

Characteristics of clinical *Helicobacter pylori* strains from Ecuador

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In Ecuador, *Helicobacter pylori* infections are highly prevalent. A total of 42 *H. pylori* clinical isolates from 86 patients attending the outpatient clinic of the gastroenterology department of the university hospital of Guayaquil in Ecuador were characterized. Their susceptibility, and *cagA* and *vacA* status were determined. Resistance to metronidazole and clarithromycin was found in 80.9% and 9.5% of strains, respectively. Neither amoxicillin- nor tetracycline-resistant strains were found. The most prevalent genotype was the *cagA*⁺, *vacA* s1b,m1 type. This genotype was associated with gastric cancer and peptic ulcer. Typing by random amplified polymorphic DNA showed no genetic relationship among the strains.

Keywords: *Helicobacter pylori*, Ecuador, antibiotic resistance, typing, virulence factors

Introduction

The incidence of gastric cancer in Ecuador is high compared with other countries and is estimated at 29 cases per 100 000 inhabitants per year. A major risk factor for gastric cancer is infection with *Helicobacter pylori*.¹ In most patients, infection leads to chronic gastritis and peptic ulcer disease. In a minority of patients, chronic gastritis evolves to gastric atrophy, a precursor of gastric cancer. The infection can be treated with antimicrobial agents, and treatment is usually successful, provided that the infecting strain is susceptible to the antibiotics of choice. Little is known about the antimicrobial susceptibility patterns in *H. pylori* in Ecuador.

H. pylori strains that possess the virulence genes *vacA* and *cagA* are associated with peptic ulcer disease and gastric cancer. The gene encoding the vacuolating cytotoxin VacA varies between strains, especially in its signal (s1a, s1b, s1c, s2) and mid region (m1, m2).² Strains with the s1,m1 genotype produce higher levels of cytotoxin than the others. Strains that carry the s1,m1 mosaic combination of *vacA* also usually carry *cagA* (cytotoxin associated gene A), which is part of a

larger genomic entity designated the pathogenicity (*cag*) island.³

We conducted the present study to assess the antimicrobial susceptibility of *H. pylori* isolates from Ecuadorian patients. In addition, we determined the prevalence of the specific virulence markers *cagA* and *vacA*, and determined possible genetic relationships by genomic typing of the strains by random amplified polymorphic DNA (RAPD).

Materials and methods

Study population

Eighty-six patients, all residents of Guayaquil, with upper gastrointestinal complaints underwent routine endoscopy in an outpatient centre of the SOLCA Hospital in Guayaquil. The endoscopies took place between August and December 1999. Biopsy specimens from gastric mucosa, one of the antrum and one of the corpus, were taken and sent for histology to the laboratory in Ecuador. Later on, all unused biopsy specimens were transported, in a jar with dry ice, to The Netherlands for culture.

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Culture of H. pylori

Both biopsy specimens of corpus and antrum were streaked on Columbia agar (CA) (Becton Dickinson, Cockeysville, MD, USA) with 10% lysed horse blood (Bio Trading, Mijdrecht, The Netherlands), referred to as Columbia agar plates, and on CA with *H. pylori* selective supplement (Oxoid, Basingstoke, UK). Plates were incubated for 72 h at 37°C in a micro-aerophilic atmosphere (5% O₂, 10% CO₂, 85% N₂). Identification was carried out by Gram's stain morphology, and catalase, oxidase and urea hydrolysis measurements.

Determination of MIC

Inocula were prepared from an *H. pylori* culture grown on CA plates. MICs of metronidazole, clarithromycin, tetracycline and amoxicillin were determined using the Etest (AB Biodisk, Solna, Sweden) on CA plates essentially as described by Glupczynski *et al.*⁴ CA plates were inoculated with a bacterial suspension with a turbidity of a 3 McFarland standard (2×10^8 cfu/mL).

Extraction of H. pylori genomic DNA

Bacterial DNA was isolated as described by Boom *et al.*⁵ Extracted DNA was resolved in 100 µL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) supplemented with 10 µg of RNase (Sigma, St Louis, MO, USA). Purified DNA was aliquotted and stored at -20°C. DNA concentrations were estimated by agarose gel electrophoresis against diluted samples of lambda DNA (New England Biolabs Inc., Beverly, MA, USA).

RAPD

Two PCR amplifications with one primer each were carried out with the Primezym DNA Polymerase Kit (Biometra, Göttingen, Germany) according to the manufacturer's instructions. The primers used were D11344 (5'-AGTGAATTCGCGGTGAGATGCCA-3') and D9355 (5'-CCGGATCCGTGATGCGGTGCG-3'). Amplification was carried out in a total volume of 25 µL, with 20 ng of template genomic DNA and 20 pmol of primer. PCR amplifications were carried out in an automated thermocycler (PE 9700; Applied Biosystems, Foster City, CA, USA). The PCR programme consisted of four cycles of 5 min at 94°C, 5 min at 40°C and 5 min at 72°C, immediately followed by 30 cycles of 1 min at 94°C, 1 min at 40°C and 1 min at 72°C. Final extension was carried out for 10 min at 72°C. Aliquots of the PCR-amplified products (5 µL) were resolved in a 2% agarose gel, containing 0.5× TBE, stained with ethidium bromide and visualized under a short wavelength ultraviolet light source.

Line probe assay

The presence of *cagA* and the subtypes of *vacA* s and m regions were determined by multiplex PCR followed by reverse hybridization by a line probe assay (LiPA), as described by van Doorn *et al.*⁶ This assay consists of a nitrocellulose strip that contains poly(dT)-tailed oligonucleotide probes for *cagA* and the *vacA* s and m region genotypes (s1a, s1b, s1c, m1, m2a and m2b) immobilized as parallel lines. Briefly, PCR products from *cagA* and the *vacA* m and s regions (containing biotin at the 5' end of each primer) were mixed and denatured by 400 mM NaOH and 10 mM EDTA. Hybridization buffer and an LiPA strip were added, hybridization was carried out and the strip was stringently washed. Hybrids were detected by addition of conjugate (streptavidin-alkaline phosphatase) and substrate (4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate). Hybrids are visible as purple probe lines. Interpretation of the hybridization patterns was carried out visually.

Results*Culture and histology*

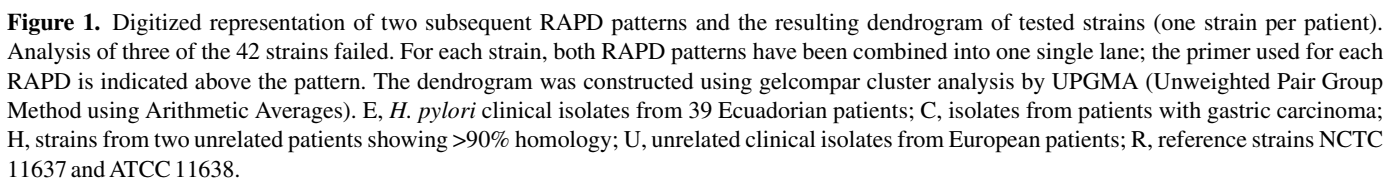
Data on histology were available for 63 of 86 patients. Three patients (4.8%) had a gastric carcinoma *in situ*. Two of these three patients had positive *H. pylori* cultures. Culture results and histology were concordant in 71.4% (45/63). Positive culture for *H. pylori* with negative histology was seen in 4.8% (3/63). Negative culture with positive histology was the case in 23.8% (15/63). Peptic ulcer was found in 10 patients.

Susceptibility testing

Resistance to metronidazole was present in 34 of 42 isolates (80.9%). All strains had MICs > 16 mg/L except for four strains with an MIC in the range 12–16 mg/L. Clarithromycin resistance was present in four of 42 isolates (9.5%), all with MICs > 256 mg/L. Three of these strains were metronidazole resistant as well. These double-resistant strains were isolated from patients who had been on antibiotic therapy for *H. pylori* before. Neither amoxicillin- nor tetracycline-resistant strains were found.

RAPD findings

Results are summarized in Figure 1. RAPD findings show a large heterogeneity of strains isolated from patients. Only two strains (E/H in Figure 1) showed 90% homology. The two strains from the patients with gastric carcinoma (E/C in Figure 1) were not genetically related.



Discussion

The prevalence of metronidazole resistance in *H. pylori* in Ecuador is high: 81% of the strains that we tested were resistant to this drug. The prevalence of clarithromycin resistance was 10%, whereas all isolates were sensitive to amoxicillin and tetracycline.

Resistance to metronidazole varies widely worldwide. In European countries resistance percentages are ~30%, in Africa ~80% and in South America resistance percentages are

Table 1. *cagA*, *vacA* allelic subtypes and correlation with disease

Tested strains (<i>n</i> = 38)	<i>cagA</i>	<i>vacA</i>		Disease
		signal	midcore	
31	+	s1b	m1	ulcus ventriculi or ulcus duodeni (<i>n</i> = 4) gastric cancer (<i>n</i> = 2) chronic gastritis (<i>n</i> = 18) not recorded (<i>n</i> = 7)
1	+	s1b/s2 ^a	m1	not recorded
1	+	s1a	m1	chronic gastritis
1	+	s1c	m2a	ulcus pylori
1	+	s2	m2a	ulcus pylori
1	–	s1b/s2 ^a	m2	not recorded
2	–	s2	m2a	chronic gastritis (<i>n</i> = 1) not recorded (<i>n</i> = 1)
ATCC (11638)	+	s1a	m1	
NCTC (11637)	+	s1a	m1	

^aMultiple genotype.

between 30% and 80%.⁷ The very high level of resistance in Ecuador precludes the use of this drug as the antimicrobial agent of first choice in this country.

Although the prevalence of resistance to clarithromycin was not that high, it is a matter of major concern, in view of reports of increasing clarithromycin resistance worldwide and combined resistance to metronidazole and clarithromycin in *H. pylori* clinical isolates.⁸ We did not find amoxicillin-resistant strains. Biopsy samples had been frozen before culturing. We are aware that because of this, strains with an unstable amoxicillin resistance type might have been missed. The low rate of successful culture (23.8%) found in this study is remarkable and probably caused by less than optimal conditions during transportation.

The SOLCA Hospital is a reference hospital for cancer patients in Guayaquil. This might explain the high prevalence of gastric cancer in the studied group. Each strain could easily be distinguished from another by RAPD fingerprinting, which was in agreement with earlier findings that *H. pylori* is an extremely diverse species.⁹ Two strains showed homology of >90% with RAPD, indicating genetic relatedness. However, these strains originated from two patients totally unrelated to each other. Both underwent an endoscopy on the same day during the same endoscopy session. We cannot rule out the possibility of cross-contamination by endoscopic procedures. We successfully classified *H. pylori* isolates from 38 patients into distinct *vacA* allelic genotypes by LiPA. Although the number of patients evaluated in this study is small, the results support the geographical distribution (s1b,m1 mosaicism more prevalent in South America) and the correlation between genotype and disease described by van Doorn *et al.*¹⁰ All the s1b,m1 strains in this study carried *cagA* and

were associated with the occurrence of peptic ulcer and gastric carcinoma. We only found one strain with the *vacA* s1c,m2a genotype. This genotype is the predominant genotype in East Asia,¹⁰ and is associated with disease. Notably, the patient infected with this strain is a native woman from Ecuador with no Asian relatives and no history of travelling. The *vacA* s2 type strains are known to be less virulent and not to carry *cagA*. One patient was infected with a *cagA*⁺, *vacA* s2,m2a type strain and had an ulcer. Virulence of this strain might be ascribed to *cagA*.

In conclusion, the high prevalence of metronidazole resistance in *H. pylori* in Ecuador precludes the use of this drug in empirical treatment of *H. pylori* infection. The vast majority of patients were infected with the more virulent type strain, namely the *cagA*⁺, *vacA* s1b,m1 genotype. This may account for the higher incidence of gastric carcinoma in Ecuador.

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References

1. Parsonnet, J., Friedman, G. D., Vandersteen, D. P., Chang, Y., Vogelstein, H., Orentreich, N. *et al.* (1991). *Helicobacter pylori* infection and the risk of gastric carcinoma. *New England Journal of Medicine* **325**, 1127–31.
2. Atherton, J. C., Cao, P., Peek, R. M., Tummuru, M. K. R., Blaser, M. J. & Cover, T. L. (1995). Mosaicism in vacuolating cytotoxin alleles of *H. pylori*: association of specific *vacA* types with cytotoxin production and peptic ulceration. *Journal of Biological Chemistry* **270**, 17771–7.

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3. Censini, S., Lange, C., Xiang, Z., Crabtree, J. E., Ghiara, P., Borodovsky, M. *et al.* (1996). *cag*, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. *Proceedings of the National Academy of Sciences, USA* **93**, 14648–53.
4. Glupczynski, Y., Labbé, M., Hansen, W., Crokaert, F. & Yourassowsky, E. (1991). Evaluation of the E-test for quantitative antimicrobial susceptibility of *Helicobacter pylori*. *Journal of Clinical Microbiology* **29**, 2072–5.
5. Boom, R., Sol, C. J. A., Salimans, M. M. M., Jansen, C. L., Wertheim-van Dillen, P. M. E. & van der Noorda, J. (1990). Rapid and simple method for purification of nucleic acids. *Journal of Clinical Microbiology* **28**, 495–503.
6. van Doorn, L. J., Figueiredo, C., Rossau, R., Jannes, G., van Asbroeck, M., Sousa, J. C. *et al.* (1998). Typing of *Helicobacter pylori vacA* gene and the detection of *cagA* gene by PCR and reverse hybridization. *Journal of Clinical Microbiology* **36**, 1271–6.
7. Alarcón, T., Domingo, D. & López-Brea, M. (1999). Antibiotic resistance problems with *Helicobacter pylori*. *International Journal of Antimicrobial Agents* **12**, 19–26.
8. Prazeres Magalhaes, P., Magalhaes Queiroz, D. M., Campos Barbosa, D. V., Aguiar Rocha, G., Nogueira Mendes, E., Santos, A. *et al.* (2002). *Helicobacter pylori* primary resistance to metronidazole and clarithromycin in Brazil. *Antimicrobial Agents and Chemotherapy* **46**, 2021–3.
9. Akopyanz, N., Bukanov, N. O., Westblom, T. U., Kresovich, S. & Berg, D. E. (1992). DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. *Nucleic Acids Research* **20**, 5137–42.
10. van Doorn, L. J., Figueiredo, C., Mégraud, F., Peña, S., Midolo, P., de Magalhaes Queiroz, D. M. *et al.* (1999). Geographic distribution of *vacA* allelic types of *Helicobacter pylori*. *Gastroenterology* **116**, 823–30.

