Detection of the bovine viral diarrhoea/mucosal disease (BVD/MD) virus in tissues from aborted ruminant foetuses using immunohistochemistry

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ABSTRACT

Various tissues from aborted ruminant foetuses were collected, fixed in formalin and embedded in paraffin wax. Sections were made and exposed to a primary monoclonal antibody against the bovine viral diarrhoea/mucosal disease (BVD/MD) virus, and subsequently to a goat anti-mouse secondary antibody conjugated to horse radish peroxidase (HRP). Diaminobenzidine (DAB) was the substrate and it released a brown pigment in the tissues on reacting with the HRP in an immunohistochemistry (IHC) procedure. Of 27 aborted foetuses, an immunoperoxidase staining reaction was observed in 1 ovine and 5 bovine foetuses. The IHC procedure located BVD/MD viral antigen in a wide variety of foetal tissues including cerebral cortical neurons, the pseudostratified columnar epithelial cells lining the bronchi, alveolar lining cells and alveolar macrophages, hepatocytes, renal tubular lining cells and the Purkinje fibres in the myocardium.

Keywords: aborted ruminant foetuses, BVD/MD virus, IHC.

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INTRODUCTION

The BVD/MD virus infects the developing foetus and is capable of causing abortions in pregnant animals[6,7]. It is one of the major causes of abortions in cattle and has a worldwide distribution. Abortions among infected cows can be a major limiting factor in milk and meat production causing economic losses to affected farmers. Part of the solution to this problem is the early and accurate detection in the herd of the specific pathogen involved in infections that result in abortion, so that efficacious interventions can be implemented to limit losses. For BVD/MD, important control measures also include the prompt identification and elimination of persistently infected (PI) animals[5], coupled with vaccination of in-contact animals.

Conventional diagnostic techniques for identifying pathogens are dependent on the isolation of the pathogen from clinical specimens. Isolation of microorganisms is a time-consuming procedure. Following isolation, other tests, usually serological, are necessary for the identification of the pathogen. Isolation, culture and serological identification of microorganisms are also expensive. Some microorganisms are also difficult to isolate from clinical specimens and to culture.

Pathology-based techniques that can offset some of these shortcomings and can give a confirmatory diagnosis while the pathogen is still in the tissues of the host are very desirable. One such technique is immunohistochemistry (IHC)[3,5]. The other advantage IHC has over conventional techniques is that it makes it possible to study the distribution of the pathogen in the tissues of the host in its histological context, that is, in relation to the tissue damage caused in the host and the reaction of the host in terms of an inflammation. Even the cells affected by the pathogen can be identified.

In this paper, the use of IHC on tissues from aborted foetuses received at the Agricultural Research Council–Onderstepoort Veterinary Institute (ARC-OVI) to detect the presence of the BVD/MD virus is reported.

Farmers throughout South Africa routinely send ruminant foetuses to the ARC-OVI to be tested for potential causes of abortion. Although the most commonly requested test is *Brucella* isolation and identification, there are a number of other important, infectious causes of abortion in cattle and other ruminant species. Such pathogens include the protozoan parasite *Neospora canis,* the BVD/MD virus, and the infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (IBR/IPV) virus. It would help greatly in the milk and meat production industries if confirmatory diagnoses of such diseases could be obtained through pathology based techniques such as IHC, without having to resort to expensive and time-consuming procedures such as isolation of the causative agent from clinical material and subsequent serological identification. Possibilities of employing IHC tests for these infections in tissues from aborted foetuses were investigated, beginning with BVD/MD.

In many parts of the world, IHC is used in BVD/MD control programmes for the prompt identification and subsequent elimination of PI animals which shed the virus without showing any clinical signs in order to to limit the infection of in-contact susceptible animals. The test is performed on a small piece of skin taken from the ear with an ear notcher[5]. The test is also used to detect the BVD/MD virus in samples from various organs and tissues taken at *post mortem* examination, including those from aborted foetuses[4].

MATERIALS AND METHODS

The survey constituted 27 foetuses received at the ARC-OVI over a period of approximately 18 months. Of these, 22 were bovine, 3 ovine and 2 caprine. A number of organ and tissue samples were taken from each foetus, including brain, skeletal muscle, myocardium, lung, liver, spleen and kidney. These were fixed in 10 % buffered formalin, trimmed and processed for histopathology and embedded in paraffin wax.

Tissue sections 3 μm thick were cut from the paraffin blocks on a microtome and transferred to glass slides. They were incubated in an oven at 37 °C overnight and then deparaffinised in xylene for 5 minutes 3 times. The tissue sections were rehydrated by placing in decreasing concentrations (100 %, 90 %, 80 % and 70 %) of alcohol for 1 minute each and washed in distilled water. To enhance antigen retrieval, the tissue sections were

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pretreated in a microwave oven at 1000 w for 13 minutes in 0.1 mol/ℓ citrate buffer at pH 6.01 and subsequently treated with trypsin pre-warmed to 37 °C prior to incubation for 1 minute, and then cooled to room temperature.

The tissue sections were placed in 3 % H_2O_2 in methanol for 10 minutes to block endogenous peroxidase activity, and rinsed in cold phosphate buffered saline (PBS) to stop enzymatic digestion. They were then incubated with the primary antibody for 1 hour at room temperature at a dilution of 1:60, rinsed in PBS for 5 minutes and incubated with the secondary antibody (Abcam goat anti mouse IgG polyclonal antibody conjugated to horse radish peroxidase [HRP] through the avidin-biotin complex) for 30 minutes at room temperature. The primary antibody was a monoclonal antibody (MAb) obtained from VMRD (Pullman, Washington, USA). It is number D89 in the VMRD catalogue and was raised in mouse ascites fluid. The use of this primary antibody to detect BVDV antigen in paraffin embedded tissues has been reported previously[2].

The peroxidase reaction was visualised with DAB substrate supplemented with substrate buffer for 1 minute, and rinsed once more in PBS for 5 minutes. The sections were then counterstained with Meyer's haematoxylin for 5 minutes, washed in running tap water for 3 minutes and rinsed in distilled water and then mounted with Permount aqueous media.

The positive control consisted of formalin fixed, paraffin embedded tissues from cattle that were known to be infected with the BVD/MD virus. The positive control paraffin blocks were obtained from B W Brodersen (USA). To demonstrate the specificity of the immunoperoxidase staining reaction, these infected tissues were processed as described, except that the primary antibody stage was omitted.

RESULTS

Out of 27 cases of abortion, 6 foetuses (22.2 %) tested positive for DVDV using immunoperoxidase staining. A case was considered positive when an immunoperoxidase staining reaction on tissue sections from the case produced a reaction comparable to that obtained with tissue sections from the positive control (Fig. 3). Location of the BVDV in each of the 6 cases was as follows:

Case number 1 (2006-D-13813)

There was an immunoperoxidase staining reaction mainly within the blood vessels in the lung and kidneys and in the Purkinje fibres in the myocardium. The pseudostratified columnar epithelium lining the bronchi was also lightly reactive. This was an aborted bovine foetus from the Heilbron district. Four abortions had occurred in the herd within 2 months.

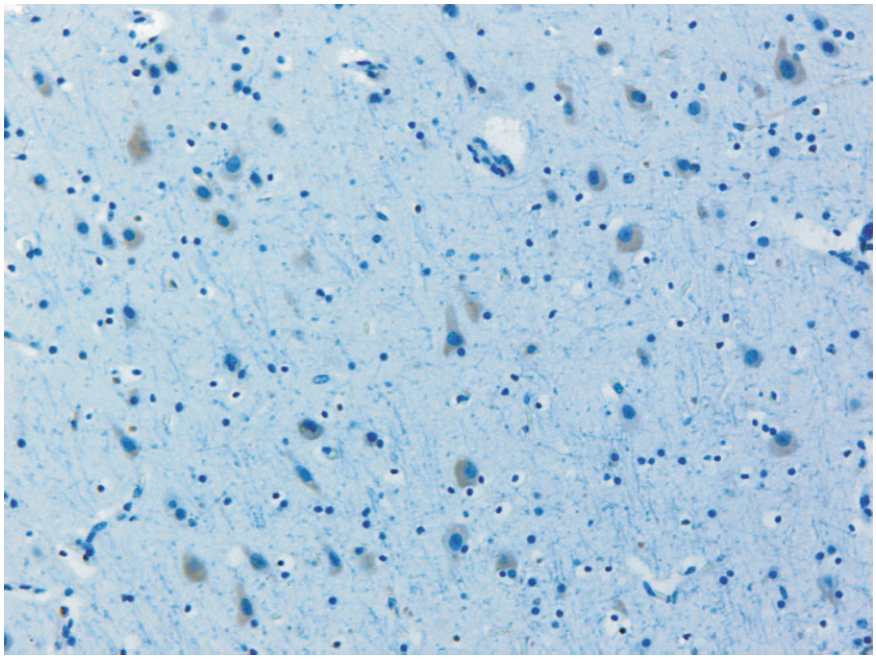

Fig. 1: **Immunoperoxidase staining reaction** (brown) **with DAB chromogen in the cytoplasm of neurons, on a section of cerebral cortex from case number 2007-D-6090.**

Case number 2 (2007-D-6090)

There was an immunoperoxidase staining reaction in the cytoplasm of some of the neurons in the cerebral cortex (see Fig. 1) and in the Purkinje fibres in the myocardium as well. This was an aborted bovine foetus from the Pretoria district. The abortion was suspected to be due to brucellosis. The brain was histologically normal.

Case number 3 (2007-D-9702)

There was an immunoperoxidase staining reaction in the cytoplasm of most of the hepatocytes. This was an aborted ovine foetus from the Kimberley district. Among the diseases suspected of being responsible for this abortion were enzootic abortion and bluetongue.

Case number 4 (2007-D-9976)

There was an immunoperoxidase staining reaction in the cytoplasm of most hepatocytes, particularly intense in the vicinity of the hepatic triad on the periphery of the hepatic lobule (see Fig. 2). A

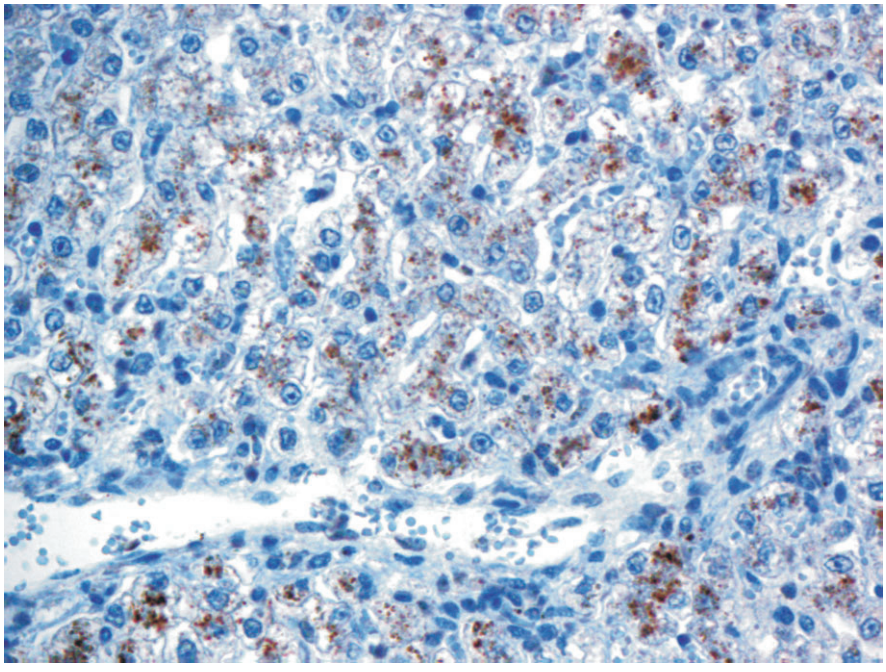

Fig. 2: **Immunoperoxidase staining reaction** (brown) **with DAB chromogen in the cytoplasm and nuclei of many hepatocytes, on a section of the liver from case number 2007-D-9976.**

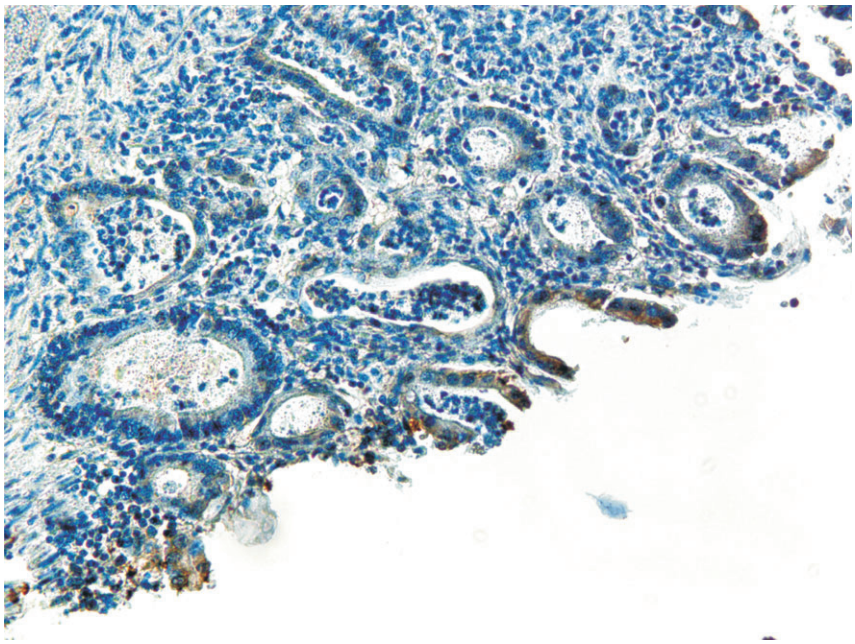

Fig. 3: **Immunoperoxidase staining reaction** (brown) **in the epithelial cells of the villi and crypts with DAB chromogen, on a section of the intestine from the positive control.**

similar reaction was seen in a number of other tissues including the cytoplasm of the pseudostratified columnar epithelium lining the bronchi, the cytoplasm of some of the tubular lining cells in the kidney and the cytoplasm of some of the neurons in the cerebral cortex. This bovine calf from the Pretoria district died soon after birth. The neonate also had a persistent *Ductus arteriosus*. The hepatocytes were vacuolated and the sinusoids mildly congested. There was moderate renal tubular epithelial degeneration and mild cerebral oedema with mild perivascular and perineuronal dilatation of space. In the lungs, moderate congestion and mild oedema were observed histologically.

Case number 5 (2007-D-15891)

There was an immunoperoxidase staining reaction within the blood vessels in the lungs, liver and kidneys. A similar reaction was seen inside the cytoplasm of some of the neurons in the cerebral cortex, inside the cytoplasm of some of the hepatocytes and in some alveolar lining cells and macrophages in the lungs. This was an aborted bovine foetus from the Bethlehem district in the Free State province. Histologically, mild congestion of blood vessels in the neuropil and meninges was observed. Alveolar capillaries were severely congested.

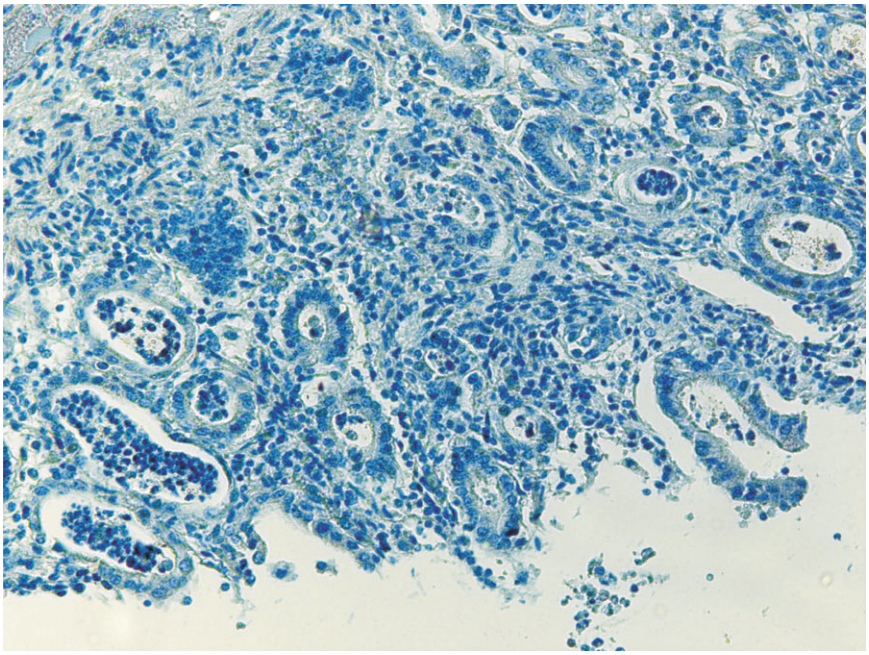

Fig. 4: **A section from the same area of tissue as in Fig. 3 above. It was treated in the same way as the others except that it was not exposed to the primary antibody. There is no immunoperoxidase staining. This section was used as the negative control.**

Case number 6 (2008-D-67476)

The immunoperoxidase staining reaction in this case was mainly within the blood vessels in the kidneys. This was an aborted bovine foetus from the Pretoria district. Three of 41 cows on this farm had aborted. Rift valley fever was suspected to be responsible.

DISCUSSION

In the 6 of 27 aborted foetuses in which the presence of the BVDV was demonstrated by IHC, an immunoperoxidase staining reaction was observed in a wide variety of tissues including:

- Inside blood vessels in the lung, kidney and liver.
- In the cytoplasm of the Purkinje fibres in the myocardium.
- In the cytoplasm of the pseudostratified columnar epithelial cells lining the bronchi.
- In the cytoplasm of neurons in the cerebral cortex.
- In the cytoplasm and nuclei of hepatocytes.
- In the cytoplasm of the tubular lining cells in the kidneys.
- In the cytoplasm of alveolar lining cells and alveolar macrophages in the lungs.

BVDV is known to cause cerebellar hypoplasia in ruminant foetuses but beyond that little is known of its neurovirulence or neuropathology[1]. Blas-Machado *et al.* (2004) reported a BVDV-induced meningoencephalitis in a heifer. It is noteworthy that BVDV antigen was encountered in the cytoplasm of neurons in the cerebral cortex of 3 of 6 cases reported in this paper in which an immunoperoxidase staining reaction was observed.

The farms from which the aborted foetuses that showed a positive immunoperoxidase staining reaction for the BVD/MD virus originated should embark on measures to control BVD/MD. These consist of the identification and elimination of PI animals and vaccination of the other animals on the farm. Both modified live virus and inactivated virus vaccines are available for BVD/MD. The use of modified live virus vaccines would lead to a positive reaction on IHC for BVD/MD and such a history must be taken into consideration when interpreting the significance of the results.

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