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Detection of *Helicobacter pylori* and the genotypes of resistance to clarithromycin and the heterogeneous genotype to this antibiotic in biopsies obtained from symptomatic children

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ABSTRACT

The aim of this study was to use a commercially available kit (GenoType® HelicoDR; Hain Life Science, Germany) to detect *Helicobacter pylori* infection and clarithromycin resistance genotype in biopsies obtained from symptomatic children. *Results*: 111 out of 136 (81.6%) biopsies were *H. pylori* positive by genotype: 47 (42.3%) showed wild-type genotype, 53 resistant genotype (47.7%) and 11 heterogeneous genotype (9.9%). Culture was negative in 27 out of the 111 genotyped biopsies. Mutation A2143G (87.5%), followed by A2142G (7.5%) and double mutant A2142C-A2143G (5%) were found. The 11 heterogeneous genotype biopsies showed wild-type plus A2143G in 9 and plus A2142G in 2. *Conclusions*: This kit is a rapid, culture-independent method for routine application in biopsies from the pediatric population that allows detection of clarithromycin resistance and heterogeneous genotypes. It is important to know the clinical impact of infection with this type of strains as well as the role in treatment success.

1. Introduction

Helicobacter pylori is a spiral-shaped microaerophilic gram-negative bacterium that colonizes the gastric mucosa of humans and it is estimated that it infects 50% of the population worldwide (Marshall, 2002). Chronic infection with *H. pylori* can cause several gastrointestinal diseases, such as severe gastritis, peptic ulcer disease and gastric mucosa-associated lymphoid tissue lymphoma (MALT) (Campuzano-Maya, 2014). Moreover, it is identified as a Group I carcinogen by the World Health Organization International Agency for Research on Cancer (Herrero et al., 2014).

Numerous treatment regimens have been used to eradicate this microorganism. Triple therapy including a proton pump inhibitor and amoxicillin and clarithromycin, is the most common treatment used (Wu et al., 2014). The main cause of treatment failure of the *H. pylori* infection is the macrolide resistance (mainly clarithromycin) (Rimbara et al., 2007). The prevalence of clarithromycin resistance varies with the population. The high clarithromycin resistance rates in strains isolated from children is strikingly alarming (Vega et al., 2007). A high level of macrolides resistance in *Streptococcus pyogenes*, has been reported in Spain since 1986 (Granizo et al., 2000) and this high resistance seems to be related to the antibiotic consumption that had risen during the previous decade (Carrasco-Garrido et al., 2009). In a European study describing the antimicrobial susceptibility in *H. pylori*, an association was found between the use of long-acting macrolides and clarithromycin resistance; resistance was higher in Western/Central and Southern Europe (higher than 20%) than in Northern European countries (lower than 10%) (Megraud et al., 2013).

Mutations in the peptidyltransferase-encoding region of 23S rRNA confer resistance to clarithromycin by decreasing the binding of clarithromycin to ribosomes in *H. pylori* (Wang et al., 1999). A2142G, A2143G or A2142C are the most frequently described (De Francesco et al., 2011), with other such as A2515G, T2717C, A2116C, G2141A, A2144T, T2182C, G2224A, C2245, C2136T, C2310A and C2428T which are described sporadically (Becerikli et al., 2014; Tanih and Ndip, 2013). Mutations in other genes, such as translation initiation factor IF-2 (*infB*) and ribosomal protein L22 (*rplV*) genes were also described (Bihn et al., 2014).

During the *H. pylori* infection, multiple strains could colonize the same host (Blaser and Kirschner, 2007) and heterogeneous genotypes refers to infection with one or multiple strains presenting different genotypes of resistance to clarithromycin (Kao et al., 2014).

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Clarithromycin resistance is mainly detected by phenotypic methods performed after culture such as E-test or agar dilution (Mégraud and Lehours, 2007). These methods are time-consuming and can take up to 2 weeks from the endoscopy (Agudo et al., 2010a). Detection of point mutations conferring resistance to clarithromycin by molecular methods may constitute a quicker approach. One commercially available test, combines polymerase chain reaction (PCR) and hybridization. It allows the molecular detection of *H. pylori* (by targeting a specific region of the 23S rRNA gene) and characterization of common mechanisms of resistance to clarithromycin (mutations in 23S rRNA gene in positions 2142 and 2143) and for fluoroquinolones (most significant mutations of the quinolone-resistance region of the *gyrA* gene, especially at codons 87 and 91) within 6 h (Becerikli et al., 2014; Cambau et al., 2009; Lee et al., 2014; Nishizawa and Suzuki, 2014). Furthermore, this test allows detecting strains with heterogeneous genotype (Bihn et al., 2014; Cambau et al., 2009; Miendje et al., 2011). The frequency of these strains in children is poorly studied as well as the clinical implication in patients infected with this type of strains.

The aim of this study was to use a commercial available kit (GenoType® HelicoDR, Hain Life Science, Germany) to detect *H. pylori* infection and the genotypes of resistance to clarithromycin and the heterogeneous genotype to this antibiotic in biopsies obtained from symptomatic children.

2. Patients and methods

2.1. Patients

A total of 136 biopsies were analyzed from symptomatic paediatric patients (aged less than 18 years). These patients were attended at the Pediatric Gastroenterology Unit of three hospitals (Hospital Infantil Universitario Niño Jesús, Hospital Universitario Doce de Octubre and Hospital Universitario de Fuenlabrada, Madrid, Spain). Paediatric patients with clinical symptoms suggestive of *H. pylori* infection were studied by a non-invasive method, Urea Breath Test, and, when the test was positive, patients were submitted to endoscopy and the biopsies were sent for culture and molecular methods. Parents signed an informed consent form. Histopatology results and treatment outcome were revised in patients infected with strains showing heterogeneous genotype.

2.2. Gastric biopsy specimens

Biopsies were placed in sterile saline solution and sent to the Department of Microbiology (Hospital Universitario de la Princesa, Madrid, Spain) and processed before 3 hours of receiving. All biopsies were cultured on nonselective (Columbia agar, with 5% sheep blood; bioMérieux, Marcy l'Etoile, France) and selective media (Pylori agar, bioMérieux, Marcy l'Etoile, France) incubated up to 14 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 and Gram stain morphology, positive reactions for urease, catalase and oxidase test. Biopsies were stored at −80 °C until used in batches for molecular methods.

2.3. Determination of minimal inhibitory concentration

Minimal inhibitory concentration (MIC) of clarithromycin was determined by Epsilon Test, E-test (bioMérieux, Marcy l'Etoile, France) as previously described (Agudo et al., 2010b). A strain was considered resistant to clarithromycin when the MIC was >0.5 mg/L and susceptible when the MIC was ≤0,25mg/L according to European Committee for Antimicrobial Susceptibility Testing (European Committee on Antimicrobial Susceptibility Testing).

2.4. DNA extraction

DNA extraction from biopsy was performed using NucliSens® easyMAG™ instrument (bioMérieux, Marcy l'Etoile, France), according to manufacturer instructions, with a previous treatment with lysis buffer containing proteinase K overnight at 37 °C (Agudo et al., 2010a). DNA obtained was stored at −20 °C until use.

2.5. GenoType® HelicoDR test

GenoType® HelicoDR tests (Hain Lifescience, Nehren, Germany) were performed following manufacturer recommendations. Probes designed to hybridize with the sequences of the wild-type alleles (WT probes) or the mutated alleles (MUT probes), A2142C, A2142G, and A2143G for 23S rRNA gene and N87K, D91N, D91G, or D91Y for *gyr*A gene are included (Lee et al., 2014). Extracted genomic DNA of the biopsies was subjected to DNA strip testing.

Conjugate and amplification control should have a positive result to validate the test. A positive band identifies *H. pylori* positivity and some bands identify the 23S rRNA and the *gyr*A genes. When there was only a faint band, test was repeated until a clear band was seen; according the recommendations of other authors (Cambau et al., 2009; Lee et al., 2014; Miendje et al., 2011).

2.6. Statistical analysis

Differences between groups were statistically evaluated by using the chi-square test with Yate's correction. Differences were considered significant at the 5% probability level. Statistical analysis was performed using specific software (Epi Info; www.cdc.gov/epiinfo).

3. Results

111 out of 136 (81.6%) biopsies analyzed were *H. pylori*-positive with Genotype® Helico DR kit: 47 (42.3%) which showed a wild-type genotype (clarithromycin susceptible), 53 resistant genotype (47.7%) and 11 heterogeneous genotype (9.9%). Culture was negative in 27 out of the 111 (24.3%) genotyped biopsies (Table 1).

Of the 47 biopsies with wild-type genotype: 33 were positive by culture, 15.15% showed resistant and 84.85% susceptible phenotype. The range of MIC was <0.016 to >256 mg/L for the biopsies genotyped and positive culture (Fig. 1).

MIC50 and MIC90 was 0.032 and 4, 2 and 32 and 1 and 8 mg/L for strains with wild-type, resistant and with heterogeneous genotype respectively

Of the 64 biopsies (*H. pylori* positive with a resistant genotype), 53 showed resistant genotype (27 boys and 26 girls) and 11 showed heterogeneous genotypes (4 boys and 7 girls) ($p = 0.8597$, no statistical differences). The mean age (mean ± S.D.) was 10.06 ± 3.86 years.

Of the 53 biopsies, 47 showed a resistant genotype and were positive by culture; 16.7% showed susceptible phenotype, while 83.3% were resistant both by phenotypic and genotypic methods. The MIC50 and

Table 1
Summary of results obtained with gastric biopsies tested by HelicoDR kit according to culture and the clarithromycin resistance detected by E-test (phenotype) in *H. pylori* positive (Number of biopsies in each group).

Culture	Genotype (HelicoDR)	Phenotype by E-test		Total
		Resistant	Susceptible	
Positive	Resistant	40	7	47
	Heterogeneous	3	1	4
	Wild type	5	28	33
	Total	48	36	84
Negative	Resistant	–	–	6
	Heterogeneous	–	–	7
	Wild type	–	–	14
	Total			27