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Diagnosis of *Helicobacter pylori*: What should be the gold standard?

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Abstract

Since the discovery of *Helicobacter pylori* (*H. pylori*) in 1983, numerous detection methods for the presence of the bacterium have been developed. Each one of them has been associated with advantages and disadvantages. Noninvasive tests such as serology, ¹³C urea breath test (UBT) and stool antigen tests are usually preferred by the clinicians. Serology has its own limitation especially in endemic areas while ¹³C UBT is technically very demanding. The stool antigen detection method, although specific, is usually associated with poor sensitivity. The ¹³C UBT is believed to be specific, but with present revelation of the fact that stomach is colonized by many other urease producing bacteria makes it questionable. Histology, culture, rapid urease

test and polymerase chain reaction (PCR) are the tests which are carried out on antral biopsies collected by invasive means. Histology has been proposed to be very sensitive and specific but the question is how by simply looking the morphology of the bacteria in the microscope, one can claim that the curved bacterium is exclusively *H. pylori*. Rapid urease test (RUT), the doctor's test, is also challenged because the presence of other urease producing bacteria in the stomach cannot be denied. Moreover, RUT has been reported with poor sensitivity specially, when density of the bacterium is low. Isolation of *H. pylori* is essential to investigate its growth requirements, antibiotic susceptibility testing, studying virulence factor to develop vaccine and many more explorations. It has also got several disadvantages *i.e.*, special condition for transporting, media, incubation and few days waiting for the colonies to appear, apart from the speed essentially needed to process the specimens. Till date, majority of the microbiological laboratories in the world are not equipped and trained to isolate such fastidious bacterium. The option left is PCR methods to detect *H. pylori*'s DNA in gastric mucosa, gastric juice, saliva, dental plaques and environmental specimens. There are speculations for false positivity due to detection of non-*pylori Helicobacters* due to genetic sharing; and false negativity due to low bacterial counts and presence of PCR inhibitors. However, specimen collection, transportation and processing do not require speed and special conditions. PCR based diagnosis may be considered as gold standard by designing primers extremely specific to *H. pylori* and targeting at least more than one conserved genes. Similarly specificity of PCR may be improved by use of internal Primers. Further, nested PCR will take care of false negatives by countering the effect of PCR inhibitors and low bacterial counts. Therefore, nested PCR based methods if performed properly, may be proposed as gold standard test.

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Key words: *Helicobacter pylori* review; Invasive tests; Non-invasive tests; Gold standard; Polymerase chain reaction detection; Nested polymerase chain reaction

Core tip: Several detection methods for *Helicobacter pylori* (*H. pylori*) have been developed. Noninvasive tests such as serology, ¹³C Urea breath test and stool antigen tests are usually preferred. Invasive test involving histology, culture, rapid urease test and polymerase chain reaction (PCR) are also available. Each one of them has been associated with one or more advantages or disadvantages. However, PCR is the best method to detect *H. pylori* in gastric mucosa, gastric juice, saliva, dental plaques and environmental specimens. PCR based diagnosis may be considered as gold standard provided that *H. pylori* specific primers targeting at least more than one conserved genes are used.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a microaerophilic fastidious human pathogen. The bacterium has been implicated in acid peptic diseases of stomach and duodenum and neoplasm of stomach^[1-7]. *H. pylori* has been detected in individuals of all ages throughout the world and its prevalence ranges between 20%-80%^[8]. The incidental discovery of this bacterium by Marshall and Warren^[9] in 1983, led to a paramount change in our understanding of acid peptic diseases. Later in 2005 they were awarded “Nobel Prize in Medicine or Physiology” for the discovery of *H. pylori*. It has been reported that approximately 80% and 95% of the gastric ulcer (GU) and duodenal ulcer (DU) respectively are caused by *H. pylori*^[10]. Of the several available diagnostic tests the detection of *H. pylori* infection, each of them has certain advantages and disadvantages. Either due to poor sensitivity or specificity, none of them can be considered as gold standard. However, combinations of more than one test *e.g.*, urease enzyme production test, microscopy, bacterial isolation and polymerase chain reaction (PCR); usually give the quite satisfactory diagnosis. However, these methods are invasive, expensive and applicable to tertiary level laboratories only. Due to the disadvantage of invasive procedure, several non-invasive tests have been developed to diagnose *H. pylori*. In this article, we intend to review both invasive and non-invasive tests so that one of them may be proposed as the gold standard.

During the early years of *H. pylori* infection, endoscopy without biopsy remained highly unsatisfactory in making diagnosis^[11]. Now days, detection of *H. pylori* in biopsy samples depends on histology, rapid urease test

(RUT), culture and PCR tests. Examination of gastric juice though not widely used may be used to assess *H. pylori* infection.

HISTOLOGY

Histology was the first method used for the detection of *H. pylori*. Presence of typical bacteria along with the inflammatory reaction in the tissue slides is considered as diagnostic test for *H. pylori* infection. Several stains like Giemsa, acridine orange, Warthin Starry, Hp silver stain, Dieterle, Gimenez, McMullen; and immunostaining are used to detect *H. pylori*. Routinely Giemsa staining is used for *H. pylori* detection. The haematoxylin and eosin stain helps in evaluation of severity of inflammation along with detection of the bacteria. However, Genta stain due to combination of silver stain, haematoxylin and eosin and Alcian blue has the advantage of visualizing both inflammatory reaction and *H. pylori*. However, this modification is technically complex and uses uranyl nitrate in its original formulation. Routinely, Giemsa stain is more feasible in detecting *H. pylori* because it is simple, highly sensitive and less expensive^[12,13]. However, the high sensitivity of histology is often influenced by the site, number and size of the biopsies material collected. Patchy colonization can sometimes cause misdiagnosis. Interestingly it has been seen that even a single biopsy taken from the lesser curve, close to the angulus, can detect *H. pylori* in majority of the cases (> 90%). Moreover, the accuracy can further be increased by multiple biopsies from the greater curve and corpus. Although, it is believed that specificity is high due to peculiar morphology of *H. pylori* and its close relation to gastric mucosa^[14], it looks unscientific in days of molecular taxonomy because presence of other bacterial species *H. heilmanni*, *H. bizozzeroni*, *Pseudomonas fluorescens* having similar morphology cannot be denied^[15]. The earliest diagnosis by histological methods takes 2-3 d and detection rate definitely varies with the expertise of examiners^[16,17]. Further, prior antibiotics and PPI may transform the typical shape of *H. pylori* from spiral to coccoid which becomes undetectable by the routine microscopy technique. However, fluorescent *in situ* hybridization is answer to it because specific detection of *H. pylori* in histological preparations can be done irrespective of the shape of bacteria^[18,19]. Fluorescent labeled oligonucleotide probes are used in this method targeting the 16S rRNA and 23S rRNA genes. This method is the fastest and takes 3 h to detect *H. pylori*. *In situ* hybridization and immunochemical methods can also be used to detect virulence factors and location of strains in the gastric mucosa^[20]. This method being independent of morphological identification usually does not have individual biasness.

UREASE TEST OR RUT

H. pylori is known for production of abundant urease. Urease enzyme hydrolyses urea to release CO₂ and NH₃. Detection of urease production has been used as a sur-

rogate marker for the detection of the bacterium in antral biopsies. Release of ammonia increases the pH of the test medium and it is detected by a colour change due to pH indicator. Tests can be done either in a solution or a solid supporting medium *e.g.*, the CLO test. However, the later test is not faster than the unbuffered urease tests. A reagent strip of rapid urease test (*Pyloritek*), where reading is taken at 1 h has been reported to have sensitivity and specificity comparable to CLO test (where results are obtained after 24 h). This strip test, therefore, is more convenient to use in the endoscopy clinics^[21]. Since, urease test basically depends on the bacterial density, a comparison of *Pyloritek* (Serim Research Corp) with Helicheck (Institute of Immunology Co.Ltd.Tochigi, Japan) has been made. The two commercial kits based on strip technique have sensitivity and specificity above 90% but when post therapy the density goes down the sensitivity of detection falls to 60%^[22]. The sensitivity and specificity of Urease test or RUT when compared with histology were 89% and 88% respectively^[23]. By using a chemiluminescent pH indicator in RUT faster results with 50-fold higher sensitivity could be achieved as compared routine RUT^[24]. The rapid urease test may also be performed per-endoscopically by using pH sensitive biosensor within a minute, giving sensitivity and specificity of 92% and 95%, respectively^[25].

The sensitivity of RUT is influenced by the bacterial density and the forms (spiral or coccoid) of bacteria present in the biopsy. The minimum of 10^4 organisms per biopsy piece are required for a positive RUT result but a good proportion of patients may harbor lower densities than this^[26]. Further, low density of the bacterium post treatment, bleeding patients and patients taking H₂-receptor antagonists or proton pump inhibitors may also adversely affect the performance of^[27]. The low cost, ease and speed of diagnosis of *H. pylori* infection gives RUT upper hand on culture and histology.

CULTURE

H. pylori may be routinely isolated by culture from human gastric biopsy samples. The organism requires a microaerophilic environment and complex media^[28-30]. Variety of basal media and different supplements have been proposed for isolation of the organism^[29,31-33]. However, Columbia Agar Base or Brain Heart Infusion (BHI) broth supplemented with blood or serum usually has been found adequate for culture^[34]. They typically grow best in freshly prepared moist media incubated in a warm (37 °C) atmosphere with 5%-10% CO₂, 80%-90% N₂ and 5%-10% O₂. Humid atmosphere enriched with H₂ (5%-8%) improves the yield of *H. pylori*. Primary isolation of the organism from gastric biopsy specimens requires 5-7 d in a microaerobic atmosphere created by a variable atmosphere incubator, partially evacuated anaerobic jars with defined gas mixtures or commercial gas generating sachets. A dedicated CO₂ incubator is useful for sub culturing *H. pylori* but is not reliable for primary

isolation. Selective and non-selective media enriched with blood/serum are recommended for the cultivation of *H. pylori* from gastric biopsy specimens, thus maximizing the sensitivity of culture. Various agar media including brain heart infusion, brucella, columbia and Skirrow's supplemented with horse blood/serum or sheep blood have been used to cultivate *H. pylori*^[35]. Antibiotic supplements (vancomycin, 10 mg/L; amphotericin B, 10 mg/L and cefsulodin or trimethoprim, 5 mg/L) are recommended for selective media to facilitate primary isolation.

The best specimens for isolation of *H. pylori* are biopsy samples obtained during endoscopy. By altering the pH by proton pump inhibitors (PPIs)^[36] which indirectly interfere with *H. pylori* distribution in the stomach. Antral part has been found to be the most affected part of the stomach by PPIs as *H. pylori* almost disappears from this niche. To avoid false negative results, Megraud and Lehours^[37] recommended not to consume these drugs 2 wk prior to endoscopy as is done for rapid urease test^[38,39]. Further, duration and dose of PPI therapy also affect the performance of *H. pylori* isolation. It is established that *H. pylori* has patchy distribution in the stomach, therefore despite the good results from biopsy taken 2 cm prior to pylorus, it is advisable to collect multiple biopsy specimens^[40]. Megraud and Lehours^[37] have recommended to take at least two biopsy specimens from the antrum and one each specimens from the anterior and posterior corpus. It has been observed that the corpus may be the only site which remains positive naturally or sometimes due to consumption of antisecretory drugs.

As per Koch's postulates culture is considered to be the most specific way to establish the *H. pylori* infection. The sensitivity of isolation the bacterium has been reported to vary greatly among laboratories because it is very fastidious in nature^[41-46]. Even the experienced laboratories recover the organism from only 50% to 70% of actually infected biopsies^[47,48]. Recovery from stool, saliva, and vomitus is very difficult because of the presence other commensal flora comprising other organisms hampering the growth of *H. pylori*^[49-53].

Bacteriological culture is a tedious, time-consuming procedure^[54], and unnecessary for the routine diagnosis of *H. pylori* infection because other noninvasive tests will detect evidence of the organism in majority of the patients^[41]. Culture allows testing of the antibiotic sensitivity of *H. pylori* to choose the appropriate agent/s for eradication. Further, isolation of bacteria has enabled us to have better understanding of the pathogens and host interaction and vaccine development. Further, detailed phenotypic and genotypic characterization facilitate the understanding of epidemiologic features relative to *H. pylori*^[55-57].

Stool culture

One of the suspected routes of transmission of *H. pylori* is oro-fecal. It means the bacterium is excreted through feces and it should be isolated from stool specimen. However, it has been found really difficult to isolate *H.*

pylori from stool specimens. The explanations given to this poor isolation are: *H. pylori* is susceptible to biliary salts^[58] and there is a great competition with other numerous bacteria present in the stool. It may also be possible that the bacterium goes into viable but not culturable form and we have still to find out the triggering functions for reverting it back to culturable form. The first culture of *H. pylori* from stool sample have been reported by Thomus *et al*^[52] from malnourished children belonging to under developed countries. Dore *et al*^[59] have reported successful isolation of *H. pylori* after treating with bile sequestering agent cholestyramine before plating on culture medium. However, the similar looking colonies *H. pylori* must be checked by molecular methods to confirm of them being really *H. pylori*. Since Namavar *et al*^[60] and our own experience (unpublished data) have indicate that phenotypically *H. pylori* like other bacteria are also present in the gastrointestinal tract. Therefore, till we really get success in isolating the bacterium from stool samples with satisfactory sensitivity, *H. pylori* isolation from stool not is used as routine diagnosis test.

PCR

PCR is used not only for the detection of bacterium but also for characterization of pathogenic genes and specific mutations associated with antimicrobial resistance. The conserved genes used for detection of *H. pylori* are urease operon: *ureA*^[61] and *glmM*, also known as *ureC*^[62], or the 16S rRNA^[63,64], 23S rRNA^[65,66] and *Hsp60*^[67] gene. It is necessary to know the DNA sequence of the target gene in as many strains of *H. pylori* and other related bacterial species as possible for designing specific primers. The highly conserved 16S rRNA gene in bacteria exhibits sequences which are shared by different species of *Helicobacter*. The 109 bp amplicon specific to *H. pylori* 16S rRNA has also been observed to be amplified sometimes in human tissues^[68], thus compromising its relevant diagnosis of *H. pylori*. Other genes with unknown function, *i.e.*, 26-kDa protein; identified as *ssaA*^[69], or random sequences have also been used^[56].

Since the introduction of string test for collection of gastric juice, *ureA* gene have been targeted in this easily available specimens with good sensitivity and specificity^[70-73]. The *cagA* gene with has also been target in Taiwan as the virulent gene is quite prevalent in for east countries^[74]. However, all the strains may not have *cagA* gene resulting into decrease in sensitivity. Since, *H. pylori* is usually restricted to oral-gastrointestinal tract, PCR is not performed in blood or serum samples. But there are reports showing the presence of *H. pylori* specific DNA in these specimens, targeting genus specific gene (C97 and C 98) and conserved region of *vacA* gene. Detection of *H. pylori* DNA in blood sample is really very surprising^[75]. Moreover, PCR based techniques have been very successfully used in specimens of stool and saliva^[76].

There are many modifications of the PCR technology for increasing the sensitivity of detection. The use

of nested or seminested PCR has been suggested using internal primer targeting conserved gene (heat shock protein; *Hsp60*) and increasing specificity and sensitivity up to 100%^[67,77]. Alternatively second PCR may also be performed using the same primers^[78]. Perkins *et al*^[79] also concluded that PCR was a more sensitive diagnostic procedure than culture techniques in the detection of *H. pylori* infection status post therapy in cats. If single step PCR technique is used, it detects 70 bacterial cells in the biopsy sample^[69]. Further, the primer pairs for the nested protocol used was able to detect as little as 30 ng of template DNA on primary amplification and 1 fg (corresponds to approximately 3 organism) after the nested cycles of amplification^[67].

By using specific probe for the amplicon detection can increase the sensitivity of detection as lower copy number can be detected by this method as compared to standard gel electrophoresis^[80,81]. New methods like liquid phase (DNA-enzyme immunoassay)^[82,83] and the reverse dot blot line probe assay (LiPA)^[84] have also been proposed to increase its specificity and sensitivity.

There are two major hurdles in the performance of PCR: Inhibitors of polymerase enzyme are present in the biological samples^[85] and another is possibility of contamination during the process of collection of specimens to amplification and documentation. To take care of PCR inhibitor, special kits are commercially available and have been found to be better than simple boiling^[86]. Very stringent precautions should be followed proper sterilization if endoscopes to performing the DNA isolation and amplification. The gel documentation of PCR amplicon is other source of contamination. Therefore, three different physically separated chambers should be used for above 3 procedures along with the separate micropipettes sets and change of gloves at every step wherever, chances of contamination is suspected.

Specificity of the PCR based detection of *H. pylori* is another issue especially in the biological samples collected from sites other than stomach. Rocha *et al*^[87] and Cirak *et al*^[88] suggested that any specimens should be designated positive for *H. pylori* when there is amplification of two different conserved target genes. On the other hand seminested or nested PCR may be used to resolve the issue of specificity. Our laboratory experience also approves the use of two different gene amplification and nested PCR. We have observed primary PCR targeting *Hsp60* gene amplified *Pseudomonas* species in antral biopsy also^[15]. However, nested primers amplified the specific sequences of *H. pylori* only. There are reports suggesting use of reverse transcription PCR (RT-PCR) successfully show the viability of the bacterium^[80,89-91]. RT-PCR is based on mRNA which determines that bacterium is in viable state, but no improvement in sensitivity has been shown.

The PCR technology may also be used to target pathogenic genes of *H. pylori*. There are two major segments implicated in pathogenesis: *cagPAI* and polymor-

phic *vacA* gene apart other virulence genes involved in adherence (*babA2*, *sabA*) or in pathogenesis (*oipA*, *dupA*, *iceA*). These genes can be targeted to assess the virulence potential of *H. pylori* in a particular individual. There are several reports stating that cagPAI possessing strains do cause more severe peptic ulceration and extra digestive diseases. This pathogenicity island has also been found to be associated with higher chances of development of precancerous lesion^[92] and gastric adenocarcinoma^[93]. There is mosaicism in the allelic distribution in *H. pylori*. The strain containing s1m1 allele of combination has been found to associate with higher production of toxin^[94]. However, *H. pylori* with *vacAs1* has been reported with higher toxin production^[84,95-99]. In this regard our laboratory findings suggest that gastric mucosa is colonized by mixed strains rather than single virulent type (unpublished data).

PCR is also used for quantification of genomic DNA of *H. pylori*. Real-time PCR is being used conventionally to quantify the *H. pylori* DNA in biopsy specimens. But Real time PCR may not be as sensitive as nested PCR and as it is usually based on the commercial kits, it may be more expensive especially when two genes are targeted.

Conventional single round PCR method to detect *H. pylori* especially after eradication therapy could not detect pathogens but it can be done by nested PCR protocol as the former methods are relatively poor in detecting the low numbers. Therefore, nested PCR may be proposed as the gold standard provided that the PCR contamination is being taken care off. Moreover, while RUT, UBT, histology cannot discriminate the re-infection or recrudescence, PCR based method is able to indicate either of the two possibilities because finger printing of the strains can be done by the amplification of different target genes of the bacterium.

Duś *et al*^[100] in their review article stated that PCR detects *H. pylori* in both forms *i.e.*, spiral or coccoid forms; that cannot be detected by other conventional diagnostic methods. However, the low sensitivity of specific culture method makes it difficult to evaluate the molecular methods. PCR was also used to detect *H. pylori* in various sites other than stomach. The bacterium could be detected from patients with appendicitis^[101], in ethmoid specimens from patients with chronic sinusitis^[102], in upper respiratory tract and oral cavity^[88]. *H. pylori* like organisms could also be detected by PCR in liver specimens reported by Rocha *et al*^[87]. Another advantage of PCR is that DNA does not require strict transport conditions, and it can be performed on specimens of urease tests sent by posts^[103-105]. Detection of *H. pylori* from formaldehyde fixed paraffin embedded material by using PCR is a quite satisfactory approach. Unfortunately, fixed specimens are not suitable for DNA isolation compare to frozen material. PCR generating short PCR products has found better in frozen/preserved materials because genomic DNA might be broken due to fixatives. Moreover, PCR can also be used to genotype the *H. pylori* isolates^[106]. Therefore, PCR based methods are the best for the specimens col-

lected by invasive methods if the test is carried out with utmost care.

In principle stool sample should be considered as one of the best sample collected by non-invasive methods for detection of *H. pylori* by PCR technology. But the earlier reports were not encouraging because of low sensitivity of detection^[107,108]. The possible reason for this poor sensitivity may be low copy number of target DNA, presence of PCR inhibitors in stool samples and single round of PCR amplification. However, PCR% inhibition could have been taken care by special techniques of DNA isolation, immune magnetic separation of DNA^[109]. But the problem here with stool samples is that the usual coccoid form becomes less antigenic^[110]. Nowadays commercial kits are available to get rid of PCR inhibitors from DNA preparation to *H. pylori* amplification in stool samples^[111].

Kabir^[112] has reviewed well on different DNA extraction methods from stool samples to reduce PCR inhibitors. The other option to care off PCR inhibitors is amplification by nested or seminested PCR. The internal amplification care of PCR inhibitors present during the first round PCR.

Nested PCR was carried out by Mishra *et al*^[77] and reported that 62.5% patients were positive for *H. pylori* targeting *Hsp60* gene. Nested PCR protocol targeting *Hsp60* gene seems to be good alternative as a non-invasive method for detecting *H. pylori* infection in fecal specimens where invasive test like endoscopy is not feasible. Several studies have been carried out to diagnose *H. pylori* targeting 16S rRNA, 26-kDa antigen, *Hsp60*, *ureA*, *glmM*, *vacA* and *cagA* gene. The development of a commercial test to detect *H. pylori* will undoubtedly provide an accurate and convenient diagnostic method^[113].

UREA BREATH TEST

Use of Urea breath test (UBT) is often considered as the gold standard test in the diagnosis of *H. pylori* infection^[114-116]. UBT consistently produces better results in comparison to many of the other available tests. In this test ¹³C or ¹⁴C labeled urea is fed to patient where in stomach is broken down by urease enzyme produces by *H. pylori* if present in the stomach. The released radioactive ¹³CO₂ or ¹⁴CO₂ diffusing in the blood released in the lungs. Here, the expired air is collected to measure the activity of labeled Carbon. Currently, improved infrared spectrometers have shown which has the extra advantage of low cost also as compared to mass spectrophotometer^[117]. Although, there is still no uniform protocol for the performance of the test but availability of certified tests have provided certain uniform results. Gisbert and Pajares^[118] have published a very good review about the UBT. In most of the studies, the sensitivity and specificity of the UBT exceed 90%^[118]. UBT is used to evaluate eradication therapy after giving anti *H. pylori* regimens. The sensitivity is quite good in post therapy and it may be explained by the fact that the UBT gives positive results when other biopsy based tests fail. It is sensitive enough

in detecting the infection even in cases of moderate colonization or patchy distribution of *H. pylori*. However, false positive results due to the presence of other urease producing microorganisms are sometimes expected as it has been established that *H. pylori* is not the only bacteria colonizing stomach. However, proton pump inhibitors anti *H. pylori* drugs (PPIs and antibiotics) may produce false-negative results. Further, metabolically inactive coccoid form of *H. pylori* present in the stomach will not give the positive UBT. The other advantage is that UBT could be also used in pediatric patients^[119].

Despite many good features of UBT, it has certain shortcomings also. UBT has been observed with poor sensitivity in patient's undergone gastric surgery or patients on drugs causing changes in neutral pH of gastric mucosa. Further, dosage of radioactive carbon also influence the performance as 15 mg dose has been reported to be better in sensitivity as *H. pylori* eradication than 10 mg of ¹³C^[120,121]. Additionally, carrier solution of urea also affect the performance because citric acid solution drink has been observed superior in performance than orange juice and water^[122]. In another study, it has been found that citric and malic acid based urea meals performed better than ascorbic acid^[123,124]. The next important issue with UBT is to decide cutoff point between positive and negative ¹³C-UBT test^[125]. Many workers have suggested some alternations to UBT and one of them is estimation of blood urea for the diagnosis and assessment of *H. pylori* eradication^[126]. In patients where biopsy is contraindicated real time ¹³C-UBT test can be done during gastroscopic examination. Although, Zagari *et al*^[127] have shown a positive correlation between UBT values and activity of gastritis, the other group (Tseng *et al*^[128]) found insignificant difference of UBT values in patients with gastritis, duodenal ulcer, gastric ulcer and gastric malignancy.

SEROLOGICAL/IMMUNOLOGICAL/ ANTIGEN-ANTIBODY DETECTION TEST

Antibody detection

Immunoblot analysis of serum from *H. pylori* infected patients has revealed that there are several antigens capable of inducing immune response. Some of the prominent antigen includes surface and secretory antigens lipopolysaccharides (LPS), cagA, different urease components, heat shock proteins, catalase *etc.*^[129-133]. Basically *H. pylori* infection is a chronic condition and therefore IgG response predominates^[134]. It is really difficult to pin point acute *H. pylori* and IgM response has rarely been reported. Although, IgG response primarily occurs at mucosal surfaces, it can be detected in all elevated in majority of the patients. Further, since in niche of the bacterium is a mucosal surface, only modest immune response is expected. This necessitates for need of good quality antigen and proper cut off value should be determined in endemic areas. Further, it has been established that *H. pylori* is a panmictic bacterium which leads to antigenic vari-

able strains colonizing the human population in different geographical areas^[135-138]. In support of this statement already diverse antibody profile by immunoblot have been reported^[139]. This is why there is suggestion that local strains should be used for preparing the kits for detection of *H. pylori* specific antibodies rather than foreign strains^[140,141]. The other alternative suggested is to find out some common antigens shared globally or strains are pooled from different region for preparing diagnostic antigens^[142,143]. Further, local titres should be decided rather than what is recommended by the manufacturer located somewhere else.

The studies have shown the sensitivity and specificity of serological methods for detection of *H. pylori* infection ranging between 80%-90%. But, these observation must be seen in the light of fact that host immune response varies from individual to individual and also duration of exposure, nutritional status, cross antigenicity with other prevalent antigenically related bacteria *e.g.*, *Campylobacter etc.* in endemic area. However, in difficult situations, where bacterial density is low due to gastric atrophy or therapy using PPI and/or antibiotics, serological methods are useful. Moreover, rapid test methods for *H. pylori* specific antibody titer detection can be used at point of care. Although initially in 1994, this rapid method gave sensitivity of 92% and specificity of 88%, more than 30 later studies could not show the satisfactory level of performance using several kits. Immunoblot assay is another alternative with better specificity but sensitivity in not higher than standard ELISA^[139,144]. As immunoblot assay involves high cost and expertise in interpretation, it is not widely used in clinical laboratory. However, immunoblot can be used as confirmatory test as a second step^[144]. The most important point which goes against serological test is that active or cured infection of *H. pylori* cannot be differentiated because it has been found that antibody levels persist for long even after cure^[145].

With all the advantages and disadvantages of serological methods already discussed researchers tried to look for anti *H. pylori* antibody titer in urine also detected by ELISA and immunoblot^[146]. It has been observed that presence of bacteria and variation in pH usually does not affect the detection *H. pylori* specific IgG. The other advantage is that looking for *H. pylori* specific antibody is definitely absolutely non-invasive, easy, rapid and inexpensive way to diagnosis the infection. But the major problem is that *H. pylori* specific IgG are excreted in very low concentration in urine. Further, high level of non-specific IgG in urine may give false positive and low concentration of IgG may give false negative results^[147,148].

Saliva

Saliva is the other specimens which can be collected non-invasively and excretion of IgG and IgA is known. There are studies which have shown that while *H. pylori* specific IgA is unable to distinguish between infected and non-infected individuals, IgG detection may be help in making diagnosis^[149-151]. Although, specificity of the anti *H. pylori*

IgG detection can be improved by immunoblotting, sensitivity is quite low and rarely reaches 90%^[152-154]. However, better results have been reported in cases of high serum titer and in children above 5 years of age^[155,156].

Antigen detection

H. pylori antigen in stool specimens has been detected successfully for the first time in 1997. Using polyclonal antibodies, the sensitivity and specificity have been found to be 88.8% and 94.5% respectively^[157]. In another study concluded by Vaira *et al.*^[158] could observed the sensitivity of 94.1% and specificity of 91.8%. The advantage of antigen detection test is to evaluate the eradication of *H. pylori* infection. However, if concentration of antigen becomes low, false negativity may also be reported. Perri *et al.*^[159] compared the performance of antigen detection *vs* UBT in 458 dyspeptic patient and reported discrepancy in 8% of the case. They suggested that antigen detection was less accurate than UBT^[159]. Later a new generation of stool antigen kits has been developed using monoclonal antibodies giving comparable accuracy as that of UBT.

Despite all the above observation on performance of antigen detection *H. pylori* in stool, it has certain disadvantage: antigen excretion may vary over the time period and antigen may degrade while passing through intestine. Further, use of N-acetylcysteine like mucolytic agent may decrease the accuracy of the diagnosis^[160]. Cut off titer, though difficult to decide but crucial to reach the conclusion by using antigen detection technique. However, stool antigen detection using monoclonal antibody has been recommended by EHSg as it gives equivalent diagnosis accuracy to UBT^[161].

DETECTION OF *H. PYLORI* IN THE ORAL CAVITY

It has been reported that oral cavity may be only transitory reservoir as *H. pylori* is coming here due to regurgitation or vomiting. There are reports showing good isolation rate from oral cavity^[162] but the isolates were never confirmed by DNA based method of their being *H. pylori*. Some workers have shown very low isolation rate from oral cavity^[53,163,164].

PCR based studies in detection of *H. pylori* in oral cavity (saliva/dental plaque) have shown variable report since many uncharacterized bacteria closely related to *H. pylori* are present in oral cavity and the pit of the gastrointestinal tract, the results should be considered positive when at least two targeted conserved genes specific to *H. pylori* give expected amplification.

WHAT TEST SHOULD BE GOLD STANDARD?

The choice of diagnostic tests to determine *H. pylori* infection status depends on the sensitivity, specificity, reproducibility, availability cost, and rapidity of the re-

sults^[26]. There a need for a reference method to be used as “gold standard” to find patients truly infected. Unfortunately, none of the currently used methods is able to further this criterion. One solution is to combine the results of two or more techniques, and compare with results of each method being evaluated. PCR may be slightly superior as compared to other diagnostic methods for detection of *H. pylori* infection and to verify *H. pylori* eradication after treatment^[26,165]. PCR is regarded as a highly sensitive method to detect DNA of *H. pylori* from different clinical samples^[110,166,167] and provide useful information concerning the presence of genes encoding specific virulence factors and antibiotic resistance^[168]. Both sensitivity and specificity of nested PCR has been reported to be 100%^[67]. In contrast, the sensitivity and specificity of serological, urea breath, fecal antigen, rapid urease tests, histopathology, PCR and culture have been found to be 85% and 79%, 75%-100% and 77%-100%, 67%-100% and 61%-100%, 75%-100% and 84%-100%, 66%-100% and 94%-100%, 75%-100% and 84%-100% and 55%-56% and 100% respectively. The sensitivity of PCR can be increased by performing nested or semi-nested approaches. However, such approaches might increase the possibility of false positive results caused by crossover contamination as well as detection of DNA from dead bacteria^[26]. The low positivity rate of the culture (69%) may be due to a low number of organisms, presence of non culturable coccoid forms^[169,170], absence of microorganisms in the gastric biopsy specimens, loss of viability during transport, fastidious growth requirements or contamination by other bacteria suppressing the growth of *H. pylori* or antibiotic intake. For these reasons, percent agreement/disagreement was preferred to compare culture results with results of corresponding samples by the other diagnostic methods. The so called false positives with real time or nested PCR may not be true false positives, rather the results obtained by the relatively less sensitive culture method may be false negatives^[40]. Although, PCR may be used as gold standard, provided that the chances of contamination are taken care off, the method to be used should be proposed on the basis of level of available diagnostic facilities. If endoscopic facility is not available in periphery or underdeveloped regions, diagnosis by SAT using monoclonal antibody based kits may be applied on stool specimens. In situation where UBT system is available but endoscopic facility is not available then this test should be considered the best option. Although, serology is often misleading but it may be the best in children where sanitary conditions are satisfactory and prevalence of the infection is low especially in pediatric age group. Therefore, utility of each of the invasive and non-invasive tests are almost equally important depending upon the given clinical situation.

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