S. Agudo[1]
T. Alarcón[1]
L. Cibrelus[1]
P. Urruzuno[2]
M. J. Martínez[3]
M. López-Brea[1]

High percentage of clarithromycin and metronidazole resistance in *Helicobacter pylori* clinical isolates obtained from Spanish children

[1] Hospital de La Princesa
Madrid (Spain)
[2] Hospital Universitario Doce de Octubre
Madrid (Spain)
[3] Hospital Infantil Universitario Niño Jesús
Madrid (Spain)

Objective. To determine the primary and secondary resistance to several antimicrobial agents in Spanish *Helicobacter pylori* clinical isolates obtained from paediatric patients from January 2002 to June 2006.

Methods. Samples were collected from gastric biopsies of symptomatic paediatric patients and *H. pylori* cultured according to standard microbiological procedures. Resistance was determined by E-test. Strains were considered resistant if minimal inhibitory concentration (MIC) ≥ 2 mg/l for amoxycillin, ≥ 4 mg/l for tetracycline, ≥ 8 mg/l for metronidazole, ≥ 1 mg/l for clarithromycin, MIC ≥ 4 mg/l for ciprofloxacin, MIC ≥ 32 mg/l for rifampicin and intermediate if MIC = 0.5 mg/l for clarithromycin, and MIC = 2 mg/l for ciprofloxacin.

Results. A total of 101 patients were included: 38 males and 63 females (sex ratio M/F: 0.6). Average age was 10 years (range: 4-18 years). All strains were susceptible to amoxycillin, tetracycline and rifampicin, 35.7% were resistant to metronidazole, 54.6% to clarithromycin and 1.8% to ciprofloxacin. 2.0% were intermediate to clarithromycin and 1.8% to ciprofloxacin. Double resistance to metronidazole and clarithromycin rated at 17.2%. Thirty-five patients (34.7%) had a history of treatment failure, and were considered as secondary *H. pylori*. Primary resistance rates to metronidazole and clarithromycin were 32.8% and 49.2%, respectively, and secondary resistance rates were 41.2% and 70.6%, respectively.

Conclusions. Resistance to clarithromycin (56.6%) was higher than to metronidazole (35.7%) in the *H. pylori* strains studied. Clarithromycin resistance was very high even in strains from paediatric patients not previously treated for *H. pylori* infection.

Key words:
Primary resistance. Paediatrics. Ciprofloxacin. Rifampicin. Treatment failure.

Rev Esp Quimioter 2009;22(2):88-92

Correspondencia:
Sonia Agudo
Departamento de Microbiología
Hospital Universitario de La Princesa
Diego de León, 62
28006 Madrid (Spain)
Correo electrónico: soniaagu@hotmail.com

Alto porcentaje de resistencia a claritromicina y metronidazol en aislamientos clínicos de *Helicobacter pylori* procedentes de pacientes pediátricos

Introducción. Determinar la resistencia primaria y secundaria a varios agentes antibióticos en aislamientos clínicos de *Helicobacter pylori* procedentes de pacientes pediátricos desde enero de 2002 a junio de 2006.

Métodos. Las muestras fueron biopsias gástricas procedentes de pacientes pediátricos sintomáticos. *H. pylori* fue cultivado acorde con los estándares de los procedimientos microbiológicos. La resistencia a antibióticos fue determinada mediante E-test. Los aislamientos fueron considerados resistentes si la CMI ≥ 2 mg/l para amoxicilina, ≥ 4 mg/l para tetraciclina, ≥ 8 mg/l para metronidazol, ≥ 1 mg/l para claritromicina, ≥ 4 mg/l para ciprofloxacino y ≥ 32 mg/l para rifampicina, y fue considerado intermedio si la concentración mínima inhibitoria (CMI) = 0,5 mg/l para claritromicina, y CMI = 2 mg/l para ciprofloxacino.

Resultados. De los 101 pacientes incluidos en el estudio: 38 fueron hombres y 63 mujeres. La media de edad fue de 10 años. Todas las cepas fueron sensibles a amoxicilina, tetraciclina y rifampicina. El 35,7% de los aislamientos fueron resistentes a metronidazol, el 54,6% fueron resistentes a claritromicina y el 1,8% a ciprofloxacino. El 17,2% de los aislamientos tuvieron doble resistencia a metronidazol y claritromicina. Treinta y cinco pacientes (34,7%) tuvieron un fallo en el tratamiento frente a *H. pylori*, previamente. La resistencia primaria a metronidazol y claritromicina fue del 32,8 y 49,2%, respectivamente, y la resistencia secundaria fue de un 41,2 y 70,6%, respectivamente.

Conclusión. La resistencia a claritromicina (56,6%) fue más alta que a metronidazol (35,7%) en los aislamientos clínicos de *H. pylori* estudiados. La resistencia a claritromicina fue alta incluso en los pacientes que no habían tenido tratamientos previos frente a *H. pylori*.

Palabras clave:
Resistencia primaria. Pediátricos. Ciprofloxacino. Rifampicina. Tratamiento fracasado.

INTRODUCTION

Helicobacter pylori is a microaerophilic spiral-shaped gram-negative bacteria, about 3 m long with a diameter about 0.5 μm with 4-6 flagellas and it is found in the gastric mucous layer or adherent to the epithelial lining of the stomach. The organism has an abundant urease enzyme production, which is important for colonization, due to the formation of ammonia on the gastric mucosa, increasing the pH of its environment. This enzyme is also important for the detection of the organism.

H. pylori has a special affinity for gastric mucosa and is associated with various digestive diseases, such as peptic ulcer (duodenal and gastric), chronic active gastritis and mucosa- associated lymphoid tissue and it is considered a risk factor in the development of the gastric cancer.[1-3] *H. pylori* is most frequently acquired during childhood and usually persists throughout life, causing different digestive diseases in childhood and adulthood unless a specific treatment is given (spontaneous eradication is rare).

Treatment with antibiotics is widely recommended for several of these diseases, such as peptic ulcer. The Maastricht III Consensus Report recommended proton pump inhibitor (PPI) or ranitidine bismuth citrate-based triple regimen with clarithromycin and amoxycillin or metronidazole as a first-line therapy.[4] However, side effects, poor compliance and resistance to the antibiotics used are common causes of treatment failure. Resistance to metronidazole and clarithromycin is population-dependent, and several studies suggest that clarithromycin resistance is higher in strains obtained from children than in those from adults.[5] The evaluation of antibiotic resistance in paediatric patients from different areas can help in optimizing therapeutic regimens to prevent treatment failure.

The aim of this study was to determine the primary and secondary resistance to several antimicrobial agents in Spanish *Helicobacter pylori* clinical isolates obtained from paediatric patients from January 2002 to June 2006.

MATERIAL AND METHODS

Patients

Samples were collected from gastric biopsies of symptomatic paediatric patients (aged 18 years old and under) attending to the Gastroenterology Unit from two children's hospitals (Hospital Infantil Universitario Niño Jesús and Hospital Universitario 12 de Octubre, Madrid). Both hospitals were *H. pylori*-reference centres and parents signed an informed consent form for the endoscopy. At the time samples were taken, patients had not received Proton Pump Inhibitors (PPIs) or antibiotics for at least two weeks. Medical records were reviewed to obtain treatment history to know if patients were previously treated for *H. pylori* infection.

Samples collection and identification

Biopsies were received at the Department of Microbiology (Hospital Universitario de La Princesa, Madrid) and processed before 3 h. Samples for culture were placed in sterile saline solution for transport. Biopsy tissue was chopped into smaller pieces and homogenised under aseptic conditions. Homogenised tissue was streaked onto both non-selective (Columbia agar, with 5% sheep blood; BioMérieux) and selective media (Pylori agar; BioMérieux) incubated 10 days at 37 °C in a microaerophilic atmosphere (5% O_2, 10% CO_2, 85% N_2). Isolates were identified as *H. pylori*-based on colony morphology (small, grey and translucent), positive biochemical reactions for urease, catalase and oxidase test, and negative Gram staining. Colonies of 48 h *H. pylori* cultures were suspended in sterile saline and adjusted to a density equal to McFarland turbidity standard.[3] The suspensions were spread onto the plates with sterile cotton swabs. The minimal inhibitory concentrations (MICs) of isolates were determined by the Epsilometer test (E-test; AB Biodisk, Solna, Sweden) on Mueller Hinton sheep blood agar (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD, USA). Plates with strips containing amoxycillin, clarithromycin, tetracycline, metronidazole ciprofloxacin and rifampicin were incubated for 72 h under microaerophilic conditions. MIC was considered as the lowest concentration of drug which inhibited visible growth and read as the intercept of the elliptical zone of inhibition with the graded strip for the E-test. Based on the CLSI[6] and other previously published data[7,8], strains were resistant if MIC ≥ 2 mg/l for amoxycillin, MIC ≥ 4 mg/l for tetracycline, MIC ≥ 8 mg/l for metronidazole, MIC ≥ 1 mg/l for clarithromycin, MIC ≥ 4 mg/l for ciprofloxacin and MIC ≥ 32mg/l for rifampicin and intermediate if MIC = 0.5 mg/l for clarithromycin and MIC = 2 mg/l for ciprofloxacin. They were susceptible below these thresholds. MIC_{50} and MIC_{90} were calculated as the MIC value that inhibited 50% or 90% of the isolates.

Statistical analysis

Data were analyzed using Stata® 8.0 (StataCorp, Texas, USA). Differences between groups were tested with Chisquare (categorical variables) and Student (continuous variables) tests. Significance was construed at $p \leq 5\%$.

RESULTS

Description of the population and symptoms

H. pylori strains were obtained from gastric biopsy specimens of 101 consecutive *H. pylori*-positive paediatric patients. There were 38 males and 69 females (sex[2] ratio M/F = 0.6). The average age was 10 years (range: 4-18 years), with digestive and non-digestive symptoms. 93% of symptoms were digestive-mainly epigastralgia (63%), perilumbilical pain (17%), vomiting (26%) and pirosis/reflux (19%). Nondigestive symptoms, including anaemia

(4.4%), halitosis (2.2%), asthma (1.1%), disorder of alimentary behaviour (1.1%) and alteration of general status (1.1%).

Previous medication and treatment failure

34.7% of the patients had been previously treated for *H. pylori* infection. They were treated by antibiotics (amoxycillin, metronidazole, clarithromycin) plus antacids (67%) or by antacid only (30%).

Antimicrobial resistance

All strains were susceptible to amoxycillin, tetracycline and rifampicin, 35.7% (35 out of 98 strains tested for this antibiotic) were resistant to metronidazole, 54.6% (54 out of 99) to clarithromycin and 1.8% (1 out of 55) to ciprofloxacin. Intermediate strains to clarithromycin and ciprofloxacin were 2% (2 out of 99) and 1.8% (1 out of 55) respectively. Double resistance to metronidazole and clarithromycin rated at 17.2%. Results can be found in the table 1.

MIC_{50} and MIC_{90} of amoxycillin, tetracycline, metronidazole, clarithromycin, ciprofloxacin and rifampicin are detailed in table 2.

Thirty-five patients (34.7%) had a history of treatment failure, and were considered secondary resistance to *H. pylori*. Primary resistance rates to metronidazole and clarithromycin were 32.8% and 49.2%, respectively and secondary resistance rates were 41.2% and 70.6%, respectively.

Primary and secondary resistance rates to metronidazole and clarithromycin (including double resistance) are detailed in the table 3 (intermediate strains to clarithromycin were considered resistant).

Table 1 Number and percentage of resistant *Helicobacter pylori* clinical isolates from paediatric children

Antibiotic	Freq resistance	% resistance	Freq intermediate	% intermediate
Amoxycillin	0/101	0	0	0
Tetracycline	0/101	0	0	0
Metronidazol	35/98	35.7	0	0
Claritromycin	54/99	54.6	2	2.0
Ciprofloxacin	1/55	1.8	1	1.8
Rifampicin	0/55	0	0	0

Table 2 Minimal inhibitory concentration $(MIC)_{50}$ MIC_{90} and range (in mg/l) of the 6 antibiotics tested against *Helicobacter pylori* clinical isolates obtained from paediatrics patients

Antibiotic	MIC_{50}	MIC_{90}	Range
Amoxycillin	0.016	0.125	<0.016-0.5
Tetracycline	0.064	0.25	<0.016-1
Metronidazole	0.094	>256	<0.016->256
Claritromycin	1.5	48	<0.008->256
Ciprofloxacin	0.094	0.5	<0.032-32
Rifampicin	0.5	2	<0.016-4

DISCUSSION

H. pylori is considered the major cause of chronic gastritis and peptic ulcer disease. Additionally, it can increase the risk of malignancies in adulthood. In general, *H. pylori* eradication usually entails a proton pump inhibitor or bismuth salts in combination with two antibiotics (metronidazole, amoxycillin, tetracycline or clarithromycin).

Resistance of *H. pylori* to antimicrobial agents is the main cause of treatment failure, even a triple regimen (two antibiotics plus antacids). *H. pylori* antimicrobial resistance varies between different geographical regions. The severity of gastric inflammation, dosage of proton pump inhibitor and the nature

Table 3 Number (n) and percentage (%) of primary and secondary resistance to amoxycillin, tetracycline, clarithromycin, metronidazole, ciprofloxacin and rifampicin in children infected by *Helicobacter pylori*. Madrid, 2002-2006*

Antibiotic	Primary resistance n (%)	Secondary resistance n (%)
Amoxycillin	0 (0)	0 (0)
Tetracycline	0 (0)	0 (0)
Metronidazole	21 (32.8)	14 (41.2)
Clarithromycin	32 (49.2)	24 (70.6)
Ciprofloxacin	2 (3.6)	0 (0)
Rifampicin	0 (0)	0 (0)
Double resistance to metronidazole and clarithromycin	10 (15.4)	9 (26.5)

* Intermediate strains to clarithromycin and ciprofloxacin were considered as resistant.

of pathology (ulcerative versus non ulcerative disease) also affect the outcome of therapy. In the last few years, guidelines of *H. pylori* infection in children have been published. They suggested that children should be treated with a twice-daily triple-drug regimen, with two antibiotics (clarithromycin, amoxycillin and metronidazole) plus a PPI during two weeks. These drugs are considered to be most effective against *H. pylori,* but drug resistance is a growing problem.

Overall, *H. pylori* resistance to clarithromycin was higher than other antimicrobial agents in the paediatric patients included in this study. It was 49.2% in primary resistance and 70.6% in secondary resistance. Macrolide resistance is based on defined point mutations in the peptidytransferase loop in both copies of 23 S rRNA genes. Monotherapy with clarithromycin could induce these mutations.[9] In Spain and other European countries, including France, Portugal, Poland, Turkey and Bulgaria an increase of resistance to clarithromycin was observed.[10,11] In Northern European countries, this increase was not observed. This difference probably depends on macrolide consumption. New macrolides were marketed in Spain at the beginning of the 1990, and clarithromycin in 1991; these children have been more exposed to macrolides. It is very frequent nowadays to treat respiratory infections in young children. Compared to with other studies in our hospital,[12] the rate of resistance to clarithromycin during 1999 and 2000 was 29.16% (28 out of 96) and the resistance to clarithromycin increased from 29.16% to 49.2% in the period 1999-2000 to 2002-2006. Resistance to this antibiotic has increased considerably during this period. The secondary resistance to clarithromycin was higher than the primary, because this antibiotic is the most frequently included in the standard triple therapies for *H. pylori* eradication and when susceptibility is studied after treatment failure was because the original strains was resistant to clarithromycin in many cases.

The resistance to metronidazole was high in our population too. The primary resistance was 32.8% and the secondary one was 41.2%. The resistance to metronidazole is very variable in different countries; it is high in underdeveloped countries because this antibiotic is widely used to treat parasitic diseases and gynaecological infections in female patients.[13] There was a study in our hospital about the evolution of the susceptibility to metronidazole during 9 years[14] and metronidazole resistance was 7.14% in 1991–1993, 20.25% in 1994–1996 and 43.90% in 1997-1999. Metronidazole resistance increased during these nine years and in that period exceeds clarithromycin resistance. In the present study the percentage of resistance to metronidazole was lower than the percentage of resistance to clarithromycin.

Clarithromycin and metronidazole are two antibiotics used in the *H. pylori* treatment very often; for this reason it is very important to know the prevalence of resistance to both antibiotics. Double resistance was 15.4% in primary resistance and 26.5% in secondary resistance. The secondary resistance was higher than primary due to the majority of the primary treatment is being with two antibiotics plus PPIs. The most useful antibiotics were clarithromycin and metronidazole. Although the rate of double resistance is low in Europe, it is high in the underdeveloped countries.

All strains were susceptible to amoxycillin and tetracycline in our study. We tested the susceptibility to tetracycline in all *H. pylori* strains to know the prevalence of resistance in our area although this antibiotic should not be advised for children. Due to this fact, there are not many studies about the susceptibility to tetracycline in *H. pylori* isolates in children, although in most of them, resistance was not found. In a study from Bulgaria 3.4% of resistant strains were found.[15]

In the rest of Europe the rate of resistance to amoxycillin is very low. For example, in a multicentre European study performed by Glupzcynski, et al.[16] Nine resistance isolates to amoxycillin were described, in Italy, Greece, Denmark and England. The percentage of amoxycillin resistance is described as less than 1% in many regions but in some other parts, as a study of Iran, a 56% of resistance to this antibiotic was described.[17] In an article from Japan, they studied the correlation between substitutions in penicillin-binding protein 1 and amoxycillin resistance in *H. pylori*.[18]

As the rates of resistance to clarithromycin and metronidazole are high, new antibiotics are investigated for treatment of *H. pylori.* For instance-triple PPI-based regimen, including rifampicin and levofloxacin have recently been used as alternative regimens to classical therapy.[19]

In our study, only 3.6% were resistant to ciprofloxacin (both intermediate and fully resistant). Rates of resistance lower than 10% have been reported from several countries such as France, Japan, The Netherlands or Germany.[7,20,21] But, there is a study from Korea,[22] where they reviewed the susceptibility to ciprofloxacin during ten years and they suggested that resistance to this antibiotic has been increasing in his population, from 13.9% in 1994 to 33.8% in 2003 were resistant strains and the resistance was mediated through point mutation in *gyr A.*

Rifampicin is normally used in the treatment of mycobacterial infections and in severe bone infections due to *Staphylococcus aureus* or legionnaire's disease, but rifabutin (a semisynthetic derivative of rifamycin) has shown to have a high efficacy against *H. pylori.* In our study all strains were susceptible to rifampicin. A study about the rate of rifampicin resistance in *H. pylor*i isolated from patients in Germany showed 1.4% of resistance to rifampicin. Resistance is still low and associated with mutations in the *rpo B* gene. This antibiotic could be recommended in drug-resistant *H. pylori* infections. However, the use of rifampicin should be restricted to avoid increasing the resistance in the future.[8]

In conclusion, resistance rates of *H. pylori* to metronidazole and clarithromycin in children are critical and induce treatment failure although they are associated to other antibiotics.

Double resistance could become a serious clinical problem. This is particularly important in areas with a high prevalence of primary metronidazole resistance, where more treatment failures and more double resistance will occur. It is very important to know local (geographical) prevalence of antimicrobial resistance before recommending a treatment for *H. pylori*. Empirical treatment in areas with identified high resistance rates should therefore be avoided. Future trials are needed to study several combinations of treatments.

ACKNOWLEDGEMENTS

This work was supported by Fondo de Investigación Sanitarias de la Seguridad Social, grant FIS PI 052442 and PI 052452.

Conflict of interests: non declared.

REFERENCES

1. Mégraud F, Lehours P. *Helicobacter pylori* detection and antimicrobial susceptibility testing. Clin Microbiol Rev 2007;20: 280-322.
2. Poddar U, Yachha S. *Helicobacter pylori* in children: an Indian perspective. Indian Pediatrics 2007;44:761-0.
3. Steven J, Czinn MD. FAAP, FACG *Helicobacter pylori* infection: Detection, investigation and management. J Pediatr 2005;146: 21-6.
4. Malfertheiner P, Megraud F, O'Morain, Bazzoli F, El-Omar E, Graham D, et al. Concepts in the management of *Helicobacter pylori* infection: the Maastricht III Consensus Report. Gut 2007; 56:772-81.
5. Megraud F. *H. pylori* antibiotic resistance: prevalence, importance and advantages in testing. Gut 2004;53:1374-84.
6. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing: seventeenth Informational Supplement M100-S17. Wayne PA: CLSI 2005.
7. Tankovic J, Lascols C, Sculo Q, Petit J, Soussy C. Single and double mutations in *gyr*A but not in *gyr*B are associated with lowand high-level fluoroquinolone resistance in *Helicobacter pylo - ri.* Antimicrob Agents Chemother 2003;47:3942-4.
8. Glocker E, Bogdan C, Kist M. Characterizacion of rifmpicin-resistant clinical *Helicobacter pylori* isolates from Germany. J Antimicrob Chemother 2007;59:874-9.
9. Peterson WL, Graham DY, Marshall B, Shall B, Blaser MJ, Gentar M, et al. Clarithromycin as monotherapy for eradication of *Helicobacter pylori:* a randomized, double blind trial. Am J Gastroenterol 1993;88:1860-4.
10. Koletzko S, Richy F, Bontems P, Crone J, Kalach, Monteiro ML, et al. Prospective multicentre study on antibiotic resistance of *Helicobacter pylori* strains obtained from children living in Europe. Gut 2006;55:1711-6.
11. Francesco V, Margiotta M, Zullo A, Asan C, Giorgio F, Burattini O, et al. Prevalence of primary clarithromycin resistance in *Helicobacer pylori* strains over a 15 year period in Italy. J Antimicrobial Chemother 2007;59:783-5.
12. Alarcón T, Vega E, Domingo D, Martínez MJ, López-Brea M. Clarithromycin resistance among *Helicobacter pylori* strains isolated from children: prevalence and study of mechanism of resistance by PCR-restriction fragment length polymorphism analysis. J Clin Microbiol 2003;41:486-8.
13. John Albert M, Al-Mekhaizeem K, Neil L, Dhar R, Dhar PM, Al- Ali M, et al. High prevalence and level of resistance to metronidazole, but lack of resistance to other antimicrobials in *Helicobacter pylori,* isolated from a multiracial population in Kuwait. Aliment Pharmacol Ther 2006;24: 1359-66.
14. López-Brea M, Martínez J, Domingo D, Alarcón T. A 9 year study of clarithromycin and metronidazole resistance in *Helicobacter pylori* from Spanish children. J Antimicrob Chemother 2001;48: 295-7.
15. Boyanova L, Nikolov R, Lazarova E, Gergova G, Katsarov N, Kamburov V, et al. Antibacterial resistance in *Helicobacter pylori* strains isolated from Bulgarian children and adult patients over 9 years. J Med Microbiol 2006;55:65-8.
16. Glupczynski Y, Mégraud F, Lopez-Brea M, Andersen LP. European multicentre survey of *in vitro* antimicrobial resistance in *Helicobacter pylori.* Eur J Clin Microbiol Infect Dis 2001;20: 820-3.
17. Raffeey H, Ghotaslau R, Nikuask S, Hafez AA. Primary resistance in *Helicobacter pylori* isolated in children from Iran. J Infect Chemother 2007;13:291-5.
18. Rimbara E, Noguchi N, Kawai T, Sasatsu M. Correlation between substitutions in penicillin binding protein 1 and amoxicillin resistance in *Helicobacter pylori.* Microbiol Inmunol 2007;51: 939-44.
19. Oderda G, Shcherbakov P, Botems P, Urruzano P, Romano C, Gottrand F, et al. Results from the pediatric European register for treatment of *Helicobacter pylori.* Helicobacter 2007;12: 150-6.
20. Fujimura S, Kato S, Iinuma K, Watanabe A. *In vitro* activity of fluoroquinolone and the gyrA gene mutation in *Helicobacter pylori* strains isolated from children. J Med Microbiol 2004;53: 1019-22.
21. Heep M, Kist M, Strobel S, Beck D, Lehn N. Secondary resistance among 554 isolates of *Helicobacter pylori* after failure of therapy. Eur J Clin Microbiol Infect Dis 2000;19:538-41.
22. Kim JM, Kim JS, Kim N, Jung HC, Song IS. Distribution of fluoroquinolone MICs in *Helicobacter pylori* strains from Korean patients. J Antimicrob Chemother 2005;56:965-7.