ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 2002, p. 2021–2023 Vol. 46, No. 6
0066-4804/02/$04.00+0 DOI: 10.1128/AAC.46.6.2021–2023.2002

Helicobacter pylori Primary Resistance to Metronidazole and Clarithromycin in Brazil

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Received 12 September 2001/Returned for modification 8 January 2002/Accepted 19 February 2002

Helicobacter pylori **resistance to metronidazole was detected in 107 (52.97%) of 202 strains. Twenty (9.85%) strains, 18 of them harboring 23S ribosomal DNA mutations, were resistant to clarithromycin. Metronidazole resistance was associated with female gender. Resistance to metronidazole and resistance to clarithromycin were associated. Increasing clarithromycin resistance rates were observed over time.**

Helicobacter pylori is associated with peptic ulcer and gastric carcinoma etiopathogenesis (3, 9). Resistance to metronidazole and clarithromycin is usually associated with a poor outcome of anti-*H. pylori* therapy involving one of these frequently employed antimicrobial drugs (5, 6). Metronidazole resistance is widespread, reaching frequencies of around 70% in Brazil (17, 20), while rising rates of clarithromycin resistance have been reported in some regions of the world (13).

We addressed *H. pylori* resistance to metronidazole and clarithromycin and also searched for an association between resistance and factors such as age, gender, type of disease, and *cagA* and *vacA* genotypes, controlling for confounding factors.

Isolates from 203 pretreatment patients, 78 (38.42%) with gastritis only, 92 (45.32%) with peptic ulcer, and 33 (16.26%) with gastric carcinoma, were studied.

Gastric specimens were transported in sodium thioglycolate broth (Difco, Detroit, Mich.) in an ice bath, ground, and plated onto Belo Horizonte medium (18). Culture was performed under microaerobiosis (Anaerocult C; Merck, Darmstadt, Germany) at 37°C. Macroscopic identification was confirmed by routine procedures (19).

DNA was extracted by a standard phenol-chloroform method (7). *cagA* and *vacA* genotypes were detected by PCR (2, 10, 16).

MICs of metronidazole and clarithromycin were determined by the agar dilution method, using twofold increments (0.5 to 32.0 μg/ml), on brain heart infusion agar plus 10% sheep blood and 0.004% 2,3,5-triphenyltetrazolium chloride. Incubation was performed under microaerobiosis at 37°C for 72 h, and strains were considered resistant when the MICs of metronidazole and clarithromycin were ≥8 and ≥2 μg/ml, respectively (11, 12).

Point mutations related to clarithromycin resistance were investigated by a PCR-restriction fragment length polymorphism analysis. 23S ribosomal DNA (rDNA) amplicons (14) were digested with *Bsa*I (New England Biolabs, Beverly, Mass.) or *Mbo*II (Life Technologies, Gaithersburg, Md.) (25, 26).

Statistical analysis was performed by the two-tailed Fisher or χ^2 tests. Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated by logistic regression (8). The changes in the frequency of resistance over the study period were analyzed by the χ^2 test for trend. The level of significance was set at $P \leq 5 \times 10^{-2}$.

Most isolates were *cagA* positive (164 [82.41%]). *cagA*-positive strains were more frequently detected for gastric carcinoma ($P < 10^{-4}$; OR, noncalculable) and ulcer ($P = 10^{-2}$, OR = 2.8, and 95% CI = 1.21 to 6.63) patients than for gastritis patients. Regarding *vacA*, s1 and m1 were more frequent among gastric carcinoma ($P < 10^{-3}$; OR, noncalculable; and $P < 10^{-8}$, OR = 26.60, and 95% CI = 5.52 to 86.04, respectively) and peptic ulcer ($P < 10^{-2}$, OR = 3.26, and 95% CI = 1.33 to 8.07 and $P = 2 \times 10^{-2}$, OR = 2.24, and 95% CI = 1.15 to 4.40, respectively) strains than among gastritis strains. Of strains isolated from gastritis patients, 28 (36.84%), 26 (34.21%), and 22 (28.95%) were genotyped as s1-m1, s1-m2, and s2-m2, respectively. Among the peptic ulcer isolates, 51 (56.67%) were typed as s1-m1, 29 (32.22%) were typed as s1-m2, and 10 (11.11%) were typed as s2-m2. Thirty-one (93.94%) gastric carcinoma strains were s1-m1, and two (6.06%) were s1-m2.

Metronidazole and clarithromycin resistance was found in 107 (52.97%) and 20 (9.85%) isolates, respectively. Resistance to both drugs was observed for 15 (7.43%) strains.

In developed countries, metronidazole resistance rates range from 10 to 50%, whereas, in developing ones, higher rates are usually observed (6, 13). This may be linked to the frequent use of metronidazole for treating parasitic infections (13), which, besides selecting resistant strains, may induce resistance due to its mutagenic effect (21). Metronidazole MICs showed a continuous distribution spectrum across the drug

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concentrations employed. As reported elsewhere (12), the yearly rates of resistance to metronidazole were similar throughout the study period. Also, a positive correlation between metronidazole resistance and female gender was found ($P = 10^{-2}$, OR = 2.10, and 95% CI = 1.19 to 3.71) (6, 24), which may be explained by the use of metronidazole to treat gynecologic infections. Resistance to metronidazole was not associated with the patient's disease and mean age. However, when the patients were stratified in groups under and above 40 years old, resistant strains were more frequently isolated from the younger group ($P < 10^{-2}$, OR = 2.40, and 95% CI = 1.29 to 4.51).

Regarding clarithromycin, the frequency of resistance in *H. pylori* usually ranges from 0 to 30% (11, 12, 13, 15). In this study, increasing resistance rates were observed: 4.48% in 1996, 7.69% in 1997, 10.00% in 1998, 12.19% in 1999, and 19.05% in 2000 ($P = 3 \times 10^{-2}$). Rising clarithromycin resistance rates have already been reported (13, 22) and seem to parallel its increasing use, not only for *H. pylori* eradication but also for treatment of respiratory tract infections (13). No association with age and gender was observed. In contrast, clarithromycin resistance was negatively and independently associated with gastric carcinoma ($P = 3 \times 10^{-2}$, OR = 0.43, and 95% CI = 0.20 to 0.93), which may be explained by the fact that clarithromycin resistance increased over the study period and the gastric carcinoma strains were collected before 1998.

Resistance to any of the drugs was not associated with *cagA* and *vacA* s and m genotypes. In contrast, as already reported, an association between resistance to metronidazole and that to clarithromycin was observed ($P = 5 \times 10^{-2}$, OR = 2.99, and 95% CI = 1.03 to 8.66). Since the mechanisms of action of these two antimicrobial agents are distinct, it is plausible to hypothesize that patients harboring metronidazole- and clarithromycin-resistant strains have been previously treated with both drugs (24).

Among the resistant isolates, 18 (90.0%) harbored 23S rDNA mutations: A2142G ($n = 3$), A2143G ($n = 12$), or both ($n = 3$). It is possible that the two clarithromycin-resistant strains that did not exhibit such alterations carry another mutation in the 23S rDNA, since the method that we employed detects only two of the five point mutations that have already been described for natural *H. pylori* strains (23). An explanation for the predominance of A2143G and A2142G changes is that these mutants exhibit an insignificant disadvantage in an environment without clarithromycin and, consequently, are not negatively selected (4, 27). Also, because strains harboring these mutations have the highest growth rates, more stable resistance, and higher MICs than do other strains, they may be preferential (4). It should be mentioned that, in employing DNA obtained directly from 10 biopsy fragments (data not shown), the results were the same as those found for DNA from the isolated bacteria, reinforcing the possibility of applying the method directly to biopsy specimens, which would render the technique more practical, simpler, and faster (1).

This work was supported by grants from CNPq, FAPEMIG, and FINEP, Brazil.

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