Detection of *Campylobacter pylori* in stomach tissue by DNA in situ hybridisation

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SUMMARY A non-radioactive DNA in situ hybridisation (DISH) protocol was developed. It requires the use of biotinylated *Campylobacter pylori* DNA as the probe to detect *C pylori* DNA in routinely embedded stomach biopsy specimens. In sequential tissue samples from a 58 year old woman with recurrent chronic active gastritis the *C pylori* probe hybridised with bacteria whenever they were histologically visible. When no bacteria were visible histologically, hybridisation was negative with one exception. In a single biopsy specimen without visible *C pylori*, hybridisation occurred with the surface of the antrum epithelium, while control hybridisation with a heterologous probe remained negative. From a parallel biopsy specimen taken at the same time the *C pylori* culture was positive.

It is concluded that DISH, although more laborious than routine staining techniques, may be more sensitive in that it detects bacteria very easily, even when sections are not counterstained or when they are present in low numbers, and that bacteria which do hybridise are unequivocally identified as *C pylori* and not *Campylobacter*-like organisms.

As early as 1893 a curved bacterium had been observed in the stomach of man,¹ but it was only in 1983 that the presence of this bacterium was linked to the development of gastritis.^{2,3} The bacterium morphologically resembling *Campylobacter* species was called *Campylobacter pyloridis* and later renamed *C pylori*. Although the bacterium still carries this name, it is suspected of belonging to another or novel genus.^{4,5}

It is now well established that *C pylori* is the major aetiological agent of chronic active gastritis.⁶⁻⁸ Whether the presence of *C pylori* after treatment for gastric ulcers confers an increased risk for ulcer recurrence remains controversial.^{4,7}

C pylori isolates seem to be extremely variable at the DNA level; each isolate has its own characteristic restriction endonuclease digestion pattern.⁹ By using restriction endonuclease digestion of purified *C pylori* DNA, it has been demonstrated that patients who were apparently successfully treated for *C pylori* infection, but who seemed to be reinfected at follow up with *C pylori*, carried the same *C pylori* type as before treatment.⁹ These results suggested that *C pylori* may be resistant to antibiotics and may survive in the stomach undetected by routine culture and microscopic techniques.

We developed a non-radioactive DNA in situ hybridisation (DISH) protocol using biotinylated *C pylori* DNA as probe to detect *C pylori* DNA in paraffin wax embedded tissue sections of stomach biopsy specimens.

Material and methods

Antral mucosal biopsy specimens were collected endoscopically at regular intervals from 1984 to 1988 from a 58 year old woman with chronic relapsing active gastritis and focal intestinal metaplasia. The patient originally presented with a *C pylori* associated gastritis. In an attempt to eradicate *C pylori* she was initially treated with colloidal bismuth subcitrate for two months without success. After she developed a benign gastric ulcer the patient was treated with ranitidine and simultaneously with a five day course of furazolidone with short term success. After a relapse she was treated successfully with amoxycillin. For the past three years she has been free of detectable *C pylori*.

At each endoscopy (n = 25), two biopsy specimens were placed in 2 ml phosphate buffered saline (PBS) at 4°C for *C pylori* culture and two specimens were fixed in formalin and embedded in paraffin wax blocks for histological examination. For in situ hybridisation (DISH) analysis three histologically *C pylori* positive

Table Detection of *C. pylori* in selected antrum biopsy specimens from one patient by DNA in situ hybridisation

Biopsy No	Probe	Culture	Histology*	DISH	
				Bacteria	Surface†
84a	<i>C. pylori</i>	+	+	++	++
	CMV			—	—
84b	<i>C. pylori</i>	+	—	—	+
	CMV			—	—
85a	<i>C. pylori</i>	++	++	++	++
	CMV			—	—
85b	<i>C. pylori</i>	++	+	++	++
	CMV			—	—
85c	<i>C. pylori</i>	—	—	—	—
	CMV			—	—
85d	—	++	+	ND	ND
87a	<i>C. pylori</i>	—	—	—	—
	CMV			—	—

*CLO visible in haematoxylin and eosin staining of tissue section, †Staining of the surface of the antrum epithelium.

biopsy specimens designated 84a, 85a, and 85b and three histologically negative biopsy specimens designated 84b, 85c, and 87a, were selected. One set of biopsy specimens designated 85d was reviewed to confirm the relapse of *C. pylori* infection (table) but was not used in the DISH experiments.

Nine different *Campylobacter* species were used: *C. jejuni*, *C. coli*, *C. consisus*, *C. fetus fetus*, *C. jejuni doyley* (GCLO2), *C. laridis*, *C. UPTC*, *C. uppsaliensis* and *C. pylori*. From the latter species two different strains (65 and 67) were used. Cultures of 50 ml were grown under microaerophilic conditions and bacterial DNA was extracted as described previously.⁹ Human DNA was isolated from cultured fetal lung cells and placenta as described previously.¹⁰

DOT BLOT HYBRIDISATION

DNA samples at 10 µg/ml in 0.2 M NaOH were heated to 100°C for five minutes, cooled quickly on ice and quenched by addition of an equal volume of 2 M NH₄Ac. Duplicate 5 µl aliquots were spotted on to nitrocellulose membranes (Schleicher & Schüll, BA84) which had been soaked in 3 × SSC (SSC = 0.15 M NaCl, 0.015 M Na₃ citrate, pH 7.0) and 1 M NH₄Ac and air dried. Dot blots were baked for two hours at 80°C and hybridised with 0.5 µg in vitro ³²P labelled *Campylobacter* DNA, essentially as described previously.¹⁰ After hybridisation overnight, duplicate filters were washed at two different stringencies (1 × SSC, 0.5% sodium dodecyl sulphate, 65°C, and 0.1 × SSC, 0.5% sodium dodecyl sulphate, 65°C) and autoradiographed with Kodak X-AR2 film for 18 hours.

DNA IN SITU HYBRIDISATION

Unless otherwise stated, all steps were performed at room temperature. Paraffin wax sections (5 µm) were mounted on slides coated with gelatin-chrome alum,

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baked at 60°C for 16–20 hours, and stored at 37°C until required. To prevent non-specific probe binding and facilitate probe penetration the following pre-treatment steps were performed: after deparaffinisation sections were treated with xylometazolin-hydrochlorid (MultiPharma) for 30 minutes. After rinsing in PBS and dehydration in graded ethanol, sections were incubated in 50 mM glucose, 25 mM TRIS-HCl, pH 7.5, 10 mM EDTA containing 30 mg/ml freshly dissolved Lysozym (Sigma) for 10 minutes, rinsed in PBS, and incubated with 50 µg/ml proteinase K (Boehringer) in 20 mM TRIS-HCl, pH 7.5, 2 mM CaCl₂ for 30 minutes at 37°C. Sections were rinsed in PBS and incubated in PBS containing 3% H₂O₂ for 30 minutes.

After dehydration and drying, sections were pre-hybridised in HybMix (=2 × SSC, pH 7.0, 50% formamid, 250 µg/ml single stranded carrier DNA, 10 × Denhardt's solution, 10% dextran sulphate, 0.5% Tween 20, 0.1% d-biotin) for one hour at 37°C and subsequently rinsed in PBS, 0.5% Tween 20, dehydrated and air dried. Biotinylated *C. pylori* DNA probe (10 ng) or human cytomegalovirus (CMV) DNA probe¹¹ in 10 µl HybMix was applied to the sections which were covered with a glass coverslip. Probe and target DNA were simultaneously denatured by placing the sections on an 80°C hotplate for 10 minutes. Hybridisation was performed for 10 hours at 37°C in a sealed humidified container.

Coverslips were removed by submerging the sections in 0.3 × SSC, 50% formamid, 0.5% Tween 20, and sections were thoroughly rinsed in the same buffer. Hybridised biotin labelled probe sequences were visualised in the sections by sequential incubations in 10% normal swine serum in PTB (=PBS containing 0.5% Tween 20 and 1% bovine serum albumin), 0.1% rabbit anti-biotin (Dako) in PTB for one hour, and 2% horseradish peroxidase (HRP) conjugated swine anti-rabbit Ig (Dako) in PTB for 30 minutes. HRP activity was developed with 0.5 µg/ml diaminobenzine (DAB), 0.02% H₂O₂ in 50 mM TRIS-HCl, pH 7.8, for four to six minutes. DAB precipitate was enhanced using an experimental silver enhancement kit (Janssen Pharmaceutica) resulting in dark brown to black deposits.

Results

To obtain maximal DISH efficiency¹¹ the total *C. pylori* genome was used as a DISH probe. To test whether *C. pylori* DNA crosshybridised with human DNA or DNA from other *Campylobacter* species, dot blot hybridisation analysis was performed as summarised in fig 1. In vitro ³²P labelled total *C. pylori* (65) DNA did not hybridise with other *Campylobacter* species and only weakly hybridised at low stringency with human DNA; in contrast, it hybridised strongly with

Fig 1 Cross hybridisation analysis of *C. pylori* DNA with DNA from various *Campylobacter* species and man. The DNA (total genomes) from the sources indicated were spotted in duplicate on six nitrocellulose strips and hybridised with in vitro ^{32}P labelled total genome probe DNA from *C. pylori* (lanes A), *C. coli* (lanes B), and *C. jejuni* doyley GCLO2 (lanes C). Post hybridisation washes were performed at low (lanes 1) and high (lanes 2) stringency.

DNA from a different *C. pylori* strain (fig 1; A1, 2). These results confirmed that *C. pylori* is not closely related to most members of the genus *Campylobacter*.⁵ When *C. coli* or *C. jejuni* doyley (GCLO2) DNA were used as probe (fig 1; B1, 2 and C1, 2, respectively) these *Campylobacter* species cross hybridised with most other species but not with *C. pylori* or human DNA.

For DISH experiments *C. pylori* DNA was biotinylated and applied to paraffin wax embedded tissue sections of a series of selected antrum biopsy specimens from a patient with recurrent *C. pylori* associated gastritis. Initial experiments with routine DISH techniques using streptavidin/polyalkaline phosphatase staining^{11,12} were only weakly positive in the morphologically visible *Campylobacter*-like organisms (CLO) in some of the stomach biopsy specimen (data not shown) and showed strong background staining. Therefore, we developed a highly sensitive DISH procedure which entailed extensive proteolytic pre-treatment, background reduction with

mucus dissolving pharmaceuticals and free d-biotin, combined with the enhancement of DAB precipitate with metallic silver. The results with this protocol are shown in fig 2 and summarised in the table. Whenever CLO were histologically visible they were strongly stained with the *C. pylori* probe (fig 2b) while cytomegalovirus probe was negative. In these cases focal *C. pylori* specific hybridisation of the epithelial surface in the vicinity of *C. pylori* organisms was often found. In biopsy 84b a similar hybridisation of the surface of the epithelium was observed without the visible presence of bacteria (fig 2d); the cytomegalovirus probe did not stain the epithelium surface in this biopsy specimen. From one of the parallel biopsy samples of 84b the *C. pylori* culture was positive (table).

Discussion

Fig 1 shows that *C. pylori* probably does not belong to

Fig 2 DNA in situ hybridisation of antrum biopsy specimens with biotinylated *C. pylori* DNA. Paraffin wax sections of antrum biopsy specimens were mounted on coated slides and processed for DNA in situ hybridisation. (a) Biopsy 85a haematoxylin and eosin stain; (b) biopsy 85a DISH with *C. pylori* probe and HRP/DAB-silver staining.

the genus *Campylobacter* as has been suggested by others^{4,5} because there is no cross-hybridisation between the total genome DNA of the different species. The results in lanes B and C could suggest, however, that *C. pylori* may be related to *C. consisus* and *C. fetus fetus* because these species hybridise to the same extent to *C. coli* and *C. jejuni* doyley probe as does *C. pylori*. The results in lanes A refute this conclusion, however, as *C. consisus* and *C. fetus fetus* do not (cross) hybridise with *C. pylori* even under low stringency conditions (lane A1).

As shown in fig 2 DISH is quite a sensitive technique to detect *C. pylori* organisms in antrum biopsy specimens. Although DISH is more laborious than haematoxylin and eosin, silver, or Giemsa staining, it may be more sensitive and has important advantages over these routine staining methods: (i) bacteria are very easily detected, especially when sections are not

counterstained, even when they are present in low numbers; (ii) bacteria that do hybridise are unequivocally identified as *C. pylori* and not merely as CLO; (iii) in biopsy 84b, of which a parallel specimen from the same region of the antrum gave positive culture results, DISH detected *C. pylori* DNA hybridisation in the epithelial surface, while histological examination did not show *C. pylori* at this location (fig 2d, table).

Regarding the issue of relapsing *C. pylori* infection we must conclude that in our patient *C. pylori* does not persist in clinically important numbers in undetectable form in the antrum mucosa after apparent eradication. An intact genome must be conserved to enable revival of infectious bacteria and such bacterial DNA molecules would theoretically be detected by DISH. In our patient *C. pylori* DISH of biopsy 85c was negative (as were the histology and culture results) while a

Fig 2 (c) Biopsy 84b haematoxylin and eosin stain; (d) biopsy 84b DISH with *C pylori* probe and HRP/DAB-silver staining.

relapsing infection with the same *C pylori* strain was seen one month later (table, biopsy 85d). This result presents us with a problem because the fact remains that patients with relapsing *C pylori* infection are generally "reinfected" with their "own" *C pylori* strain.⁹ An explanation for this phenomenon may be that some *C pylori* organisms survive eradication and persist in a different part of the stomach (such as the fundus) from which biopsy specimens are not normally taken.

The DISH result with biopsy 84b (table), in which *C pylori* probe hybridised to the periepithelial mucous layer of the antrum (fig 2d), could suggest the presence of latent bacteria at this site in a morphologically obscure form. In our opinion it is more likely that the probe detects debris, including DNA fragments, of recently eradicated bacilli. Interestingly, the epithelial surface staining was not present in other parts of the

same biopsy specimen with intestinal metaplasia. Obviously these observations need to be substantiated with additional experiments using tissue samples from several different patients.

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