Localisation of enteropathogens in paraffin embedded tissue by immunoperoxidase

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SUMMARY An indirect immunoperoxidase technique has been used to identify enteropathogens in formol-sublimate fixed paraffin embedded sections of calf intestine. Infections with bovine rotavirus, bovine coronavirus, Newbury agent SRV-1, and K99⁺ *Escherichia coli* have been detected in the intestines from experimentally infected and conventionally reared diarrhoeic or normal calves. The ability to visualise enteropathogenic agents in histological sections resulted in the demonstration of virus infected cells at sites not previously shown to be infected using the immunofluorescence technique.

Diarrhoea frequently occurs in neonates and has been ascribed to a variety of infectious causes including viruses, bacteria, and protozoa. Excretion of an infectious agent in diarrhoeic faeces does not prove a causal association because enteropathogens may be excreted by normal animals.¹ Direct localisation of an agent in association with characteristic lesions, however, provides a more definite diagnosis.

Most investigations have shown viral and bacterial antigens by immunofluorescence in cryostat sections of intestinal tissues. Collection of frozen material is inconvenient, however, and it dehydrates during storage; cryostat sections stained by immunofluorescence methods are of little value for detailed histological study. Fixed material has a number of advantages: it can be processed at a convenient time, thinner sections may be cut, it provides good preservation of cellular architecture, and it is excellent for detailed histological investigations.

This paper describes the application of immunoperoxidase staining to demonstrate the location and extent of bacterial and virus infections in paraffin embedded intestinal calf tissues so that infectious agents and characteristic lesions may be detected and studied in the same section.

Material and methods

In the indirect immunoperoxidase method antibody (primary antibody) is attached to the antigen under investigation, followed by an antiglobulin against

immunoglobulins of the species providing the primary antibody. The antispecies antiglobulin is conjugated with horseradish peroxidase (peroxidase conjugated antibody).

The attached peroxidase is visualised by a histochemical reaction, usually employing Graham and Karnovsky's diaminobenzidine method.²

INFECTED TISSUES AND THEIR FIXATION

Infected tissues stained by the immunoperoxidase method were:

- 1 Monolayers of monkey kidney cells (MA 104) infected with the tissue culture adapted bovine rotavirus³
- 2 Intestinal tissues from five gnotobiotic calves experimentally infected with rotavirus, 14 infected with Newbury agent,⁴ and two infected with *Escherichia coli* B41
- 3 Intestinal tissues from three conventionally reared calves infected with *E coli* B44
- 4 Intestinal tissues from 14 conventionally reared calves which were excreting rotavirus, detected by enzyme linked immunosorbent assay (ELISA)⁵ of faeces
- 5 Intestinal tissues from eight conventionally reared diarrhoeic calves.

Fixatives tested using infected monolayers were acetone, ethanol, formol-calcium, and formol-sublimate. Fixation was by immersion for 10 min. Formol-sublimate was replaced by 80% ethanol for 10 min before mercuric chloride deposits were removed by immersion for 5 min in Gram's iodine, followed by 5 min in 0.5% sodium thiosulphate.

Intestinal samples were removed under pentobarbitone sodium (Sagatal, May and Baker Ltd) anaesthesia* and fixatives assessed using infected intestine were formol-sublimate and 12% neutral buffered formalin, the former being replaced after 24 h with 80% ethanol. Blocks were embedded in paraffin wax and sections cut at 5 μ m.

PREPARATION OF ANTISERA

Rotavirus hyperimmune antiserum was raised by oral administration of 3.0 ml of a cell-culture grown bovine rotavirus to a 15 day old gnotobiotic calf. This was followed by a second oral dose at 33 days and two intramuscular injections at 63 and 105 days with the same rotavirus, purified from MA-104 cells and emulsified with Freund's incomplete adjuvant; antiserum was obtained at 139 days of age.³

Coronavirus hyperimmune serum was prepared as described previously.⁶

Antiserum to the Newbury agent was raised by oral administration of 3.0 ml of a bacteria free 0.45 μ m faecal filtrate containing the isolate SRV-1⁴ to a gnotobiotic calf at 22 and 42 days old. Antiserum was obtained at 57 days of age.

Antisera to live *E coli* B41, grown on TGX medium,⁷ was prepared by the method of Sojka.⁸ Antisera to purified K99 adhesin⁹ was prepared by subcutaneous injection of a rabbit with 200 μ g of antigen in 2 ml of Freund's incomplete adjuvant. Four weeks later the rabbit was injected intravenously with 200 μ g of antigen and bled after a further 10 days.

IMMUNOPEROXIDASE STAINING PROCEDURE

Sections were dewaxed by successive immersion for 1 min in xylene, absolute alcohol, 90% alcohol, and 70% alcohol. Mercuric chloride deposit was removed as described for cell monolayers.

Inhibition of endogenous peroxidase was compared using 0.5% H₂O₂ in methanol, 10% H₂O₂ in methanol, 0.074% HCl in ethanol, and periodic acid followed by sodium borohydride.¹⁰

To eliminate background staining sections were incubated with 20% non-immune serum for 10 min. Non-immune serum was serum from uninfected rabbits or goats containing no cross reacting antibodies and corresponding to the animal species in which the peroxidase conjugated antibody was raised. Slides were then drained but not washed.

Antisera containing primary antibody diluted to 1/20, 1/40, 1/80, and 1/160 and peroxidase conjugated antisera diluted to 1/160, 1/320, 1/640, and 1/1280 in sterile phosphate buffered saline pH 7.0 (PBS) were tested at different temperatures and various incubation times.

Monolayers infected with rotavirus were

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incubated with primary antiserum for 30 or 120 min at room temperature followed by rabbit antiovine gamma globulin conjugated with peroxidase (RAB/PO, Nordic Immunological Laboratories Ltd) for 30 or 120 min at room temperature.

Sections of virus infected intestine were incubated with primary antiserum for 30 min followed by RAB/PO for 30 min at room temperature, or with antiserum for 120 min at room temperature or 18 h at 4°C followed by RAB/PO for 120 min at room temperature.

E coli infected sections were incubated with antisera to whole *E coli* (B41) or K99 adhesin for 60 min at room temperature or 30 min at 37°C followed by 30 min exposure to 4°C. This was followed by goat antirabbit peroxidase conjugate (Nordic Immunological Laboratories Ltd) for 30 min at room temperature.

Sections were washed with three changes of PBS for 5 min each before and after treatment with peroxidase conjugate.

The peroxidase was detected by incubation for 5 min with freshly prepared 0.05% 3, 3'-diaminobenzidine tetrahydrochloride (Koch-Light Laboratories Ltd), containing 0.01% hydrogen peroxide in PBS. Slides were washed for 10 min in running tap water to stop the reaction and counter-stained briefly in Gills-3 haematoxylin (Lerner Laboratories), differentiated in acid alcohol, blued in tap water, dehydrated, and mounted in DPX (Gurr).

Specificity of viral antisera was demonstrated by use of each preimmune serum and antiserum on intestinal tissue of gnotobiotic calves infected with each of the viruses. Specificity of *E coli* and K99 antisera was shown by staining tissues infected with K88 and an atypical *E coli*.¹¹

Results

STAINING CONDITIONS

Acetone, formol-sublimate, formol-calcium, and ethanol fixation were all excellent for immunoperoxidase staining of rotavirus in cell monolayers. Formol-sublimate increased the contrast between viral antigen and background by reducing non-specific staining of tissue. Formol-sublimate fixed, paraffin wax embedded tissues gave more intense specific staining of virus infected enterocytes than 12% neutral buffered formalin.

All methods examined inhibited endogenous peroxidase in erythrocytes, leucocytes, and enterocytes satisfactorily, but there was variation in the degree of background staining. Treatment with hydrogen peroxide gave intense staining of viral antigen, but also dark non-specific background

Table 1 Procedure for indirect immunoperoxidase staining

1	Dewax thoroughly with xylene and hydrate.
2	Remove mercuric chloride deposit with Gram's iodine and sodium thiosulphate.
3	Inhibit endogenous peroxidase with 0.01M periodic acid in distilled water for 10 min followed by 0.003M sodium borohydride in distilled water for 30 min. Wash with three changes of PBS, 5 min each.
4	Treat with 20% non-immune serum for 10 min; do not wash; drain serum from slides.
5	Incubate with optimally diluted primary antisera. Viral antisera for 18 h at 4°C. <i>E coli</i> antisera for 60 min at room temperature. Wash with three changes of PBS, 5 min each.
6	Treat with optimally diluted peroxidase conjugate, 2 h on virus sections and 30 min on <i>E coli</i> . Wash with three changes of PBS, 5 min each.
7	Incubate with freshly prepared 0.05% diaminobenzidine containing 0.01% hydrogen peroxide for 5 min. Wash well in running tap water for 5 min.
8	Counterstain briefly in Gills-3 haematoxylin, blue, dehydrate, clear, and mount in DPX (Gurr).

staining. Hydrochloric acid in ethanol reduced background staining, but virus infected enterocytes stained weakly. Best results were obtained by treatment with 0.01M periodic acid for 10 min followed by 30 min in 0.003M sodium borohydride; this gave pale background staining of leucocytes, erythrocytes, and mature enterocytes, and dark staining viral antigen in infected enterocytes. Non-specific background staining of tissue was reduced by incubation of sections with 20% non-immune serum before treatment with primary antisera.

Optimal dilutions of primary antisera determined by titration on positive sections were 1/80 for rotavirus, 1/40 for Newbury agent, 1/80 for coronavirus, and 1/160 for *E coli*. Optimal dilutions of peroxidase conjugated antibody were 1/320 for rotavirus, Newbury agent, and *E coli* and 1/640 for coronavirus.

Incubation of cell culture monolayers with rotavirus antiserum followed by RAB/PO for 2 h each at room temperature gave dark background staining, but non-specific staining was reduced by incubation with antiserum and conjugate for only 30 min each. Specific staining of viral antigens in

paraffin sections was obtained only after 18 h or more incubation at 4°C with primary antisera. Incubation for 2 h with RAB/PO gave more intense specific staining but also caused higher non-specific background staining. Incubation of sections with *E coli* antiserum for 30 min at 37°C followed by 30 min at 4°C gave results similar to those obtained with incubation for 60 min at room temperature. The optimal conditions and recommended procedure for indirect immunoperoxidase staining of formol-sublimate fixed paraffin-embedded tissues are given in Table 1.

SPECIFICITY OF ANTISERA

Preinoculation sera used at 1/40 dilution gave no positive immunoperoxidase staining of antigen in infected tissues. Postinoculation sera showed specific staining for the virus under investigation (Table 2). Antisera to live *E coli* B41 and K99 adhesin stained bacteria in tissue from B41 and B44 infected calves, but failed to stain K88⁺ *E coli* in pig tissues and an atypical *E coli* in calf tissues.

LOCATION OF INFECTIONS

The results of immunoperoxidase staining of calf tissues with antisera are shown in Table 3. Enterocytes infected with Newbury agent were detected in only 3/14 calves, at 18 and 24 h after inoculation. Rotavirus infected enterocytes were demonstrated in two of three conventional calves infected with *E coli*, confirming the previous finding of rotavirus in the faeces of these two calves by ELISA. Rotavirus infected enterocytes were seen in all 14 calves in which faecal excretion of rotavirus had been detected by ELISA. Small numbers of colonic enterocytes infected with coronavirus were found in three of these calves; coronavirus in the faeces of these calves had not been detected by ELISA. Of the eight conventionally reared diarrhoeic calves examined by immunoperoxidase for enteropathogens one was infected with rotavirus, two with coronavirus, four with rotavirus and coronavirus, and one with rotavirus and Newbury agent. Bacteria were found in association with the mucosa of the colon of the eight animals but were not K99⁺ *E coli*.

Rotavirus infected enterocytes were detected by immunoperoxidase with a distribution similar to that described in studies using immunofluorescence^{1,2}—that is, on the surface of normal length villi. They were most numerous on the apical half of the villus, although infected cells were seen along its whole length (Fig. 1). Additionally, small numbers of rotavirus infected cells were detected in caecal crypts (Fig. 2).

Coronavirus infected cells were found with a distribution similar to that described in studies using

Table 2 Demonstration of specificity of antisera using germ free calves mono-infected with enteropathogenic viruses

Infection	Primary antiserum					
	Rotavirus		Coronavirus		Newbury agent	
	Pre†	Post	Pre	Post	Pre	Post
Rotavirus (5)*	—	+	NT	—	NT	—
Coronavirus (1)	NT	—	—	—	NT	—
Newbury agent (3)	NT	—	NT	+	—	+

*Number of calves shown in parentheses.

†Pre- or post-inoculation sera.

NT = not tested.

Table 3 Immunoperoxidase staining in infected calf tissues

Calves		Infection	Antiserum				
No	Type		Rotavirus	Coronavirus	Newbury agent	<i>E coli</i> B41	K99 adhesin
2	Gnotobiotic	None	0	0	0	NT	NT
5	Gnotobiotic	Rotavirus	5*	0	0	NT	NT
14	Gnotobiotic	Newbury agent	0	0	3	NT	NT
2	Gnotobiotic	<i>E coli</i> B41	0	0	0	2	2
3	Conventional	<i>E coli</i> B44	2	0	0	3	3
14	Conventional	Rotavirus	14	3	0	NT	NT
8	Conventional	Unknown	6	6	1	0	0

*Number of animals positive.

NT = not tested as no adherent bacteria observed.

Fig. 1 Rotavirus infected enterocytes distributed along the whole length of the villus of the mid-ileum. Original magnification $\times 200$.

immunofluorescence¹³—that is, on the surface of ileal villi, caecum, and colon and in caecal and colonic crypts (Fig. 3). Additionally, small numbers of crypt cells were seen to be infected in the ileum.

Newbury agent was found to infect enterocytes primarily near the base of the villi (Fig. 4) in the upper small intestine. In experimentally infected calves adherent *E coli* B41 and B44 were demonstrated using B41 antiserum and shown to possess the K99 adhesin (Fig. 5).

Fig. 2 Rotavirus infected crypt enterocytes of the caecum. Original magnification $\times 500$.

Discussion

The ease with which antigens may be detected in fixed tissue by the immunoperoxidase system is dependent on the accessibility of the antigen to the primary antibody. Antigen availability is affected by the length of exposure fixative,¹⁴ choice of fixative,¹⁵ and presumably by the thickness of tissue. In this study viral antigen in monolayer cell cultures was stained satisfactorily by a short incubation with primary antisera, probably because of the short fixation time. Overnight incubation of paraffin sections with primary antiserum was necessary to stain virus infected tissues, presumably due to slower penetration of primary antibody into tissue sections,

Fig. 3 Severe infection of surface and crypt enterocytes of the colon with coronavirus. Original magnification $\times 200$.

Fig. 4 Enterocytes at the base of a villus of the mid-ileum infected with Newbury agent SRV-1. Original magnification $\times 500$.

Fig. 5 *Escherichia coli* B41 stained for the K99⁺ adhesin, attached to the villus enterocytes of proximal ileum. Original magnification $\times 800$.

which are much thicker than monolayers, and the decreased immunoreactivity of viral antigens after prolonged fixation. Demonstration of rotavirus antigen in paraffin sections of Zenker fixed ileum¹⁶ has been reported, but the incubation times used were not.

Fixation is an important factor in the demonstration of antigens in paraffin embedded tissues by the immunoperoxidase technique.¹⁵ Formalin is used for routine fixation in most laboratories, but improved immunoperoxidase staining with mercury based fixatives has been reported.¹⁵ Our observations confirmed these findings: formol-sublimate fixation reduced non-specific background staining of tissues and produced more intense staining of viral antigens in enterocytes. Viral antigens in paraffin embedded tissues have been detected by the immunoperoxidase method following Zenker fixation¹⁶ (rotavirus), fixation in cold 98% alcohol¹⁷

(bovine virus diarrhoea virus), and formalin^{18,19} (hepatitis B virus,²⁰ herpes simplex virus).

Endogenous peroxidase was sufficiently inhibited by all methods examined, but non-specific attachment of peroxidase conjugate caused dark background staining which interfered with the interpretation of results. Background staining was affected by the fixative, method of inhibiting endogenous peroxidase, and treatment of sections with non-immune serum, but could be completely eliminated

only by high dilution of the peroxidase conjugate. Thus where an optimal dilution of conjugate was used, strong positive staining of virus infected enterocytes and adherent bacteria was achieved with minimal non-specific background staining.

The specificity of the antisera was confirmed by use of tissues from mono-infected gnotobiotic calves. Poor results were obtained from calves infected with Newbury agent and there are three possible reasons for this. Newbury agent particles are seen in intestinal contents in lower numbers than rotavirus and coronavirus,²¹ so it is probable that the amount of antigen available for detection in enterocytes was low. Secondly, convalescent antiserum was used in the immunoperoxidase test for Newbury agent which was likely to be of low titre; failure of the virus to grow in tissue culture prevents hyperimmunisation. Thirdly, Newbury agent calves were killed at intervals of 0.5 to 10 days after inoculation so that not all were expected to be infected at the time of slaughter. Presumably the calves which stained positively were those at the height of infection.

Detection of mixed infections in tissues from naturally infected calves showed the value of the method in investigating farm cases, and identification of coronavirus infection in three calves with faeces negative by ELISA suggested a superior sensitivity by the immunoperoxidase method.

It can be difficult to identify, with confidence, the precise position of small numbers of cells stained by immunofluorescence, but virus infected cells stained by immunoperoxidase can be examined in sections where the cellular architecture is well preserved and single infected cells can be identified as an integral part of the sections and distinguished from exfoliated, infected enterocytes present in luminal contents. This study has shown a distribution of rotavirus and coronavirus infected cells similar to that described in studies using immunofluorescence.¹²⁻¹³ However, small numbers of cells infected with rotavirus were detected in the colonic mucosa, small numbers of crypt cells infected with coronavirus were detected in the ileum, and a few cells infected with Newbury agent were detected at the base of jejunal villi; none of these sites has been shown previously to be infected in calves using the immunofluorescence technique. These observations and those of Newbury agent infection illustrate the value of antigen detection in good histological sections.

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