In situ localisation of *Yersinia enterocolitica* by catalysed reporter deposition signal amplification

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Abstract

Aim—The sensitive detection of pathogenic *Yersinia enterocolitica* in paraffin embedded tissue sections by in situ hybridisation (ISH).

Methods—*Y enterocolitica* infected cell lines, rat spleens, and patient biopsy specimens were used to compare conventional ISH, immune fluorescence assay (IFA) detection, and catalysed reporter deposition (CARD) signal amplification ISH.

Results—CARD-ISH was shown to be more sensitive than conventional ISH and had a comparable sensitivity to IFA. In contrast to IFA, CARD-ISH preserved good tissue morphology.

Conclusions—CARD-ISH appeared to be a fast and sensitive ISH method for detecting *Y enterocolitica* in routinely processed tissue sections. Application of this method allows the combination of routine detection and cellular localisation of the pathogen within the infected tissue.

(J Clin Pathol 1998;51:444-449)

Keywords: *Yersinia enterocolitica*; in situ hybridisation; in situ localisation

Yersinia enterocolitica is an enteric pathogen which usually causes self limiting gastrointestinal infection.^{1 2} In some cases infection with this agent can be responsible for chronic and recurrent disorders in humans, such as chronic ileitis, hepatitis, and arthritis.³ The presence of serum antibodies against *Y enterocolitica* (IgG, IgM, and IgA) may reflect the difference between a recently acquired infection, the persistence of the microorganism, or a past infection,^{4 5} but does not give information on the actual presence of the bacterium. Therefore, the detection of *Y enterocolitica* in tissue specimens might be important because this could eventually decide which treatment strategy should be followed. Several different detection methods have been described. Most of the techniques rely on culturing the bacterium from the specimen, which is difficult in the chronic stage of the infection, or on the multiplication of specific DNA sequences.

Polymerase chain reaction (PCR) detection of the bacterium is relatively sensitive and specific, although in the case of *Y enterocolitica* a combination of culture and PCR is often required to increase the sensitivity of detection.⁶⁻¹⁰

An alternative is immune fluorescence assay (IFA) detection with serotype specific antibodies.¹ The IFA technique is highly sensi-

tive, and detection of the pathogen in routinely fixed and paraffin embedded sections can be performed. Despite the disadvantages of IFA, such as loss of tissue morphology and the need for several different antibodies to detect the different *Y enterocolitica* serotypes, it is the most commonly used technique for detecting *Y enterocolitica*.

The advantage of in situ hybridisation (ISH) is the detection of the pathogen within the tissue while the tissue morphology remains preserved. In spite of the fact that conventional ISH is very specific, its sensitivity is limited and additional amplification of the signal is often required. Several techniques have been described to increase the sensitivity of ISH. Amplification of ISH signal can be performed with normal immunohistochemical amplification methods such as avidin-biotin complexes or enzyme labelled antibodies, but sensitivity is often still not sufficient.¹¹ PCR-ISH uses the principles of PCR by amplifying the target sequence directly in tissue sections, and it appears to be highly sensitive.^{12 13} The catalysed reporter deposition (CARD) amplification method creates a deposition of biotinylated tyramine at the place of the hybridised probe and amplifies the hybridisation signal. The biotinylated tyramine can be visualised with a fluorochrome or enzyme labelled avidin.¹⁴ We applied the CARD-ISH technique for the detection of *Y enterocolitica* in paraffin embedded tissue sections.

Methods

BACTERIA

Y enterocolitica serotype O:8 strain WA (ATCC 9610) harbouring the virulence plasmid was grown overnight at 26°C in brain-heart infusion broth followed by growth for four hours at 37°C. The culture was subsequently washed with phosphate buffered saline (PBS) before infection of Caco-2 cells and Fischer 344 rats. In addition, an overnight culture was directly used for the isolation of the 70 kb *Y enterocolitica* virulence plasmid (pYV).¹⁵ Isolation was performed with the Wizard Plus minipreps DNA purification system according to the manufacturer's instructions (Promega, Madison, Wisconsin, USA).

CACO-2 CELL LINE

Human colon carcinoma derived Caco-2 cells¹⁶ were grown to confluency on surfactant-free nitrocellulose membrane filters (12 mm diameter, 0.4 µm pore size, Costar, Cambridge, Massachusetts, USA) placed in 24 well tissue culture plates (Costar) in Dulbecco's modified Eagle's medium (DMEM, Sigma, St Louis,

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Accepted for publication
4 February 1998

Missouri, USA) without antibiotics, supplemented with 20% heat inactivated fetal calf serum, and 1% non-essential amino acids. Cells were incubated at 37°C and 90% relative humidity in air containing 5% CO₂. The medium was added to both the apical and basolateral sides of the cells and was changed daily.

PBS washed bacterial suspensions of 2×10^8 CFU/ml in DMEM without supplements were made and 0.5 ml of this suspension was added to the apical side (brush border) of Caco-2 cells. Cells with bacteria were incubated at 37°C for six hours and formalin fixed and paraffin embedded for light microscopy.

EXPERIMENTALLY INFECTED RATS

Fischer 344 rats (Central Animal Laboratory, Nijmegen, The Netherlands) were intravenously infected with 10^4 CFU in 0.2 ml 0.9% saline *Y. enterocolitica* WA O:8 (ATCC 9610).¹⁷ A control Fischer 344 rat was injected with saline. Fourteen days after infection, rats were killed and their spleens were removed. The tissues were formalin fixed and paraffin embedded for light microscopy.

HUMAN BIOPSY SPECIMENS

Biopsy specimens from two patients with a proven *Y. enterocolitica* infection were formalin fixed and paraffin embedded for light microscopy. Positive *Y. enterocolitica* infection was proven by positive anti-Yop serology (as described earlier¹⁸) and positive immune fluorescence assay (IFA, see below). An abdominal lymph node biopsy from a patient with a mild infection (*Y. enterocolitica* serotype O:5,27 or O:9), and a stomach biopsy from the other patient who had a severe infection (*Y. enterocolitica* O:3) were available. Colon biopsies of two patients with Crohn's disease were used as negative control biopsy specimens.

DNA PROBES FOR IN SITU HYBRIDISATION

The pYV plasmid and the pBR322 plasmid were biotin-14-dATP labelled by nick translation with the BioNick Labelling System according to the manufacturer's instructions (GibcoBRL, Life Technologies, Gaithersburg, Maryland, USA), generating biotin labelled DNA fragments of 100 to 500 bp in size.

PREPARATION AND PRETREATMENT OF SAMPLES FOR IN SITU HYBRIDISATION

Formalin fixed, paraffin embedded 4 µm tissue sections of the Caco-2 cell line, rat spleens, and human biopsy specimens were mounted on SuperFrost Plus coated and positively charged glass slides (Menzel-Gläser, Germany) and heated overnight at 60°C. After dewaxing in xylene and rinsing in methanol the endogenous peroxidase activity was blocked for 15 minutes at room temperature in 1% H₂O₂-methanol. Slides were then rinsed in methanol and air dried. Samples were incubated for five minutes in 1 M NaSCN at 80°C and five minutes in demineralised water at 37°C, and then with 8000 U/ml pepsin (Sigma) in 0.2 M HCl at 37°C to create good accessible target DNA for the ISH probes. The optimal pepsin digestion

time was checked for each paraffin embedded tissue sample individually, starting with a time range of 1 to 10 minutes. The digestion times are critical as loss of morphology owing to section digestion can occur. Next, the slides were rinsed in demineralised water and dehydrated in an increasing alcohol range. Again endogenous peroxidase was blocked with 1% H₂O₂ in methanol, mainly to inactivate the endogenous enzymes within tissue red blood cells, polymorphonuclear leucocytes, and macrophages. Subsequently the sections were air dried after rinsing in methanol.

IN SITU HYBRIDISATION AND IMMUNOCHEMICAL DETECTION

The *Y. enterocolitica* plasmid probe was hybridised to the paraffin sections at a probe concentration of 2 ng/µl hybridisation mixture (60% deionised formamide (Merck, Darmstadt, Germany), 10% dextran sulphate (Sigma), $2 \times$ SSC pH 7.0 ($1 \times$ SSC = 0.15 M NaCl/0.015 M Na citrate)), and 50 ng/µl herring sperm DNA (Boehringer Mannheim, Mannheim, Germany). Twenty microlitres of probe mixture were applied to the sections under a coverslip (24 × 50 mm) and sealed with rubber cement. Probe and target DNA were denatured at 80°C for 10 minutes in a moist chamber. After overnight hybridisation at 37°C, the coverslips were removed by immersing the slides in $2 \times$ SSC at 42°C. Post-hybridisation washes were carried out twice in 60% deionised formamide in $2 \times$ SSC for five minutes and twice in $2 \times$ SSC for five minutes at 42°C. Next, the sections were rinsed in phosphate buffered saline with 0.05% Tween-20 (Sigma) (PBST) at room temperature.

The horse radish peroxidase labelled ABC method was applied for the detection of the biotinylated probe in the sections,¹¹ using the Elite ABC kit (Vector Laboratories, Burlingame, California, USA). Incubations were performed for 30 minutes at 37°C. PBST containing 1% non-fat dry milk (Protifar, Nutricia, The Netherlands) (PBSTM) was used for all incubation steps. All washing steps between the incubations were three times in PBST for five minutes at room temperature. For blocking non-specific signals the tissue sections were preincubated with PBSTM for 15 minutes at 37°C. Next, mouse anti-biotin (1:100, Boehringer Mannheim), biotinylated horse antimouse (1:200, HsaM-bio, Vector), and ABC (avidin 1:100, biotin-peroxidase 1:100, Vector) were applied. After rinsing in PBST and PBS, demonstration of horse radish peroxidase was accomplished by precipitating 3,3'-diaminobenzidine tetrahydrochloride (DAB, Pierce, Rockford, Illinois, USA) through the reaction of H₂O₂ (Merck) with horse radish peroxidase. Sections were incubated with 0.05% DAB/0.15% H₂O₂ in PBS with 0.65% imidazole (Merck) for five minutes. After rinsing in demineralised water, the DAB signal was amplified with 0.5% CuSO₄ in 0.9% NaCl for one minute and sections were again rinsed in demineralised water. Sections were counterstained with Mayer's haematoxylin and eosin. Finally, the sections were

Figure 1 Results of ISH, CARD-ISH, and IFA on paraffin wax sections of infected Caco-2 cells and rat spleen. (A)–(D), *Y. enterocolitica* serotype O:8 strain WA (ATCC 9610) infected Caco-2 cell line; (E)–(H), *Y. enterocolitica* serotype O:8 strain WA (ATCC 9610) infected rat spleen; (A) and (E), control CARD-ISH with pBR322 probe; (B) and (F), normal ISH with pYV probe; (C) and (G), CARD-ISH with pYV probe; (D) and (H), IFA with a *Y. enterocolitica* serotype O:8 mouse monoclonal antibody. All preparations were briefly counterstained with Mayer's haematoxylin and eosin.

mounted in Permount (Fisher Scientific, Fair Lawn, New Jersey, USA).

CATALYSED REPORTER DEPOSITION (CARD)–ISH SIGNAL AMPLIFICATION

A normal ISH and ABC method (as described above) was used up to the DAB detection. Before amplification with the CARD-ISH signal amplification method, biotinylated

tyramine (BT) was made up as previously described by Kerstens *et al.*¹⁴ For the CARD-ISH signal amplification, BT was precipitated through the enzymatic reaction of horse radish peroxidase. The specimens were incubated with BT-PBS and 0.01% H₂O₂ for five minutes at room temperature. The BT concentration was 0.7 μM. After rinsing in PBST, the biotin precipitate was detected with a second incuba-

Figure 2 Results of CARD-ISH and IFA on paraffin wax sections of clinical biopsy specimens from two patients with a *Y. enterocolitica* infection. (A)–(C), lymph node biopsy specimen from a patient with a mild *Y. enterocolitica* infection; (D)–(F), stomach biopsy specimen from a patient with a more severe *Y. enterocolitica* infection; (A) and (D), control CARD-ISH with pBR322 probe; (B) and (E), CARD-ISH with pYV probe; (C) and (F), IFA with a *Y. enterocolitica* serotype cross reactive antibody against serotypes O:3, O:5,27, and O:9. All preparations were briefly counterstained with Mayer's haematoxylin and eosin.

tion of ABC (dilute ABC complex 1:4) for 20 minutes at 37°C. After rinsing in PBST and PBS, horse radish peroxidase was demonstrated as described above.

IMMUNE FLUORESCENCE ASSAY

Sections from the same tissues as described above were deparaffinated and treated with protease XIV (0.0125%, Sigma) in PBS for 30 minutes at 37°C. Mouse monoclonal antibodies against *Y. enterocolitica* serotypes O:8 and O:3, and a cross reactive antibody against serotypes O:3, O:5,27, and O:9, in dilutions of 1:25, 1:20, and 1:20 respectively in PBS were added, followed by addition of fluorescein isothiocyanate (FITC) conjugated rabbit anti-mouse immunoglobulins (1:100, Dakopatts, Glostrup, Denmark).¹ Both monoclonal antibodies showed clear, sharp cell wall fluorescence.

Results

The CARD-ISH detection of *Y. enterocolitica* in infected Caco-2 cells and rat spleen was compared with ISH and IFA on parallel sections (fig 1). When using ISH with the conventional ABC method, weak hybridisation signals were observed in both infected Caco-2 cells and rat spleen (fig 1, B and F). After application of the CARD-ISH method, clear hybridisation signals and additional hybridisation signals were observed (fig 1, C and G). IFA showed a similar sensitivity as CARD-ISH, although with IFA a loss of tissue morphology was observed (fig 1, D and H). When biotinylated pBR322 was used as control probe no hybridisation signals were observed in infected Caco-2 cells and rat spleen (fig 1, A and E).

Figure 2 shows the results of CARD-ISH and IFA on parallel human biopsy tissue sections of two patients infected with *Y. entero-*

colitica. Patient 1 (fig 2, A–C) had a mild infection in an abdominal lymph node, and patient 2 (fig 2, D–F) had a severe infection in the stomach. A CARD–ISH control with biotinylated pBR322 as probe is shown in fig 2, panels A and D. No hybridisation signal was observed in either panel. The CARD–ISH with the *Y enterocolitica* virulence plasmid as probe is shown in fig 2, panels B and E. Panel B shows a small focus of infection and some solitary bacteria, while panel E shows a large focus of infection. Panels C and F in fig 2 show the IFA detection of *Y enterocolitica*. This IFA detection showed similar results to the CARD–ISH detection, but with loss of tissue morphology.

As negative controls, colon biopsies of two patients with Crohn's disease were analysed. IFA was performed to compare the signals with the CARD–ISH. Both IFA and CARD–ISH were negative. No CARD–ISH signal was observed in the surrounding tissue or in normal intestinal flora (data not shown).

Discussion

The detection of bacteria in routinely fixed tissue samples for light microscopy is still hampered by the low sensitivity and specificity of the methods available. IFA is still the most commonly used routine method for detecting *Y enterocolitica*. However, IFA requires different *Y enterocolitica* serotype specific antibodies,¹ and tissue morphology is completely lost. For ISH a single probe can be used to detect different serotypes, while tissue morphology is generally well preserved. On the other hand, the sensitivity of ISH is limited. To improve the sensitivity of this method several immunohistochemical amplification methods have been developed. Sequential incubations with various antibodies are performed or else the most sensitive current immunohistochemical signal amplification technique—the horseradish peroxidase labelled avidin–biotin complex (ABC) technique—is applied to increase the sensitivity.¹¹ Although both methods result in higher sensitivity, it is still often insufficient.

Another option is to combine immunohistochemical amplification with a multicopy target or with probes ranging from several kilobases up to the size of the total bacterial genome.^{19–20} This combined ISH method has already been applied successfully for the detection of different human pathogens, for example *Helicobacter pylori*, *Chlamydia trachomatis*, *Leishmania*, *Plasmodium falciparum*, and various different viruses.^{20–24} However, in cases when only probes of 30 to 50 bases can be used or when the accessibility of the DNA target is poor, the sensitivity of ISH remains a limiting factor. In addition, in order to detect bacteria long enzyme digestions are generally required to obtain good accessible target DNA; while this results in optimal hybridisation it also leads to a loss of tissue morphology. To circumvent these problems enhanced amplification is necessary.

Recently, amplification methods have been described which increase the sensitivity of the ISH. The PCR–ISH is based on the amplification of nucleic acids in the tissue section,

directly followed by a normal ISH.¹² The CARD–ISH on the other hand, starts with a normal ISH, after which the detection signal is amplified.^{14–25} Both methods allow the detection of a single copy of the human papilloma virus (HPV) 16 in SiHa cells with similar sensitivity, while normal ISH was limited by a threshold of 20 copies per cell.^{12–25} Furthermore, both CARD–ISH and PCR–ISH can be performed within 24 hours. Although the sensitivity of both methods is similar and both are rapid, expensive equipment and reagents are required for PCR–ISH while for CARD–ISH, normal ISH and routine immunohistochemical reagents and equipment can be used, making the CARD–ISH method more applicable for routine settings.

Zehbe *et al* recently described a modified CARD–ISH method in which they used a nanogold label with silver acetate autometallography to visualise the probe.²⁶ With this modified CARD–ISH method the sensitivity for the detection of HPV 16 in SiHa cells was similar to that achieved when enzymatic detection was used for visualisation. However, the signals were sharper and contrast was increased compared with enzymatic detection, resulting in better ability to judge positivity. Moreover, for pathophysiological studies, this modified CARD–ISH can be very useful since gold and silver are very electron-dense, which allows the use of the CARD–ISH in electron microscopy.

Detection of *Y enterocolitica* in cases of chronic infection and extraintestinal complications is almost impossible.^{1–27} In these cases the organism disappears from the circulation and hides within the gut associated lymphoid tissue and other lymphoid tissues.³ The only direct detection of the bacterium then possible is in biopsy samples. The CARD–ISH system appears to be a good molecular tool to demonstrate *Y enterocolitica* within infected tissues and provides direct information of the presence of *Y enterocolitica* in such tissues. Furthermore, the CARD–ISH system can be used to gain knowledge about the pathophysiology of *Y enterocolitica* infections.

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