Faecal antigen – Molecular techniques

Athanasios Makristathis

During recent years, faecal antigen detection for the diagnosis of the infection with *H. pylori* has been well established world-wide. Analysis can easily be conducted in all age groups; yet there is still an age-dependency with specificity being lower among younger children. The monoclonal enzyme linked immunofluorescence assay (monoclonal EIA, Amplified IDEIA HpStAR former Femtolab *H. pylori*) approaches the diagnostic accuracy of the UBT. A systematic review by Gisbert and Pajares[1] clearly demonstrated that the diagnostic accuracy of the monoclonal EIA was statistically significantly higher than that of the polyclonal (Premier Platinum HpSA) both in pre-treatment diagnosis and post-treatment control (Table 1). As shown during the first clinical evaluation of the monoclonal EIA in the year 2000 using stool specimens of paediatric patients,[2] with only few exceptions positive samples gave values far above and negative samples values clearly below the cut-off value (Figure 1). In contrast, in many cases values obtained with the polyclonal assay did not considerably differ from that of the cut-off value, which may in part be the explanation for the minor performance of this test in comparison to the monoclonal one.

In spite a satisfactory overall performance of the monoclonal EIA, a recent meta-analysis of 22 publications (2,499 patients) showed to some extent a stronger variation of the test sensitivity post-treatment as compared to that demonstrated before the initiation of therapy (89%-96% vs. 93%-95%, respectively).[3]

Treatment with proton pump inhibitors (PPI) was shown to affect the performance of the polyclonal EIA causing a considerable loss of sensitivity (98% vs. 83%).[4] A similar effect

Division of Clinical Microbiology, Department of Hygiene and Medical Microbiology, Medical University of Vienna

Table 1. Stool antigen EIA for the diagnosis of *Helicobacter pylori* infection: a systematic review.

Pre-treatment	**EIA**		
	monoclonal	**polyclonal**	
Sensitivity (%)	96	90	p<.001
Specificity (%)	97	94	
PPV (%)	96	91	
NPV (%)	97	85	
Pre-treatment			
Sensitivity (%)	95	84	p<.001
Specificity (%)	97	91	
PPV (%)	91	74	
NPV (%)	98	92	

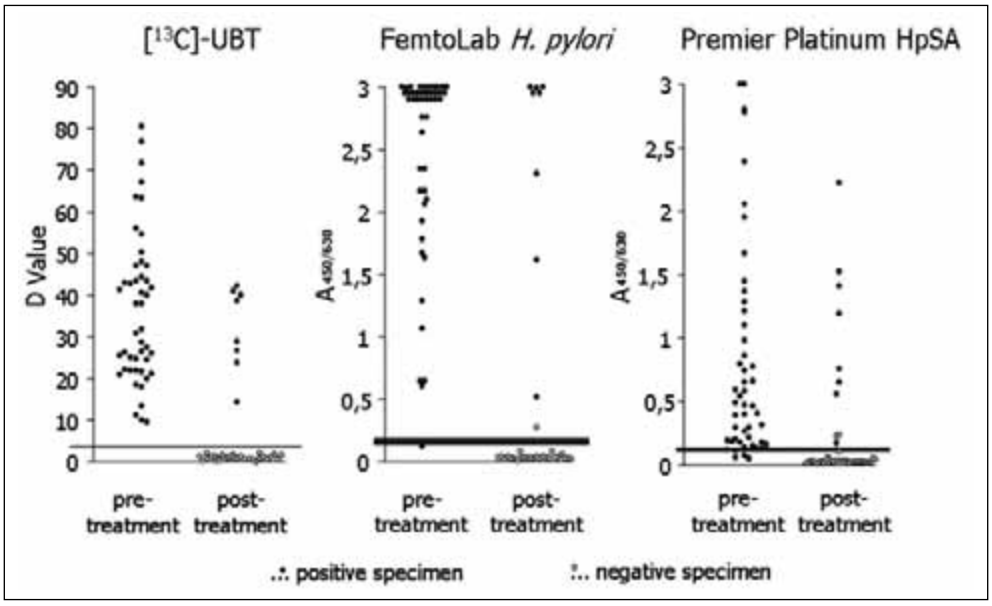

Figure 1. Results of [^{13}C]UBT, FemtoLab *H. pylori* and Premier Platinum HpSA with specimens collected from paediatric patients before and 4 weeks after discontinuation of therapy. Before treatment, a positive *H. pylori* status was assumed if both [^{13}C]UBT and serology were positive. A post-treatment negative *H. pylori* status was assumed if [^{13}C]UBT was negative both at 4 and 12 weeks after the end of treatment. Positive and negative patients´ *H. pylori* status is indicated by black and white dots, respectively.

of the PPI treatment may also be assumed for the performance of the monoclonal test. A recent systematic review and meta-analysis showed that the polyclonal EIA is improper for use in patients with upper gastrointestinal bleeding;[5] with respect to the suitability of the monoclonal test only a few published data exist showing controversial results.[6,7]

Commercially available office based stool antigen tests (Immunocard STAT HpSA and Rapid HpStAR) use monoclonal antibodies. In recent literature, a number of published data exist on the suitability of Immunocard STAT HpSA showing considerable controversy. Thus, the sensitivity varies between 58% and 100% and the specificity between 76% and 97% (Table 2). Published data on Rapid HpStAR are sparse and controversial as well. More data are needed in order to be able to assess the practicability of this test in the clinical routine. However, there is evidence to suggest that the diagnostic accuracy of the laboratory tests is superior to that of rapid assays.

During recent years, several attempts have been made to detect *H. pylori* DNA in stool samples by conventional PCR. However, most of these reports have revealed low sensitivity. Nested PCR generally increases sensitivity, but has a high risk of contamination. Real-time PCR has several advantages over conventional PCR, such as short working time, high sensitivity and specificity, and low risk of contamination. FRET (Fluorescence Resonance Energy Transfer) technology allows for the specific detection of *H. pylori* 23S

Table 2. Immunocard STAT!HpSA.

Reference	Number of patients	Sensitivity (%)	Specificity (%)
Trevisani 2007	122	**58**	**76**
Lu 2006	120	97	**83**
Queseda 2006	79	91	97
Kaklikkaya 2006	65	**78**	**79**
Hooton 2006	102	**79**	96
Wu 2006	254	95	**83**
Hauser 2006	43*	100	**76**
Veijola 2006	185	93	**89**
Kalach 2005	128*	**86**	93
Trevisani 2005	105	**85**	93
Kato 2004	182*	91	94
Cheng 2004	80	100	93
Chrisholm 2004	87	**88**	**89**
Li 2004	53	93	**89**
Wu 2003	253	96	91
Calvet 2003	63	**89**-91	**86**-93

rDNA and point mutations responsible for resistance to clarithromycin (A2142G, A2143G and A2142C). On the basis of this technology, Schabereiter-Gurtner et al developed a real-time PCR hybridization assay enabling for both specific detection of *H. pylori* infection and clarithromycin susceptibility testing using a biprobe.8 Biprobes are sequence-specific probes labeled with the fluorophore Cy5. The probe includes the sites of the mutations on the *H. pylori* 23S rRNA gene responsible for resistance to clarithromycin and has 100% homology to the sensitive wild-type genotype. When the probe hybridizes to the target sequence (complementary strand of the amplicon), Cy5 is excited by the energy transfer due to the inclusion of SybrGreen I in the double-stranded DNA resulting in an increase of emitted light. During thermal analysis, light emission decreases dramatically at the melting temperature of the biprobe-amplicon duplex. In the presence of a mutation, thermal analysis reveals a lower melting temperature as compared to the wild-type sequence. A melting temperature of 63˚C corresponds to the clarithromycin sensitive wild-type genotype; resistant genotypes show melting temperatures of 54˚C (both mutations A2142G and A2143G) and 58˚C (mutation A2142C). In *H. pylori* negative samples, the presence of a melting peak at 47˚C (internal control) was indicative of PCR conditions allowing amplification (Figure 2).

With respect to detection of *H. pylori* infection this test showed an excellent diagnostic accuracy not only in gastric biopsies but also in stool samples.[8] In detecting resistance

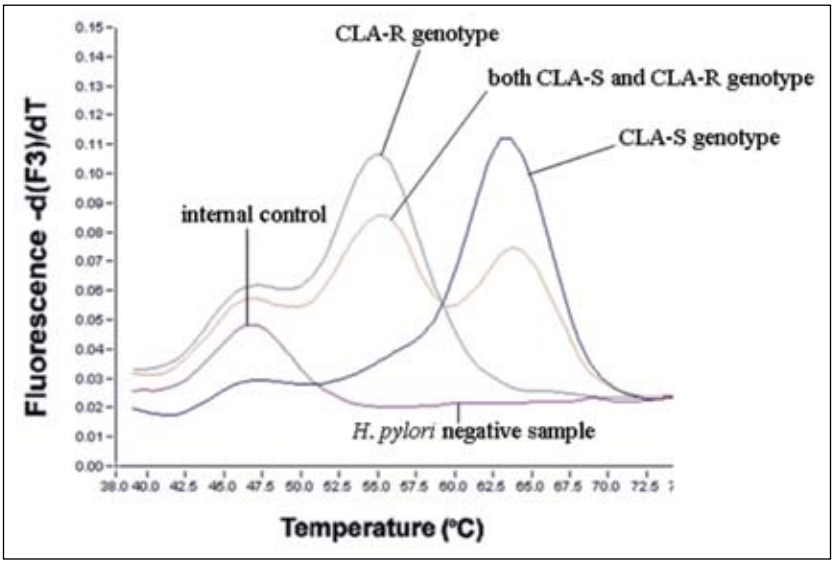

Figure 2. Examination of stool DNA extracts by *H. pylori* ClariRes assay; melting curves of representive samples.

Table 3. Real-time PCR for detection of *H. pylori* infection (*ureA* and 23S rRNA gene assays) and clarithromycin susceptibility testing (23S rRNA gene assay); specificity, sensitivity, positive predictive values (PPV) and negative predictive values (NPV) for biopsy and stool samples.

	Detection of *H. pylori* infection		**Clarithromycin susceptibility testing**	
	Biopsy	**Stool**	**Biopsy**	**Stool**
Specificity (%)	98	98	100	100
Sensitivity (%)	100	98	82	73
PPV (%)	98	98	100	100
NPV (%)	100	98	94	92

to clarithromycin the specificity of PCR was 100%; however, the sensitivity was 82% in biopsies and 73% in stool specimens (Table 3). Thus, PCR tends to underestimate the true rate of resistance to clarithromycin. In the case of a concurrent infection with both a sensitive and a resistant strain, PCR may fail to detect the resistant one, if a 10:1 ratio of the wild-type to the mutant bacterial population is exceeded.[8] The higher affinity of the biprobe to the wild-type amplicon and the preponderance of the latter as compared to the mutant amplicon is likely to be the reason for this phenomenon.

Recently, on the basis of the test by Schabereiter-Gurtner et al, *H. pylori* ClariRes assay has been developed, which is a commercially available CE certified test for in-vitro diagnostic on a LightCycler instrument. Since the introduction of this test in the market, it has been validated in a number of studies. In a routine setting using gastric biopsies and stool specimens of 108 consecutive dyspeptic adult outpatients of a tertiary center, sensitivity of detection of resistance to clarithromycin with almost 95% was considerably higher than that shown with the precursor test (data not published yet). This may, to some extent, be the consequence of the higher rate of patients with one or more previous *H. pylori* eradication attempts among the infected patients in this study in comparison to the previous one.[8] Due to selective pressure during therapy, the great majority of these patients were shown by cultural methods to harbour only the clarithromycin resistant genotype.

Furthermore, more than 200 stool specimens of dyspeptic children referred to a paediatric gastroenterology outpatient clinic had been examined by both ClariRes assay and the monoclonal stool EIA; a high degree of concordance (96%) was found between the two tests. As a matter of fact, in case of discordance using UBT and serology as reference, real-time PCR was shown to be at least as sensitive as and definitely more specific than the stool EIA, which performed as reported in literature. Therefore, stool PCR was implemented as a screening method and the diagnostic reliability of this test in the clinical routine was analysed retrospectively. Thus, all consecutive children presenting between March 2006 and February 2008 with dyspepsia and/or iron deficiency or undergoing follow-up

examination after eradication treatment for *H. pylori* were examined by stool PCR. All results were counterchecked by at least one other invasive or non-invasive standard method. The data collected within the 24-month evaluation period showed excellent specificity and acceptable sensitivity in terms of detection of *H. pylori* infection and determination of clarithromycin resistance (data not published yet).

In a recent publication,[9] the sensitivity of ClariRes assay was only 63% in stool specimens of symptomatic children whose *H. pylori* infection status had been analysed by histology, culture, UBT, and the monoclonal stool EIA. This may reflect inappropriate pre-analytic and laboratory practice; the good performance of the monoclonal stool antigen test in that study[9] raises the question whether the sample aliquots used for each of the tests were of equal quality and both tests were performed on the same or different occasions.

For molecular tests aimed at the detection of small amounts of microbial DNA, sensitivity strongly depends on the quality of the DNA. Prolonged storage/transportation of feces at room temperature, repeated freezing and thawing, or prolonged storage of the DNA extract prior to PCR may impair the test sensitivity due to enzymatic or mechanical degradation of the DNA. Furthermore, it proved to be of importance to run samples in duplicate in the PCR. Since Lottspeich et al performed only single sample runs with the ClariRes assay, this may be a further explanation for the low test sensitivity shown in their study.[9]

In conclusion, the use of a non-invasive *H. pylori* diagnostic test that allows for simultaneous clarithromycin susceptibility testing would definitely facilitate therapy decisions, considering the limited availability of alternative treatment regimens, especially for children, and the growing rates of resistance to alternative drugs. At present, stool PCR is the only non-invasive means for investigation of clarithromycin susceptibility. The costs of the method are definitely lower than those of conventional methods of obtaining information on antibiotic susceptibility, i.e. endoscopy including culture and susceptibility testing; while costs of stool PCR including DNA extraction and run in duplicate are 130 Euro, expenses for oesophagogastroduodenoscopy with culture and E-test will be 350 Euro, costs for general anaesthesia and hospital admission, which is the usual procedure in children, not included. It remains to be seen if the stool PCR should be applied for screening purposes, as confirmatory test or in terms of assessing eradication success.

REFERENCES

1. Gisbert JP, Pajares JM. Stool antigen test for the diagnosis of *Helicobacter pylori* infection: a systematic review. Helicobacter 2004;9:347-368.
2. Makristathis A, Barousch W, Pasching E, et al. Two enzyme immunoassays and PCR for detection of *Helicobacter pylori* in stool specimens from pediatric patients before and after eradication therapy. J Clin Microbiol 2000;38:3710-3714.
3. Gisbert JP, de la Morena F, Abraira V. Accuracy of monoclonal stool antigen test for the diagnosis of *H. pylori* infection: a systematic review and meta-analysis. Am J Gastroenterol 2006;101:1921-1930.
4. Gatta L, Vakil N, Ricci C, et al. Effect of proton pump inhibitors and antacid therapy on 13C urea breath tests and stool test for *Helicobacter pylori* infection. Am J Gastroenterol 2004;99:823-829.
5. Gisbert JP, Abraira V. Accuracy of *Helicobacter pylori* diagnostic tests in patients with bleeding peptic ulcer: a systematic review and meta-analysis. Am J Gastroenterol 2006;101:848-863.
6. Hanvivatvong O, Thong-Ngam D, Kuakarn S, Mahachai V. Evaluation of *Helicobacter pylori* stool antigen test in Thai patients with upper gastrointestinal bleeding. J Med Assoc Thai 2006;89:S98-103.
7. Gisbert JP, Trapero M, Calvet X, et al. Evaluation of three different tests for the detection of stool antigens to diagnose *Helicobacter pylori* infection in patients with upper gastrointestinal bleeding. Aliment Pharmacol Ther 2004;19:923-929.
8. Schabereiter-Gurtner C, Hirschl AM, Dragosics B, et al. Novel real-time PCR assay for detection of *Helicobacter pylori* infection and simultaneous clarithromycin susceptibility testing of stool and biopsy specimens. J Clin Microbiol 2004;42:4512-4518.
9. Lottspeich C, Schwarzer A, Panthel K, et al. Evaluation of the novel *Helicobacter pylori* ClariRes real-time PCR assay for detection and clarithromycin susceptibility testing of *H. pylori* in stool specimens from symptomatic children. J Clin Microbiol 2007;45:1718-1722.