization reaction.

Statistical analysis

Data were analysed by the scientific Geigy tables indicating the exact 95% confidential borders for binomial distributions (Documenta Geigy, scientific tables, Basel 1980).

Results

We first analysed 21 patients with persistent Helicobacter pylori infection after a clarithromycin containing eradication regimen in order to assess the local rate of H. pylori 2142/43 23s rRNA mutations in this population. Nineteen patients had obtained a triple therapy with clarithromycin and PPI plus amoxicillin (15 times) or metronidazol (3 times) or ornidazol (once). Seven
Figure 1
Patient MR with eradication failure between consecutive biopsies in year 2000 and 2002. a) Restriction digest with Mbo2 (M, A2142G) and Bsa1 (B, A2143), U undigested fragment, C undigested WT control, L 100 bp ladder fragment, very left ClaI strain V15-37400 (A2143G). b) SSCP analysis: Left, single band (asterisk) for the WT strain V15–37469 and a control patient (PC). Middle, shifted band towards the bottom pole with widely spaced minor band above (asterisks) for the patient MR and a positive control patient (PC). Right, narrowly spaced double bands (asterisks) for patient BB presented in Fig. 2 and the strain V15–4434 (both A2143G).

Figure 2
Patient BB with H. pylori gastritis, subcardial ulcer, treatment failure and persistent infection eight weeks later. Sequencing analysis (left, reverse strand) and clarithromycin-stain (right) of the antrum biopsy in March (a, upper panels) and May 2002 (b, lower panels), indicating a mixed WT/mutant population before therapy for the locus 2143, with A/T roughly equal to G/C and a pure mutant situation after therapy.

of them were treated repetitively. The other 2 patients were treated with a double therapy of clarithromycin and PPI. All patients were diagnosed positive for H. pylori microbes on conventional H&E and Giemsa-C sections. These 21 samples of the antrum were first analysed by restriction enzyme digestion with Mbo2 and Bsa1, single strand conformational polymorphism (SSCP), DNA sequencing and line probe reverse hybridisation (Figures 1 and 2). The restriction enzymes Mbo2 and Bsa1 cut when 2142 or 2143 are mutated from A to G, respectively. SSCP yielded specific band shifts for the mutations A2142/43G in the polyacrylamide gel (downward band shift and a widely spaced minor band above for A2142G and narrowly spaced double bands for A2143G). Both methods do not resolve the mutation A2142C. We thus decided to first apply DNA sequencing with the advantage of an internal control by the reverse strand, and to use the line probe kit assay for confirmation of positive results for all further patients. In 19 of these 21 patients (90%; 95%-confidence interval: 69.6–98.8%) the H. pylori strain displayed a point mutation of the genomic 23s rRNA at position 2142/43 after therapy. The remaining two patients, thoroughly analysed in both pre- and post-therapeutic antrum and corpus biopsies, did not harbour mutated microbes. In the second series, 8 of 93 patients (9%, 95%-CI: 3.8–16.3%) with untreated infection carried mutated strains. Thirdly, a series of 16 successfully eradicated patients was investigated. Post-therapy biopsies were scored H. pylori negative on H&E and Giemsa-C staining and controlled by immunohistochemistry using a polyclonal anti-H. pylori antibody (DAKO-Cytomation) and PCR amplification for absence of microbes. 15 of these 16 patients were eradicated with a triple therapy regimen, always including clarithromycin and a PPI. Nine patients obtained in addition amoxicillin, 4 metronidazole and 2 ornidazole. One patient was eradicated only with clarithromycin. In all 16 pre-eradication biopsies H. pylori strains were WT for loci 2142/43 without any minimal mutated moiety (resistance rate 0%; 95%-CI: 0–20.6%).

The mutation frequency distribution for the three series is given in Table 1. For 13 of the 19 patients with failed eradication and mutated microbes, an available pre-therapy biopsy was also investigated (Table 2). A pure WT strain was found in 5 of the 13 patients (38%) and a pure mutant one in 3 (24%). The other 5 patients (38%) harboured a mixture mutant/WT, 3 of them with only a minor mutated moiety. The evolution of the H. pylori population after therapy is also demonstrated on Table 2: the already pure mutated strains remained constant post eradication and the mixed mutant/WT ones changed to pure mutant. The 5 pure WT ones changed to pure mutant. Interestingly, in one patient (Nr. 85) the 2142 A to G mutation was completely lost after therapy. In the total

Table 1
Summary of the mutation frequency and spectrum in the 3 study populations analysed.

<table>
<thead>
<tr>
<th></th>
<th>n (enrolled)</th>
<th>n (analysed)</th>
<th>WT 2142G</th>
<th>2142C</th>
<th>2143G</th>
<th>mixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>a failed eradication</td>
<td>22</td>
<td>21</td>
<td>2</td>
<td>4</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>b untreated infection</td>
<td>98</td>
<td>93</td>
<td>85</td>
<td></td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>c successful eradication</td>
<td>21</td>
<td>16</td>
<td>16</td>
<td>4</td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>Total</td>
<td>141</td>
<td>130</td>
<td>103</td>
<td>4</td>
<td>3</td>
<td>14</td>
</tr>
</tbody>
</table>
of the first and second series, the A2143G mutation was predominant with 52% followed by mixed populations (22%), A2142G (15%) and A2142C (11%). Mutations at position 2115 (A to G), 2141 (G to A), 2142 (A to T) and recently at 2717 (T to C) in Italy as well as 2224 (G to A), 2245 (C to T) and 2289 (T to C) in China, conferring re-

Discussion

We have used formalin-fixed and paraffin-embedded archival gastric biopsy samples from daily routine pathological practice because of the possibility of combining morphological, immunohistochemical and molecular biological data of the \textit{H. pylori} gastritis and of following the disease over years. In our region, the diagnosis is nowadays mostly done by endoscopy with biopsy, followed by histology.

The clarithromycin resistance rate in patients with untreated \textit{H. pylori} gastritis is 9% and corre-

<table>
<thead>
<tr>
<th>Pat. Nr.</th>
<th>before therapy</th>
<th>after therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sequencing</td>
<td>line probe</td>
</tr>
<tr>
<td>72</td>
<td>2143G</td>
<td>2143G&gt;A</td>
</tr>
<tr>
<td>78</td>
<td>2143A&gt;G</td>
<td>2143A&gt;G</td>
</tr>
<tr>
<td>79</td>
<td>2142G</td>
<td>2142G</td>
</tr>
<tr>
<td>81</td>
<td>2143A&gt;G</td>
<td>2143A&gt;G</td>
</tr>
<tr>
<td>83</td>
<td>2143A&gt;G</td>
<td>2143A&gt;G</td>
</tr>
<tr>
<td>84</td>
<td>2143G</td>
<td>2143G</td>
</tr>
<tr>
<td>85</td>
<td>2142A&gt;G, 2143A&gt;G</td>
<td>2142G, 2143G</td>
</tr>
<tr>
<td>91</td>
<td>2142C</td>
<td>2142C</td>
</tr>
<tr>
<td>74</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>75</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>86</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>90</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>92</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>73</td>
<td>no biopsy available</td>
<td>no biopsy available</td>
</tr>
<tr>
<td>77</td>
<td>no biopsy available</td>
<td>no biopsy available</td>
</tr>
<tr>
<td>87</td>
<td>no biopsy available</td>
<td>no biopsy available</td>
</tr>
<tr>
<td>88</td>
<td>no biopsy available</td>
<td>no biopsy available</td>
</tr>
<tr>
<td>89</td>
<td>no biopsy available</td>
<td>no biopsy available</td>
</tr>
<tr>
<td>93</td>
<td>no biopsy available</td>
<td>no biopsy available</td>
</tr>
<tr>
<td>94</td>
<td>2143G</td>
<td>2143G</td>
</tr>
<tr>
<td>99</td>
<td>2143A&gt;G</td>
<td>2143A&gt;G</td>
</tr>
<tr>
<td>105</td>
<td>2142G&gt;A</td>
<td>2142G&gt;A</td>
</tr>
<tr>
<td>5</td>
<td>2143G</td>
<td>2143G</td>
</tr>
<tr>
<td>21</td>
<td>2143G</td>
<td>2143G</td>
</tr>
<tr>
<td>30</td>
<td>2143G</td>
<td>2143G</td>
</tr>
<tr>
<td>34</td>
<td>2143G&gt;A</td>
<td>2143G&gt;A</td>
</tr>
<tr>
<td>38</td>
<td>2143G</td>
<td>2143G</td>
</tr>
</tbody>
</table>

Table 2: Comparison of the two molecular biological methods sequencing and line probe hybridisation for resolution of uniform mutated and mixed mutated/WT populations in either pre- or post-therapeutic antrum biopsies (bold: one moiety detected by only one technique).

H. pylori populations were found in a total of 35 antrum biopsies from 27 patients. All 24 biopsies with uniform mutated and 8 of 11 biopsies with mixed mutant/WT populations yielded concordant results for the two molecular biological methods (100%; 95%-CI: 86–100% and 73%; 95%-CI: 39–94%, respectively). In the remaining 3 of these 11 biopsies, the mixed state was detected only by sequencing (2 biopsies), or only by line probe hybridisation (1 biopsy).

Table 2: Comparison of the two molecular biological methods sequencing and line probe hybridisation for resolution of uniform mutated and mixed mutated/WT populations in either pre- or post-therapeutic antrum biopsies (bold: one moiety detected by only one technique).
metronidazol, the _H. pylori_ resistance rate is currently about 25% in Switzerland [11]. Therefore, this antibiotic should not be used for eradication.

When evaluating the different methods for detection of clarithromycin mutations, we have found good concordance between the DNA sequencing approach and the clarithromycin resistance kit INNO-liPA with a line probe reverse hybridisation technique. Both methods seem to be equally valid for assessment of either pure mutated or mixed mutated/WT _H. pylori_ populations in formalin-fixed and paraffin-embedded gastric biopsy samples, and are able to indicate a valid overview of the population’s resistance status. For precise resolution of mixed populations, repetitive PCR reactions, use of multiple locations and single colony isolation out of cultures are needed. In particular, the interesting issue of heteroresistance between antrum and corpus has been addressed recently [20]. It raises the question how many biopsies from how many locations are required to obtain a full overview of the _H. pylori_ resistance status in the stomach.

We like to thank the gastroenterologists Dr. D. Angwerd-Wicki, L. Clerc, C. Urech and W. Hess for providing gastric biopsy samples and Dr. R. Zbinden (Institute of Microbiology, University of Zürich) for _H. pylori_ control strains. R. Frank and G. Frei are acknowledged for excellent technical assistance with the Ventana IHC automat and Dr. H. R. Schmid (Institute of Clinical Chemistry, Cantonal Hospital Baden) for his kind help with _H. pylori_ culture techniques.

Correspondence:
Dr. Alex Soltermann
Institute of Clinical Pathology
University Hospital Zürich
CH-8091 Zürich
E-Mail: alex.soltermann@usz.ch

References


The many reasons why you should choose SMW to publish your research

What Swiss Medical Weekly has to offer:

- SMW’s impact factor has been steadily rising, to the current 1.537
- Open access to the publication via the Internet, therefore wide audience and impact
- Rapid listing in Medline
- LinkOut-button from PubMed with link to the full text website http://www.smw.ch (direct link from each SMW record in PubMed)
- No-nonsense submission – you submit a single copy of your manuscript by e-mail attachment
- Peer review based on a broad spectrum of international academic referees
- Assistance of our professional statistician for every article with statistical analyses
- Fast peer review, by e-mail exchange with the referees
- Prompt decisions based on weekly conferences of the Editorial Board
- Prompt notification on the status of your manuscript by e-mail
- Professional English copy editing
- No page charges and attractive colour offprints at no extra cost

Editorial Board
Prof. Jean-Michel Dayer, Geneva
Prof. Peter Gehr, Berne
Prof. André P. Perruchoud, Basel
Prof. Andreas Schaffner, Zurich
( Editor in chief)
Prof. Werner Straub, Berne
Prof. Ludwig von Segesser, Lausanne

International Advisory Committee
Prof. K. E. Juhani Airaksinen, Turku, Finland
Prof. Anthony Bayes de Luna, Barcelona, Spain
Prof. Hubert E. Blum, Freiburg, Germany
Prof. Walter E. Haefeli, Heidelberg, Germany
Prof. Nino Kuenzli, Los Angeles, USA
Prof. René Lutter, Amsterdam, The Netherlands
Prof. Claude Martin, Marseille, France
Prof. Josef Patsch, Innsbruck, Austria
Prof. Luigi Tavazzi, Pavia, Italy

We evaluate manuscripts of broad clinical interest from all specialities, including experimental medicine and clinical investigation.

We look forward to receiving your paper!

Guidelines for authors:
http://www.smw.ch/set_authors.html

EMH Swiss Medical Publishers Ltd.
SMW Editorial Secretariat
Farnburgerstrasse 8
CH-4132 Muttenz

Manuscripts: submission@smw.ch
Letters to the editor: letters@smw.ch
Editorial Board: red@smw.ch
Internet: http://www.smw.ch