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Diagnosis of *Helicobacter pylori*

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There are numerous well-tried methods for the diagnosis of *Helicobacter pylori*. Patients should avoid antibacterial agents for 2–4 weeks prior to direct testing lest a false negative result be obtained. Direct tests include biopsy for Gram stain, culture, rapid urease test and histology, urea breath test, stool antigen test and various molecular methods on gastric material. Indirect (antibody-based) tests are also very accurate for initial diagnosis but are difficult to interpret in the previously treated patient. In most cases, the combination of a sensitive and a specific test (for example urea breath test with serology) results in high accuracy with minimal cost.

Recent advances have been based on new molecular-based techniques such as polymerase chain reaction (PCR) which yield quicker results but have been difficult to introduce into the routine diagnostic laboratory.

Key words: urease test; culture; antibiotic sensitivity; microbiology; histology; serology; antigen; antibody; urea breath test; PCR.

The diagnosis of *Helicobacter pylori* has evolved rapidly since the original isolation of the organism (Warren and Marshall, 1983). For several years

endoscopy was always necessary because Gram stain of mucus smears, culture and histology were the only available detection methods. The rapid urease test still required a biopsy but at least it allowed gastroenterologists to diagnose *H. pylori* rapidly with little expense. Serology allowed initial diagnosis by any physician, but follow-up after treatment was still difficult to do. Management was simplified by the urea breath test which determined the presence of active infection, and was as accurate as the invasive biopsy methods. More recently, molecular methods have been developed which allow us to study the virulence and pathogenicity of *H. pylori* without the tedium of long (5-day) culture times.

This chapter introduces the reader to a comprehensive array of methods for *H. pylori* diagnosis. The *H. pylori* novice should use it as a starting point and is advised to obtain more detail from the references used.

DIRECT AND INDIRECT METHODS FOR DETECTION OF *HELICOBACTER PYLORI*

Helicobacter diagnosis can broadly be described as direct and indirect, invasive and non-invasive. Invasive tests rely on the detection of the bacteria or its products in a gastric specimen collected during an endoscopy. For example, the presence of *H. pylori* can be demonstrated by culture or histological examination of biopsied tissue (Jones et al, 1984; Marshall et al, 1985) (direct and invasive), the urease activity test using Christensen urea broth (Christensen, 1946; McNulty and Wise, 1985; Hazell et al, 1987) (direct and invasive), by the detection of specific serum antibodies (Steer and Newell, 1985; Jones et al, 1986; Morris et al, 1986) (indirect, non-invasive) or by the urea breath test (UBT) (Bell et al, 1987; Graham et al, 1987) (direct, non-invasive). Newer methods using non-invasive techniques include the string test (Patchett et al, 1995; Perez-Trallero et al, 1995) and the detection of *H. pylori* in faeces and saliva (Thomas et al, 1992; Ferguson et al, 1993). It is also possible to detect *H. pylori* indirectly by targeting certain antibodies (Steer and Newell, 1985) and/or DNA (Lin et al, 1996a; Westblom, 1997). These methods have proven to be quick, sensitive, specific and in most cases cheaper than traditional invasive diagnostic methods. There may never be one definitive diagnostic test for *H. pylori*; instead, the use of more than one test may be more suitable. The accuracy of these invasive and non-invasive tests has been compared and the results are summarized in Table 1 (Cutler et al, 1995).

NOTES FOR THE ENDOSCOPIST

Patient preparation

As with any microbiological diagnostic test, thoughtful preparation of the patient is necessary lest a *H. pylori* infection is missed or a *H. pylori*-negative patient is needlessly subjected to antibiotic therapy.

Table 1. Accuracy of diagnostic tests: sensitivity, specificity, negative predictive value and positive predictive value of seven diagnostic assays for *H. pylori* infection among 268 Detroit patients undergoing oesophagogastroduodenoscopy.

Parameter value (%)	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Invasive				
Biopsy: chronic Inflammation*	100	66.3	84.4	100
Biopsy: acute Inflammation†	86.7	93.7	96.2	79.5
Biopsy: Warthin–Starry silver stain‡	93.1	99.0	99.4	88.7
CLOtest™ Rapid Urease test§	89.6	100	100	84.1
Non-invasive				
^{13}C Breath test¶	90.2	95.8	97.5	84.3
Serum IgG‖	91.3	91.6	95.2	85.3
Serum IgA‖	71.1	85.3	89.8	61.8

* Chronic inflammation present in gastric antral biopsies.
† Acute inflammation present in gastric antral biopsies.
‡ Warthin–Starry stain of gastric antral biopsy.
§ Urease test conducted on gastric antral biopsy with results ascertained at 24 hours.
¶ [^{13}C] urea breath test 60 minutes after administration of 150 mg ^{13}C-labelled urea.
‖ Serum antibodies to *H. pylori*.

Direct tests (histology, urease, UBT) will fail to detect very low numbers of *H. pylori*, so medications which suppress the organism should be ceased well before gastric mucosal samples are taken. Inhibitory medications of concern are bismuth (Pepto-Bismol, De-Nol), antibiotics and proton-pump inhibitors (PPI). H2 receptor antagonists (H2RA) do not usually inhibit *H. pylori*, so patients taking PPI may be switched to H2RA for the week prior to endoscopy. After attempted eradication of *H. pylori*, it is usual to wait 28 days before repeating tests to confirm eradication.

Taking biopsies

It is common-sense that collecting extra biopsies will improve the detection rate for *H. pylori*. However, each pass of the biopsy forceps adds two minutes to the procedure time.

In patients with non-ulcer dyspepsia, there may be little disadvantage to a failed diagnosis of *H. pylori* so a rapid urease test may be a sufficient and cost-effective screening test.

In patients with complicated peptic ulcer disease or high cancer risk, detection of *H. pylori* is essential for optimal management. Therefore, a rapid urease test and/or multiple biopsies for histology are required (total three or four biopsies). If the patient has been treated before and may have failed therapy, a culture of *H. pylori* should be added to the above tests (total four biopsies). In this situation it is now possible to replace endoscopic follow-up with a UBT 4 weeks post-therapy.

For research studies the following specimens are recommended: rapid urease test (one antrum), histology (two antrum, one corpus), culture (one antrum).

False-positive and false-negative rates

The predictive value of each diagnostic method determines its usefulness in various populations. If most patients are *H. pylori*-positive, then a sensitive test is required. Where most patients are *H. pylori*-negative, a specific test is required. To achieve almost 100% diagnostic accuracy one may conveniently combine a sensitive and a specific test. In my experience, a CLOtest® rapid urease biopsy test (positive predictive value 98%) can be used in conjunction with a sensitive serological test (negative predictive value 95%).

If two tests are used, it is best that different types of test be chosen. In the above example the rapid urease test could be replaced by any direct method (histology, culture or UBT). If two direct methods are used, low numbers of *H. pylori* may go undetected by both tests (the sensitivity is not additive).

RAPID UREASE TEST

H. pylori possess many enzymes, which assist in its survival in the gastric mucosa (Nilius and Malfertheiner, 1996). Of these, urease (urea amidohydrolase, EC 3.5.1.5) stands out as the most significant, not only for its ability to hydrolyse urea to form a basic micro-environment for *H. pylori* to survive in the gastric mucosa (Mobley, 1996), but also as a means of identifying the organism in gastric biopsies. *H. pylori* produces an extraordinarily high amount of urease (Langenberg et al, 1984) and it has been estimated that this enzyme represents over 5% of the total cell protein (Hu and Mobley, 1990). There are no other known urease-producing bacteria that inhabit the gastric mucosa, therefore it is possible to detect the presence of *H. pylori* in gastric biopsies by the detection of urease (Marshall et al, 1987). The initial urea medium used was the Christensen's urea media (Christensen, 1946), a colorimetric assay medium designed to detect the urease enzyme. This enzyme chemically hydrolyses urea to yield ammonia with a net effect of an increase in pH of the solution, which is indicated by a change in colour of the indicator (Hamilton-Miller and Gargan, 1979); hydrolysis is as follows:

$$H_2NCONH_2 + 2H_2O + H^+ \leftrightarrow 2NH_4^+ + HCO_3^-$$

There have been many modifications to Christensen's urea media, in order to improve the sensitivity and specificity (Boyanova et al, 1996). There are three urease tests that are commercially available: CLOtest®, Hpfast® and Pyloritek®. All three tests are quick, highly sensitive and specific in

comparison to culture, histology and polymerase chain reaction (PCR) highly sensitive and specific (Laine et al, 1996). Rapid urease tests are highly specific to *H. pylori*, however, under certain circumstances they fail to be sensitive enough. This is due to several factors such as density of *H. pylori* in the gastric mucosa and, most importantly, the patchy distribution of *H. pylori* in the stomach (Kohli et al, 1993). As a result, the density of *H. pylori* is different between biopsy samples taken from different sites (Bayerdorffer et al, 1989). Recently this problem has been overcome by performing PCR on biopsy samples placed in the CLOtest® in order to detect *H. pylori* (Lin et al, 1996b). Using primers targeting *H. pylori*-specific genes, PCR is sensitive and specific for the detection of *H. pylori* (Valentine et al, 1991; Clayton et al, 1992; Fabre et al, 1994).

DIAGNOSIS OF *H. PYLORI* USING CULTURE

Culture from biopsy specimens

Although many gastroenterologists do not send biopsy specimens to the bacteriology laboratory for culture of *H. pylori*, this method of diagnosis is still one of importance, especially for determining antibiotic sensitivities and for epidemiological studies.

H. pylori is a Gram-negative spiral-shaped bacterium with fastidious nutritional requirements. In 1982 the organism was originally isolated from gastric biopsies on non-selective chocolate agar and in microaerobic conditions developed for *Campylobacter* species (Warren and Marshall, 1983) but since then various improved media have been described for transport and isolation.

Transport and storage

Although *H. pylori* will survive in water and moist areas for several hours, atmospheric oxygen is toxic to fresh isolates of *H. pylori* on gastric mucosa. Therefore the biopsy specimens must be transported to the laboratory as quickly as possible.

The organisms will remain viable in 0.5 ml physiological saline for up to 6 hours, but if a longer delay is unavoidable, Stuart's semi-solid transport medium is recommended (Stuart et al, 1954). Optimal recovery is achieved if the specimens are kept below 15°C for 4–5 hours, but for longer periods they need to be held at 4°C (Soltesz et al, 1992). Alternatively, the biopsy can be frozen at –80°C in Brucella or brain heart infusion (BHI) broth plus 15% glycerol, or snap-frozen in liquid nitrogen and thawed just prior to culturing (Megraud, 1996).

Culture media used

In the laboratory, the biopsy tissue is aseptically macerated in 0.5 ml saline

with a sterile scalpel blade or homogenized with a tissue grinder. A few drops of this cellular mixture are plated onto at least two agar plates—one selective and one non-selective. Numerous different media have been proposed for the isolation of *H. pylori* during the past 15 years (Goodwin et al, 1985; Buck and Smith, 1987; Queiroz et al, 1987; Dent and McNulty, 1988). In general, researchers have concluded that the highest rate of isolation is achieved when at least one selective and one non-selective medium is used (Ansorg et al, 1991; Tee et al, 1991; Piccolomini et al, 1997). All of these media have a rich agar base (Columbia, BHI or Brucella) with either the addition of sheep or horse blood, or serum. Usually a vitamin supplement such as IsoVitalex or Yeast extract is also needed. Some authors have suggested the use of media without blood products and have proposed culturing *H. pylori* on plates containing charcoal (Glupczynski et al, 1989) or egg yolk (Westblom et al, 1991). The antibiotics most commonly added to the selective plates to inhibit contaminants are vancomycin, trimethoprim and the antifungal agent, amphotericin. The plates should be as freshly made as possible (preferably less than 2 weeks old).

Incubation

The inoculated plates are incubated at 37°C in a microaerobic atmosphere of between 5 and 10% O_2 5 and 10% CO_2, and 80 and 90% N_2 with a relative humidity of at least 95%. This can be in jars filled directly with the mixture of gases described above, with gas-generating sachets added, or even a candle jar, but all of these must have moist filter paper in the base to maintain a high level of humidity. If a large amount of *Helicobacter* work is being done, a CO_2 incubator set at 10% CO_2 and 95% humidity can be used exclusively for *Helicobacter*. Colonies of *H. pylori* appear on the surface of the agar after 3–7 days (some say that if the patient is being investigated post-treatment then incubation should be longer, even as long as 10–14 days) (Glupczynski, 1996).

Identification

On blood-based plates, colonies are usually small (1–2 mm) and transparent in colour. Some of the newer media have triphenyltetrazolium chloride as an additive which gives the *H. pylori* colonies a characteristic yellow golden colour which aids the differentiation from other commensals (Queiroz et al, 1987; Glupczynski et al, 1989; Westblom et al, 1991).

Once cultured, the identification of *H. pylori* is based on cellular morphology using either a Gram stain or phase-contrast microscopy. The organisms appear as curved or comma-shaped Gram-negative rods and spiral or helical shapes are less evident. Positive urease, catalase and oxidase reactions are needed to confirm that the organism is *H. pylori*. Care must be taken to ensure that colonies of contaminants that appear similar do not have the same three biochemical characteristics listed above.

Differentiation from other organisms, including other campylobacters and helicobacters

Helicobacter pylori grows best at 37°C and forms discrete transparent colonies that seem like water droplets on the surface of blood agar after 3–4 days. The multi-spiralled helicobacters such as *H. felis* tend to grow more slowly and form a film over the plate which is often difficult to see except with oblique lighting. *H. mustelae* grows best at 42°C and rather poorly at 37°C.

Broth culture

Growth in liquid culture can be used if a large amount of *H. pylori* or its cytotoxins are required but is seldom used in diagnosis. Similar rich basal media such as Brucella and BHI supplemented with 3–10% fetal calf or horse serum is used. Broth culture requires good gaseous exchange which is achieved by continuous agitation in a thin layer culture, for example, a small volume in a conical flask (Goodwin and Worsley, 1993) or gas-permeable tissue culture flasks (Secker et al, 1991), in anaerobic jars with gas packs or in a CO_2 incubator. Recent research has shown that human serum is superior to horse or bovine serum in promoting growth of *H. pylori* in broth (Westblom et al, 1995). A defined medium has now been developed for *H. pylori* which has allowed the determination of its amino acid requirements (Reynolds and Penn, 1994) and will enable researchers to study its growth patterns further.

Non-invasive specimen collection for culture

Endoscopy for the collection of gastric biopsies is invasive, expensive and not free of discomfort for the patient. The swallowed string test (Perez-Trallero et al, 1995) or culture of *H. pylori* from faeces (Thomas et al, 1992) or saliva (Ferguson et al, 1993) offer alternative sources for the collection of specimens to culture *H. pylori*.

The string test involves the patient swallowing a length of nylon string, pulling it back up and the string being sent to the laboratory for culture and urease testing. Culturing *H. pylori* from faeces has been reported but it is not widely used because the isolation rate is low and only a handful of laboratories around the world have had success using it.

Sensitivity testing: its role and correlation with clinical outcome

It is well known that *H. pylori* rapidly becomes resistant to metronidazole and ciprofloxacin during therapy with these drugs. Similarly, macrolide resistance is found in most (50–90%) isolates that survive treatment with clarithromycin.

The cure rate for second therapies is usually less than 50% when *H. pylori* has developed resistance to one of the treatment components (especially metronidazole). For this reason, several investigators have

seen a good correlation between treatment failure and in vitro resistance.

Helicobacter pylori almost never becomes resistant to amoxicillin, tetracycline or bismuth. Thus, sensitivity to these agents can usually be assumed. Metronidazole sensitivity can be performed using Kirby–Bauer 10 mm discs containing 5 μg metronidazole. Zone sizes >7 mm radius warrant use of metronidazole. Similar testing can be used for clarithromycin or (if clarithromycin discs are not available) erythromycin. It has been said that slow-growing organisms do not give reliable results when disc testing methods are used. For this reason the 'E-test' has been recommended as a more quantitative means of measuring the MIC of *H. pylori* (Glupczynski et al, 1991). Interpretation is usually simple, although the process takes at least 14 days because several growth cycles of *H. pylori* are required. The reliability of disc diffusion tests and even the E-test has been challenged and has been claimed to be responsible for the great variation in test results even seen in one country. Agar diluting methods are considered to be the standard for testing of metronidazole. Currently the FDA is drafting standard protocols for the sensitivity testing of *H. pylori* which should alleviate these problems (L. Utrup, personal communication). Rapid determination of antimicrobial sensitivity can be carried out by performing primary isolation onto a multiwell plate containing various concentrations of antibiotic (Vasquez et al, 1996).

HISTOLOGICAL DIAGNOSIS

Biopsy methods

The technique used for taking biopsies for *H. pylori* diagnosis can affect the accuracy of the test. If the pathologist has large or many pieces of tissue to examine, accurate diagnosis is easier. Therefore, it has been recommended that 'jumbo' biopsies be taken (Engstrand et al, 1997). Since these are not the norm outside research studies, the endoscopist should attempt to take the largest biopsy possible with the available equipment. To do this, the forcep is exposed only 1–2 cm from the end of the endoscope, opened, and directed perpendicularly into the stomach wall by bending the endoscope. Before closing the forcep, air is sucked from the stomach to relax the gastric mucosa and allow it to fold into the jaws of the forcep. This technique allows good-sized biopsies to be taken in all areas of the stomach and is also very useful for examination of the duodenum and oesophagus.

The highest detection rate is achieved by biopsying the incisura (angle of the lesser curve). Almost 100% detection can be achieved by sending two antral biopsies (one from the greater and one from the lesser curve) and one corpus biopsy (greater curve) for histological examination.

Biopsies for assessment of gastritis and *H. pylori* should be taken away from, and in addition to, visible lesions. This is because all erosions and ulcers have an inflammatory response present in their margins as part of normal healing, even when the lesions are caused by NSAIDs. Thus, to use

active or chronic gastritis as an indicator of *H. pylori* infection, the pathologist must examine biopsy specimens from normal-appearing mucosa. Inflammation in this tissue will trigger a careful look for the bacterium, whereas normal histology rules against *H. pylori*.

Helicobacter pylori and gastritis may not be uniformly present in all areas of the stomach. Therefore, special stains should always be used when gastric biopsies are being examined. *Helicobacter pylori* may be present on normal mucosa when it contaminates the specimen from nearby inflamed mucosa.

Location and appearance in sections

Anatomy

The normal gastric antral mucosa consists of a loose lamina propria with very few inflammatory cells. The epithelium in the antrum is folded into mucus-secreting glands. The epithelial cells are of gastric mucus type, supposedly having an alkaline mucus that takes up the periodic acid–Schiff (PAS) stain. The major distal parts of these columnar cells are filled with PAS-positive mucus. There are no cilia, but sparse microvilli are usually present.

In the antrum, surface mucus cells are folded into mucus-secreting glands. Thus, *H. pylori* can live within the mucosa as well as on its surface. As a result, *H. pylori* is more numerous and may be easier to detect. Similarly, inflammation in the antrum is more likely to be deep.

In the corpus, the lamina propria is filled with tightly packed parietal cell glands (secreting acid) and the surface epithelium is flat with pits spotted over its surface. This means that *H. pylori* has relatively less mucus-cell surface area to which it can attach. As a consequence, *H. pylori* is usually less numerous in the corpus, and the inflammation is more often limited to the superficial part of the mucosa. The organism is almost never invasive, so one merely examines the surface of the gastric epithelial cells for bacteria.

Histology

Epithelial cells. When *H. pylori* infects the cells, the bacteria are visible attached to their surfaces. In addition, cells may become stunted and lose much of their intracellular mucus. The cytoskeleton of the epithelial cells is also affected such that they lose their flat 'picket fence' luminal profiles and develop a 'cobblestone' appearance. In the corpus, *H. pylori* is less numerous, and the features of cytotoxicity are usually less obvious.

Active gastritis. The lamina propria usually contains increased numbers of polymorphonuclear neutrophil leukocytes (PMNs), most typically in groups of three to five, infiltrating necks of the mucus glands. Occasional neutrophils are present in all biopsies because of those present in blood within the capillaries. The experienced histopathologist will soon spot extra

PMNs and be alerted to the possibility of *H. pylori*. Because they are an acute response, PMNs arrive very soon after the infection starts, and disappear within a few days of starting effective antibiotic therapy.

Chronic gastritis. Mononuclear cells (lymphocytes, macrophages and plasma cells) appear in the mucosa after a few days and remain (albeit in gradually decreasing numbers) long after *H. pylori* is cured. Thus, the presence of 'active chronic gastritis' is a good indicator of *H. pylori* (PMNs and monocytes are both present). However, the presence of 'chronic gastritis' is an imperfect indicator of *H. pylori* and may just signal an *H. pylori* infection several years before.

Atrophy and intestinal metaplasia. If the patient has had *H. pylori* for many years, especially in Asians, the inflamed gastric mucosa may be replaced gradually by intestinal mucosa. This is called 'intestinal metaplasia' and if widespread, the patient tends to have a smooth unfolded mucosa in which the veins beneath it are visible through the endoscope. Histologically, the mucosa may have fewer glands, and patches of chronic inflammation. Because *H. pylori* cannot attach to the intestinal-type mucosa, biopsies from patients with gastric atrophy may fail to detect the organism. If histology shows widespread intestinal metaplasia, serology should be used to exclude the infection.

Special stains

Silver (Warthin–Starry, Steiner, Genta)

These stain the *H. pylori* organisms black with a silver precipitate. *Helicobacter pylori* is actually increased in size by the silver deposit on its surface; for this reason, *H. pylori* is more easily seen. Imperfect technique, however, leads to the accumulation of many small black granules that can resemble *H. pylori*.

The Genta stain has the advantage that it also stains mucus-secreting cells so that metaplasia can also be easily identified.

Blue (toluidine blue, Giemsa)

Blue stains are almost as sensitive a detection method as the silver stains (by a percent or so), but are far simpler to do in a one-step technique. Thus, they can be prepared routinely, with little extra cost. The normal cells appear clear with pale blue membranes, and *H. pylori* appears as a blue bacterium in the clear mucus. PMNs and other cells can also be seen quite well, so this type of stain may be the simplest 'all purpose' stain.

SEROLOGY IN DIAGNOSIS

Following the isolation of *H. pylori* in 1982, a variety of serological techniques has been employed to detect antibodies to *H. pylori*. These include

haemagglutination, complement fixation, bacterial agglutination, indirect-immunofluorescence, immunoblot-techniques (IBTs) and enzyme-linked immunosorbent assays (ELISAs) (Jones et al, 1984; Newell and Rathbone, 1989; Newell and Stacey, 1989; Morris et al; 1991; Glupczynski, 1993). ELISAs have the advantage of great sensitivity, rapidity, replicability, cost-effectiveness and adaptability to most laboratory situations, whether they be high-throughput commercial laboratories or low-throughput research situations. ELISAs of diverse methodologies have contributed to our understanding of the epidemiology of *H. pylori* infections (Glupczynski, 1993; Fauchere, 1996). Various antigens have been included in ELISA kits. Typically, these should be surface exposed, highly antigenic and produced by all *H. pylori* isolates in large amounts (Glupczynski, 1993). Therefore, water-soluble whole-cell extracts have given way to highly purified specific proteins such as 'high-molecular-weight cell-associated protein' (HM-CAP), flagellar protein and native urease, which are listed in Table 2.

The rapid development of commercial serological kits for the diagnosis of *H. pylori* has resulted in the use of such kits in initial diagnosis of gastritis. However, the usefulness of a positive ELISA deteriorates in the post-treatment situation, where positive serology is not proof of current infection. A methodological vacuum exists in this area at the present time. The reason is two-fold: (a) the long duration required for the decline of antibody titres, and (b) the great range of variability in titres observed

Table 2. Antigens employed in the development of ELISAs.

Antigen used		Reference
H. pylori whole-cell sonicate	A*	Jones et al (1984), Newell and Rathbone (1989), von Wulffen (1992), Mitchell et al (1989)
H. pylori acid glycine extract		Hirschl et al (1990), Newell and Stacey (1989), Goodwin et al (1987)
N-acetylneuraminyl lactose-binding haemagglutinin (NLBH)		Evans et al (1989b)
Native urease fraction	B, C	Newell and Stacey (1989)
Flagella protein fraction		Talley et al (1991)
120 kDa protein		Hirschl et al (1990)
High-molecular-weight cell-associated protein (HM-CAP)	D	Evans et al (1989a)
120 kDa cytotoxin-associated vacuolizing factor	E	Danielli (1993)
Surface-exposed 30 kDa protein		Bolin et al (1995)

A: Urease of *H. pylori* antigenically related to that of *Helicobacter heilmanii*.
B: A component antigen used in the development of 'Cobas-Core' kit, anti-*H. pylori* ELISA.
C: A component antigen used in the development of 'Mala Kit'.
D: A component antigen used in the development of 'Premier *H. pylori*' kit.
E: Obtained following zwitterionic and non-ionic detergent extraction and of HPCCUG17874 but not present in those obtained by acid–glycine or guanidine–HCl extraction; faintly identifiable in Triton X-405 extracts.
* Cross-reactive with flagellar proteins of *Campylobacter jejunii* in the 54–59 kDa region.

between individuals. Nevertheless, there are reports (Kosunen et al, 1992), where the decline in serum IgG titres correlated well with successful treatment of patients with duodenal ulcer (26, 43 and 55% depreciation of IgG titre at 3, 6 and 12 months respectively). Although the potential of serology to monitor treatment success or failure exists in its incipient stages, it must be acknowledged that confirmation of *H. pylori* eradication following antimicrobial therapy is possible by serological techniques ONLY if titre quantification is conducted no sooner than 6 months post-treatment, and only if comparative assessments are made using pre-treatment serum samples of the same patient. Highly specific immunological markers are urgently needed which will reflect, quantitatively, the outcome of antimicrobial chemotherapy.

Antigen detection methods

An answer to the post-treatment inaccuracy of serology may lie in future direct tests for *H. pylori* in clinical specimens such as gastric juice, or stools (Enroth and Engstrand, 1995). Commercial stool antigen tests are not generally available but show considerable promise with accuracy superior to serology and comparable to breath testing. Such novel serological modalities are eagerly awaited.

UREA BREATH TEST (UBT)

Information common to ^{14}C-urea and ^{13}C-urea tests

The ^{13}C- and ^{14}C-urea breath tests are now well established diagnostic tests, and their use is especially valuable for following up patients after therapy (Bell, 1993; van de Wouw et al, 1997). Sensitivity and specificity of these tests have proved to be excellent, and in most cases results are obtained in less than an hour. The greatest advantage of UBTs is that they are non-invasive and the speed with which a result may be obtained.

The fundamental principle of the ^{14}C-UBT is precisely identical to that employed for the ^{13}C-UBT, the single difference being the use of different isotopes of carbon. In the presence of an *H. pylori* infection a significant quantity of the *H. pylori* urease is produced in the gastric mucosa of an infected individual. The ingested ^{14}C- or ^{13}C-labelled urea is enzymatically hydrolysed by the urease of *H. pylori* on contact with the gastric mucosa. The resultant products are HCO_3^- and NH_4^+ ions. Since the solubility of CO_2 in acidic gastric juice is low, $^{14}CO_2$ is driven towards the mucosa, where it rapidly enters the circulation, as shown in Figure 1.

^{14}C urea versus ^{13}C urea breath tests

^{14}C is a low-energy β-emitter which can easily be detected by scintillation counting of 'captured' $^{14}CO_2$ in expired breath (Marshall and Surveyor, 1988; Surveyor et al, 1989). In this rapid method, ^{14}C was given in water,

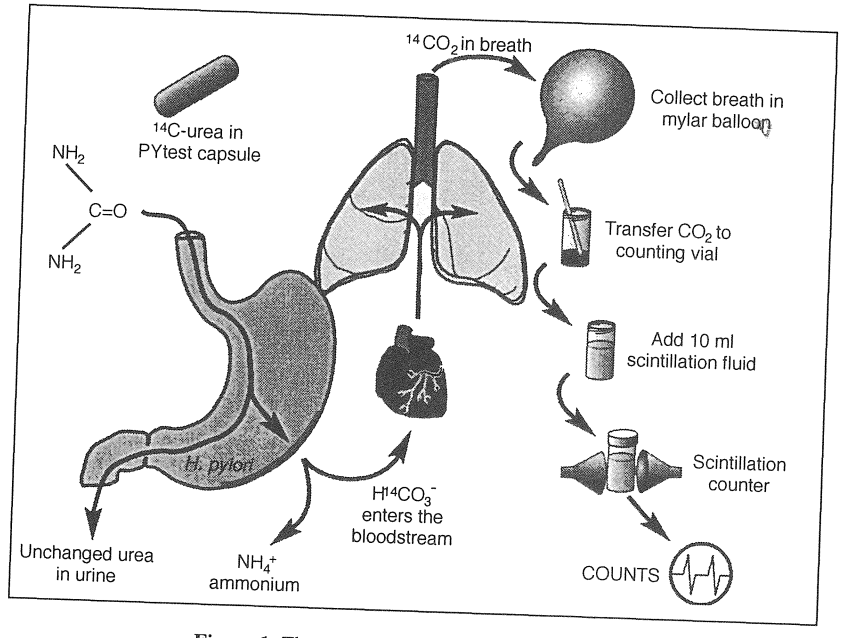

Figure 1. The principle of the ^{14}C urea breath test.

and diagnostic samples were taken after 20 minutes. As an alternative to ^{14}C, Graham et al (1987) reported the use of a ^{13}C-UBT where they measured the exhaled $^{13}CO_2$ using a mass spectrometer. Due to inherent limitations with the ^{13}C urea method, a 'liquid meal' was incorporated into the protocol to delay gastric emptying and ^{13}C was quantified in breath samples collected from the patients for 60–90 minutes (see Figure 2). Bell et al (1987) and Rauws et al (1989) used ^{14}C urea in an almost identical manner with a 'meal' and quantified cumulative $^{14}CO_2$ excretion. Theoretically, meal-based tests are more sensitive than rapid tests since the ^{14}C urea cannot be rapidly emptied from the stomach, an event which could give a false-negative result (Marshall, 1996). However, in practice, meal-based tests are no more sensitive than rapid tests, although direct comparisons of the two protocols have not been reported. Meal-based tests require a longer collection time (20–90 minutes) and give lower breath activity than tests in which the isotope is given in a drink to a fasting patient (Eggers et al, 1990; van Zanten Veldhuyzen et al, 1990; Marshall, 1991). In the early studies using ^{14}C urea, 3 to 10 μCi of ^{14}C urea was preferred (Marshall et al, 1991b). In order to minimize the dose of ^{14}C urea while maintaining diagnostic accuracy, Marshall et al (1991a) have described a capsule-based method which maintains rapidity of the test, utilizes only 1 μCi of ^{14}C urea and avoids spurious urea hydrolysis due to contact between the isotopically labelled urea and urease in the oropharyngeal flora. The encapsulation of the ^{14}C urea within a 'quick-dissolve' capsule delivers urea directly onto the gastric mucosa, and allows the use of 'as low as reasonably achievable' (ALARA) quantities of ^{14}C urea. The 1 μCi

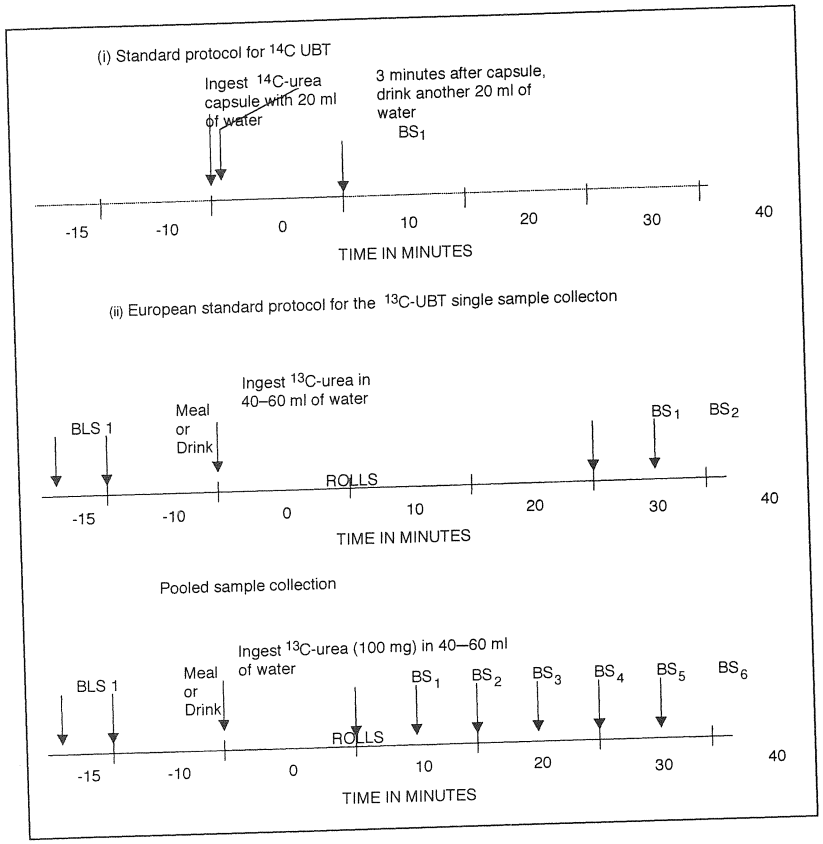

Figure 2. UBT protocols. BLS = base line sample, BS = breath sample, ROLLS = patient turns from side to side on examination table.

^{14}C-UBT capsule (PYtest® manufactured by Tri-Med Specialties Inc., a division of Ballard Medical Products, USA) is inexpensive (costs *c.* twice that of laboratory-based serological tests for *H. pylori*), convenient, can be completed in 15 to 20 minutes, and a positive or negative diagnosis can be arrived at within 10 minutes if a scintillation counter is at hand (Marshall et al, 1991a).

The only drawback of the ^{14}C-UBT is the fact that it is radioactive and thus is subject to regulations concerning its use. While some of these regulations relate to sensible handling of radioisotopes, most are designed to regulate the handling of large dosages of other radioisotopes. In contrast, residual ^{14}C from the 1 μCi ^{14}C-UBT is about equal to the natural body content of ^{14}C and exposes patients to no more radiation than that received from the normal environment in 24 hours.

In recognizing this, the 1 μCi-UBT is used without restriction in the United States where the Nuclear Regulatory Commission (NRC) has made it 'exempt' from the handling restrictions. The extremely low dose of ^{14}C

urea and the advantages of the 1 μCi capsule indicate that the PYtest® will be a useful and rapid diagnostic tool in the future.

MOLECULAR DIAGNOSIS

Background

Since the invention of the polymerase chain reaction (PCR) by Kary Mullis in 1988 (Saiki et al, 1988), a large number of laboratories around the world have successfully applied this molecular-based method for the detection and characterization of not only *H. pylori* but other bacteria, viruses, fungi etc. PCR is a powerful gene amplification procedure involving the in vitro production of exponential amounts of a specific target DNA or RNA. It involves repeated cycles of primer annealing, extension and denaturation in the presence of a thermolabile DNA polymerase (*Taq*) and deoxynucleoside triphosphates (dNTPs). Following amplification, PCR products are commonly analysed by ethidium bromide-stained agarose gel electrophoresis.

Prior to the invention of PCR, molecular-based approaches for the detection of *H. pylori* have included the use of radiolabelled or antigenically labelled chromosomal fragments (Wetherall et al, 1988), in situ hybridization (Vanderberg et al, 1989), restriction enzyme analysis (REA) (Langenberg et al, 1986; Li et al, 1993), and hybridization with an oligonucleotide (Morotomi et al, 1989). These methods required the use of fresh clinical materials or cultivated *H. pylori* isolates, and the procedures are relatively straightforward since good quality nucleic acid can be easily extracted. However, the nucleic acid from archival or processed materials, for example, paraffin-embedded or chemically preserved tissues, tends to be impure, of low molecular weight and contain single-stranded nicks, hence is not as amenable to analysis with these procedures. With the high sensitivity of PCR, small quantities of poor quality DNA can be tolerated and amplified. Therefore, PCR can be applied to retrospective studies of stored archival materials that may prove highly useful in epidemiological studies and numerous retrospective investigations. A study by Jackson et al (1989) has found that PCR could successfully amplify DNA extracted from archival haematoxylin and eosin (H&E) stained sections of up to 30 years old.

PCR

Currently, many laboratories around the world are utilizing PCR for the detection of *H. pylori* in numerous clinical and environmental specimens (see Table 3) with excellent results.

The principles and rationale of PCR for the detection of *H. pylori* are well documented and they differ from each other mostly in their choice of primers.

Table 3. Sites where *H. pylori* has been successfully detected by PCR.

Clinical specimens	References
Biopsy specimens	
fresh	Valentine et al (1991), Clayton et al (1992), van Zwet et al (1993), Fabre et al (1994)
paraffin embedded	Ho et al (1991), Clayton et al (1992)
in CLOtest™	Lin et al (1996b)
Gastric juice	Westblom et al (1993), Basso et al (1996)
Saliva	Hammar et al (1992), Li et al (1995)
Dental plaque	Mapstone et al (1993), Namavar et al (1995)
Bile	Lin et al (1995)
Faeces	Kelly et al (1994), Namavar et al (1995)
Environmental sources, for example, water supply	Enroth and Engstrand (1995)

Primer design

The specificity of PCR is dependent upon the design of the primer sequences. Thus, the *H. pylori* target gene of interest has to be selected carefully to ensure that the chosen gene is present in all *H. pylori* isolates to avoid false-negative results. On the other hand, the primer sequences need to be unique so that they do not cross-react with other bacterial species that could be present in clinical material.

The first published primer sequences of *H. pylori* were from the DNA sequence of the 16S ribosomal RNA (rRNA) (Hoshina et al, 1990). Since then, other nucleic acid sequences employed have included the 23S rRNA gene (Versalovic et al, 1996), urease A (Clayton et al, 1992; Kawamata et al, 1996) and urease B genes (Lopez et al, 1993; Akashi et al, 1996), 26 kDa species-specific antigen of *H. pylori* (Hammar et al, 1992), polymorphic random sequences of the chromosomal DNA (Valentine et al, 1991), ure C (phosphoglucosamine mutase gene) (Moore et al, 1993), *cagA* (cytotoxin-associated gene) (Tummuru et al, 1993) and hpaA (adhesin gene) (Evans et al, 1995). Other primers are designed specifically to target the RNA, for example, the 16S rRNA (Engstrand et al, 1992). In general, the most commonly applied are the urease A and 16S rRNA gene. At present more primers are being designed and tested.

Practical applications

The literature abounds with conflicting reports of *H. pylori* detection by PCR. As PCR is extremely sensitive, a single contaminating cell of *H. pylori* may lead to a false-positive reaction. Hence, any laboratory that wishes to perform PCR for diagnostic purposes must develop strict guidelines for the physical separation of clinical specimens and the post-PCR products (Persing, 1991). Theoretically, a PCR laboratory is required to have the following areas: sample preparation area, pre-PCR area, PCR machine area, and a post-PCR area. However, this is usually unattainable for most small laboratories, but

with careful planning and sample preparation, contamination can be prevented. Another important point to consider is the disinfection of endoscope, forceps and the handling of clinical specimens in the endoscopy room by physicians to minimize intermittent cross-contamination.

The set-up of PCR, amplification, and detection takes less than 8 hours to complete as opposed to a few days for culture and histological analysis. PCR, compared with histology (Lin et al, 1996a) and culture (van Zwet et al, 1993; Fabre et al, 1994), was found to be the most sensitive and specific test for detecting *H. pylori* with the detection rate of 10–100 *H. pylori* cells. An interesting study by Enroth and Engstrand (1995) found that the number of bacteria needed for a positive PCR result increased with the age of the cultured sample, suggesting that coccoidal forms of *H. pylori* may have different antigenicity and DNA content. This finding may be of significance if one is to evaluate the detection rate of PCR by assessing the colony-forming units (c.f.u.) of *H. pylori*, or by the use of microscopy, if we assume that the coccoidal form is non-culturable.

Specimen preparation

The stringent transport conditions and time constraint (>4 days) experienced with culturing *H. pylori* can be easily overcome by PCR. This is because there are no special requirements for specimen transport and the use of 'transport medium' is not essential as *H. pylori* is not required to be alive when tested. DNA is also very stable chemically and has the capability to withstand changes in the environment for long periods of time (Doran et al, 1986). These characteristics make PCR suitable for both clinical and/or environmental sampling, and specimens can be shipped between hospitals or laboratories without compromising the results of the test. Alternatively, specimens in 'transport medium' will not affect the PCR results. Furthermore, several laboratories have reported the successful detection of *H. pylori* from biopsy placed (Hua et al, 1994) and transported by mail in the CLOtest® (Lin et al, 1996b).

However, conflicting data by various investigators (Engstrand et al, 1992; Lamouliatte et al, 1993; van Zwet et al, 1993) indicate that the sensitivity of PCR is close to that of culture, with PCR being only slightly superior if applied for the verification of *H. pylori* eradication. PCR techniques, although fast, sensitive and specific, are hindered by complicated and time-consuming sample processing procedures (e.g. *H. pylori* enrichment by immunomagnetic beads (Enroth and Engstrand, 1995), proteinase K lysis and phenol/chloroform extraction to purify the *H. pylori* DNA). In the case of gastric biopsies and juice, specimen processing has been reduced to just simple proteinase K treatment or 10 minutes boiling with little or no post-processing (Clayton et al, 1992).

In spite of the fact that PCR is cost effective and yields excellent results, it is difficult to implement in routine clinical laboratories. This is due to the requirement for specific laboratory equipment, the use of carcinogenic ethidium bromide and the need to photograph the gel to document results.

However, low sensitivity of PCR can occur, especially when dealing with poor quality or quantity of starting target DNA. Using reverse transcriptase-PCR (RT-PCR), nested PCR or other post-PCR modifications, the sensitivity and specificity of both DNA and RNA amplification can be considerable improved.

RT-PCR

Amplification of DNA from RNA, for example, 16S rRNA, is possible by RT-PCR. RT-PCR comprises the combination of a standard PCR protocol with an initial incubation with a reverse transcriptase (RT) enzyme to generate complementary DNA (cDNA). The detection of RNA by RT-PCR has an advantage in that the sensitivity of detection of *H. pylori* is enhanced compared to that with DNA-PCR alone. This is because of the higher numbers of RNA transcripts present per bacterial cell as opposed to one DNA copy present per cell. Engstrand et al (1992) and Wahlfors et al (1995) noted that the sensitivity of the RT-PCR is 1–10 *H. pylori* cells per sample by comparison with 10 or more cells for the standard DNA-PCR (Clayton et al, 1992).

The use of 16S rRNA has caused some confusion in the laboratory. Due to the nomenclature of '16S rRNA', one invariably assumes the isolation and detection of RNA. However, this is not correct as it could mean either the detection of 16S rRNA by RT-PCR or standard DNA-PCR for detection of the DNA that codes for 16S rRNA. Several laboratories have designed primers for both the detection of RNA (Engstrand et al, 1992) and DNA (Hoshina et al, 1990; Ho et al, 1991) of *H. pylori* 16S rRNA. Nevertheless, the laborious technique involved in RNA extraction and purification, and the labile nature of RNA (short half-life), has limited the use of RT-PCR to *H. pylori* fingerprinting and epidemiological studies. An important aspect of RNA detection is the strict requirement of fresh materials (usually requires flash-freezing in liquid nitrogen) or freshly cultivated *H. pylori* isolates, and thus it is not used for routine diagnosis. Another potential setback in the detection of 16S rRNA is its relative conservation in many species, since 16S rRNA is found in most living cells and phylogenetically has common roots. All these factors have rendered the use of 16S rRNA less valuable for the diagnosis of *H. pylori* in clinical specimens, although it may have a role in screening environmental samples for the presence of other *Helicobacter* species.

Nested PCR

Nested PCR is a second PCR performed on the first amplified products using a different set of primers internal to the first sequence amplified. Nested PCR will increase the sensitivity of a standard PCR with the detection rate of one to three *H. pylori* cells (Shiada et al, 1994). However, nested PCR is prone to the production of multiple bands or artifacts (Froham, 1993). To prevent this, the first PCR product is usually purified from the residual primers and nucleotides before the second round of PCR. Due to a high risk of contamination linked with manipulation of the first amplified PCR products, the use of this procedure is problematic.

Others

Nevertheless, there are other potential approaches for increasing the sensitivity of PCR. Monteiro et al (1997b) indicated that human error could be a problem in the detection of PCR products by gel electrophoresis due to subjective evaluation. Improving the detection methods for the PCR products may be one way to minimize this. Besides solely performing PCR and visualizing the PCR products by agarose gel electrophoresis, the PCR products can be subjected to additional post-PCR detection techniques, for example, Southern blotting (Clayton et al, 1992) or dot blotting (Li et al, 1995).

Post-PCR investigations

Transfer of PCR products from an agarose gel to a nylon membrane makes use of the technique perfected in 1975 by Professor E. M. Southern and referred to as Southern blotting. Southern blotting produces a membrane that carries a replica of the DNA bands from the agarose gel. The DNA is usually transferred by capillary action and linked covalently on the membrane by UV irradiation or by heating. The PCR products are then visualized by hybridization with the aid of a fragment of DNA (probe) isolated from the same region of the gene selected for PCR amplification. The probe is usually radiolabelled by the enzymatic incorporation of radioactive nucleotides. However, recently several investigators have employed non-radioactive labels such as colorimetric probes, for example, digoxigenin (Karttunen et al, 1996) or chemiluminescent labelled probes (Anderson et al, 1993; Yu et al, 1995). Southern hybridization offers greater resolution (pg) than agarose gels, and visual inspection has a lower limit of resolution of 10 ng of DNA. Southern hybridization is difficult to perform and is not suitable for large numbers of samples or adapted for automation; therefore, it is not practical in most laboratories. An alternative to Southern blot is the 'dot blot' method where the PCR products are directly dotted and cross-linked onto a nylon membrane. The principle and rationale of 'dot blot' is comparable to that of Southern blot, and although dot blotting can accommodate a larger number of samples, the technique, like Southern blot, is cumbersome and thus not practical in diagnostic laboratories.

Recently, approaches incorporating a colorimetric hybridization assay similar to that of conventional enzyme immunoassay has been proposed (Lage et al, 1996). The method involves the detection of PCR products by hybridization onto a solid phase (e.g. 96-well microtitre plate). Detection of the PCR products can be attained by the use of a monoclonal antibody directed against double-stranded DNA (dsDNA), i.e. PCR products, or using specific labelled probes that will result in the formation of specific hybrids following hybridization. The last step involves a conventional enzyme immunoassay. This innovation makes it feasible and practicable in

clinical laboratories. The procedure takes 4 hours to perform, offers excellent results and has proved to be 100-fold more sensitive than agarose gel detection. Currently, there are three commercial kits employing this concept in the market.

Molecular screening for antimicrobial resistance

Because of the time required to culture *H. pylori*, PCR and DNA-based methods are attractive alternatives for antimicrobial susceptibility testing. This has recently been used to determine macrolide resistance in *H. pylori* (Versalovic et al, 1996; Szczebara et al, 1997).

Point mutations lead to decreased binding of the macrolide to the ribosome and hence reflect a high minimal inhibitory concentration (MIC) for macrolides on *H. pylori* (usually in the range of 8–128 mg/l), which is too toxic to be achievable in normal therapy.

PCR can be applied to amplify the 23S rRNA gene using primers flanking the nucleotides at 1406 and 2807:

5′-AGTCGGGACCTAGGCGAG-3′ (pos 1406 to 1424)
and
5′-TTCCCGCTTAGATGCTTTCAG-3′ (pos 2787 to 2807)

The amplification utilizing the primers above will yield amplicons of approximately 1400 base pairs (bp) when compared with molecular size markers which can be visualized under UV light following 1.5% agarose gel electrophoresis. Amplicons may then be subjected to restriction endonuclease (RE) digestion with either *Bsa*I or *Mbo*II. The point mutation in the 23S rRNA either in nucleotide 2143 or 2144 forms an extra RE site for *Bsa*I or *Mbo*II respectively. The non-mutated amplicons yield two DNA fragments of 1000 bp and 400 bp if digested with *Bsa*I when visualized after gel electrophoresis. However, if a point mutation occurs in 2143, three DNA fragments of 700, 400 and 300 bp will be observed. As for *Mbo*II enzyme, normal DNA amplicons will remain undigested at 1400 bp and a point mutation at nucleotide 2144 will yield two fragments of approximately 850 bp and 750 bp. The concept is summarized in Figure 3.

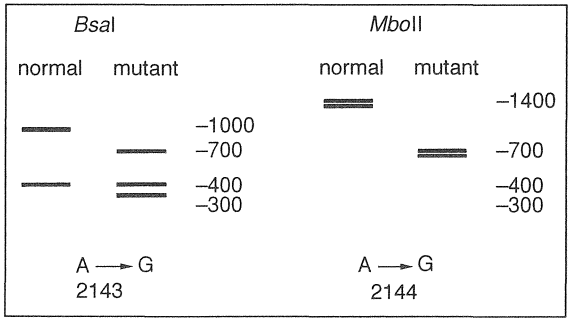

Figure 3. DNA fragments after digestion of 23S rRNA amplicon.

PCR inhibitors

A major problem with detection of *H. pylori* by conventional PCR is false-negative results. When the result of PCR is negative it is possible that the PCR amplification is suppressed. Inhibitors of *Taq* polymerases (*Taq*) which may be present in the sample DNA mixture prevent positive results from being obtained. Therefore, it is important to evaluate the entities present in clinical specimens such as inhibitors and contaminants from the saliva, faeces, blood, paraffin wax embedded tissues and gastric juices.

Different approaches have been taken to characterize and eliminate inhibitors. Monterio et al (1997a) carried out experiments to characterize these PCR inhibitors in faecal samples and established that they are complex polysaccharides possibly originating from vegetable materials in the diet. Elimination of these inhibitors is possible by using a filtration step with a polypropylene filter or by performing serial dilutions of the specimen prior to PCR. Nilsson et al (1996) have used magnetic beads coated with rabbit anti-*H. pylori* antibodies in order to enrich *H. pylori* from faecal samples. Both authors could detect less than 10^6 organism/gram of faeces and reported a good correlation with *H. pylori* status as determined by serology. A similar experiment by Enroth and Engstrand (1995) adopted magnetic immunoseparation to enrich for *H. pylori* in ground water.

Recently, Furuta et al (1996) have used competitive PCR (cPCR) to determine the presence of PCR inhibitors. Briefly, a competitive template (sDNA) (66 bp) containing the same primer-binding capability and a subset of the target DNA sequences is incorporated in the PCR mix. This acts as a competitor for primer binding and amplification with the *H. pylori* target DNA (132 bp). If PCR inhibitors are present, both of the 66 bp and 132 bp PCR products will not be present. However, in the situation of a true negative, i.e. absence of *H. pylori* DNA, only products of size 66 bp will be amplified.

Attempts to quantify the load of *H. pylori* using PCR is also possible using this competitive technique with slight modification (Monteiro et al, 1997c). An internal standard of known concentration is co-amplified with the same primers as the target *H. pylori* DNA and the results are compared. However, this procedure is long and complex so that it is not used routinely. Normally, quantification of the *H. pylori* load is obtained by performing serial dilution of *H. pylori* suspensions in which the number of organisms is known exactly by measurement of total DNA concentration or by accessing the colony forming units (c.f.u.).

Other recommendations

PCR, like UBT, is another method that can be used to determine eradication of infection, and specimens should be analysed in the follow-up post-treatment period when a sensitive and objective test must be used. The testing of gastric juice is recommended as it shows the organism's overall presence in the entire stomach (Westblom et al, 1993). However, if the

follow-up period is too brief, care has to be taken in interpreting the results as PCR may falsely amplify DNA from *H. pylori* that has been killed by the antibiotic therapy but remained in the stomach.

Monteiro et al (1996) propose that the gold standard for PCR is to test two biopsies, from each part of the stomach and also to use two sets of primers derived from different genes. The biopsy is considered positive only when at least one of the two PCR tests is positive.

FUTURE OUTLOOK

PCR or similar technology will certainly expand in the future when automation and commercialized kits are available to most laboratories.

A straightforward PCR that can determine the pathogenic potential of various *H. pylori* strains would be useful, if researchers in the future could design the correct primers.

The further development of the non-invasive techniques mentioned above (breath tests, antigen detection and culture) will alter the diagnostic methods used to detect *H. pylori* in the next decade, just as major advances have altered the techniques used in the past 15 years.

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