Helicobacter pylori amoxicillin heteroresistance due to point mutations in PBP-1A in isogenic isolates

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Objectives: To investigate the Helicobacter pylori amoxicillin resistance rate, the occurrence of heteroresistance, and their related molecular mechanisms.

Methods: Eighty-seven H. pylori-positive patients were included: 45/87 with single biopsy and 42/87 with multiple biopsies. MICs were determined, and sequencing analysis of pbp1A gene and the variable regions of seven hop porins was performed in resistant and susceptible isolates. Clonal relationships were determined by Ispa-glmM-RFLP and by random amplification of polymorphic DNA–PCR. An isogenic amoxicillin-susceptible isolate was transformed with pbp1A PCR products from the resistant isolates.

Results: Amoxicillin-resistant (MIC 2 mg/L) and amoxicillin-susceptible (MIC 0.06 mg/L) isolates, belonging to the same strain, were observed in different biopsies in one patient (inter-niche heteroresistance). Isolates from the remaining patients were amoxicillin-susceptible. Sequencing analysis of the pbp1A of two amoxicillin-resistant isolates and their susceptible partners revealed the same two point mutations: (i) in the third PBP motif of the resistant isolates (C1667G); (ii) a nonsense mutation at the 3' end of the gene. Replacement of pbp1A of a susceptible isolate by pbp1A from a resistant isolate increased the transformants MICs (2 mg/L). A similar MIC was observed when a pbp1A DNA fragment including both point mutations was transformed. Transfer of the smallest fragment (C1667G region only) yielded slightly lower MICs (0.5–1 mg/L). Identical hop gene sequences were observed in paired susceptible and resistant isolates.

Conclusions: A low resistance rate was observed. However, inter-niche heteroresistance could hinder amoxicillin resistance detection when only one biopsy is obtained. Alteration in PBP-1A seems to be enough to reach an MIC of 2 mg/L in our resistant isolates.

Keywords: beta-lactams, resistance, Gram-negative, bacilli

Introduction

Amoxicillin is one of the first-line antimicrobial agents used for Helicobacter pylori eradication, and the emergence of resistant isolates may be related to treatment failure.¹ The prevalence of H. pylori resistance to amoxicillin is very low (0.8% to 1.4%),¹ except in Taiwan and Brazil, where 20% to 30% resistance has been reported.²,³ The investigation of amoxicillin resistance mechanisms has been frequently performed by transformation of susceptible strains with: (i) genomic DNA of clinical resistant isolates; (ii) PCR products of putative related genes of mutants generated in vitro by successive passage on plates containing amoxicillin.⁴,⁵ Resistance has been attributed to alterations in the penicillin-binding protein 1A (pbp1A) gene, even though the role of other genes cannot be excluded.⁴,⁵ It has also been demonstrated that amoxicillin-resistant isolates accumulated ~40% less ¹⁴C-labelled penicillin G than equal numbers of amoxicillin-susceptible isolates, suggesting that porins such as HopA, HopB, HopC, HopD and HopE could be involved in the resistance.³ Comparison of the amino acid substitutions in
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PBP-1A and the Hop porin family between in vitro amoxicillin-resistant isolates and their susceptible parental counterparts suggested that changes in PBP-1A, HopB and HopC accounted for all the resistance mechanisms found. These results were confirmed by transformation analysis with the construction of a triple mutant possessing altered pbp1A and hop genes showing an amoxicillin MIC of 4 mg/L.

Heteroresistance, defined as the co-existence of susceptible and resistant isolates in the same patient for the same antimicrobial agent, is a common phenomenon in the H. pylori population. Heteroresistance can be explained by: (i) mixed infection (unrelated isolates); or (ii) susceptible and resistant variants of the same strain. Heteroresistance in clonally related isolates has been described as either intra-niche (susceptible and resistant isolates are present in the same site of gastric mucosa) or inter-niche (susceptible and resistant isolates are located in different anatomic sites within the stomach). Inter-niche heteroresistance can lead to the underestimation of antimicrobial resistance because it is more difficult to detect resistance when it is not uniformly distributed in the stomach mucosa. It has been reported that the H. pylori population in a patient can be heterogeneous with respect to metronidazole, clarithromycin, tetracycline and ciprofloxacin susceptibility. To the best of our knowledge, amoxicillin heteroresistance has not been described in vivo. In this study, we investigated the amoxicillin resistance rate and the probable existence of heteroresistance in H. pylori-positive patients, with the aim to find susceptible and resistant isogenic isolates in order to analyse resistance mechanisms found in vivo.

Materials and methods

The study included 87 H. pylori-positive patients. By upper endoscopy, antral biopsies were obtained from 45 of 87 patients and multiple simultaneous biopsies (three from antrum and three from corpus) from the remaining 42 of 87 patients. All patients provided written consent and Human Research Committees of both participating hospitals approved this study. Biopsies were cultured as previously described. Amoxicillin resistance was identified with PBP-4F primer and PBP-4TR (TGAAATACGAAAGAGTCC) and 72°C for 1 min. Amplified DNA was purified using Wizard-PCR Preps (Promega, Madison, WI, USA), according to the manufacturer’s instructions. Sequencing was performed on both DNA strands, using an ABI 373 DNA sequencer (Applied Biosystems, SA, Argentina). The nucleotide sequences were analysed by BioEdit version 5.0.9 and the Blast program at the National Center for Biotechnology Information. The amino acid sequences were confirmed by transformation analysis with the construction of a pbp1A gene amplified from a resistant isolate, or with 100–200 ng of DNA fragment amplified with primers PBP-4F and PBP-4R including the third pbp1A conserved motif region, or with 200 ng of the smallest DNA fragment of this conserved motif region, amplified with PBP-4F primer and PBP-4TR (TGAAATACGAAAGAGTCC). Transformants were selected on blood agar plates with 0.5 mg/L amoxicillin. As controls, bacteria were transformed with DNA from another amoxicillin-susceptible isolate, or TE (10 mM Tris–HCl, 1 mM EDTA) without DNA. MICs of transformants were determined, as described earlier.

Results and discussion

In agreement with the low amoxicillin resistance observed worldwide, <1% of our patients showed amoxicillin resistance (1/87). Resistant isolates were recovered in 1 of the 42 patients with multiple biopsies, a 73-year-old male patient with peptic
ulcer and failure of eradication treatment with clarithromycin/amoxicillin/omeprazole. In addition, resistance was not homogeneously found in the multiple biopsies; resistant isolates were only obtained from one of three antrum and from one of three corpus biopsies (MIC 2 mg/L). Instead, all isolates obtained from expanded single colonies of the remaining four biopsies showed an MIC of 0.06 mg/L.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{PBP-1A amino acid sequences in paired susceptible and resistant isolates. Partial sequence of 3' end \textit{pbp1A} gene, differences between susceptible and resistant isolates are indicated by boxes (GenBank accession numbers EF583173 and EF583174, respectively). HP26695 reference strain (GenBank accession number AE000511). 16-Amx-S-C3: amoxicillin-susceptible isolate recovered from one corpus biopsy; 16-Amx-R-A2: amoxicillin-resistant isolate recovered from one antral biopsy.}
\end{figure}

\textit{A}--\textit{glm} \textit{M} and RAPD fingerprints demonstrated that resistant and susceptible isolates belonged to the same strain (Figure 1). Comparing the whole \textit{pbp1A} sequence of two amoxicillin-resistant isolates with their susceptible partners, the same two differences were found. One of them was located in the third conserved penicillin-binding protein motif, where resistant isolates showed one point mutation (C1667G) resulting in a T556S shift (Figure 2). Gerrits \textit{et al.}\textsuperscript{4} demonstrated that the replacement of the wild-type \textit{pbp1A} gene of reference strain 26695 by the \textit{pbp1A} gene of amoxicillin-resistant clinical isolates resulted in an increased MIC (0.125 to 0.5–1 mg/L). Sequence analysis indicated that amino acid substitutions in or adjacent to the second (SKN402-404) and third (KTG555-557) conserved \textit{PBP}-1A motifs mediated the resistance. Therefore, the KTG→KSG substitution in our isolates might be the cause of resistance. The nonsense mutation T1911G that creates a truncated PBP-1A might affect protein folding with an additional increase in MIC value.

Analysis of \textit{pbp1A} and \textit{hop} gene sequences in our paired susceptible and resistant isolates revealed that, except for the point mutations mentioned above, they were absolutely identical. This supports the results of \textit{ispa}-\textit{glmM} and RAPD and allows us to assert that the isolates are isogenic, indicating an inter-niche amoxicillin heteroresistance.

The presence of isogenic isolates implies that amoxicillin resistance emerged \textit{in vivo} due to point mutations, probably through exposure to subinhibitory amoxicillin concentrations during eradication treatment.

In conclusion, our results indicated that \textit{pbp1A} point mutations are responsible for amoxicillin resistance. However, mutations in porin genes could contribute to MIC increases in other resistant isolates. \textit{In vivo} inter-niche heteroresistance adds a new complexity in the detection of amoxicillin resistance when only one biopsy is obtained.

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\section*{Transparency declarations}
None to declare.
References


