Differentiation of Metronidazole-Sensitive and -Resistant Clinical Isolates of *Helicobacter pylori* by Immunoblotting with Antisera to the RdxA Protein

STEPHANIE R. LATHAM, ROBERT J. OWEN, NICOLA C. ELVISS, AGNÈS LABIGNE, AND PETER J. JENKS

Department of Medical Microbiology, Royal Free and University College Medical School, and Helicobacter Reference Unit, Central Public Health Laboratory, London, and Institute of Infections and Immunity, University of Nottingham, Nottingham, United Kingdom, and Unité de Pathogénie Bactérienne des Muqueuses, Institut Pasteur, Paris, France

Received 27 March 2001/Returned for modification 23 April 2001/Accepted 14 June 2001

Antimicrobial resistance in *Helicobacter pylori* is a serious and increasing problem, and the development of rapid, reliable methods for detecting resistance would greatly improve the selection of antibiotics used to treat gastric infection with this organism. We assessed whether detection of the RdxA protein could provide the basis for determining the susceptibility of *H. pylori* to metronidazole. In order to raise polyclonal antisera to RdxA, we cloned the *rdxA* gene from *H. pylori* strain 26695 into the commercial expression vector pMAL-c2, purified the resultant fusion protein by affinity chromatography, and used this recombinant RdxA preparation to immunize rabbits. We then used this specific anti-RdxA antibody to perform immunoblotting on whole bacterial cell lysates of 17 metronidazole-sensitive and 27 metronidazole-resistant clinical isolates of *H. pylori*. While a 24-kDa immunoreactive band corresponding to the RdxA protein was observed in all metronidazole-sensitive strains, this band was absent in 25 of 27 resistant isolates. Our results indicate that testing for the absence of the RdxA protein would identify the majority of clinical isolates that will respond poorly to metronidazole-containing eradication regimens and have implications for the development of assays capable of detecting metronidazole resistance in *H. pylori*.

*Helicobacter pylori* is a gram-negative, microaerobic, spiral bacterium that colonizes the stomachs of approximately half the world’s population (7). Infection with *H. pylori* is associated with chronic gastritis and peptic ulceration, and the bacterium is also considered a risk factor for the development of gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (2, 25, 26). Modern triple-drug regimens are highly effective for treating *H. pylori* infection, but bacterial resistance to the two most effective antibiotics, metronidazole and clarithromycin, is a serious and increasing problem. It has been estimated that 11 to 70% of clinical strains isolated in western Europe and the United States are resistant to the 5-nitroimidazoles, and this prevalence is far higher in developing countries and in certain immigrant populations (7). Although there have been conflicting reports concerning the clinical impact of metronidazole resistance in *H. pylori*, many studies have now demonstrated that resistance to this class of antibiotics does reduce the efficacy of metronidazole-containing eradication regimens and is therefore an important predictor of treatment failure (3, 6, 11, 12, 15, 16). Several reports also suggest that the prevalence of metronidazole resistance is rising and is likely to become an increasingly important problem in the clinical management of *H. pylori* infection (23, 34).

Because *H. pylori* is slow growing, susceptibility testing by culture-based methods is cumbersome and in practice rarely performed before empirical antibiotic treatment is commenced (24). However, many centers are reassessing the importance of routine susceptibility testing, appreciating that this will provide a far more rational approach to the use of antibiotics. However, cost implications, ease of access to noninvasive tests, and practical problems, such as exist for the determination of metronidazole resistance, mean that it is unlikely that routine testing for antimicrobial susceptibility will be universally adopted. A rapid and useful alternative is to identify resistance markers directly in gastric biopsy specimens, and several tests have been developed to detect the limited number of the point mutations within the peptidyltransferase region of 23S rRNA that are associated with macrolide resistance in this organism (27, 30, 33). However, it has not been possible to develop similar genotype-based tests for metronidazole, since resistance is associated with many different alterations of the *rdxA* gene (which encodes an oxygen-insensitive NADPH nitroreductase), including missense and frameshift mutations and deletions and insertion of transposable elements (5, 10, 14, 20, 29, 31). Furthermore, recent reports have demonstrated that inactivation of other reductase-encoding genes, including *fdxB* (which encodes ferredoxin-like protein) and *frxA* (which encodes NADPH flavin oxidoreductase), are also associated with resistance to metronidazole (17–19). While the precise contribution of other mechanisms to the resistant phenotype remains unclear, current evidence suggests that secondary mutations in these genes result in transition to high-level resistance once inactivation of *rdxA* has occurred. Although the development of a simple assay capable of detecting metronidazole resistance does not appear straightforward, it would represent a major advance in the antibiotic management of patients with *H. pylori*.
infection. We hypothesized that such a system could be developed based on the detection of the RdxA protein of *H. pylori*. Our strategy for detection of the RdxA protein was to use immunoblotting with specific anti-RdxA antibody.

**MATERIALS AND METHODS**

**Bacteria and growth conditions.** *Escherichia coli* strain TG1 (9) was grown at 37°C in L broth (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter, pH 7.0) or on L agar plates (1.5% agar) at 37°C. The antibiotic carbenicillin (100 µg/ml) was added as required.

In the first part of the study we used the metronidazole-sensitive *H. pylori* strains SS1, G27, and HAS-141 (4, 13, 22) and isogenic strains in which the rdxA gene had been disrupted and which were resistant to metronidazole (15). For the investigation of production of RdxA in clinical isolates, we used 47 strains that had been isolated from patients who had undergone upper gastrointestinal endoscopy for duodenal ulceration and nonulcer dyspepsia. These included 30 strains (10 metronidazole sensitive and 20 metronidazole resistant) isolated from patients in the United Kingdom and 14 strains (7 metronidazole sensitive and 7 metronidazole resistant) from French and North African patients (31).

**Expression and purification of recombinant RdxA protein.** Recombinant *H. pylori* RdxA protein was expressed as a MalE-RdxA fusion protein (66 kDa) and purified as described previously (8). Briefly, fresh 500-ml volumes of L broth, containing carbenicillin (100 µg/ml) and 30% (wt/vol) glucose, were inoculated with overnight cultures (5 ml) of strain TG1 harboring the recombinant plasmid and incubated with shaking at 37°C. When the optical density at 600 nm of the culture reached 0.5, isopropyl β-D-thiogalactopyranoside (IPTG; final concentration, 1 mM) was added, and the cells were incubated for an additional 4 h. Following induction with IPTG, the cells were harvested by centrifugation, washed with phosphate-buffered saline and incubated for 4 h at room temperature to remove nonspecific antibodies to *E. coli*. Immunoreactivities were detected using an ECL Western blotting detection system (Amersham).

**Protein analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting.** Two-day cultures of *H. pylori* were harvested, washed in sterile distilled water, and suspended in double-strength sample buffer (62.5 mmol of Tris base/liter [pH 6.8], 4.0% sodium dodecyl sulfate, 5.0% mercaptoethanol, 30% glycerol, 0.025% bromophenol blue) prior to solubilization by boiling for 5 min. Protein concentrations were estimated using a commercial version of the Bradford assay (Sigma Chemicals). Solubilized bacterial cell extracts, containing 20 µg of protein, were analyzed on slab gels, comprising a 4.5% acrylamide stacking gel and a 17.5% resolving gel, according to the procedure of Laemmli (21). Electrophoresis was performed at 200 V in a Mini-PROTEAN II electrophoresis cell (Bio-Rad Laboratories, Hemel Hempstead, United Kingdom). Molecular weight standards from Bio-Rad were run on each gel.

**RESULTS**

**Generation of polyclonal rabbit antisera against RdxA.** To assess the specificity of the antisera we performed immunoblot analysis of the MalE-RdxA fusion protein as well as whole-cell extracts of the well-characterized, metronidazole-sensitive *H. pylori* strains SS1, G27, and HAS-141. The antisera reacted strongly with the MalE-RdxA fusion protein, and in *H. pylori* strains SS1, G27, and HAS-141 a band of approximately 24 kDa (equivalent to the predicted molecular mass of the RdxA protein) was observed (Fig. 1). In contrast, this 24-kDa band was absent from solubilized protein preparations prepared from isogenic *H. pylori* strains in which the rdxA gene had been disrupted by mutagenesis (15) and which were resistant to metronidazole (Fig. 1).
In some metronidazole-resistant strains a faint parasite band was observed at approximately 22 kDa. We were able to remove this in the majority of strains by preadsorption of the antisera with *E. coli* TG1 cells prior to immunoblotting.

**DISCUSSION**

The development of rapid, genotype-based tests to detect resistance to metronidazole in *H. pylori* has been hindered by the fact that resistance is associated with many different mutations within *rdxA* and possibly other reductase-encoding genes. Our results demonstrate a high correlation between production of the RdxA protein and susceptibility of *H. pylori* to metronidazole, confirming that the *rdxA* gene is inactivated in the vast majority of resistant isolates and that mutations in other genes are either rare or involved in transition to high-level resistance. We, and other groups, have demonstrated that resistant strains frequently contain frameshift mutations within their *rdxA* gene that result in the creation of a translational stop codon in the region immediately downstream of the mutation, and such strains would be predicted to produce a truncated RdxA protein. (10, 14, 20, 29, 31). A particularly important observation of this study was that production of the RdxA protein was completely abrogated in all but one of the resistant strains; none of the examined strains produced a truncated protein. We therefore conclude that in the majority of resistant strains, mutational inactivation of the *rdxA* gene prevents production of the protein or results in production of an abnormal polypeptide which is subsequently degraded. This suggests that testing for the absence of the RdxA protein would identify the majority of clinical isolates that will respond poorly to metronidazole-containing eradication regimens and has important implications for the development of assays capable of detecting metronidazole resistance in *H. pylori*. The advantage of using this approach is that it will identify all resistant strains that carry mutations affecting expression of the *rdxA* gene, including those that have not yet been identified by sequence analysis.

Although a small number of metronidazole-resistant strains of *H. pylori* have been reported in which the nucleotide sequence of the *rdxA* gene has been unchanged there has been no further analysis of these mutants to confirm whether they have decreased RdxA activity or synthesis (1, 14, 31, 35). We are now examining whether the RdxA enzymes produced by the two resistant strains are functionally inactive or whether other mechanisms are responsible for their resistant phenotype. We are also using a larger collection of strains to assess how common such isolates are in clinical practice. In addition, we plan to refine our approach to develop a rapid and simple test that will allow the detection of metronidazole resistance in *H. pylori*. Such an assay would represent an important advance in the clinical management of *H. pylori* infection, allowing a more rational approach to the use of this antibiotic.

**ACKNOWLEDGMENT**

P. J. Jenks is supported by an Advanced Fellowship for Medical, Dental, and Veterinary Graduates from the Wellcome Trust, United Kingdom (reference 061599).

**REFERENCES**


IMMUNOBLOTTING WITH ANTISERA TO H. PYLORI RdxA


