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SHORT COMMUNICATION

Helicobacter pylori infection: antibiotic resistance and eradication rate in patients with gastritis showing previous treatment failures

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SUMMARY

Forty patients infected by *Helicobacter pylori* were studied. The treatment was based on the positivity or negativity of cultures (tailored therapy or empiric therapy). The eradication rate was 68% and 82% respectively. Genotypic susceptibility testing proved very useful in case of heteroresistance or mixed infections that represent a real problem possibly leading to a resistance underestimation. Real-time PCR detected the resistant population at a very low concentration not detectable by phenotypic tests. Bismuth quadruple therapy (PPI, bismuth, metronidazole, tetracycline, PBMT) was effective in the *Hp* eradication rate consistent with a high level of clarithromycin resistance.

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Helicobacter pylori (*Hp*) is involved in different diseases such as chronic active gastritis, peptic ulcer disease, gastric carcinoma, mucosa-associated lymphoid tissue lymphoma (MALT) and other endothelial dysfunctions leading to vascular diseases (Atherton *et al.,* 1997). *Hp* infection is widespread especially in developing countries with a prevalence of about 80% whereas in the developed areas such as the USA, Canada, Japan and Western Europe the prevalence is much lower (25-30%) with about 50% of world population infected (Goh *et al.,* 2011). *Hp* infection can be permanent in the absence of an appropriate treatment, and *Hp* intrinsic antibiotic resistance is an important obstacle to eradication.

This microorganism is in fact increasingly difficult to eradicate because the treatment regimens are declining in efficacy and the therapy of *Hp* infection is bedevilled by drug-resistant strains. Antibiotics such as clarithromycin and metronidazole once regarded as the first choice for *Hp* therapy are now considered obsolete considering their high rate of resistance due mainly to their widespread use for other pathologies in most countries (Graham and Fishbach, 2010). Therefore, it is crucial to identify effective therapies capable of curing *Hp* infection.

Aim of our research was to take into account a group of *Hp*-infected patients with gastritis who had failed previous treatments and to evaluate their eradication rate following either empiric therapy or specific tailored therapy. The problem of mixed infections and heteroresistance was also examined.

Forty patients attending an Academic Hospital with upper gastric symptoms that required assessment with diagnostic endoscopy (33 women and 7 men, mean age 56 years) previously treated with one or more eradication attempts were taken into consideration. They were divided into two groups depending on the presence or absence of *Hp* strains in culture media. All patients were UBT (Urea Breath Test) positive so definitely infected by *Hp* as reported in the literature (Gisbert *et al.,* 2004; Tonkic *et al.,* 2018). They were asked to sign their informed consent to undergo an oesophago-gastro-duodenoscopy with multiple biopsies. Intake of antibiotics, proton-pump inhibitors (PPI), bismuth or H_2-antagonists was interrupted during the four weeks before endoscopy. Biopsies taken from antrum, corpus and fundus were collected and submitted separately to rapid urease test (Tonkic *et al.,* 2018; Tseng *et al.,* 2005), culture and susceptibility to antibiotics by both phenotypic and genotypic tests. Essential conditions for *Hp* culture were the following: microaerophilic atmosphere, temperature of 37° (range 33°-40°), presence of 0.5% glycine. The culture media used were:

a) Blood agar Columbia with addition of cyclodextrane, 10% of horse blood, antibiotics and hemine;
b) Pylori Selective Agar (bio-Merieux, Marcy L'Etoile France) with 5% sheep blood and antibiotics (amphotericin, vancomycin and trimethoprim).

The microorganisms were identified through the following tests: colony morphology, characteristic spiral-shaped Gram-negative bacteria and positive findings on oxydase, urease and catalase tests. A sub-culture of the isolated colonies was performed in order to obtain a secondary isolation used for antibiotic sensitivity tests and for their

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preservation. The antibiotics under consideration were the following: metronidazole (MZ), levofloxacin (LEV), tetracycline (TE), clarithromycin (CLA) and amoxicillin (AMX). The methods used for antimicrobial agents susceptibility testing were the E-test method and the genotypic tests only for CLA and TE.

E-test strips were aseptically placed onto the dried surfaces of the inoculated plates for 72 h. In order to define strain resistance, the following MIC breakpoints were used: S < and R ≥0.12 mcg/ml for AMX; S < and R ≥0.25 and 0.5 respectively for CLA; S < and R≥1 mcg/ml for both TE and LEV; S < and R ≥8 mcg/ml for MZ (European Committee on Antimicrobial Susceptibility Testing, 2018). Two quality control reference strains were used throughout the testing: *Hp* ATCC43504 and *Hp* RD26. Genotypic susceptibility testing was carried out as previously described (Glocker *et al.,* 2005; Oleastro *et al.,* 2003). In brief, DNA from either gastric tissue samples or *H. pylori* colonies was extracted using a DNA extraction kit (Qiagen, Germany) in line with manufacturer's instructions. Real-time PCRs followed by melting curve analyses using fluorochrome-labeled hybridization probes were then performed to identify 23S rRNA with its mutation A2144G and/or 16S rRNA mutations conferring resistance to CLA and TE respectively. Molecular studies were carried out by a random amplified polymorphism DNA (RAPD profile) to investigate the virulence genes (cagA and vacA) in the process of heteroresistance as previously described (Mascellino et al., 2010).

The individual treatment was based on the positivity or negativity of *Hp* culture. The patients with *Hp* growth in culture media were treated following the susceptibility tests *in vitro* and submitted to antibiotic susceptibility-tailored therapy. Generally they underwent a treatment with a proton-pump inhibitor (PPI) and antibiotics following the susceptibility tests among these antimicrobials: AMX (1 gr t.d.s.), CLA (250 mg t.d.s.), MZ (250 mg t.d.s.), LEV (250 mg t.d.s.) and TE (250 mg t.d.s), whereas patients with more severe symptoms were given a quadruple therapy including bismuth salts. MZ was given at higher doses (from 250 mg to 500 mg) in patients showing MZ-resistance. Even the group of patients with no *Hp* growth detected but with UBT positive underwent an empirical therapy being considered infected by *Hp*. In fact, due to the high specificity and sensitivity of this test (~100% specificity and above 97% sensitivity) (Gisbert *et al.,* 2004; Gisbert *et al.,* 2003), we assumed that patients with gastritis, previously infected by *Hp* and showing prior treatment failures, still yielded *Hp* despite the negative cultures. They were generally treated with antibiotics never taken before, otherwise with quadruple therapy (PPI-bismuth-MZ-TE, PBMT) for 14 days as well as omeoprazole, AMX and rifabutin (after three treatment failures) or LEV-containing therapy as a rescue therapy. *H. pylori* eradication for both groups was tested by a validated ^{13}C-urea breath test three months after therapy completion (Gisbert *et al.,* 2003). The cut-off of this test in our study was <4.5‰ DOB (Delta Over Baseline) (Breath Quality UBT, AB ANALITICA Srl, Padua, Italy).

Out of 40 patients, culture and susceptibility tests were obtained in 30 patients (75%) whereas in 10 (25%) no *Hp* growth was detected. The biopsies taken from patients with negative cultures as well as the biopsies taken from patients with *Hp* positive cultures were all positive to RUT (rapid urease test) performed before sample processing. Considering the high specificity and sensitivity of this test (Tonkic *et al.,* 2018; Tseng *et al.,* 2005; Uotani *et al.,* 2015), we can affirm that even the individuals with no evident *Hp* growth were infected. Two of these patients had very small colonies possibly related to coccoid forms of the microorganism.

The eradication rates corresponded to 68% and 82% respectively with an average 75% eradication. Out of 30 patients with positive *Hp* cultures, the percentage of cure was strictly dependent on the number of gastric regions (antrum, corpus or fundus) infected by *Hp* in a single patient. In fact when only one region is infected this percentage is higher than in patients with two or three infected regions (82%, 70% and 52% respectively).

Demographic data, and endoscopic and histological findings of patients are reported in *Tables 1a*, *1b* and *1c*, respectively. Smoking, alcohol consumption, NSAIDs, type and dosage of PPI prescribed did not seem to affect the eradication rates. As far as endoscopic findings are concerned, we found 15/40 patients (37.5%) with PUD (peptic ulcer disease), of whom 5/15 (33%) with active ulcer and 10/15 (66%) with healed ulcer (scar) whereas the remaining 23/40 (57.5%) showed NUD (non-ulcer disease) and 2/40 (5%) erosions. As for histological findings, chronic gastritis was detected in 31/40 individuals (77.5%), severe gastritis in 7/40 (17.5%) and intestinal metaplasia in 2/40 (5%).

Table 1 - *Characteristics of the 40 patients included in the study.*

1a - *Demographic data*	
Age (Mean)	54 yrs
>50 yrs (N)	24
Gender	
Male (N)	7
Female (N)	33
Familiarity [N (%)]	16 (40%)
Smoking	
Smokers [N (%)]	8 (20%)
Non Smokers [N (%)]	25 (62,5%)
Ex-smokers [N (%)]	7 (17,5 %)
Alcohol consumption [N (%)]	10 (25%)
NSAID	6 (15%)
Hp positive culture	30 (75%)
PPI	Omeprazole 20 mg BID
1b - *Endoscopic findings [N (%)]*	
PUD	15 (37,5%)
active ulcer	5/15 (33%)
healed ulcer scar	10/15 (66%)
NUD	23 (57,5%)
EROSIONS	2 (5%)
1c - *Histological findings [N (%)]*	
Chronic gastritis	31 (77,5%)
Severe gastritis	7 (17,5%)
Intestinal metaplasia	2 (5%)

Hp: Helicobacter pylori; PUD: peptic ulcer disease; NUD: non-ulcer disease; NSAID: non-steroidal anti-inflammatory drugs; PPI: Proton-Pump Inhibitors.

The resistance to CLA and MZ in the *Hp* strains included in this study was very high (50% and 68% respectively). In particular, MZ resulted particularly ineffective, with 2 strains showing a MIC value ≥256 mcg/ml. AMX was the most active antibiotic as well as TET (only 4% and 6% of resistance respectively). The resistance rate of LEV was about 25%. Double resistance to MZ and CLA, which can be demonstrated in about half of the strains under study, may lead to a worse course of the disease and poor eradication outcome.

Heteroresistance, defined as the concomitant presence of a different susceptibility patterns in the different districts of a single stomach (Norazach *et al.,* 2009), was detected in 4 patients out of the 30 positive ones (13%). For each of these patients, we found a different antimicrobial pattern in the strains isolated from antrum (susceptible) and corpus (resistant) towards CLA (four patients) and MZ (one patient showing a double heteroresistance towards MZ and CLA). The strain genotypes, identified on the basis of virulence genes (cagA and vacA), were the same for both loci in pairs of isolates (susceptible and resistant) obtained from different regions of a single stomach (cagA + s1m2 in one patient and cagA + s1m1 in three patients) (*Table 2*).

Mixed infections were demonstrated in patients concomitantly yielding both wild type and mutant strains in a single gastric region. We studied the resistance with both methods (phenotypic tests and PCR molecular tests) for CLA and TE (*Table 3*). Three patients (10%) out of 30 with *Hp* positive culture were found to yield both wild type strains and strains carrying the A21444G mutation for CLA, whereas 12 (40%) only yielded A2144G mutated strains. Among these 15 individuals with CLA resistance, only 9 (60%) were found to show resistance by E-test. As far as TE is concerned, two polymorphisms (rrnA and rrnB) affecting the nucleotides from 926 and 928 were found in 2 out of the 30 patients (≈6%). Instead, only one strain was resistant by E-test.

On the whole we can say that the treatment of *H. pylori* infections is becoming more and more problematic. The increasing resistance to the most common antibiotics represents a crucial issue in the management of this disease. It is reported that this trend is rising mainly for CLA and MZ all over the world (Graham and Fishbach, 2010). CLA resistance in particular is now at such high levels that the use of clarithromycin-triple therapy is no longer recommended. Consequently, new strategies have been reported for *Hp* treatment in order to achieve an acceptable eradication rate. The Toronto Consensus Group suggests prolonging eradication therapy up to 14 days replacing the old triple therapy with the quadruple therapy (unaffected by CLA-resistance) based on PPI, bismuth, MZ and TE (PBMT) for most patients (this treatment is recommended even for first-line therapy) or alternatively the variant quadruple therapy without bismuth including PPI, AMX, MZ and CLA (PAMC) in countries where the CLA-R is low (less than 15%) (Fallone *et al.,* 2016; O'Connor *et al.,* 2017). A new drug, Vonoprazan (potassium-competitive acid blocker P-CAB), recently approved in Japan, has become available for *Hp* treatment even for CLA-resistant strains (Akazawa *et al.,* 2016)

In any case, it should be emphasized that for the eradication of *Hp* infection, the appropriate treatment should be based mostly on the prevalence of antibiotic resistance in a local situation, over than on susceptibility tests. As a matter of fact, the prevalence of antibiotic resistance for a specific regimen as well as the eradication patterns in a definite country have proved to be crucial in the management of *Hp* infections (Fallone *et al.,* 2016).

In our patients, the eradication rate (68%) following the susceptibility tests in those patients where *Hp* was isolated in culture were lower than those related to a tailored empirical therapy (82%) even if this difference was not statistically significant (p-value >0.5). This could be due to the fact that the bacteria present in the ten empirically treated patients where *Hp* was not able to grow *in vitro* were in a less virulent or dormant phase or in a very low number to be cultured. Coccoid forms were detected in two patients out of ten. These metabolically inactive forms may be able to cause a chronic active gastritis even in the absence of spiral bacillary forms as reported by Balakrishna (2013). This emphasizes the role of coccoid forms in causing active gastritis. As a result, these patients were more likely to respond to therapy even without the susceptibility tests. Empiric therapy based on the regional resistance patterns may achieve higher eradication rates. In this way the sus-

Table 2 - *Heteroresistance to Clarithromycin and Metronidazole of Hp isolates in different districts of the stomach of 4 patients.*

	Pt 1	*Pt 2*	*Pt 3*	*Pt 4*
Clarithromycin Genotype (same in A and C)	S(A)→R(C) cagA+s1m2	S(A)→R(C) cagA+s1m1	S(A)→R(C) cagA+s1m1	S(A)→R(C) cagA+s1m1
Metronidazole Genotype (same in A and C)			S(A)→R(C) cagA+s1m1	

C=corpus; A=antrum; S=susceptible; R=resistant.

Table 3 - *Helicobacter pylori Resistance to clarithromycin (CLA) and tetracycline (TE): comparison between real time PCR and E-test method.*

CLA Resistance (15 patients) (Total patients 30) 15/30 (50%)		*TE Resistance (2 patients) (Total patients 30) 2/30 (6%)*	
PCR	*E-test*	*PCR*	*E-test*
3 Wildtype + A2144G 12 A2144G	0 R 9 R	2 rrnA-rrnB (polymorphisms)	1R
15/15 (100%)	9/15 (60%)	2/2 (100%)	½ (50%)

ceptibility testing is not essential in guiding therapeutic strategies. This question is still controversial in the literature considering also that it has economic implications since susceptibility testing is an expensive and time-consuming method that requires endoscopy which is generally reserved to a limited number of patients (Liou *et al.,* 2013; Wenzhen *et al.,* 2010). The heteroresistance detected in 4 of our patients worsens the situation being an important issue as an isolate could be mistakenly considered susceptible if a single biopsy is used for antimicrobial tests possibly leading to a resistance underestimation (Norzah *et al.,* 2009).

Genotypic resistance is very useful for identifying mixed infections (Tursi *et al.,* 2017). Quite frequently, both wild types and mutant strains may occur at the same time. Some differences in the detection of antibiotic resistance may be observed in several strains when comparing E-test with PCR. If there are only a few resistant bacteria, they are difficult to be identified among many susceptible ones through phenotypic testing where the susceptible bacteria are primarily seen. On the contrary, a mixed infection with resistant and susceptible strains can easily be detected at the same time through a real-time PCR. Furthermore, genotypic tests are very useful in the absence of live bacteria or in the presence of contamination as well as when used directly in the gastric specimens for predicting CLA resistance or for changing previous treatments that had failed.

Conflict of interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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