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Graphical abstract

Methods of diagnosis of *Helicobacter pylori* in children: Current status and prospects

Abstract

Infection by *H. pylori* is everywhere at worldwide, this is acquired mainly in childhood being clinically important because it can cause a variety of diseases (gastritis, gastric or duodenal ulcer, mucosa-associated lymphoid tissue lymphoma, and gastric cancer throughout the patient's lifetime, subnormal growth, malnutrition, and iron deficiency anemia, especially in children). It is widely recognized that the results obtained from studies carried out in adults cannot be generalized to studies performed to children. The objective of this review is to show the state of the art in the diagnosis of infection by *H. pylori* in children, trends and perspectives in the light of new developments in the analytical sciences. In order to carry out a correct description about the diagnosis, different

Keywords

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Diagnosis-in-children

aspects related with *H. pylori* are described (morphology, metabolisms, growth requirements, among other).

Métodos de diagnóstico de *Helicobacter pylori* en niños: Estado actual y perspectivas

Resumen

La infección por *H. pylori* está en todas partes a lo largo del mundo, esta se adquiere principalmente en la infancia siendo clínicamente importante porque puede causar una variedad de enfermedades (gastritis, úlcera gástrica o duodenal, linfoma del tejido linfoide asociado a mucosa y cáncer gástrico a lo largo de toda la vida del paciente, crecimiento subnormal, desnutrición y anemia por deficiencia de hierro, especialmente en niños). Es ampliamente reconocido que los resultados obtenidos de los estudios realizados en adultos no pueden generalizarse a los estudios realizados con niños. El objetivo de esta revisión es mostrar el estado del arte en el diagnóstico de la infección por *H. pylori* en niños, así como las tendencias y perspectivas a la luz de los nuevos desarrollos en las ciencias analíticas. Además, con el fin de realizar una descripción correcta del diagnóstico, se describen diferentes aspectos relacionados con *H. pylori* (morfología, metabolismo, requerimientos de crecimiento, entre otros).

Palabras claves

Helicobacter pylori
Métodos invasivos
Métodos no-invasivos
Diagnóstico en niños

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Abstract

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1. Introduction

Helicobacter pylori (*H. pylori*) is a bacterium which has been formally recognized to be a bacterial carcinogen and, in addition, it is one of the most successful human pathogens, being estimated that around half of the world's population is colonized with this microorganism [1].

The infection by *H. pylori* is acquired mainly in childhood, especially in developing countries; however, its clinical significance in children remains largely unknown, but also, the rate of acquisition at different ages has not been ascertained using consistent tests on gastric biopsies [1, 2]. *H. pylori* infection is clinically important because it can cause a variety of diseases. At the gastrointestinal level, these can be gastritis, gastric or duodenal ulcer, mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric cancer throughout the patient's lifetime; and at the extra-intestinal level, it is possible the identification of subnormal growth, malnutrition, and iron deficiency anemia (IDA), especially in children [2-3]. It is important to indicate that most of the infected individuals are initially asymptomatic [3]. For all reasons exposed above, the diagnosis and the treatment of *H. pylori* infection in children are of great importance because there is evidence indicating that in children the infection is different to that in adults [3-7]. Between the identified differences are: epidemiology, pathogenesis and host response, clinical features, related diseases, diagnosis, as well as treatment strategies [3]. In consequence, the results obtained from studies carried out in adults cannot be generalized to studies performed to children.

The objective of this review is to show the state of the art in the diagnosis of infection by *H. pylori* in

children, trends and perspectives in the light of new developments in the analytical and computational sciences. In order to carry out a correct description about the diagnosis, different aspects related with *H. pylori* are described (morphology, metabolisms, growth requirements, among other).

2. *H. pylori*: Historical background, microbiology and epidemiology

2.1. Historical background

Kusters *et al*, (2006) published an interesting review showing a historical development about *H. pylori* [1]. Thus, for the reader interested in a complete historical description we suggest to start by previous reference. In brief, since 19th and 20th centuries were reported the presence of spiral microorganisms in the stomachs of animals and humans [1, 8-10]. In addition, it is known that *H. pylori* was cultured in 1982 at Royal Perth Hospital in Western Australia [8]. A first publication in Germany was performed by Krienitz in 1906, *i.e.*, 111 years ago to 2017 ("Ueber das auftreten von mageninhalten bei carcinoma ventriculi" or "on the occurrence of gastric contents in carcinoma ventriculi"), a previous work appears to be 1899 by P.K. Pel also in Germany [1]. On the other hand, the link between *H. pylori* and peptic ulcer disease and gastric cancer was not relevant in the beginning because of the high prevalence of *Helicobacter* species in the stomachs of people without any clinical signs. Despite this, it has been reported that some patients were sometimes even treated with high doses of the bismuth-based antimicrobial compound [8]. In 1984, Dr. Barry Marshall and Dr. Robin Warren reported the association of *H. pylori* with peptic ulcers in The Lancet and they obtained the Nobel

Figure 1. Photo of *H. pylori* cultured in vitro (A) and scattering electron micrograph of *H. pylori* biofilm on reactive polymer surfaces (Courtesy of the Research Group on Science with Technological Applications, GI-CAT, Universidad del Valle, Cali-Colombia).

Figure 2. Statistical information of publications related with *H. pylori* [17]

Prize in Medicine in 2005 [11]. In the 90s, with the advance on genomics and proteomics techniques, it was possible to differentiate between *H. pylori* strains from different geographic locations, thus, it was discovered that people in South America were infected with “Spanish” strains of *H. pylori*, which by sequence analysis of the *vacA* toxin gene were designated as “Europe 1” strains. However, new genomic studies of hundreds of isolates evidence that all human strains of *H. pylori* had a common ancestor more than 60,000 years ago. In conclusion, *H. pylori* colonized to the humans, according to Ming *et al.*, (2013), since they walked out of Africa [8]. A short illustration of historical evolution of *H.*

pylori is shown in the Figure 1.

The number of publications on *Helicobacter* has rapidly increased. Kuster *et al.*, (2006) indicated the number of publications change from less than 200 in 1990 to approximately 1500 per year in 2006 [1]. However, it is clear that this information has changed and is limited to consulted database (PubMed, www.pubmed.gov).

In order to evidence a more current picture, and a broader view of related information, specifically with *H. pylori*, statistical information of publications related to this topic is shown in the Figure 2 (www.springerlink.com [17]) and Figure 3 (www.sciencedirect.com [18]).

Figure 3. Statistical information of publications related with *H. pylori* [18]

2.2 Microbiology and pathogenesis

H. pylori is a Gram-negative bacterium, with spiral-shape, with 2-4 microns long and 1 micron wide, and with high mobility since it has 2-6 flagella of about 3 microns in length (these confer motility and allow a rapid movement in viscous environments such as the mucus layer overlying the gastric epithelial cells). An electronic micrograph of *H. pylori* grown *in vitro* is shown in the Figure 1. In addition, *H. pylori* can be classified to belong to *Helicobacter* genus, *Helicobacteraceae* family, *Campylobacterales* order and *Proteobacteria* subdivision. This bacterium can change into a coccoid form, especially after antibiotic treatment or prolonged *in vitro* subculturing. Whether the coccoid forms are viable and whether microorganisms have the capacity to return into the spiral shape is still being discussed. The existence of bacteriophages capable to infect the *H. pylori* has been reported, but their detailed characterization is lacking [14]. Recently, it has been published the possible biological interaction with other microorganisms, in particular, *C. albicans*; however, the consequences of such interaction and their nature are still research subjects, being the internalization interaction a current research topic [15-18]. The genus *Helicobacter* is constituted of over 20 recognized species, with many species awaiting

formal recognition [19]. Bacteria of the genus *Helicobacter* are all microaerophilic organisms and in most cases are catalase and oxidase positive, and many but not all species are also urease positive [1, 17]. In addition, *Helicobacter* species, that are either associated with human disease or have relevance for animal models of human *Helicobacter* infections, can be classified to be: (a) Gastric *Helicobacter* species (*e.g.*, *H. felis*, *H. mustelae*, *H. acinonychis* and *H. heilmannii*) and (b) Enterohepatic *Helicobacter* species (*e.g.*, *H. hepaticus*, *H. bilis*, *H. muridarum*, and *H. trogonum*) [1, 17].

H. pylori is genetically heterogeneous, being the genetic heterogeneity possibly an adaptation of *H. pylori* to the gastric conditions of its host, as well as to the distinct patterns of the host-mediated immune response to *H. pylori* infection [1, 14, 17]. For example, the size of the two sequenced *H. pylori* genomes is approximately 1.7 Megabase Pair (Mbp), with guanine and cytosine content (G+C content) of 35 to 40 %. Strains of *H. pylori* can have different amounts of genes (*e.g.*, for the strain 26695 of *H. pylori* its genome includes 1587 genes, whereas the genome of strain J99 includes only 1,491 genes), in addition, both genomes contain two copies of the 16S rRNA (it is the component of the 30S small subunit of a prokaryotic ribosome), 23S rRNA (*i.e.*, 2904 nucleotides in length being a component of the large subunit (50S) of the

bacterial ribosome), and 5S rRNA genes (*i.e.*, a rRNA molecule of approximately 120 nucleotides in length and a mass of 40 kDa). Many strains carry one or more cryptic plasmids, which do not seem to carry antibiotic resistance genes or virulence genes [14].

A main attribute of *H. pylori* is its microaerophilicity, with optimal growth at O₂ levels of 2 to 5 % and the additional need of 5 to 10 % CO₂ and high humidity. Many laboratories make use of standard microaerobic conditions of N₂ (85 %), CO₂ (10 %), and O₂ (5 %) for *H. pylori* culture. The growth occurs at 34 to 40 °C, with an optimum of 37 °C. Although its natural habitat is the acidic gastric mucosa, *H. pylori* is considered to be a neutrophile. The bacterium will survive brief exposure to pHs of 4, but its growth occurs only at the relatively narrow pH range of 5.5 to 8.0, with optimal growth at neutral pH [1].

H. Pylori has urease, catalase, and oxidase activity, these characteristics are vital to its survival in the harsh acidic gastric environment. The urease helps convert urea to ammonia, which aids in neutralizing gastric acid and promoting bacterial protein synthesis. *H. pylori* infection triggers an intense release of phagocytic cells that attempt to kill the organism by releasing oxygen metabolites. However, catalase activity enables *H. pylori* to survive this oxidative stress. Then, the inflamma-

tory response damages the gastric epithelial lining and allows *H. pylori* to flourish. *H. Pylori* infection predominantly affecting the gastric corpus is associated with hypochlorhydria because of damage to parietal cells, whereas infection localized to the gastric antrum is associated with duodenal ulceration given an increase in gastrin production may be seen [1, 20]

2.3 Epidemiology

Infection by *H. pylori* is everywhere at worldwide, however, the overall prevalence of *H. pylori* infection in developed countries has been reported to be lower than that in developing countries; this difference in prevalence of infection was attributed to the rate of acquisition of *H. pylori* in childhood (according with Mobley *et al.*, the prevalence of *H. pylori* infection in children less than 10 years in developed countries was approximately 0 to 5 % compared with 13 to 60 % in children resident in developing countries [12, 21-22]; however, the epidemiology of *H. pylori* has been changing over the last decades, with a decline of the prevalence of the infection in most countries) [21, 23]. Some studies from Asian countries also have shown decreasing rates of *H. pylori* infection over the past 40–50 years [24]. A short illustration is shown in the Figure 4.

Figure 4. Prevalence of *H. pylori* infection in some countries (adaptation from [23])

Table 1. Summarize of methods for the detection of *H. pylori* infection (Adapted from [25, 26]).

Type of test	Test	Sensitivity (%)	Specificity (%)	Advantages	Limitations
Invasive	Upper gastrointestinal endoscopy				
	Histology	82-95	99-100	Good sensitivity	High inter-observer variability, time consuming
	Rapid urease test	85-90	98-100	Cheaper, simple, rapid	Requires high bacterial burden
	Microbiological culture	70-80	100	Antibiotic sensitivity profile	Limited availability, slow, technically challenging
	Polymerase chain reaction	> 95	> 95	Quick, antibiotic sensitivity profiling	Expensive, limited availability, contamination,
Non-invasive	Serology	76-84	79-90	Widely available, identifies virulence factors	Remains positive after eradication, low sensitivity
	Carbon 13-labeled urea breath test	> 95	> 95	Reliable, simple, widely available, rapid	Special equipment required for analysis
	Stool antigen test	94	97	Reliable, simple, rapid	Patient reluctance

The prevalence of infection generally increases with age and is higher in blacks and Hispanics than in whites. The seroprevalence of *H. pylori* is approximately 30 % in individuals younger than 30 years and 63 % for individuals aged 55 to 65 years [27].

It has been reported that the acquisition of *H. pylori* infection mainly occurs in children, with a prevalence of infection between 4.9 and 73.3 % worldwide depending on different countries, target population, range of ages, years of specimen collection, sample size and detection methods used in the studies [24]. Ding *et al.*, (2015) concluded that the *H. pylori* infection rate displayed a linear growth trend with increasing age in school age children. This study was performed with a total of 3491 children selected from the three Chinese cities. They ranged in age from 1 day to 18 years (1760 males and 1731 females, including 330 newborns, 319 infants of 1-12 months, 289 children of 1-3 years old, 624 of 4-6 years old, 528 of 7-9 years old, 308 of 10-12 years old, 685 of 13-15 years old and 408 of 16-18 years old) [24].

Different factors have been identified to have influence on the transmission of *H. pylori*: socioeconomic status, genetic predisposition, and route of transmission. A complete discussion related with routes of transmission is analyzed by H. M. Mitchell (2001). He shows the evidence for and

against of gastro-oral transmission, fecal-oral transmission, oral-oral transmission, and transmission associated with water and animals as a potential source of *H. pylori* [14].

3. Detection methods of *H. pylori*

The techniques used for *H. pylori* diagnostic can be divided in two groups: (a) invasive techniques or biopsy-based tests, which mostly require a gastric endoscopic for the gastric biopsies (*e.g.*, histology, rapid urease test or RUT, microbiological culture and polymerase chain reaction or PCR) and (b) non-invasive techniques, which are considered to be less aggressive, (*e.g.*, stool antigen test, serology and urea breath test or UBT) [28]. A summarize of methods for the detection of *H. pylori* infection is shown in the Table 1.

3.1 Invasive techniques

The invasive techniques are based on the direct detection of the pathogen in the gastric mucous membrane, their performing implies the obtaining of mucosal fragments through esophagus-gastro-duodenoscopy or upper gastrointestinal endoscopic (UGIE). This consists in the examination of the lining of the esophagus, stomach, and upper

duodenum with a small camera (flexible endoscope) which is inserted down the throat [29]. The main invasive techniques are described below.

3.1.1. Upper gastrointestinal endoscopy (UGIE)

UGIE is a test that allows visual exploration of the inner walls (or mucous membrane) of the esophagus, the stomach and the duodenum to detect anomalies, and collect tissue samples (biopsies) for histological, RUT, microbiological culture and PCR study. The gastric antrum is the preferred site for biopsies and to detect *H. pylori* infection in most cases, however, in patients with antrum atrophy or intestinal metaplasia, biopsy is suggested to be performed using a greater gastric body curvature to avoid false negatives [30]. The endoscope contains a video camera which allows the visual examining of upper gastrointestinal tract. Though it was introduced in the 1970s, the development of clinically useful instruments was in the 1990s. From there, pediatrics endoscopic procedures have now become standard tools in the developed world for the management of GE disorders [21-34].

The findings obtained by conventional endoscopy such as edema, erythema and nodularity are not sufficiently specific for the diagnosis of *H. pylori*, therefore, for the initial diagnosis, rapid urease tests, microbiological culture and tissue staining using gastric tissues obtained by UGIE are preferred in both adults and children [31, 32, 36]. Thus, some researchers recommend that diagnostic tests for *H. pylori* infection be done in children who are first-degree patients with gastric cancer [32]. However, it is important to note that, for the performing of UGIE in children, it is necessary to sedate them to perform the procedure easier and painless [35]; obviously, sedation is procedure also carried out in adults; however, there are special considerations for infants and children. For example, most analgesics, including opioids and local anesthetics, are conjugated in the liver and, the hepatic enzyme systems are not mature in newborns and premature infants; also, neonates have reduced plasma concentrations of both albumin and alpha-1 acid glycoprotein than older children and adults. This may result in higher concentration of active drug leading to greater drug effect or drug toxicity [36]. UGIE-based studies included stool testing for ova, parasites, occult blood, and fecal antigen for *Helicobacter pylori* have evidenced that children

with gastritis were *H. pylori* positive [31]. But also, UGIE have been useful for the identification of changes associated with *H. pylori* infection. Thus, it is known that the chronic inflammation induced by *H. pylori* interferes with the normal physiology of the gastric acid secretion in different degrees, producing chronic gastritis, which, in most individuals, remains asymptomatic and does not progress. However, in some cases, the development of peptic ulcer can be induced, whereas in other cases, gastritis progresses to atrophy, intestinal metaplasia, and eventually to gastric carcinoma, or rarely, to gastric lymphoma due to the persistent immune stimulation of the lymphoid tissue [37, 32]. Though UGIE is expensive and invasive, it has demonstrated to be of great usefulness for the establishing the diagnosis, the locating the ulcer and the obtaining biopsy fragments for histopathological analysis and for *H. pylori* investigation [25]. Thus, UGIE remains as the method of choice for the diagnosis in very young children.

Adeyini *et al.*, (2016) showed that the symptoms for the UGIE indication were abdominal pain, upper gastrointestinal bleeding, recurrent and intractable vomiting, dyspepsia, acidity and dysphagia (difficulty or discomfort in swallowing, as a symptom of disease). According to authors, the most recurrent symptom is abdominal pain. A more detailed description is shown in the Figure 5 [31]. Other studies in children do not support a role for *H. pylori* infection in functional disorders such as recurrent abdominal pain [38]. Therefore, in children the decision making to investigate and treat the infection should be supported by a clear benefit for the individual child.

Figure 5. Effects produced by UGIE on patients (Adapted from [31]).

The guidelines of European Society for Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) and North American Society for Paediatric Gastroenterology, Hepatology, and Nutrition (NASPGHAN) recommends that the primary goal of clinical investigation of gastro-intestinal symptoms should be to determine the underlying cause of the symptoms and not solely the presence of *H. pylori* infection [39].

3.1.2. Histology

The histology can be defined as the study of the microanatomy of cells and tissues; thus, histological studies are used in forensic investigations, autopsy, and diagnosis. Also, histology is used extensively in medicine especially in the study of diseased tissues and it is usually considered to be the “gold standard” in the direct detection of *H. pylori* infection [40]. Several factors can influence the histological diagnostic accuracy, such as: site, size and number of biopsies, staining methods, proton pump inhibitor (PPI), antibiotics and experience of the examining pathologist [42, 43]. One advantage of the histological test over other modalities is that it allows for the examination of inflammation, metaplasia, dysplasia, and malignant neoplasm [41]. Some aspects are shortly analyzed below.

In general, biopsy is a procedure based in the collection of one or more samples from a tissue for their analysis; in consequence, the sampling procedure is very important and, by the nature of the points under examination, it is clear that an appropriate collection site for the biopsy samples can decrease sampling error, false negative results and congruency between different methods.

Standard histopathological investigation of the gastric tissue includes observation for the presence and severity of inflammation, atrophy, and intestinal metaplasia. To carry out the histological examination, it is necessary to introduce a flexible tube of optical fiber or video endoscope through the child's mouth to transport light into the esophagus cavity and in the stomach allowing that the gastric interior to be observed. Additionally, it is possible to collect tissue samples (biopsy) to be analyzed (see Figure 6), but, it has been found that to obtain favorable results at least two tissue samples from the antrum and from the body are required [32].

After of sampling stage, the staining is the critical part of histological exam. Several dyes have been

used, like routine methods, to detect *H. pylori*. Although the immunohistochemical stain is the most sensitive and specific stain method, HE stain, or haematoxylin and eosin stain, is usually sufficient for diagnosis of *H. pylori* infection in routine clinical practice [42, 43]. Details of some strain methods are summarized in the Table 2. In addition, an illustration of visual features of samples during histopathological analysis is shown in the Figure 7.

Figure 6. Illustration to procedure to carry out the histological analysis [34].

Figure 7. (A) Histological features of the gastric mucous membrane in children with chronic gastritis. Comparison among endoscopic features and histological features (B and C) (Adapted from [32]).

Table 2. Summarized of some strain methods used in histological analysis [42, 43].

Strain method	Description	Remarks
HE staining or haematoxylin and eosin stain	The staining method involves application of a complex formed from aluminum ions and hematein, which is an oxidation product of hematoxylin, which colors nuclei of cells.	It is the most widely used stain in medical diagnosis and is often the standard method.
Giemsa strain	It is a mixture of methylene blue, eosin, and Azure and it attaches itself to regions of DNA where there are high amounts of adenine-thymine bonding.	It is specific for the phosphate groups of DNA and also used to visualize chromosomes.
Warthin–Starry stain	It is a silver nitrate-based staining method (a silver stain).	Warthin–Starry method stains organisms dark brown to black, and the background light golden brown/golden yellow.
Toluidine blue strain	Toluidine blue is a basic thiazine metachromatic dye with high affinity for acidic tissue components	It stains nucleic acids blue and polysaccharides purple, also increases the sharpness of histology slide images. It is especially useful today for staining chromosomes in animal tissues.
Acridine orange strain	It is used as a nucleic acid-selective fluorescent dye useful for cell cycle determination. Being cell-permeable, it interacts with DNA and RNA by intercalation or electrostatic attractions respectively.	When bound to DNA, it is very similar spectrally to fluorescein, with an excitation maximum at 502 nm and an emission maximum at 525 nm (green). When it associates with RNA, the excitation maximum shifts to 460 nm (blue) and the emission maximum shifts to 650 nm (red).
Immunohistochemical stain	It refers to the process of selectively imaging antigens in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues.	It is widely used in the diagnosis of abnormal cells such as those found in cancerous tumors. Specific molecular markers are characteristic of particular cellular events such as proliferation or cell death

3.1.3 Rapid urease test (RUT)

RUT is a rapid diagnostic test for diagnosis of *H. pylori* which is based on the ability of *H. pylori* to secrete the urease enzyme, which catalyzes the conversion of urea to ammonia and carbon dioxide [43, 44]. The accuracy of the test is dependent on the number of tissue specimens tested, the location from which the biopsy is obtained, the bacterial load and previous use of antibiotics and PPI as well as the prevalence of the infection in the community [42]. In clinical practice, when there is an indication for endoscopy, and there is no contraindication for biopsy, the RUT is recommended as a first-line diagnostic test. One biopsy should be taken from the corpus and one from the antrum; in addition, RUT is not recommended as a test for *H. pylori* eradication assessment after treatment [45].

To carry out the RUT, a specimen is placed on a commercially available urea-containing medium. The hydrolysis of the urea by urease brings about a color change in the pH-sensitive chemicals in the

medium allowing the identification of the presence of the organism [43–45]. However, several commercial urease tests including gel-based tests (CLOtest, HpFast), paper-based tests (PyloriTek, ProntoDry) and liquid-based tests (UFT300, EndoscHp) are available, and different commercial RUTs have different reaction time to provide results. For example, CLOtest usually takes 24 h to obtain accurate result, whereas PyloriTek takes 1 h and UFT 300 takes 5 min to provide more rapid results. Reading the urease tests earlier than recommended time may lead to false negative results. In addition to the designs of commercial kits, the density of bacteria present in the biopsy specimen also affects the reaction time and diagnostic accuracy of RUT, while the minimum of 10000 organisms are usually required for a positive RUT result. Other factors influencing the diagnostic accuracy of the urease tests include H₂-receptor antagonists, PPI, bismuth compounds, antibiotics, achlorhydria and presence of blood, all of which increase the possibility of false negative results.

Two weeks before is recommended to suspend the PPI and the antibiotics 4 weeks before to perform the RUT in order to avoid altering the activity and population density of the bacteria. Furthermore, contamination of biopsy samples with formalin also decreases the sensitivity of RUTs [44-46].

3.1.4. Microbiological culture

A microbiological culture is a method of multiplying microbial organisms by growth in culture media under controlled laboratory conditions. Microbial cultures are used to determine the type of organism and its abundance. It is one of the primary diagnostic methods of microbiology and is used as a tool to determine the cause of infectious disease.

In general, culturing has almost 100% specificity, but the sensitivity of culture shows significant variation, between 85-95 %. Due of the delicate nature of *H. pylori*, the cultivation *in vitro* requires particular transport medium, growth medium and incubation environment. Biopsy specimens can be kept in a transport medium, like Portagerm pylori or Stuart's transport medium, for up to 24 h at 4 °C. The isolation of *H. pylori* from gastric mucosal fragments allows detecting virulence factors and susceptibility to antimicrobials. The use of at least two fragments from the gastric mucous membrane, one from the antrum and another one from the gastric body, adequate specimen transportation, use of a selective and indicator medium, and the team's experience are essential for the successful isolation of *H. pylori* [2].

Several types of agar can be used for culture as *H. pylori* are isolated. The commonly used media include Pylori agar, Skirrow agar, Columbia blood agar, Brucella agar, Brain heart infusion or Trypticase soy agar, supplemented with sheep or horse blood. The agar plates are usually incubated in a microaerobic environment at 35 to 37 °C for at least 5-7 days because *H. pylori* has been considered a microaerophile. However, a recent study showed growth of *H. pylori* is promoted by atmospheric oxygen levels with the presence of 10 % CO₂, bringing a novel concept that *H. pylori* may be a capnophilic aerobe (*i.e.*, microorganisms that thrive in the presence of high concentrations of carbon dioxide). Diagnosis of *H. pylori* from culture medium is based on morphological characteristics as well as positive urease, catalase,

and oxidase reactions, which mean the microbiological laboratories, should be equipped and trained to isolate this bacterium [42-43]. In addition, it is important to note that conditions such as poor quality of specimens, delayed transport, exposure to aerobic environment or inexperienced microbiologist have adverse influence on the performance of culture and reduce the diagnostic accuracy. The influence of transport time as well as temperature shows a decrease on culture rate. Since studies have shown that an increase of the temperature of 24.4 °C causes a decrease of 12.6 % culture rate. For this reason, new culture media have been developed. GESA transport medium is a new transport semi-solid medium which can store gastric biopsy specimens at 4 °C for up to 10 days and provide a quantifiable recovery rate of *H. pylori* (90.7 %) [43].

Researches indicate that a high quality in the data is due to the results made with GBC. For example, Montes *et al.*, (2014) studied the presence of *H. pylori* in 143 children younger than 15 years with an indication for upper gastrointestinal endoscopy: by gastric biopsy culture (GBC), ¹³C-urea breath test (UBT) and stool antigen immunochromatography test (SAIT) in 56 children; by GBC and UBT in 20, by GBC and SAIT in 18, and by GBC alone in 49. Antimicrobial susceptibility was determined by E-test. Infection was defined as a positive culture or positivity in both UBT and SAIT. Children were considered to be infected if the results of GBC were positive or if the results of GBC were negative but those of both the UBT and SAIT were positive. 74 children resulted infect (74/143, 51.74%) and in all cases GBC showed positives results. While 71.62 % of the total infected had a positive result for UBT, for SAIT it was 33.78 % [42]. Maastricht *et al.*, (2017) recommend that after a first failure of treatment, if an endoscopy is carried out, then culture and standard antimicrobial susceptibility testing are suggested to tailor the treatment [46].

3.1.5. Polymerase chain reaction (PCR)

PCR is a technique used in molecular biology to amplify a single copy or a few copies of a segment of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The vast majority of PCR methods are performing using a system of thermal cycling, which involves exposing of the reactants to

cycles of repeated heating and cooling, permitting different temperature-dependent reactions, specifically, DNA melting and enzyme-driven DNA replication, to quickly proceed many times in sequence. Also, PCR allows for rapid and highly specific diagnosis of infectious diseases, including those caused by bacteria or viruses. The basis for PCR diagnostic applications in microbiology is the detection of infectious agents and the discrimination of non-pathogenic from pathogenic strains by virtue of specific genes [47].

PCR has been used extensively for the diagnosis of *H. pylori* from gastric biopsy specimens, saliva, stool, gastric juice and variable specimens [43] because this technique provides excellent sensitivity and specificity (greater than 95 %) [29, 43]. Several target genes including *UreA*, *glmM*, *UreC*, 16S rRNA, 23S rRNA, HSP60, and *VacA* genes, have been used for detection of *H. pylori* and using two different conserved target genes the specificity can be increased, which in turn avoids false positive result, especially for samples other than gastric biopsy specimens.

Other advantages of PCR are: fewer bacteria are required in sample, faster results, and no need for special processing supplies or transportation, enable clinicians to make quicker and more accurate decision on patient's treatment. Furthermore, PCR also allows concurrent detection of specific mutations leading to antibiotic resistance. In addition, detection of virulence factors by PCR helps to evaluate the genetic variation within virulence factors of *H. pylori* and gives more information to understand the clinical discrepancies between patients infected with different strains of *H. pylori* [44, 40].

Several reasons have made the tendency to avoid the endoscopic diagnostic methods [43, 44]:

- a) Endoscopy is an invasive method which produces discomfort and not suitable for patients with severe co-morbidities or contraindications.
- b) Cost of endoscopy and additional cost adding on endoscopy, such as disposable probes and anesthesia, may be high.
- c) Sampling bias is almost inevitably encountered in biopsy-based methods due to uneven distribution of *H. pylori* in stomach.
- d) There are limitations to using endoscopy on children, especially after the eradication of *H. pylori*, due the endoscopy is a method that may cause complications or inflict psychological bur-

den on children and their parents.

3.2 Non-invasive techniques

The non-invasive techniques, or indirect methods, consist of detection of *H. pylori* antigens in serum, urine and saliva samples, in stool samples, and samples of breath by detection of carbon 13 non-radioactive isotopes. It has been recommended that at least two tests be used for a more accurate diagnosis of the infection. Besides, the non-invasive tests, Carbon 13-labeled urea breath test (UBT) and Stool antigen test (SAT), are ideal for confirming *H. pylori* eradication after appropriate treatment because these are able to detect an active infection. It is also necessary that noninvasive methods be validated for the population to be assessed [29, 41].

3.2.1 Serology

Serology is the study of serum and other bodily fluids. In practice, the term usually refers to the diagnostic identification of antibodies formed in response to an infection (against a given micro-organism), against other foreign proteins or to one's own proteins. As in any other infection, *H. pylori* infection of the stomach exerts an immunological response in the body, which triggers the production of anti-*H. pylori* antibodies (IgM, IgA and IgG) [29, 31]. While both IgA and IgG antibodies on take up to 60 days to develop, the antibody response in children is associated with low molecular weight antigens (15-30 kDa), and while IgM antibodies can be detected very early, in comparison with others which only can be detected between 3 weeks to 3 months after the onset of acute infection, and for up to 2 years after bacterial eradication [29].

Tests of serum immunoglobulin for IgA or IgM antibodies are unreliable to detect gastric colonization and, therefore, only IgG antibodies are used in clinical laboratory practice, which is measured using an enzymelinked immunosorbent assay (ELISA) since it is quick, easy-to-use, reproducible, inexpensive, and highly accurate; several kits have been commercially available. The presence of IgG antibodies against *H. pylori* denotes an active infection because once an individual is colonized, the infection continues throughout life unless a course of appropriate eradication therapy is instituted. Also immunoenzyme assays for the detection of antibodies in the urine and saliva are

even less sensitive and specific, they are not recommended for the diagnosis neither management of infection in children [29]. However, it should not be used for the diagnosis of infection in children aged less than 12 years, because its sensitivity is too low in this age group (44.4 % for children aged 2 to 6 years and 76.7 % for those aged 7 to 11 years) [29] due to low antibody titers as a result of relatively short infectious period and immature immune response to *H. pylori* in childhood [42].

The *H. pylori* strains are different in the different places of the world, for this reason, the choice of the antigen to be used in this test in order to detect the antibody is a critical stage. It is observed that commercially available serum based immunoassays have a range of sensitivity from 63 to 78 % for adults; however, the sensitivity of serological assays is poor in children (less than adults), due the mean antibody levels in young children are significantly lower than in older children and adults and these age-related standard values have not been established for children. In addition, the most commercial kits have not been validated for children and the cutoff point for adults may be too high for children, resulting in a low sensitivity for the test [29, 42, 43].

An advantage of serological test is that the accuracy of serological tests is not affected by ulcer bleeding, gastric atrophy as well as the use of PPI or antibiotics, which cause false negative results in other invasive or noninvasive tests. However, serological test is should not be used as a method to assess eradication therapy because antibody levels can persist in the blood for long periods of time even after successful eradication. Serological methods are mostly used in population-based seroprevalence studies, although in the populations with high seroprevalence rates, these methods have been suggested to be used as the first method if urea breath test and fecal antigen test are not available. Because the serological tests do not distinguish between active infection and past exposure to *H. pylori*, further confirmation by other tests is required before eradication therapy [31, 40, 46].

3.2.2 Carbon 13-labeled urea breath test (UBT)

Urea breath test (UBT) is the most popular and accurate non-invasive test for diagnosis of *H. pylori* infection. By the urease activity of *H. pylori*, the

^{13}C - or ^{14}C -labeled urea ingested by the patient is hydrolyzed to labeled CO_2 in stomach, then labeled CO_2 is absorbed in the blood and exhaled by breathing in which labeled CO_2 [42] can be measured in expired air using a mass spectrometer. The sensitivity, specificity, positive predictive value and negative predictive value of UBT have been reported to be approximately 96, 93-97, 91-97 and 97 %, respectively in children and adolescents [43]. Thus, UBT is a good example of a non-invasive diagnostic test that can be performed safely and easily, for it does not require an experienced examiner and costs is low when compared to endoscopy. The test is simple to perform as it requires only two breath samples, before and 15–30 min after drinking the test solution which contains the ^{13}C -urea substrate [26].

It is known as a convenient and accurate test for confirming the presence of *H. pylori* infection a non-invasive manner in both adults and children [29]. However, the accuracy of UBT in pediatric patients is not as good as it used in adult patients, especially for children younger than 6 years old [42].

Although several factors including patient, bacteria and the test itself influence the results of UBT, the UBT is a highly accurate and reproducible test with near 95 % sensitivity and specificity under standardized procedures. Patient should stop taking PPI two weeks and antibiotic four weeks before exam to avoid false negative results. UBT is also useful for epidemiological studies and for assessing the efficacy of eradication therapy, as it is indicated in the guidelines of ESPGHAN and NASPGHAN, seeing that ^{13}C -UBT is one of the most reliable non-invasive method in lieu of endoscopy to confirm the eradication of *H. pylori* in children [29, 39].

^{13}C -UBT is preferable to the ^{14}C -UBT to avoid exposure to radiation, even though ^{14}C -UBT is safe for children and pregnant women because radiation from ^{14}C -UBT is lower than radiation acquired from the natural environment. In the absence of expensive equipment and ability to pay high cost of ^{13}C -UBT, however, ^{14}C -UBT is more popular in the developing countries. The diagnostic accuracy between ^{13}C -UBT and ^{14}C -UBT is not different and both tests can be considered to be gold standard among the various noninvasive tests for the diagnosis of *H. pylori* infection. There are two protocols for the oral administration of ^{14}C -urea to patients for *H. pylori* diagnosis: non-encapsulated

and encapsulated (this method was developed to avoid the problem of ^{14}C -urea hydrolysis by the action of urease-producing). It is important to note that, the non-encapsulated protocol has higher sensitivity than encapsulated protocol as a result of rapid transit of the ^{14}C -urea containing capsule from the gastric tract or its incomplete resolution in the stomach during the phase of breath collection [43].

3.3.3 Stool antigen test (SAT)

Stool antigen tests (SATs) or *H. pylori* stool antigen (HpSA) are non-invasive diagnostic methods for *H. pylori* infection. Two types of SATs exist for the diagnosis of *H. pylori* infection, one based on enzyme immunoassay (EIA) and another on immunochromatography (ICA). SATs do not require expensive chemical agents or specified equipment; hence, they are less expensive compared with the UBT [29]. The accuracy of SAT is seen to be influenced by several factors, like the use of antibiotics, bowel movement and upper gastrointestinal bleeding. In patients with diarrhea the sensitivity is reduced as the antigen concentration is diluted. The preservation of the specimens, temperature and transport time before testing are very important factors, and cut-off value also have impacts on the diagnostic accuracy of SAT [26, 31].

4. Clinical test for children: Analysis and prospects

As it was previously indicated, there is much evidence indicating that *H. pylori* infection in children differs from that in adults. A short description of this differentiation on diagnosis is shown below:

4.1. Gastrointestinal endoscopy

The analysis of *H. pylori* infection by gastrointestinal endoscopy shows a varied range of situations. Thus, if *H. pylori* infection occurs during childhood, it coexists with epithelial cells during the patient's lifetime and leads to chronic gastritis; however, if colonization continues even in asymptomatic children, then the severity of lesions that appear in the gastric mucous membrane can get worse [31, 38-41]. On the other hand, if there are nodular changes in the antrum or erosions and ulcers in the duodenum on endoscope, *H. pylori* infection

should be suspected in children [32], it is accepted that, in children, if nodular gastritis on endoscopy of the upper gastrointestinal tract is identified, then it is a clear indication that *H. pylori* will be identified with a probability of ~90%. In addition, there are several common endoscopic manifestations of *H. pylori* infection in children (e.g., nodular arthritis, gastric lymphoid hyperplasia, follicular gastritis, or lymphofollicular gastritis) [31, 52,53]. In chronic *H. pylori* infection of childhood, numerous small nodules are observed on the endoscope in 44-67 % of *H. pylori*-infected children [9], in contrast to 0.19% of adults according to a previous study [55]. During upper endoscopy it is recommended that at least 6 gastric biopsies should be obtained for the diagnosis of *H. pylori* infection. Two biopsies should be obtained from the antrum and two biopsies from the corpus for the histopathological evaluation [39].

The guidelines of ESPGHAN and NASPGHAN, newly published, recommend against diagnostic testing for *H. pylori* infection as part of the initial investigation in children with IDA and suggest that in children with refractory IDA in which other causes have been ruled out, testing for *H. pylori* during upper endoscopy may be considered. Besides, suggest that if *H. pylori* infection is an incidental finding at endoscopy, treatment may be considered following careful discussion with the patient/parents [39].

4.2. Histopathologic test, rapid urease test, and culture for *H. pylori* using gastric tissues

Uhlig *et al.*, (2003) concluded that no significant difference was identified between histopathological parameters of *H. pylori*-associated gastritis in children with those findings in adults [55]. But, children with antral nodularity had significantly higher histological scores, suggesting that antral nodularity may be the important sign of higher grade gastritis in young children. These differences were explained by differences in immune response to *H. pylori* infection between children and adults. On the other hand, acute active inflammation (infiltration of neutrophils) is less prominent in children compared to adults, while chronic inflammation (infiltration of lymphocytes and plasma cells) is more prominent [56-58]. Besides, atrophy, intestinal metaplasia, gastric cancer and MALT lymphoma are rare in children [42].

It is recommended that the initial diagnosis of *H. pylori* infection be performed using invasive gastric biopsy-based methods including: positive bacterial culture or *H. pylori* gastritis on histopathology with at least 1 other positive biopsy-based test (RUT, or molecular-based assays where available, including PCR or fluorescent in situ hybridization). However, a positive noninvasive test supports the diagnosis in cases in which positive histology is the only invasive test available [56-58].

4.3. UBT

In children less than 6 years of age, clinical application of UBT is seen to be limited because of relatively low specificity and high rate of false positive results (*i.e.*, ~8.3 %) compared to adults and older children (children larger than 6 years of age show false positive rate about 0.85 %) [31, 59]. There are several possible explanations for high false positive results in children younger than 6 years: Differences in the metabolic production of isotopic carbon depending on physiological parameters and a tendency to retain ¹³C-urea in the mouth as a result of microorganisms such as *S. salivarius*, *P. mirabilis*, and *K. pneumonia* [59].

4.4. *H. pylori* antibody tests in serum, urine, or saliva

Previously it was indicated that IgM antibodies may rise during the early stages of *H. pylori* infection, but in the cases of chronic infection, IgA and IgG antibodies are detected in blood, urine, and saliva. The diagnosis based on serological analysis of *H. pylori* infection may be inappropriate in young children because of relatively short infection period and immature immune response (low sensitivity of 79.2 % with a high specificity of 92.4% for young children). In addition, serum *H. pylori* IgG antibody test is also inappropriate to monitor *H. pylori* status after treatment because elevated serum titers of *H. pylori* IgG antibody persist for a long time [60]. It is accepted that the antibody tests for *H. pylori* in serum, urine, or saliva are not reliable for use in children and should not be used to detect the presence of active *H. pylori* infection or for use in post-treatment evaluation in children or adolescents [39].

Figure 8 shows results positive (+) for the evaluation of *H. pylori* infection by two methods:

Stool Antigen test and IgG antibody. It shows that for IgG antibody the percentage of infection in children increases with increasing age, whereas in stool antigen test age was not a relevant factor for both techniques, different results were found [48]. On the other hand, in terms of biological sex, a higher percentage of girls (95 %) were found with *H. pylori* compared to 91 % for boys using stool antigen test, while in IgG the percentages of infected male children were 89 % compared to 81 % infected girls (Figure 9) [48].

Figure 8. Results positive (+) for the evaluation of *H. pylori* infection by two methods: Stool Antigen test and IgG antibody (Adapted from [48]).

Figure 9. Comparison between Stool Antigen test and IgG antibody in girls and boys tested (Adapted from [48]).

4.5. HpSA test

Clearly an important advantage of HpSA test using monoclonal antibody is that this test can be considered an useful office-based test which can be easily available anytime and anywhere. Diagnostic accuracy for the initial diagnosis of *H. pylori* infection in children is high (sensitivity of 87-100 % and specificity of 82-100 %) [61,62]. However, differences between mono- and polyclonal tests have been identified. Thus, by meta-analyses, for HpSA tests using monoclonal antibody showed a sensitivity of 96.2-97 % and specificity of 94.7-97 % in children, whereas polyclonal HpSA tests using antibody revealed a lower diagnostic accuracy with a sensitivity of 88-92 % and specificity of 93% [63, 64]. In addition, in children, after the elimination treatment of *H. pylori* infection, the sensitivity of HpSA decreased from a mean sensitivity of 92.6 % before treatment to 80.9 % after treatment, whereas the sensitivity of HpSA was still high with a mean specificity of 97.2 % after treatment [65]. Endoscopy and biopsy-based tests to confirm eradication are rarely needed in pediatric patients with uncomplicated peptic ulcer disease. It is recommended that the ¹³C-UBT or 2-step monoclonal stool *H. pylori* antigen test be used to determine whether *H. pylori* treatment was successful [66].

5. Conclusions

At present, there are several diagnostic tests with high sensitivity and specificity to detect *H. pylori* infection, however, the choice of one method or another depends on several aspects such as: age of the patient, probability to obtain a positive or negative result in different clinical circumstances, as well as, the accessibility and the particular advantages and limitations of each test.

From available information, invasive or non-invasive tests can be applied to children to detect *H. pylori* infection, however, medical team should be careful and take in consideration the differences between children and adults to carry out a proper diagnostic.

Finally, for the diagnosis of acute *H. pylori* infection in children, it is recommended to perform invasive methods based on gastric biopsy; however, a positive non-invasive test can support diagnosis in cases where only histology is available. While to corroborate post-treatment bacterial eradication, it

is recommended to perform non-invasive methods, specifically exhaled breath test or antigen test in feces.

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Conflict of interests

The authors declare no conflict of interest.

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