1130-0108/2009/101/11/743-756
Revista Española de Enfermedades Digestivas

Rev Esp Enferm Dig (Madrid)
Vol. 101, N.° 11, pp. 743-756, 2009

Editorial

Infection with *Helicobacter pylori*. Prevalence, research and impact of antibiotic resistance

Antibiotic resistance is probably the primary cause of failed therapy for infectious disease, particularly for infection with *Helicobacter pylori*. Since the relevance of infection with *H. pylori* in the pathogenesis of peptic gastroduodenal disease and gastric neoplasms was unveiled, the effectiveness of treatment with a number of antibiotics and therapeutic schemes has been investigated (1). *H. pylori* is intrinsically resistant to glycopeptides, cefsulodin, polymyxines, nalidixic acid, trimetoprim, sulfonamides, nystatin, amphotericin B, and cycloheximide, whereas wild-type strains are susceptible to β-lactams (except cefsulodin), phosphomycin, macrolides, aminoglycosides, tetracyclines, chloramphenicol, rifampicin, fluoroquinolones, 5-nitroimidazoles, and nitrofurans (2). So-called quadruple therapy with a proton-pump inhibitor (PPI) in combination with bismuth, tetracyclines and metronidazole, and most particularly triple therapy with a PPI associated with clarithromycin and amoxicillin or metronidazole represent the most widely accepted therapies. The European Helicobacter Study Group (EHSG) reported in 1997 the conclusions of the 1st Consensus Conference held in Maastricht, and recommended triple therapy as first-line treatment for infection with *H. pylori*, with quadruple therapy being held in reserve for potential first-line treatment failures (3). Triple therapy has remained the first-choice regimen during the past decade, and has been recommended by most consensus meetings and both European and American scientific societies (4-8). The initial effectiveness of this regimen, around 80-90%, has progressively declined to below 80-70% in the last few years, which has led to consider novel therapeutic approaches (9-19). Acquired resistance to commonly-used antibiotics, particularly clarithromycin and metronidazole, is thought to be the primary cause of treatment failure, but other causes such as non-compliance or prescriptions with inadequate dosage or duration should also be taken into account (20-22).

Prevalence of antibiotic resistance of *H. pylori*

During the period 1999-2003 multiple studies were reported on the prevalence of primary antibiotic resistance in *H. pylori*, and significant variations were seen among countries or even regions and ethnic groups within countries. In Europe, a low prevalence of resistance to clarithromycin was reported in the Netherlands, Germany, and Sweden (1.7-2.9%), which was higher in Spain, France and Portugal (12-22%) (22-27). Interestingly, prevalence in northern Italy was 1.8 *versus* 23.4% in central Italy (28-29). It was 10-12% in the United States, 25% in Mexico, in 9.8% in Brazil, 11-13% in Japan, and 5-6% in Korea (30-37). The prevalence of resis-

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tance to metronidazole was 15-40% in Europe, 20-35% in the United States, 76% in Mexico, 53% in Brazil, 9-12% in Japan and 40% in Korea, while that of resistance to amoxicillin and tetracyclines was below 1% (22-37). A meta-analysis of 20 clinical trials in the USA acknowledges an increased prevalence of resistance to clarithromycin, from 4.9% in 1993-95 to 10.1% in 1995-99 (38). A multicenter European study with homogeneous methodology performed in 22 countries during 1998 found that the prevalence of primary resistance to clarithromycin was 9.9%, to metronidazole was 33.1%, and to amoxicillin was 0.8%. Resistance to clarithromycin was more common in children versus adults, and in southern (18%) *versus* central (9.3%) or northern (4.2%) European countries. Simultaneous resistance to clarithromycin and metronidazole was also more frequent in southern European countries (39).

A multicenter European study was carried out in 14 countries during 1999-2002 to assess *H. pylori* antibiotic resistance in the pediatric population. The prevalence of primary resistance to clarithromycin was 20%, higher in younger children (< 6 *vs*. > 12 years) and in people residing in southern Europe; that of primary resistance to metronidazole was 23%, to both clarithromycin and metronidazole was 5.3%, and to amoxicillin was 0.6% (40).

In Spain several studies on the primary antibiotic resistance of *H. pylori* were published during 1999-2004, which revealed that the prevalence of resistance to clarithromycin ranged from 8.7 to 13%, and to metronidazole from 13.8 to 42% (26,41-44). In Seville, in our healthcare area, we observed that the prevalence of resistance to clarithromycin was 10%, and to metronidazole was 28.6%, during the period 1996-1998, consistent with previously reported data (45).

More recent publications (2006-09) show that, in the last decade, primary resistance to clarithromycin has either remained stable or increased, which would account for the progressively decreased effectiveness of triple therapy. Zullo et al., during 2004-06, identified in Italy a prevalence of primary resistance to clarithromycin of 16.9%, more common in patients with non-ulcer dyspepsia (19.1%) (46). Boyanova et al., in Bulgaria, observed a prevalence of primary resistance to clarithromycin of 10% during the period 1996-99, and of 17.9% during the period 2005-07 (47), and such an increase was also seen in other studies in Great Britain, Korea and Japan (48-50). Storskurubb et al., in Sweden, saw a low prevalence of primary resistance to clarithromycin (1.5%) and metronidazole (16.2%), which they relate to low use because of restrictive antibiotic policies (51). Aboderin et al. reported the opposite in Nigeria upon detection of 100% resistance to clarithromycin, ampicillin, and metronidazole in 32 study cases, which they related to uncontrolled use of these antibiotics (52). Agudo et al., in Madrid (Spain), during the period 2002-06, identified in children a high primary and secondary resistance to clarithromycin (49.2 and 70.6%), superior to that seen with metronidazole (32.8 and 41.2%), and dual resistance to clarithromycin and metronidazole, both primary and secondary, in 15.4 and 26.5% of cases, respectively; the authors highlighted the huge negative effect of such a high prevalence on the efficacy of triple therapy for *H. pylori* eradication (53). Kalach, during 1999-2005 in France, also detected in children a high prevalence of primary resistance to clarithromycin (23.2%), lower than that seen in Spain and that remained stable since 1994-98 (22.1%) (54). Antibiotic resistance and its negative influence on the effectiveness of eradicating therapies for *H. pylori* was a most relevant topic at the Third Maastricht Consensus Conference (6). Increased resistance to clarithromycin was endorsed in association with a greater use, and triple therapy with a PPI, amoxicillin or metronidazole, and clar-

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ithromycin was recommended for populations with resistance prevalence lower than 15-20%, whereas clarithromycin should otherwise be withheld. Quadruple therapy with a PPI, bismuth, tetracyclines and metronidazole may be an alternative to triple therapy as first-line regimen. The impact on triple therapy effectiveness is higher in cases with resistance to clarithromycin versus resistance to metronidazole (21,55). In the review by Megraud, published in 2004 (21), the effectiveness of triple therapy with a PPI, amoxicillin and clarithromycin is seen to decrease from 87.8 to 18.3% depending on sensitivity or resistance to the latter antibiotic, with a loss of efficacy at virtually 70%. That study also revealed that the efficacy of triple therapy with a PPI, amoxicillin and metronidazole, depending on the presence of susceptibility or resistance to the latter antimicrobial, has decreased from 89.4 to 64.4%, with a loss of efficacy at 25%, and that the effectiveness of triple therapy with a PPI, clarithromycin and metronidazole has decrease from 97 to 0%, according to susceptibility or resistance to both antibiotics, with an efficacy of 72.6% in case of clarithromycin susceptibility and metronidazole resistance, and of 50% in the opposite situation. It is thought that treatment for *H. pylori* infection should be recommended when efficacy is higher than 80% (intention-to-treat) or 85% (per protocol) (3,11,56). The current effectiveness of triple therapy as first-choice regimen is usually below 80%, even 70% in some studies (9-12), and cannot be therefore recommended.

Mechanisms of antibiotic resistance in *H. pylori*

Evidence suggests that the mechanisms of antimicrobial resistance in *H. pylori* mainly result from chromosomal mutations (22,57) primarily acquired through vertical transmission among bacteria. Chromosomal mutations will show up in the offspring, which will consequently lead to a progressive increase in the prevalence of antimicrobial resistance. Horizontal transference from a resistant to a susceptible strain, both of them in the stomach, cannot be ruled out.

Clarithromycin is a bacteriostatic antibiotic; a semisynthetic derivative of erythromycin, it belongs in the macrolide class, and is the most powerful drug against *H. pylori*. It targets ribosomes, thereby blocking bacterial protein synthesis. Resistance to clarithromycin results from point mutations that occur in the 23S rRNA gene and modify ribosome structure, impairing antibiotic binding. Most common mutations include adenine replacement by cytosine or guanine at position 2142 (A2142C, A2142G), or by guanine at position 2143 (A2143G); A2115G and G2141A occur less commonly. These mutations enable resistant strains to tolerate increasing antibiotic concentrations, from 1 to 64 μg/mL (58,59).

Amoxicillin is a bactericidal antibiotic inhibiting bacterial wall synthesis. The prevalence of *H. pylori* resistance to amoxicillin is very low, and this antibiotic requires the presence of penicillin-binding proteins (PBPs) for proper functioning. The mechanism of resistance generated by *H. pylori* consists of mutations in gene *pbp* that result in changed PBPs with greater antibiotic affinity. Resistant strains do not generate amoxicillin-PBP complexes, and thus preclude bactericidal effects (57).

Tetracyclines are bacteriostatic antibiotics that target ribosomes to block bacterial protein synthesis. The prevalence of resistance to tetracyclines in *H. pylori* is very low, and is acquired via mutations in the 16S rRNA gene, primarily in adenine-guanine-adenine triplets at positions 926 to 928 (AGA 926-928); single, dual or even

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triple substitutions may occur. A triple replacement of AGA926-928 by thymine, thymine and cytosine, respectively (AGA926-928→TTC), in the 16S rRNA gene enables strains to tolerate antibiotic concentrations up to 64 μg/mL; dual mutations allow the tolerance of concentrations up to 8 μg/mL, and single replacements enable tolerance to 2 μg/mL (60,61). The presence of other potential mechanisms of resistance has been discussed in recent years, involving either TetA efflux protein (62) or the presence of a membrane permeability inhibitor.

Metronidazole is a bactericidal antibiotic within the nitroimidazole class that has been widely and indiscrimanetely used in the management of multiple infections, thus conditioning a high prevalence of resistance in *H. pylori*. Nitroimidazoles, on entering target cells, are activated via a reduction process mediated by the enzyme NADPH-nitroreductase, which damages DNA and bacterial structures. The mechanisms of resistance currently known are based on mutations in genes *rdxA* and *frxA*, which would block cell-related nitroimidazole activation.

Levofloxacin and ciprofloxacin are bactericidal antibiotics in the quinolone class; their mechanism of action is similar and consists of blocking bacterial DNA replication by binding the enzyme DNA-gyrase, coded for by genes *gyrA* and *gyrB*. *H. pylori* resistance to these antibiotics is conditioned by mutations in *gyrA* QRDR *(quinolone resistance-determining region)* (63).

Methods for the study of *H. pylori* antibiotic susceptibility

Multiple methods are available for the study of *H. pylori* antibiotic susceptibility, which may be categorized as phenotypical methods, most commonly used and based on gastric biopsy cultures, including agar dilution and E-test (epsilometer test), and genotypical methods, including fluorescent in situ hybridation, PCR (polymerase chain reaction), both conventional and with sequencing, RFLP *(restriction fragment length polymorphism)* PCR, and real-time PCR, which allows to directly detect chromosomal mutations in gastric biopsies and feces. The Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical Laboratory Standards (NCCLS), recommends agar dilution as gold standard for the study of *H. pylori* susceptibility to antibiotics, specifically for minimum inhibitory concentration (MIC) estimates, and establishes cutoff points for clarithromycin (64). The British Society for Antimicrobial Chemotherapy (BSAC) recommends in turn E-test diffusion, which has a high correlation with agar dilution. E-test has the benefit of being a quantitative method with direct MIC expression; it also adapts to slow-growing bacteria such as *H. pylori* (65). Other available methods include broth dilution and disk diffusion (66,67). Cutoff points suggested for clarithromycin include: ≤ 0.25 μg/mL for susceptible strains, ≥1 μg/mL for resistant strains, and 0,5 μg/mL for intermediate strains. These cutoffs have allowed excellent predicting values for the success of triple therapy with a PPI, clarithromycin and amoxicillin. No standardized criteria are available for antibiotics, including amoxicillin, tetracyclines, rifabutin, and levofloxacin. However, agar dilution may be used, and literature-endorsed cutoff points are used (47,68). Metronidazole is special in that most studies have shown an absence of interlab and intralab reproducibility on unknown grounds (2). Absent correlation between *H. pylori* susceptibility and eradicating therapy outcomes should also be counted in. *H. pylori* strains with a high MIC may be eradicated, possibly due to a variable redox potential within the stomach. The cutoff point usually considered to define resistance to metron-

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idazole is ≥ 8 μg/mL. EHSG, at the 3rd Maastricht Consensus Conference, discourages the use of metronidazole susceptibility tests in clinical practice for want of clinico-bacteriological correlation (6).

Genotypical methods are particularly used for the study of clarithromycin resistance, but may also be applied for other antibiotics. Chromosomal mutations responsible for antibiotic resistance may be easily detected with molecular testing based on fluorescent *in situ* hybridation (69) or genomic amplification, mainly using PCR-related techniques (22). The advantages of such molecular tests include a shorter time for results, and an excellent correlation with susceptibility as assessed using phenotypical methods. Their main shortcoming is that they require a molecular microbiology laboratory. The first genotypical method used was RFLP PCR. This test amplifies the region harboring mutations, and synthesized fragments are then treated with restriction endonucleases that recognize specific mutation-generated sites. The size of the resulting fragments indicates the presence or absence of a mutation (22). RFLP PCR is a simple method but uses conventional PCR, which delays results and requires amplicon manipulation because of contamination risks. PCR with subsequent amplified fragment sequencing has been used on gastric bacteria and biopsies to assess resistance to clarithromycin and quinolones. It may also be used for other antibiotics such as amoxicillin. The technique is somewhat complex but automation has allowed its development (22). Lastly, real-time PCR has been successfully used (22,70) to detect any mutations in the amplified region, and represents a relevant advance as results may be obtained within 2 hours. It has been used to directly identify *H. pylori* in biopsy and fecal samples, as well as in cultured bacteria. Real-time PCR for fecal samples has become a promising diagnostic technique as it represents a rapid, non-invasive method allowing direct bacterial identification and an assessment of clarithromycin susceptibility. A higher degree of standardization demands a commercially available kit with high sensitivity and specificity.

The study by Torres Debat et al. included in this issue of the *Spanish Journal of Gastroenterology* (71) is first to inform on the prevalence of *H. pylori* antibiotic resistance in Uruguay, and also investigates the molecular mechanisms of clarithromycin resistance using PCR. The authors research the prevalence of antibiotic resistance using the E-test in two time periods and two adult populations with gastric biopsy cultures positive for *H. pylori*, and diverse ethnic descent. In 2001 they studied 19 patients representative of the general population, and in 2006 they studied 31 patients of African descent. The prevalence of resistance to metronidazole was 36.8 and 35.5%, respectively; to clarithromycin, 0 and 19.4%; to levofloxacin, 5.2 and 3.2%; and to amoxicillin and tetracyclines, 0% for both groups. Of note is the variable prevalence of clarithromycin resistance in these two periods of time and/or population groups. The fact that antibiotic – and specifically clarithromycin – resistance prevalence varies among countries or among regions, social groups, or ethnic groups within one same country is well known. Information available on antibiotic resistance prevalence in African populations or Latin American countries is insufficient. In Mexico, during the years 1995-97, the prevalence reported for clarithromycin resistance was 25% (32); recently, a prevalence of 2% was acknowledged in Paraguay (72). Aboderin, during the period 2002-06 in Nigeria, detected a 100% prevalence of clarithromycin resistance, and that for other antibiotics was also very high (52). Available data are anyway inadequate for drawing conclusions. The prevalence of clarithromycin resistance found in 2006 by Torres Debat et al. in Uruguay is high, and these authors also state that whether this reflects the current

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status in the general population, with a rapid increase during the last few years, or the current status in a specific Uruguayan ethnic group is uncertain, the latter possibility being potentially supported by the finding of one single mutation at position 2143 in all cases (71). The high prevalence of resistance to metronidazole is similar to that currently seen in some developed rather than – as claimed by the authors – developing countries (39).

As previously stated, it is accepted that triple therapy should not be used to manage *H. pylori* infection when the prevalence of clarithromycin resistance is higher than 15-20%, as in the above study; in such cases other therapeutic alternatives should be considered. It would be desirable to have regularly updated, reliable information on the prevalence of antibiotic resistance in *H. pylori*, specifically regarding clarithromycin, for the various countries, regions or healthcare areas, so that the potentially most effective eradicating regimen could be assessed on an individual basis. The study of antibiotic resistance is particularly necessary when low effectiveness is found regarding triple therapy for *H. pylori* eradication, and in certain patients following the failure of an initial or second eradicating therapy.

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