In situ hybridisation for the identification of *Helicobacter pylori* in paraffin wax embedded tissue

M S Bashir, F A Lewis, P Quirke, A Lee, M F Dixon

**Abstract**

A method for identifying *Helicobacter pylori* using a non-isotopic in situ hybridisation technique is described. A probe generated by polymerase chain reaction (PCR) with primers directed against parts of the *Helicobacter pylori* 16SrRNA sequence was used. Paraffin wax embedded gastric biopsy specimens from patients with and without gastritis were hybridised with the probe, and the method was shown to be sensitive and specific for *H pylori*.

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The association of a bacterial agent with gastritis was first recognised in 1983, although a curved bacterium had been observed in the stomach of man as early as 1893. The bacterium, initially called *Campylobacter pyloridis*, has been reclassified and named *Helicobacter pylori*, and is now well established as the major aetiologic agent in gastritis and peptic ulcer. Several biopsy based methods have been used to identify Helicobacter including culture, histochemical stains, urease tests, hybridisation techniques, and the polymerase chain reaction.

After eradication of *H pylori*, early relapse is generally attributed to proliferation of small numbers of residual organisms which escape detection by conventional means so that histology and culture after treatment can be falsely negative. The use of a sensitive and specific method for the detection of small numbers of organisms that might be present is required in these cases.

**Methods**

The primers used for preparing the 109 base pair polymerase chain reaction (PCR) product were selected by comparing the sequence for *H pylori* and related organisms—that is, *Wolinella succinogenes*, *H felis*, *H mustelae* and other *Campylobacter* species—which have been shown to be specific for *H pylori*. A biotinylated 109 base pair PCR product of a 16SrRNA sequence (fig 1) of *H pylori* was generated by mixing together 10 μl of 10× PCR reaction buffer (100 mM TRIS HCl (pH 9-2), 500 mM KCl, 0-1% gelatin, and 1-0% Triton-X100), 20 pmol each of dATP, dCTP, dGTP, 20 pmol of a mixture of dTTP and biotin-11-dUTP (Sigma Chemical Co Ltd, Poole, Dorset) in a ratio of 3 to 1, 50 pmol each of primers HP1 and HP2, 1 ng of DNA extracted from an *H pylori* culture and 1 unit of Supertaq DNA polymerase (HT Biotech, Cambridge) in a final volume of 100 μl in molecular biology grade water (Merck Ltd, Lutterworth, Leicestershire). The reaction mixture was subjected to thermal cycling at 94°C for five minutes and 60°C for 30 seconds, for one cycle, 94°C for 30 seconds and 60°C for 30 seconds for 39 cycles, followed by a final incubation at 72°C for five minutes in an MJ Research Programmable Thermal Controller (Genetic Research Instrumentation Ltd, Felsted, Essex). The products from several PCR reactions were pooled and spun through a Microcon microcentrifugor (Amicon Ltd, Stonehouse, Gloucestershire) at 12 000 × g for five minutes to remove excess primer. The PCR product was precipitated with 0-1 volume of 3M sodium acetate (pH 5-3) and 2-5 volumes of ethanol at −20°C overnight. The DNA was recovered by centrifugation at 15 000 × g for 10 minutes, washed with 70% ethanol, and dried in a vacuum desiccator for 10 minutes. The biotinylated PCR product was dissolved in 50 μl of molecular biology grade water; 2 μl were used for quantitation by fluorometry using a mini-DNA fluorometer (Hoefer Scientific Newcastle-under-Lyme, Staffordshire) and 5 μl were analysed on a 2% agarose gel to ensure that the 109 base pair product was present and free of contaminating bands. The remainder of the pooled product was adjusted to a final concentration of 10 μg/ml with molecular biology grade water and stored in 100 ng aliquots at −20°C.

For in situ hybridisation gastric biopsy specimens from eight cases of *Helicobacter*-associated gastritis and five histologically normal *H pylori* negative cases were used. The biopsy specimens had been routinely fixed in 10% formalin. Sections (5 μm thick) were mounted on to aminalkylsilane coated single well slides and hot plated for three days before dewaxing and rehydration through graded alcohols to water. The sections were treated sequentially with 0-02 M HCl for 10 minutes, 0-01% Triton-X100 for three minutes, 10% hexadecyltrimethylammonium chloride and a vacuum desiccator for 5 minutes.

A 10× working solution of biotinylated probe was made up in 10 μl of molecular biology grade water (2 μl of the pooled product used for quantitation and 8 μl of water). For in situ hybridisation the gastric biopsy specimens were digested according to standard methods and treated with proteinase K at 42°C for 10 minutes, 0-02% Triton-X100 for 10 minutes, and 0-01% Triton-X100 for three minutes. The slides were then dehydrated through alcohol and xylene.

A stringency wash was performed at 42°C for 10 minutes by incubation in a mixture of 2× saline sodium citrate (SSC) at 2× SSC and 100 μg/ml of RNAse for 5 minutes, followed by washing in 0-2× SSC at 42°C for 10 minutes.

A third wash was performed at 65°C for 10 minutes by incubation in a mixture of 2× SSC at 2× SSC and 20 μg/ml of RNAse at 65°C for 10 minutes. The slides were then warmed at 70°C for 5 minutes, dried under vacuum for 10 minutes, and dehydrated in a series of alcohols.

An avidin biotin complex was added to the slides for 10 minutes at room temperature, washed in a mixture of 2× SSC and 0-1% Tween 20 at room temperature for 5 minutes.

Diaminobenzidine was added to the slides for 5 minutes at room temperature and then rinsed in tap water for 1 minute.

The slides were then counterstained with haematoxylin for 5 minutes at room temperature and rinsed in tap water for 1 minute.

The sections were then dehydrated in a series of alcohols and xylene and mounted in DPX.

Figure 1. Primer sequences from *H pylori* 16S rRNA region used for generating a 109 base pair probe by PCR.
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bromide (CTAB) in 0.7M sodium chloride for five minutes and digested with 0.5 mg/ml proteinase K in 50 mM TRIS- HCl (pH7.6), 5 mm EDTA at 37°C for 10 minutes. The sections were incubated in 20% acetic acid at 4°C for 15 seconds and postfixed in 4% paraformaldehyde in phosphate buffered saline before being dehydrated through graded alcohols to ethanol. The dried sections were covered with 75 μl of the biotinylated PCR generated probe for H pylori containing 200 ng probe/ml in a hybridisation buffer containing 2 x SSC (1 x SSC = 0.14 M sodium chloride, 0.014 M sodium citrate), 5% dextran sulphate, and 0.2% milk powder. The slides were covered with a piece of Gel Bond (Flowgen Instruments Ltd, Sittingbourne, Kent), sealed with nail varnish, and incubated at 98°C for 10 minutes to denature the probe and cellular DNA. The slides were incubated overnight at 37°C and washed three times in 2 x SSC at room temperature for 10 minutes—each wash to remove excess probe and mismatched hybrids. The biotin labelled hybridisation product was detected by using a biotin-strep-tavidin-alkaline phosphatase sandwich technique, as described before. Negative and positive controls were run with each batch. The specificity was confirmed by running parallel controls of paraffin wax embedded mouse gastric mucosa infected with the closely related organisms H felis and G hominis.

Results
Preliminary experiments were carried out to optimise digestion times with proteinase K and CTAB. Digestion times ranging from five to 30 minutes were tried, and five minutes with 10% CTAB and 10 minutes with 0.5 mg/ml proteinase K were found to give optimal signal with minimal background. A total human DNA probe was used as a positive control for the method; the negative control was treated with the hybridisation buffer only.

All eight cases of Helicobacter-associated gastritis demonstrated positive staining organisms with the morphology of H pylori on the luminal aspect of surface epithelial cells and in the pits (fig 2). No signal was detected in the lamina propria. All five histologically normal gastric biopsy specimens failed to demonstrate any signal on in situ hybridisation.

The specificity was confirmed by the absence of signal in the negative controls and the paraffin wax embedded mouse gastric mucosa infected with H felis and G hominis.

Discussion
Several molecular biological techniques have been described for the identification of H pylori, including hybridisation with radioactively and non-radioactively labelled genomic and cloned DNA probes, in situ hybridisation, and PCR. In situ hybridisation offers the advantages of preserving tissue morphology and being sensitive enough to permit detection of even a small number of organisms. Van den Berg et al described an ISH method for H pylori. They used the entire H pylori genome as a DNA probe and studied multiple biopsy specimens from one patient. In addition to using this restricted material, the probe was not tested against other Helicobacter sp in tissue sections and cross-reacted with surface epithelial membrane. We used a biotinylated 109 base pair probe directed against part of the H pylori 16S rRNA sequence as H pylori was classified on the basis of such sequences. This probe is more specific and shows no cross-reaction with gastric epithelial cells. We studied a number of patients with and without Helicobacter-associated gastritis, and tested the specificity of the probe by running parallel controls of paraffin wax embedded mouse gastric mucosa infected with the closely related organisms H felis and G hominis.

Helicobacter pylori organisms were demonstrated on the luminal aspect of the surface and foveolar epithelium in all eight cases of H pylori associated gastritis. The digestion times with CTAB and proteinase K are critical as loss of morphology due to section defacement can occur. These were optimised for maximal signal with minimal background. The specificity of the technique was confirmed by the absence of a signal in the infected mouse gastric mucosa. Therefore, the in situ hybridisation technique that we used offers a sensitive and specific method for the detection of H pylori in tissue sections. In situ hybridisation may thus be suitable for identifying the small number of H pylori organisms that may be present in patients who relapse after apparent eradication, and which are currently deemed to be “negative” using routine histological stains.

Figure 2 H pylori on the luminal aspect of gastric epithelial cells (in situ hybridisation using H pylori probe).
Mesenchymal hamartoma of liver in a man: Comparison with cases in infants

K Y Chau, J W C Ho, P C Wu, W K Yuen

Abstract
A 53 year old man with a large mesenchymal hamartoma is reported. Only a few bile ducts could be found in the periphery of the lesion and no hepatocytes were identified within the lesion. As far as is known, this is the only adult male patient reported to date. On the basis of the reported findings of mesenchymal hamartomas in other adults, it is suggested that there could be changes in the morphology of this lesion with age: progressive loss of hepatocytes; degeneration of bile duct epithelium; and cystic change of the mesenchymal component. The haematopoietic element is considered to be part of the fetal hepatic haematopoiesis that occurs in the hamartoma.

Case report
A 53 year old Chinese man was admitted into Queen Mary Hospital, Hong Kong, complaining of dull-aching right upper quadrant pain with fever which he had had for one day. His health had been good. He was found to have an enlarged liver about 10 cm below the xiphisternum. There was tenderness but no guarding or rebound. Laboratory investigations showed no abnormalities. Abdominal ultrasonography and a computed tomography scan showed a 20 × 14 × 10 cm heterogeneous hypodense lesion in the right lobe of the liver with well defined, irregular, curved band-shaped density areas. The caudate lobe was affected and the left lobe was displaced laterally. Contrast injection showed irregular marginal enhancement. Angiography (hepatic, superior mesenteric, and right renal) showed that the mass was hypovascular with no arterio-venous shunting. The main portal vein, inferior vena cava, and the right kidney were displaced but not affected.
A laparotomy was performed and a large soft mass replacing the right lobe was resected. The patient was well two years after the operation with no evidence of recurrence on ultrasound examination. The resected lobe weighed 1520 g and measured 27 × 15 × 13 cm. There was a well circumscribed tumour measuring 20 cm at its largest diameter. The cut surface showed soft, greyish, oedematous tumour tissue with extensive cystic change. The cysts were filled with clear fluid. There was no necrosis, calcification, or haemorrhage (fig 1).

Histological examination showed loosely