



Polyacrylamide Gel Electrophoresis of Proteins Method *versus* Enzyme-Linked Immunosorbent Assay and Immunochromatography with Monoclonal Antibodies in Pediatric Infection with *Helicobacter Pylori*

RALUCA SUZANA IFTIMI¹, ANA MARIA ALEXANDRA STANESCU²,
CARMEN ANTON^{3*}, SMARANDA DIACONESCU^{1,3*}, NICOLETA GIMIGA^{1,3},
OANA-MARIA ROSU⁴

¹St. Mary Clinical Emergency Hospital for Children, 62 Vasile Lupu Str., 700309, Iasi, Romania

²Carol Davila University of Medicine and Pharmacy, 8 Eroii Sanitari Str., 050474, Bucharest, Romania

³Gr. T. Popa University of Medicine and Pharmacy, 16 Universitatii Str., 700115, Iasi, Romania

⁴University of Medicine, Pharmacy, Science and Technology, 38 Gh. Marinescu Str., 540072, Targu Mures, Romania

Abstract. *The current methods for the diagnosis of Helicobacter pylori infection in children include invasive (direct) methods, that enable the detection of the bacteria or the bacterial urease in gastric biopsies, or noninvasive (indirect) methods, which consist of researching specific antibodies, antigens and urease in other different samples (serum, saliva, urine, stool, exhaled air). The urease functions in H. pylori infection is to neutralize gastric acid by producing NH₃ through the hydrolysis of urea. Monochloramine, a NH₃-derived compounds has cytotoxic effects on host cells. The Western Blot method refers to the extraction of a sodium dodecyl sulphate from a Helicobacter pylori strain followed by a separation of the solubilised protein using discontinuous polyacrylamide gel electrophoresis according to molecular mass and transfer of the separated proteins to nitrocellulose. The ELISA (enzyme-linked immunosorbent assay) technique refers to the detection of Anti-Helicobacter pylori antibodies type Ig G. We also used an immunochromatographic system with the lateral leakage test strip based on the principle of the sandwich technique with monoclonal antibodies. As a result, more than two types of reactions can be detected on a single assay device by the combination of colloidal gold –labelled antibodies and complementary oligonucleotide-labelled immobilized at different places on a nitrocellulose membrane. Our study aims to assess the performances of the Western Blot method for determining anti-Helicobacter pylori Ig A and Ig G antibodies in correlation with clinical data and other available diagnosis methods. For validation purposes, the results were compared to those obtained using the enzyme-linked immunosorbent assay technique, gastric biopsy and immunochromatography.*

Keywords: *Helicobacter pylori, Western Blot, ELISA, biopsy, children*

1. Introduction

The fundamental research in the pediatric infection with *Helicobacter pylori* gather clinical aspects with endoscopic and immunological aspects.

According to ESPGHAN guidelines (2016), the diagnosis of *Helicobacter pylori* infection in children is made based on the following recommendations:

- The initial diagnosis of *Helicobacter pylori* infection is based on a positive histopathology, a positive rapid urease test or a positive culture;
- Stool antigen testing for *Helicobacter pylori* is a non-invasive test that is not recommended for the initial diagnosis of the infection in children;

*email: carmen_ro2008@yahoo.com, turti23@yahoo.com



- Serum or whole blood anti-*Helicobacter pylori* (Ig G, Ig A) antibody tests are not reliable for use in clinical conditions;

- It is recommended to test *Helicobacter pylori* using stool samples or using a biopsy at least 2 weeks after stopping proton pump inhibitor (PPI) therapy and minimum 4 weeks after stopping the antibiotic treatment;

- Diagnosis tests for the *Helicobacter pylori* infection are not recommended to children with functional abdominal pain. (1)

With the exception of molecular biology techniques that are available only in research laboratories, all these methods can be used for current diagnosis.

The role of serological testing was limited owing to cross-reactivity with antibodies against other bacterial species which may lead to false-positive reactions. Also, high titers that can persist up to 4-6 months (sometimes even up to several years) after eradicating the infection may lead to false-positive reactions. False-negative results may occur in the early stages of the infection (serum IgG are detectable in approximately 20 days after the infection, depending on the patient). (4) Stool antigen detection using rapid qualitative tests is an efficient method that indicates a current infection, the occurrence of a reinfection and monitoring treatment effectiveness, but it has the disadvantage of low reproducibility; the method is acceptable for adult patients. (2, 3)

The profile of serum antibodies compared to different antigens of the *Helicobacter pylori* bacterium can be monitored using the (Immunoblot Test). This is a quantitative, non-invasive method allowing the simultaneous study of antibodies against several antigen fractions. It is a well-known fact that less than 2% of infected patients present with seroconversion, therefore an optimal and sensitive Western Blot system should be able to detect approximately 98% of active infections. (5)

The largest part of enzyme linked immunoabsorbed assays (the ELISA technique) is based on the detection of anti-*Helicobacter pylori* antibodies type IgG. Such preparations include antigens that may have cross-reactions with other bacterial species, resulting in a specificity that rarely exceeds 90%. Even 4th generation ELISA systems that contain highly purified substances still entail a risk for cross-reactions. (4)

The Western Blot technique allows for direct visualization of antibodies binding to specific antigens. Compared to the ELISA technique, this method is more cost and labor-intensive, but it has the advantage of accurately characterizing the humoral immune response to different types of bacterial virulence antigens. This method is also used to confirm positive and equivocal results obtained using another technique. (5)

Western Blot helps detect both recurrent and ongoing *Helicobacter pylori* infections. Results are correlated with those of invasive tests. The kit contains the ready-to-use test strips, the control matrix (Figure 1), enzyme conjugate, universal buffer, and sublayer solution. Kits provide a qualitative “in vitro” test to determine IgA and IgG class antibodies, and a quantitative test for the bacterial virulence antigens included on the strip. They contain ready-to-use strips with electrophoretically separated *Helicobacter pylori* antigens. The proteins in polyacrylamide gels interact with antibodies, and the membranes used for blotting are made of nitrocellulose.

There are 2 phases identified in this technique: during the first phase proteins are separated and blocked, and during the second phase the immobilized protein can interact with the antibody. The binding of specific antibodies occurs after incubation with enzyme conjugate, which contains alkaline phosphatase. The complex is identified following an enzymatic reaction that will generate colored reaction product, the intensity of which shall be measured. After carrying out the work procedure, after the blot strips are dried, they were scanned using a densitometric scanner and analyzed using a specialized program for the analysis of dimensional separations – EUROLin Scan. (Figures 2, 3)

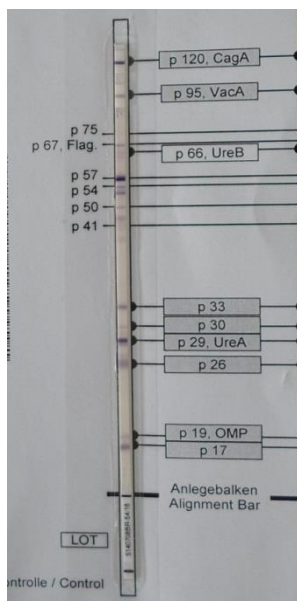


Figure 1. Anti-*Helicobacter pylori* Euroline Western Blot control matrix

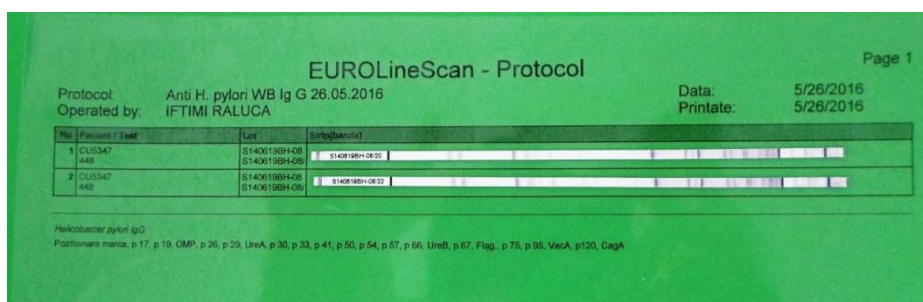


Figure 2. *Helicobacter pylori* IgG Western Blot workflow model

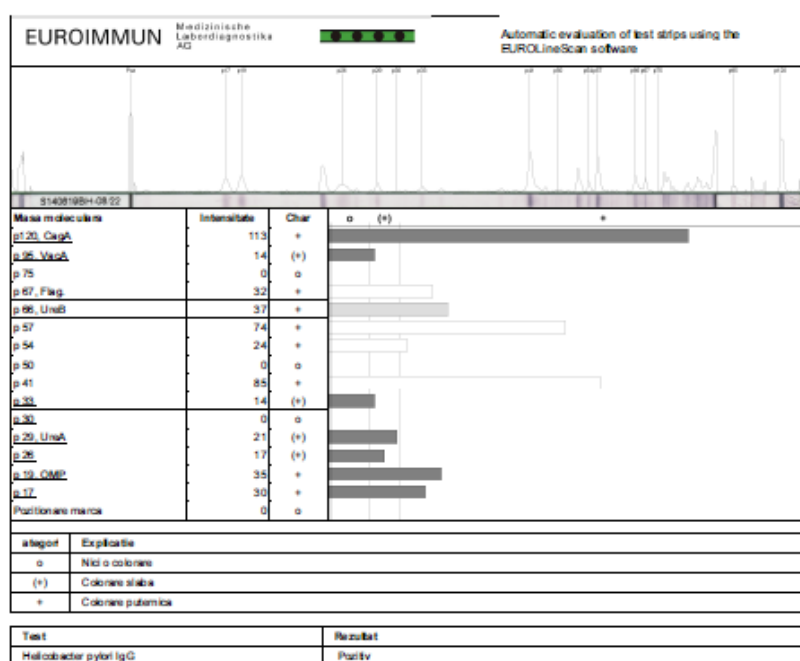


Figure 3. EUROLineScan strip analysis model

The specificity of antigens included on the strip is presented in Table 1.

**Table 1.** Specificity of antigens included on the strip:

Antigen	Specificity
Cag A- p120	Cytotoxin-associated gene A – High specificity
Vac A- p95	Vacuolating cytotoxin gene A – High specificity
p75	Nonspecific
FSH, p67	Flagellar sheath protein – Nonspecific due to cross-reactivity with another bacterium
UreB- p66	Urease heavy subunit – Cross-reactivity with another bacterium containing urease
HSP, p57	Nonspecific
Flagellin	Nonspecific due to cross-reactivity with another bacterium having flagella
p50	Nonspecific
p41	Nonspecific
p33	Probably specific
OMP- p30	Outer membrane protein – Species specificity
UreA- p29	Urease light subunit – High specificity
p26	High specificity
OMP- p19	Species specificity
p17	Probably specific

Helicobacter pylori antigens were divided into three categories:

Class 1: nonspecific antigens that have cross-reactivity, with a molecular weight of 41 kDa (p41), 50 kDa (p50), 54 kDa (flagellin), 57 kDa (HSP), 67 kDa (FSH) and 75 kDa (p75).

Class 2: antigens with a molecular weight of 66 kDa (urease B)

Class 3: antigens with species specificity and high specificity with a molecular weight of 17 kDa (p17), 19 kDa (OMP), 26 kDa (p26), 29 kDa (urease A), 30 kDa (OMP), 33 kDa (p33), 95 kDa (Vac A) and 120 kDa (Cag A).

2. Materials and methods

The two-year study included 73 patients, with ages between 2 and 17 years old, admitted to the Gastroenterology Department at the “Sf. Maria” Clinical Emergency Children’s Hospital in Iași, who underwent upper gastrointestinal endoscopy for the accepted indications. The primary selection criterion was gastric biopsy positivity, with a microscopic smear test and rapid urease test for *Helicobacter pylori*. Another inclusion criterion was the absence of a recent treatment with antibiotics or proton pump inhibitors. Exclusion criteria: patients with negative gastric biopsy for *Helicobacter pylori*, patients whose biopsy samples were improperly collected, and lack of informed consent.

Up to four fragments of gastric biopsy and venous blood samples were collected from all the patients. Serums were collected after clotting of the blood, separated after centrifugation, and stored at -20°C.

The serums were then examined based on the Western Blot technique using the *Helicobacter pylori* IgA Western Blot and *Helicobacter pylori* IgG Western Blot kits in accordance with the manufacturer’s protocol, i.e. Euroimmun Labordiagnostika ® (Germany).

The Western Blot test results were divided into 3 categories: **negative**, **uncertain** and **positive**, as presented in Table 2.

Table 2. Interpretation of results for *Helicobacter pylori* IgA Western Blot and *Helicobacter pylori* IgG Western Blot

Result	Characteristics
Negative	No antigen bands recognisable or some weak antigen bands of categories 1 and 2 or one single weak antigen band from category 3
Borderline	A minimum of two weak antigen bands from categories 2 or 3 or one distinctive antigen band from category 3 – <i>Helicobacter pylori</i> IgA Western Blot
	A distinctive antigen band from category 3 or a minimum of two weak antigen bands from category 3 – <i>Helicobacter pylori</i> IgG Western Blot
Positive	A minimum of two distinctive antigen bands from categories 2 or 3 – <i>Helicobacter pylori</i> IgA Western Blot
	A minimum of two distinctive antigen bands from category 3 – <i>Helicobacter pylori</i> IgG Western Blot



The results obtained using the Western Blot technique were compared to those obtained using the ELISA technique for determining anti-*Helicobacter pylori* IgG antibodies (kit by Euroimmun Labordiagnostika®, Germany), as well as to those obtained via invasive methods (gastric biopsy). The study was approved by the Ethic Commission of our hospital (no. 5033/24.02.2016)

3. Results and discussions

The age distribution of children in the study sample was as follows: 4.11% cases aged 2 to 5 years old, 15.07% cases aged 6 to 10 years old, and 80.82% cases aged 11 to 17 years old (Table 3).

Table 3. Distribution of patients infected with *Helicobacter pylori* by age groups

Age	No. of cases	%	Age	No. of cases	%
2 years	1	1.37	11 years	5	6.85
3 years	1	1.37	12 years	4	5.48
5 years	1	1.37	13 years	6	8.22
6 years	2	2.74	14 years	7	9.59
7 years	3	4.11	15 years	10	13.70
8 years	1	1.37	16 years	11	15.07
9 years	2	2.74	17 years	16	21.92
10 years	3	4.11			
Total	14	19.18%	Total	59	80.82%

The distribution of patients infected with *Helicobacter pylori* according to gender indicated a 34.25% frequency in male patients and a 65.75% frequency in female patients (Table 4).

Table 4. Distribution of patients infected with *Helicobacter pylori*, by gender

Patient's sex	No. of cases	%
Female	48	65.75
Male	25	34.25
TOTAL	73	100.00

Patients with rapid urease test and microscopic test directly positive for *Helicobacter pylori* in fragments of gastric biopsy had endoscopic modifications for gastritis at a rate of 91.78% (58.97% nodular gastritis, 19.58% erythematous gastritis and 13.23% hemorrhagic gastritis), 68.49% for duodenitis, 41.10% for esophagitis, and 26.03% for duodenal-gastric reflux. We registered 3 cases of duodenal ulcer and 2 cases of gastric ulcer. (Table 5)

Table 5. Endoscopic modifications in the sample patients:

ENDOSCOPIC FINDINGS	No. of cases	%
ESOPHAGITIS	30	41.10
GASTRITIS	67	91.78
GASTRIC POLYP	1	1.37
HIATAL HERNIA	1	1.37
DUODENAL ULCER	3	4.11
GASTRIC ULCER	2	2.74
DUODENAL-GASTRIC REFLUX	19	26.03
DUODENITIS	50	68.49

Based on the analysis of results obtained using the Western Blot technique on the serums from patients with *Helicobacter pylori* infection diagnosed via invasive methods, depending on the intensity of identifiable antigen strips and classification thereof in the three classes (positive, uncertain and



negative) for determining the presence or absence of IgA and IgG class antibodies we recorded the following: (Table 6)

- 36.99% positive results for anti-Helicobacter pylori IgA antibodies and 58.90% positive anti-Helicobacter pylori IgG;
- 54.79% negative anti-Helicobacter pylori IgA Western Blot and 28.77% negative anti-Helicobacter pylori IgG Western Blot

Table 6. Western Blot test performances in patients included in the study

Biopsy patients			
	Anti-Helicobacter pylori IgA Western Blot	No. of cases	%
1	POSITIVE	27	36.99
2	NEGATIVE	40	54.79
3	UNCLEAR	6	8.22
	TOTAL	73	
	Anti-Helicobacter pylori IgG Western Blot	No. of cases	%
1	POSITIVE	43	58.90
2	NEGATIVE	21	28.77
3	UNCLEAR	9	12.33
	TOTAL	73	

Analyzing the correlations between Ig A and Ig G Western Blot results and the endoscopic aspects we recorded a ratio of 39.13% positive IgA results and 62.32% positive IgG results in patients with gastritis, 40.38% positive IgA results and 67.31% positive IgG results in patients with duodenitis, 48.39% positive IgA results and 61.29% positive IgG results in patients with esophagitis. (Table 7) In patients with gastric ulcer and duodenal ulcer we recorded 100% positive IgA and IgG results. We noted that the positivity rate of Helicobacter pylori IgG Western Blot correlated to a larger extent with the endoscopic aspects compared to Helicobacter pylori IgA Western Blot.

Table 7. Correlations between IgA and IgG Western Blot and endoscopic aspects:

	Anti-Helicobacter pylori IgA Western Blot	Esophagitis		Gastritis		Duodenitis	
		No. of cases	%	No. of cases	%	No. of cases	%
1	POSITIVE	15	48.39	21	39,13	20	40.38
2	NEGATIVE	13	45.16	37	55,07	27	53.85
3	UNCLEAR	2	6.45	4	5,8	3	5.77
	TOTAL	30		67		50	
	Anti-Helicobacter pylori IgG Western Blot	No. of cases	%	No. of cases	%	No. of cases	%
1	POSITIVE	18	61.29	42	62,32	34	67.31
2	NEGATIVE	8	25.81	18	27,54	10	21.15
3	UNCLEAR	4	12.9	7	10,14	6	11.54
	TOTAL	30		67		50	

We analysed the results anti-Helicobacter pylori IgG antibodies identified using the ELISA technique. Of the 73 patients included in the study, with positive biopsy, 66 (90.4%) had positive titer



for anti-*Helicobacter pylori* IgG antibodies, 6 (8.2%) had negative results for anti-*Helicobacter pylori* IgG antibodies, and there was 1 equivocal result (1.36%). The gastric biopsy results correlate at a rate of 90.4% with the titer for anti-*Helicobacter pylori* IgG antibodies obtained using the ELISA technique. The confirmation of these results and elimination of false-positive results is carried out using the Western Blot technique (elimination of cross-reactivity).

The results obtained using the Western Blot technique and those obtained using the ELISA technique were compared in Table 8.

Table 8. Correspondence between the results obtained using the Western Blot technique and those obtained using the ELISA technique

Anti- <i>Helicobacter pylori</i> IgA Western Blot						Anti- <i>Helicobacter pylori</i> IgG antibody ELISA RU/ ml					
Positive result		Negative result		Unclear result		Negative result: <16		Equivocal result: 16-22		Positive result: >22	
No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
27	36.99					2	7.4	0	0	25	92.5
		40	54.79			3	7.5	1	2.5	36	90
				6	8.22	1	16.6	0	0	5	83.3
Anti- <i>Helicobacter pylori</i> IgG Western Blot											
43	58.90					2	4.65	1	2.32	40	93.02
		21	28.77			3	14.2	0	0	18	85.7
				9	12.33	1	11.1	0	0	8	88.8

Twenty-seven patients (36.99%) positive for anti-*Helicobacter pylori* IgA Western Blot had 7.4% negative and 92.5% positive result for anti-*Helicobacter pylori* IgG antibodies via the ELISA technique. Forty-three patients (58.90%) positive for anti-*Helicobacter pylori* IgG Western Blot had 4.65% negative and 93.02% positive result for anti-*Helicobacter pylori* IgG antibodies via the ELISA technique. The forty patients with negative anti-*Helicobacter pylori* IgA Western Blot had 90% positive and 7.5% negative result for anti-*Helicobacter pylori* IgG antibodies via the ELISA technique.

The 21 patients with negative anti-*Helicobacter pylori* IgG Western Blot had 85.7% positive and 14.2% negative result for anti-*Helicobacter pylori* IgG antibodies via the ELISA technique.

For a part of the study group we used immunochromatography to determine the presence of *Helicobacter pylori* stool antigen. Of the 20 patients that were tested, only one returned a positive result, which was unconfirmed via the Western Blot technique (Table 9).

Table 9. Correspondence between the results obtained using the Western Blot technique and those obtained via immunochromatography (stool antigen)

	<i>Helicobacter pylori</i> IgA Western Blot	<i>Helicobacter pylori</i> IgG Western Blot	<i>Helicobacter pylori</i> Antigen
1	Negative	Negative	Negative
2	Negative	Positive	Negative
3	Negative	Positive	Negative
4	Negative	Positive	Negative
5	Negative	Negative	Negative
6	Positive	Positive	Negative
7	Negative	Unclear	Negative
8	Negative	Positive	Negative
9	Negative	Negative	Negative
10	Negative	Positive	Negative
11	Positive	Positive	Negative
12	Positive	Positive	Negative
13	Negative	Positive	Negative
14	Negative	Positive	Negative
15	Negative	Positive	Negative
16	Negative	Negative	Negative
17	Negative	Unclear	Positive
18	Negative	Negative	Negative
19	Negative	Positive	Negative
20	Negative	Negative	Negative



In the study group we noticed a statistically significant increase of *Helicobacter pylori* infection cases with age, ($p=0.00042$, $\chi^2=15.92$). Comparing the 2 to 10 years old and 11 to 17 years old age groups, we obtained the value $p=0.0000533 (<0.01)$, respectively $p_{Yates}=0.0000639$ which reflects a significant difference ($RR=0.14$ in the group 2-10 years compared to $RR=0.72$ in the group 11-17 years), children aged >11 years old being more affected.

In our study we noticed that older patients had a higher number of immunoreactive strips than younger ones. Hunt et al. report that the prevalence of *Helicobacter pylori* infection in Ethiopian children aged 2 to 4 years old was 48%, while in adults it exceeded 95%; in Nigeria, the prevalence of the infection in children aged 5 to 9 years old was 82%, while in adults it ranged between 70 to 90% and similar data was reported in Mexico. Percentages are lower in developed countries. In Canada, children aged 5 to 18 years old had a 7.1% recorded prevalence of infection, while the percentage recorded for adults was of 23.1%. In Europe, the highest prevalence of infection was found in children in Bulgaria (61.7%), while the lowest number of infected children were from the Netherlands (1.2%). (6)

We also note a similar distribution of positive cases according to gender ($p=0.427$, $\chi^2=0.22$, $p_{Yates}=0.483$). We can state that there is no statistically significant correlation between the presence of infection and patients' sex. This data is consistent with those reported by other authors. A study conducted on 487 patients showed that the incidence of *H.pylori* infection was higher in women than in men (38.7% compared to 34.1%); however, the difference was not statistically significant ($p=0.301$). (7)

Using the Mann–Whitney U statistical test, we note that the degree of positivity for *Helicobacter pylori* IgG Western Blot is correlated in a higher percentage with the positive biopsy compared to *Helicobacter pylori* IgA Western Blot. The Z-Score is 5.71233 and the p-value is 0.0027 for Ig A, and the the Z-Score is 2.99804, the p-value is < 0.00001 for IgG.

Gastric biopsy results correlate at a rate of 90.4% with the titer for anti-*Helicobacter pylori* IgG antibodies obtained using the ELISA technique, but only approximately 58.9% of the total patients included in the study are confirmed using the anti-*Helicobacter pylori* Western Blot IgG method. The titer for anti-*Helicobacter pylori* antibodies identified using the ELISA technique may remain high for a long period of time, up to 6 months, even after the bacterial eradication. The ELISA technique can now be deemed a screening test in adults. Sensitivity and specificity differences were reported for the ELISA technique, using the same commercial kit in different adult populations. The mean antibody titers in infected pediatric patients are much lower compared to mean titers in infected adults. (10)

ESPGHAN does not recommend using serological testing in children neither for initial diagnosis of infection, not for the monitoring of post-therapeutic results. (1)

The Chi-Square independence test statistics showed no statistically significant association between IgA Western Blot and ELISA results, $\chi^2 (1, N=73)=4.2$, $p=0.37$, nor between the IgG Western Blot and ELISA results, $\chi^2 (1, N=73)=2.42$, $p=0.65$. The ELISA test is not a specific diagnosis method for *Helicobacter pylori* infection in children. The humoral immune response to different types of *Helicobacter pylori* antigens can only be accurately assessed using the Western Blot technique.

A meta-analysis for assessing the accuracy of antibody-based detection tests for diagnosing *Helicobacter pylori* infection in children included 68 studies, having the following selection criteria: minimum 30 children in the study sample and the use of a gold standard for diagnosing *H. pylori* infection. The conclusions of this meta-analysis highlighted the fact that Western Blot tests have high performances, with an 89% specificity and 91% sensitivity, while ELISA IgG tests had a low sensitivity of 79.2% and a high specificity of 92.4%. The clinical implications are significant, as a negative ELISA serological test cannot rule out the infection with *Helicobacter pylori*. (11)

A sample of 73 patients with positive biopsy was selected for the study; 20 patients were tested for *Helicobacter pylori* stool antigen, obtaining 95% negative results via immunochromatography.



Therefore, our results are consistent with international recommendations, which state that the initial diagnosis of infection should not be based on non-invasive methods, namely stool antigen. The test may be recommended for children when endoscopic examination is not possible.(1) There are studies that proved stool antigen could be a useful method for determining pediatric H. pylori infection in developing countries; further research is necessary, including larger patient samples.(12)

Applying Spearman's statistical test for correlating the results obtained via the Helicobacter pylori IgA Western Blot and those obtained via immunochromatography, we obtain the values $r_s = -0.096$, p (2-tailed) = 0.68, which proves the absence of a statistically significant association.

Applying Spearman's statistical test for correlating the results obtained via the Helicobacter pylori IgG Western Blot and those obtained via immunochromatography, we obtain the values $r_s = -0.280$, p (2-tailed) = 0.23, which proves the absence of a statistically significant association.

We can note that there is no statistically significant correlation between the immunochromatography-based determination of stool antigen and the Western Blot technique, proving that the immunochromatography cannot be used as a specific diagnosis method. Authors report that stool antigen detection tests have a lower accuracy when stool samples are loose or watery, because the specific antigens of Helicobacter pylori in the stool samples are diluted. The temperature and time elapsed between stool sample collection and measurements also impact the results. (13)

Another recent study showed that currently available immunochromatography-based tests return less reliable results than those based on anti-Helicobacter pylori antibody detection, the latter also including the Western Blot technique. (14)

4. Conclusions

Although non-invasive diagnosis methods are more easily accepted in the pediatric practice by children and parents alike, in the case of H. pylori infections invasive tests have high sensitivity and specificity levels, being recommended by current guidelines. This study supports the utility of the Western Blot method as the preferred method instead of current non-invasive tests; this method has the advantage of avoiding cross-reactivity and enables the detection of low antibody concentrations. In our country, due to limited resources in pediatric endoscopy, the Western Blot technique should be preferred in selected pediatric patients.

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Manuscript received: 4.12.2019