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Detection of Bovine Virus Diarrhoea Virus RNA by in situ Hybridisation with Digoxigenin-Labelled Riboprobes

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Summary
A non-isotopic in situ hybridisation (ISH) method has been developed for the detection of bovine virus diarrhoea virus (BVDV) RNA. The technique is highly specific and allows the detection of BVDV in formalin-fixed paraffin-wax-embedded sections. Digoxigenin-labelled riboprobes were generated to the coding region for the p125 non-structural protein and consequently it was possible to locate the virus positive-sense RNA in BVDV-infected tissues. Formalin-fixed tissues are ideal for ISH, combining the possibility of a retrospective analysis of tissues for virus distribution with excellent morphological preservation. In situ hybridisation has facilitated a precise examination, at the cellular level, of viral nucleic acid distribution during pestiviral infections, with an increased sensitivity and wider applicability than immunocytochemistry.

Introduction

Bovine virus diarrhoea virus (BVDV) is a member of the Pestivirus genus; a genus it shares with classical swine fever virus (CSFV) of pigs (also known as hog cholera virus) and Border disease virus of sheep [1]. The pestivirus genome is a positive sense RNA strand of approximately 12.5 kb [2] which is translated as a large polyprotein which is co- and post-translationally processed into smaller proteins [3]. Comparison of the RNA and deduced amino acid sequences of the different pestivirus isolates [4–8] reveals several regions with a high degree of homology, while other areas are more variable. BVDV is the causative agent of a range of disease syndromes in cattle, from a subclinical transient viraemia through to fatal mucosal disease (MD). There are many antigenically distinct isolates of BVDV and, furthermore, two biotypes of the virus are recoverable from field cases of mucosal disease which can be differentiated by the presence or absence of cytopathology in cell culture [9–11]. The non-cytopathogenic biotype is the most prevalent form of the virus and is particularly associated with the in utero infection of calves during the first trimester of pregnancy [12]. Calves infected at this time may spontaneously abort or, if the pregnancy continues to full term, be born with congenital abnormalities, particularly of the central nervous system. A proportion of calves infected in utero during the first trimester are born, often apparently normal, but in fact, persistently infected (PI) with the non-cytopathogenic BVDV. These animals remain infected throughout their life and appear to be immunologically tolerant to the persisting virus. They are, however, able to clear superin-

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fections with antigenically distinct 'heterologous' isolates of BVDV [13]. It is these PI animals that are susceptible to the fatal form of BVDV infection known as MD following superinfection with an antigenically 'homologous' cytopathogenic form of BVDV [14]. The origin of the cytopathogenic virus has been an enigma but recent work has suggested a possible derivation from the non-cytopathogenic form by recombination with host cellular RNAs [15] or duplication and rearrangement of virus genomic RNAs [16]. The exact cause of death in mucosal disease remains unknown but one of the major diagnostic symptoms is the dramatic depletion of lymphoid tissue throughout the animal, particularly in the gut-associated mucosa. Cattle persistently infected with BVDV are estimated to make up approximately 1% of the UK national herd. They are a constant source of virus with the potential to infect other animals with which they are in contact. Virus can be isolated from the blood of PI animals at titres of up to 10^6 logTCID/ml and from most other body fluids. Using conventional virus isolation techniques to examine the tissues of PI animals, BVDV has been identified in all tissues so far tested at a range of different titres, not surprisingly, some of the highest values are found in lymphoid tissues. In vitro the virus will grow in all bovine tissues tested, again indicating that the virus has a wide tropism. This ability of the virus to infect many tissues and derived cell lines contrasts with the specific and very restricted tissue damage observed at postmortem examination of MD animals. This observation suggests a particular association of the virus with lymphoid tissues. Examination of this association, both in PI animals and in MD animals, with the use of virus-specific monoclonal antibodies has been hampered by the antigenic variability of different virus isolates, the relatively poor morphological preservation of cryopreserved tissues and the paucity of antibodies suitable for formalin-fixed, wax-embedded tissues. The application of the technique of in situ hybridisation (ISH) would overcome many of these difficulties. This method is particularly suited to the examination of formalin-fixed, paraffin-embedded tissues and has been developed and applied to a wide range of investigations [17–20]. In this paper we describe the development of a non-isotopic technique using digoxigenin-labelled riboprobes to precisely locate BVDV RNA in paraffin sections. The probe used was chosen because it corresponds to one of the most highly conserved regions of the pestivirus genome, that encoding the non-structural protein designated p80 in the cytopathogenic virus biotype (corresponding to the C-terminal region of the p125 protein in the non-cytopathogenic viruses) [8]. Although methods already exist for detecting virus antigen in postmortem tissue [21–23], these either require cryopreservation of the tissues or are limited to a handful of monoclonal antibodies which recognise virus antigen in fixed, wax-embedded tissues. The methods described in this paper have been developed to allow sequential studies of virus migration following infection and for analysis of archival material. Further, we describe the evaluation of a range of pretreatments during the optimisation of this procedure and compare the results obtained by ISH with those obtained using conventional immunocytochemistry.

Materials and Methods

Cattle, diagnosed as persistently infected with BVDV, were obtained from screening field outbreaks of mucosal disease. They were humanely killed by intravenous injection of pentobarbitone (Euthatal). At postmortem examination, a range of lymphoid and brain tissues were taken and immediately fixed in 10% neutral buffered formalin.

Preparation of Riboprobes

Riboprobes were synthesised from a conserved portion of the p80 coding region of BVDV to maximise the chance of hybridisation with field strains of the virus. A 758-bp fragment (corresponding to 6,524–7,282 on the NADL genome) was obtained by reverse transcription and PCR amplification of RNA purified from the Pe 515 cytopathogenic isolate. The fragment was cloned into plasmid pSPT19. The recombinant plasmid was linearised with EcoRI for antisense transcription. Digoxigenin-11-UTP was incorporated into riboprobes synthesized from 1 µg of plasmid DNA using the methods described by Holtke and Kessler [24]. The probes were ethanol precipitated and checked for purity by agarose gel electrophoresis and dot-blot hybridisation.

In situ Hybridisation

Sections were cut at thicknesses of 3 µm for lymphoid and 6 µm for brain tissues and mounted in duplicate onto 3-aminopropyltriethoxysilane-coated slides [25]. The sections were incubated at 37 °C overnight to ensure adequate adherence of the sections to the slides and stored at room temperature until use. Immediately prior to dewaxing in safeclear, the slides were heated to 75 °C for 15 min and rehydrated to distilled water. In order to improve the probe penetration, a variety of protease treatments were tested:

(i) pepsin 1 mg ml^{-1} in 0.2 *M* HCl at 37 °C for 15 min;
(ii) protease VIII 0.25 mg ml^{-1} at 25 °C for 5–30 min;
(iii) pronase E (protease XIV) 0.2 mg ml^{-1} at 4 °C for 18 and 40 h;
(iv) proteinase K 0.1 mg ml^{-1} at 37 °C for 15–30 min;
(v) microwave treatment in distilled water.

Following digestion, the slides were washed twice in distilled water, dehydrated in alcohol and air dried. The hybridisation solution was 50% formamide (BRL), 5% dextran sulphate, 2 × SSC (1 × = 0.15 *M* sodium chloride, 0.015 *M* sodium citrate), 0.1 m*M* EDTA, 1 m*M* Tris·HCl pH 7.5 and denatured salmon sperm DNA to a final

concentration of 4 mg ml^{-1}. All solutions were prepared in diethylpyrocarbonate-treated distilled water.

50 µl of hybridisation mix containing 25 ng of freshly denatured probe was used per section and overlaid with a glass coverslip. The slides were placed in pairs in Teresaki plates moistened with 2 × SSC and were heated in a water bath at 65 °C for 15 min to denature the viral RNA. The hybridisation was performed at 55 °C in an incubator for 2 h.

An antisense probe specific for BVDV-positive strand RNA and a negative control probe (provided in the labelling kit) were used on paired serial sections of BVDV-infected tissues mounted on the same slide. Uninfected tissues were also used as negative controls.

Post-Hybridisation Treatment

The coverslips were carefully washed off in 4 × SSC and the slides washed in fresh 4 × SSC for 5 min. The slides were rinsed in two changes of 1 × SSC at room temperature before a more stringent washing in 0.1 × SSC at 55 °C for 15 min to remove any non-specifically hybridised probe. The slides were not allowed to dry out at any time during or following these post hybridisation washes.

Detection of Signal

Prior to detection of the DIG-labelled probe the slides were incubated in a blocking solution of 3% (w/v) bovine serum albumin and 0.05% (v/v) Triton X-100 in buffer 1 (100 m*M* Tris-HCl, 150 m*M* NaCl pH 7.5) for 30 min at room temperature in a humidified chamber. Slides were then incubated for 30 min with 1:500 dilution of alkaline phosphatase-conjugated polyclonal sheep anti-digoxigenin antibody in blocking solution. Excess conjugate was removed by washing twice in buffer 1 for 5 min. The slides were equilibrated in buffer 3 (10 m*M* Tris-HCl, 100 m*M* NaCl, 50 m*M* $MgCl_2$ pH 9.5) for 2 min and then incubated with the substrate, NBT/BCIP, in the dark for 1–16 h. The reaction was stopped by immersing the slides in distilled water and, if required, the slides were counterstained in 1% methyl green for 1 min. Excess stain was removed by washing thoroughly in tap water and the slides were mounted in Kaiser jelly.

The alkaline phosphatase substrates naphthol fast red and the substrate provided in the Serotec AS/AP Plus immunostaining kit (which produced a brown-coloured precipitate and was used according to the manufacturer's instructions) were also tested. Alternative DIG detection systems were also tried according to the manufacturer's recommended procedures including sheep IgG Fab fragments directly conjugated with horseradish peroxidase and gold-conjugated anti-DIG IgG antibodies. These systems were visualised with the colourimetric substrate diamino benzidine (DAB) or silver enhancement, respectively.

Immunocytochemistry

The sections were processed as for in situ hybridisation. After dewaxing and rehydration, the sections were incubated at 4 °C in 0.2 mg ml^{-1} pronase E diluted in PBS for 18 h [22]. The sections were stained using the reagents and protocol supplied in the Serotec AS/AP Plus kit. Briefly, a gp48 specific monoclonal antibody C42 (Hannover) was diluted 1:10 in buffer 1 and incubated with the sections for 1 h. An appropriate negative control monoclonal antibody TRT I (specific for a turkey rhinotracheitis virus protein) was used for the duplicate section on the slides. After washing, the biotinylated link antibody supplied in the kit was applied to both sections for 1 h followed by the alkaline phosphatase-conjugated anti-biotin antibody for a further 1 h. The slides were again washed and the enzyme substrate (supplied with the kit) was applied for up to 1 h. This reaction was stopped by immersion in water, the slides were washed, counterstained in haematoxylin and mounted in the kit mounting medium.

Results

In situ Hybridisation

In lymphoid tissues of persistently viraemic animals, hybridised with the riboprobe designed to detect positive strand viral genomic RNA, a characteristic staining was observed, as illustrated in the sections of palatine tonsil and mesenteric lymph node in figures 1a and 2a. Intense staining was evident in many of the germinal centres and, moreover, appeared to be exclusively located in crescent-shaped areas of the follicle, recently designated the light zone [26]. There was little or no specific signal in the perifollicular areas of either tissues or around the tonsillar ducts.

The tissues taken from the brain also gave a clearly differentiated signal following ISH; intense staining was found in many neurones whereas there was little or no staining in the peri-neuronal cells (fig. 3a). In those neurones staining for BVDV, the cellular distribution was clearly cytoplasmic; such a localisation is typical of an RNA virus infection.

In the development of this procedure, two factors emerged as being crucial for good in situ hybridisation results. The first was the length of fixation of the tissue and the second was the type and duration of protease treatment required to unmask the nucleic acids. The use of protease VIII gave a good compromise between the requirements for unmasking BVDV RNA and maintaining good tissue morphology in routinely fixed tissues. Proteinase K digestion resulted in a similar intensity of signal as the protease VIII-treated sections but gave a higher generalised background. These treatments were only optimal, however, for tissues that had been fixed for less than 1 month. Tissues left in fixative for longer than 4 weeks before processing required stronger protease treatments while in tissues which had been fixed for 6 months or longer, viral RNA was not easily unmasked. When more extensive protease treatment was used for these sections, there was a consequent loss in the resolution of cellular morphology.

Pepsin-HCl treatment was initially tested on optimally fixed tissues but the morphology was sometimes poor. Despite this limitation pepsin-HCl digestion was able to unmask the target RNA in some over-fixed tissues. The pronase treatment of tissues prior to ISH generally gave a

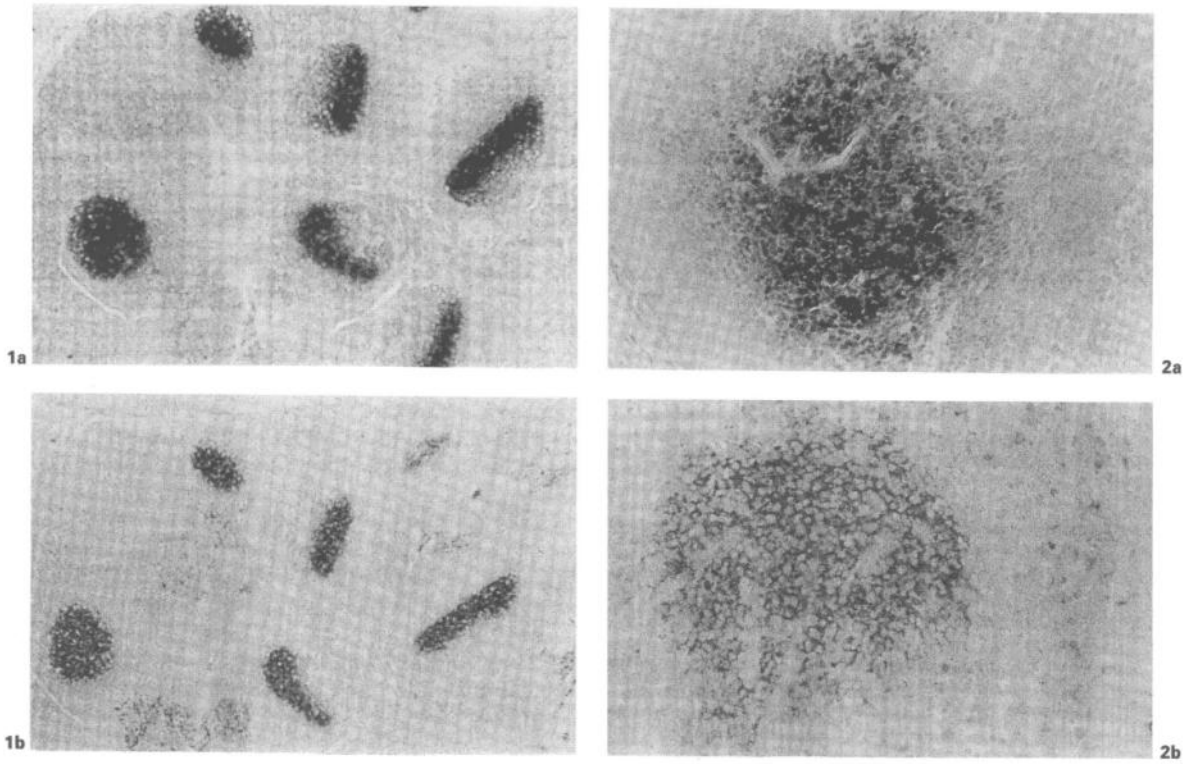


Fig. 1–3. Sections of post-mortem tissues taken from a calf persistently infected with BVDV. **a** In situ hybridisation (ISH) of viral RNA with a digoxigenin labelled riboprobe detected with a sheep anti-DIG alkaline phosphatase antibody. **b** Immunocytochemical (ICC) staining of viral antigen with a BVDV specific monoclonal antibody (C42) detected using an alkaline phosphatase-conjugated antibody system. **1a, b** Palatine tonsil – serial sections. Intense signal in the light zones of several germinal centres. × 40. **2a, b** Mesen-

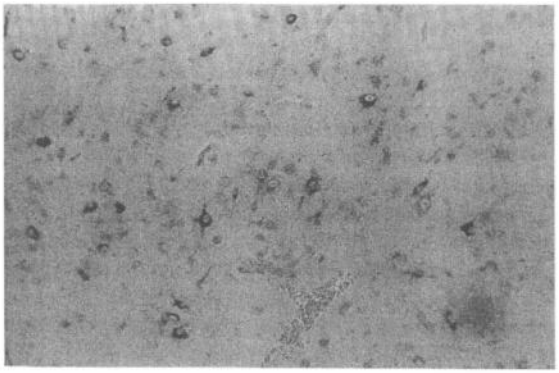
weaker signal than those previously described, particularly when used on optimally fixed tissues. This enzyme did, however, give a positive signal for ISH on some of the longer-fixed sections. Indeed, by increasing the pronase digestion time to 48 h, a strong signal was obtained where little or none had resulted from the other methods.

Microwave treatment of fixed sections in heavy metal solutions and even in distilled water has been reported to enhance antigen retrieval [27] and therefore sections microwaved in distilled water were tested for both ISH and ICC. The results were extremely variable with regions of the sections drying out and it was concluded that this treatment had no advantage over protease digestion and was not suitable for routine use with either technique.

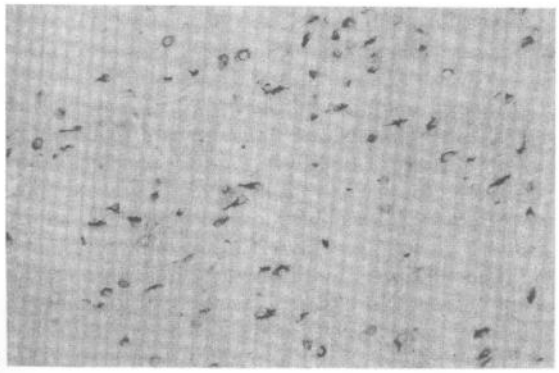
In some cases the blue colour of the NBT/BCIP reaction product compromised the choice of counterstain (it was not visible against a counterstain of haematoxylin for example). To overcome this, other detection systems were tested including alternative alkaline phosphatase substrates, horseradish peroxidase-linked antibodies and gold-conjugated antibodies. In all cases the signal strength obtained was very significantly reduced in comparison with the alkaline phosphatase-NBT/BCIP detection system.

Immunocytochemistry

The distribution of viral antigen in those tissues described above was investigated with a monoclonal antibody (C42), which binds to the structural glycoprotein gp48 of BVDV. This antibody is one of the few that detect virus in formalin-fixed, paraffin-embedded tissues. Allan et al. [22] have reported the use of 0.02% pronase E at 4 °C for



3a



3b

teric lymph node. Germinal centre strongly positive for virus RNA and antigen. × 200. **3a, b** Cerebral cortex. Neurones staining intensely in the cytoplasm with both techniques. × 100.

demonstrating BVDV antigen in formalin fixed tissues using hyperimmune antiserum, streptavidin/biotin and peroxidase. Although treatment with this enzyme was not suitable for routine use in ISH, pronase E was optimal for the immunocytochemistry and gave a strong signal with good morphology. In the lymphoid tissues of the palatine tonsil and mesenteric lymph node the staining pattern observed (fig. 1b, 2b) was similar to that seen by ISH staining of BVDV RNA described above. Intense staining of many of the secondary follicles was evident with little staining in the peri-follicular and medullary areas.

Antigen staining in brain tissues again showed strong neuronal staining with little or no background signal (fig. 3b). As would be expected the antigen distribution was clearly cytoplasmic.

Discussion

Several methods have been described for the detection of viral nucleic acid by in situ hybridisation. This paper details our attempts to apply these to fixed tissues for the detection of BVDV viral RNA in tissues of persistently infected animals. Of the many variations tested only a relatively small range of treatments were found to give an acceptable balance between signal strength and tissue morphology. Several factors were found to be of critical importance. These included the choice of protease treatment for unmasking, the length of fixation of the tissue in a suitable neutral buffered formalin solution and the choice of reporter system and enzyme substrate for the final colorimetric detection. The use of protease VIII was routinely adopted for unmasking viral RNA in tissues that had been fixed, ideally for periods between 1 and 7 days. Longer fixation times necessitated the use of stronger protease treatments and resulted in losses in tissue morphology. The use of NBT/BCIP as the alkaline phosphatase substrate was very definitely the most sensitive of the DIG detection systems tested. The resulting blue reaction product did compromise the subsequent staining of the tissues with several of the routinely used counterstains. However, when other substrates which produced different colour reaction products were used, the signal strength obtained was markedly reduced and sometimes absent. For these reasons NBT/BCIP was the substrate system of choice together with the methyl green counterstain.

Viral RNA was clearly demonstrable in both lymphoid and brain tissues by ISH staining. There was a clear differentiation between areas of intense staining for the viral RNA and the unstained background (fig. 1a–3a). Moreover, there was adequate morphology remaining in the tissues after ISH to ascribe the cellular location of the virus. It was evident that individual cells or groups of cells which had a high viral load were in close proximity to other cells with little or no RNA. These photographs represent the virus present in a persistently infected animal at a given point in time. Whether adjacent cells would have shown infection at some other time point is impossible to predict. An in vitro observation is that it is possible to infect completely a growing cell sheet and many cell lines are (accidentally and unknowingly) persistently infected with noncytopathogenic BVDV. This situation does not seem to be reflected by the in vivo snapshots depicted here. In other cases, cell cultures have been shown to maintain a persistent infection although individual cells within the culture undergo cycles of infection

followed by virus clearance [28]. Whether this occurs in BVDV-infected cultures has not been investigated. However, this does raise the possibility that cells in vivo may show similar cycles of infection and virus clearance. Alternatively, cells may only be susceptible to persistent infection at particular developmental or physiological stages of growth.

In the blood of persistently infected cattle, all major B- and T-cell populations and monocytes are infected with BVDV to some degree although the percentage of cells infected may vary from animal to animal [29]. In the lymph node and tonsillar samples examined here, there was a heavy concentration of BVDV RNA in the germinal centres. The preferential localisation of the virus in these predominantly B-cell areas was surprising given the widespread distribution of virus in all subpopulations of peripheral blood mononuclear cells. In the tissue sections, individual cells in the T-cell areas surrounding the germinal centres were also virus positive but to a less striking degree. The gp48 specific monoclonal antibody staining confirmed that the observed RNA distribution was not an artifact of the ISH procedure. Indeed, by comparing the patterns of staining in figures 1a and 1b which are serial sections of the same tissues, it is apparent that the viral RNA and the gp48 antigen are found in precisely the same areas of the germinal centre. Figures 2a and 2b illustrate the high levels of RNA and antigen observed in a mesenteric lymph node follicle. Furthermore, during the course of classical swine fever infection, Susa et al. [30] have also observed the localisation of CSFV RNA in the germinal centres of porcine lymph node sections using radioactive ISH techniques. The localisation of high concentrations of virus in precisely those areas which are subject to massive tissue damage and erosion during the fatal course of disease (either mucosal disease or classical swine fever) would seem to be far from coincidental although the exact nature or significance of this pattern remains to be determined. It is true that tissue damage and the onset of mucosal disease in cattle requires the superinfecting presence of an 'homologous' cytopathogenic variant of the persisting virus [14] and it is not unreasonable to suppose that this cytopathogenic virus has some role in causing tissue damage. However, in classical swine fever, similar destruction is seen even though in this case the disease results from a rapid, acute infection with a noncytopathogenic virus. In this disease there is no prior requirement for the establishment of a persistent viraemia. It is certainly true that the B cells in germinal centres of lymphoid tissues will have a central role in the initiation of an immune response. The possible effects of the localisation of such high levels of virus in precisely these centres can, at the moment, only be a subject for speculation although it would seem safe to assume that this would have profound consequences for the immunological competence of infected animals.

Another RNA virus which is known to establish persistent infections following in utero or neonatal challenge of immuno-incompetent mice is LCMV (lymphocytic choriomeningitis virus). A thorough analysis of many of the tissues of persistently infected mice was undertaken using ISH with a radioactively labelled DNA probe [31]. In this case although viral RNA was readily detected in the T-cell areas of lymphoid tissues, no staining was observed in the germinal centres. This is in marked contrast to the observations with BVDV and might suggest that the tropism or the mechanisms of persistence of these two viruses (LCMV and BVDV) are very different.

The demonstration of viral antigen distribution, by the use of a monoclonal antibody (C42) directed to the glycoprotein gp48, provided a useful confirmation that the ISH results were not artifactual. Both the intense germinal centre staining in lymphoid tissues and exclusively cytoplasmic neuronal staining in brain tissues were duplicated by both of these different techniques. This is an important observation and provides further information on the possible tropism of this virus. The presence of both viral genomic RNA and viral glycoprotein in the mature neurones indicates the likelihood of a productive viral infection. Persistent infection of neurones by LCMV is accompanied by a decrease in the expression of viral glycoproteins [32]. This has resulted in the suggestion that LCMV infection of the neurones in mice is predominantly unproductive and that the positive signals obtained either by ISH or by antibodies to the viral nucleoprotein are due to the persistence and slow decay of ribonucleoprotein complexes in these cells [31]. This would not seem to be the case with BVDV particularly with respect to infection of the neurones although further investigation of these possibilities is warranted as some inconsistency has arisen when monoclonals specific for other pestivirus viral proteins have been used for ICC staining. It was also observed that in some cases a positive hybridisation signal was obtained with ISH where the antibody, C42, had failed to detect virus. It was initially assumed that this failure was a feature of antigenic variation between different virus strains. However, in this instance, cryopreserved tissues were available from the same animal. When these were examined with this antibody, virus was successfully identified. As the tissue samples in question were known to have been optimally fixed and processed, this indicates

that some aspect of formalin fixation modifies the epitope recognised by C42 on at least one virus isolate but not on others, presumably in a virus-specific manner.

The riboprobe described was also tested on lymphoid tissues taken from animals with persistent BVDV infection that had been identified from several different outbreaks of disease. Good hybridisation signals were obtained in the majority of these cases, the patterns of staining obtained reflected those shown above. However, there were tissue samples from some animals that were consistently negative, failing to give a signal in ISH. This was of some concern as it may have indicated that the genomic region chosen for probe construction was not sufficiently conserved. Fortunately, virus isolation from blood samples of the animals prior to postmortem provided an alternative source of viral RNA for testing. When a preparation of viral RNA obtained from the particular field strain was blotted onto a membrane, the probe hybridised strongly confirming that it was not sequence heterogeneity that was causing the lack of hybridisation to the tissue sections. The use of buffered formalin has been found to be important in the preservation of nucleic acid [unpubl. observations] and after further investigation it was unclear whether all tissues submitted to us for ISH has been fixed in this way. It is likely that this explanation accounts for the lack of hybridisation signal in some field tissue samples.

In conclusion, the results presented here show that a DIG-labelled riboprobe derived from the coding region for the conserved p80 protein of BVDV can be used to detect virus in tissues of persistently infected cattle. In fact, this probe has successfully hybridised to virus in all of the appropriately fixed tissues so far examined, confirming this region to be one of the best conserved in the viral genome. Several variations of the basic ISH methodology were tried and the optimal system has been described in detail. After comparative trials of several riboprobe detection systems, the most sensitive methodology was judged to be that using alkaline phosphatase conjugated anti-DIG FAb fragments and the NBT/BCIP substrate. The results obtained using this system for the detection of viral genome were supported by immunocytochemistry using the antibody C42. This confirmed that in paraffin fixed tissue samples the distribution of the viral glycoprotein, gp48, was identical to that of the viral genomic RNA. It is expected that this riboprobe and the ISH methodology will be more widely applicable and more flexible than the currently available antigen detection systems for formalin fixed tissues.

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