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Indirect immunohistochemistry on skin biopsy for the detection of persistently infected cattle with bovine viral diarrhoea virus in Italian dairy herds

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SUMMARY

Indirect immunohistochemistry (IHC) on skin biopsies for identification of persistently infected (PI) animals has been used as a parallel test to antigen and antibody ELISAs in a bovine viral diarrhoea (BVD) voluntary control program. The aim was to evaluate the reliability and feasibility of IHC on ear skin tissues to detect PI animals in field conditions, including both adult and calves under 6 months of age. In animals over 6 months of age skin biopsy and blood sample were collected at the same time, whereas in young calves blood sampling was performed when animals reached 6 months of age. One hundred and sixty-five animals were tested and immunohistochemical results were compared with those of antigen ELISA. In case of inconclusive results virus isolation and virus neutralization assays were performed. Agreement K value was 0,96. Immunohistochemical staining in positive animals was clearly detectable in the keratinocytes of the epidermis and adnexa.

KEY WORDS: Pestivirus, BVD, diagnosis, immunohistochemistry, skin biopsy

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INTRODUCTION

Bovine viral diarrhoea (BVD) is a common viral infection of cattle worldwide. BVD virus (BVDV) belongs to the *Pestivirus* genus, *Flaviviridae* family, which also includes two other common viruses of livestock: classical swine fever virus (CSFV) and border disease virus (BDV). BVDV spreads easily by direct contact between cattle but the maintenance strategy of the virus is mainly based on vertical transmission followed by persistent infection. Indeed, infection of naive dams with a non-cytopathogenic BVDV biotype, within the first 120 days of gestation, can result in the birth of persistently infected (PI) calves. These animals are life-long virus carriers, shedding large amounts of virus in the environment through excretions and secretions, and are the most important source of virus for in contact susceptible cattle. Their early identification and removal is consequently pivotal to reduce virus transmission rate.

Several methods to detect the virus from PI live animals are available and blood samples are still used, due to BVDV tropism for lymphocytes and monocytes (Lopez *et al.*,1993; Sopp *et al.*, 1994). Virus isolation (VI) on bovine cell cultures is usually performed using buffy coat cells, whole blood and/or serum. Different methods for antigen detection have been identified and a number of com-

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mercial kits are available, most based on sandwich ELISA with a capture antibody bound to the solid phase for virus detection in serum samples. The antigen detection ELISA is a rapid test and easy to perform and for these reasons can be used for testing large cattle populations during control programmes. The reverse transcriptase-polymerase chain reaction (RT-PCR) has been applied to detect viral sequences directly from blood and milk samples (reviewed by Saliki and Dubovi 2004). The reliability of diagnostic tests is optimised by choosing the appropriate sampling strategy on the basis of animal age. In neonatal calves the presence of maternal antibodies can neutralize BVDV, therefore tests that are not influenced by the presence of these antibodies, like VI and PCR, have to be applied to avoid false negative results (Brock *et al.*, 1998). Since BVDV is a pantropic virus with a prominent epitheliotropism (Bielefeldt Ohmann and Block, 1982; Thur *et al.*, 1996), enzyme labelled methods to detect BVDV antigens in tissue section by means of indirect immunohistochemistry (IHC) have been developed. For detection of PI cattle several tissues can be used, but in live animals particularly good results have been achieved with skin biopsies (Thur *et al.*, 1996). Investigations by IHC on formalin fixed samples have been carried out in North America to detect acute infections and PI animals (Njaa *et al.*, 2000; Grooms and Keilen, 2002; Brodersen, 2004). A good agreement, with VI performed on white blood cells of the same animals, was recorded. The aim of this study is to evaluate the reliability and feasibility of IHC to detect PI cattle in field conditions, including samples with a prolonged fixation. The investigation was performed both on adult animals and on calves under 6 months of age.

MATERIALS AND METHODS

Cattle selection and sampling

One hundred and sixty-five animals were collected from twenty-three dairy farms located in the Lombardy region in northern Italy from April 2002 to April 2004. In these farms, according to a voluntary control program, calves from 6 to 15 months of age were subjected to BVDV tests. In addition, a follow-up on calves under 6 months of age was performed in herds with recent cases of infection. All the sampled animals were divided into two groups: <6 months old (n=68) and >6 months old (n=97). The younger group of animals was subjected to skin biopsy only, blood sampling was performed when calves reached 6 months of age; in the older animals, skin biopsy and blood sample were collected at the same time.
Bleeding was repeated at least 3 weeks apart, in all subjects with the exception of animals resulted BVDV seropositive and virus negative at the first control.

Samples

The skin biopsies were collected from the distal part of the ear using a notcher to obtain a 0.5 mm cylindrical piece of skin. The tool was washed and disinfected with a solution of sodium hypochlorite between each collection. Samples were fixed in 10% buffered formalin solution for at least 48 h to a maximum of 9 months. Blood samples were collected in 10 ml vacutainer tubes with and without EDTA and delivered to the laboratory within 24 h . Buffy coat cells and serum obtained from each sample were subsequently stored at –70°C.

Virus and antibody detection in blood samples

As routine diagnostic methods for the control programme, serum samples were tested for BVDV antigen and antibody by ELISA kits (Chekit-BVD-Virus-III, Chekit-BVD- Sero-II, Bommeli Diagnostics), according to the manufacturer's instructions. The antigen ELISA detects E^{rns} glycoprotein and antibody test is an indirect ELISA. In case of inconclusive results by ELISAs or discordance between antigen ELISA and IHC tests, VI and virus neutralization (VN) test were applied respectively to detect BVDV and specific antibody. VI was performed in microtiter plates on bovine kidney cells (MDBK) followed by viral antigen detection by immunoperoxidase method using the monoclonal antibody 20-10-6 (Dr. E. Dubovi, Cornell University, Ithaca, NY, USA) (Corapi *et al.*, 1990); VN test was carried out on MDBK using strain Nadl (ATCC VR-534) (Edwards, 1990).

Indirect immunohistochemistry

Skin tissue biopsies were routinely processed for paraffin embedding. Five-micrometer sections

were mounted on SuperFrost®Plus slides. Endogenous tissue peroxidases were inactivated by immersion in a solution of 3% H_2O_2 for 10 minutes at room temperature. The sections were digested by immersion in a solution of protease K, 2 mg in 100 ml of phosphate buffer solution pH 7.4, at 37°C for 15 minutes. The sections were incubated with BVDV monoclonal antibody 15C5 (Haines *et al.*, 1992), overnight at 4°C. As immunoperoxidase system a commercially available kit was used according to manufacturer's instructions (Vectastain® Universal ABC kit Elite; Vector Laboratories, Inc., Burlingame, CA, USA).

Data analysis

BVD reference status of sampled animals was defined on the basis of ELISA results, integrated by VI or VN results if inconclusive. In particular, the following criteria were applied: animals negative for antibodies and virus on two samples, collected at least 3 weeks apart, were considered uninfected; animals seropositive and virus negative on a single sample were considered immune; animals seronegative and virus positive on two samples, collected at least 3 weeks apart, were considered PI.

Relative sensitivity, specificity and positive and negative predictive values for IHC were calculated at 95% level of confidence referring to the animal status. Agreement was determined by using K value.

RESULTS

The results of the diagnostic tests carried out on blood samples and skin biopsies are reported in table 1. The tested animals were classified as follows: 79 uninfected, 55 immune, and 31 PI. PI animals were detected in 12 different herds, the medium age was 13 months (1-42), and 4 were calves of age ≤4 months.

Three animals negative both to VI and IHC resulted positive (two) or doubtful (one) to antigen ELISA. IHC detected 29/31 PI animals and signal was not identified in ear notches of the virus negative animals (uninfected plus immune n=134). The 2 animals negative to IHC, 8 months and 2 years old respectively, were both positive by antigen ELISA and VI; they came from 2 different herds and in one of them another 5 PI animals were correctly detected by IHC. False negative results for IHC were observed in a group of 10 PI samples fixed over 3 months.

The skin biopsy was collected twice at different times in 5 PI subjects and in all cases the IHC gave positive results.

Relative sensitivity and specificity of IHC were 94.4 % and 100% respectively, positive and negative predictive values were 100% and 98.5% respectively. Agreement K value was 0.96.

The IHC positive signal was always clearly detectable and comparable with signal of control positive slides, with absence of background

TABLE 1 - *Results of BVDV diagnostic tests on blood samples and skin biopsies.*

BVDV animal		*Blood samples*				*skin*
categories	*n*	*ELISA Ag 1st - 2nd*	*ELISA Ab 1st - 2nd*	*VI 1st - 2nd*	*VN*	*IHC*
immune	55	neg - n.d.	pos - n.d.	n.d. - n.d.	n.d.	neg
uninfected	75	neg - neg	neg - neg	n.d. - n.d.	n.d.	neg
uninfected	2	pos - neg	neg - neg	neg - neg	n.d.	neg
uninfected	1	neg - inc.	neg - neg	neg - neg	n.d.	neg
uninfected	1	neg - neg	inc. - neg	n.d. - n.d.	neg	neg
PI	29	pos - pos	neg - neg	n.d. - n.d.	n.d.	pos
PI	2	pos - pos	neg - neg	pos - pos	n.d.	neg

1st - 2nd: results of first and second test on samples collected at least 3 weeks apart; pos = positive; neg = negative; n.d. = not done; inc. = inconclusive.

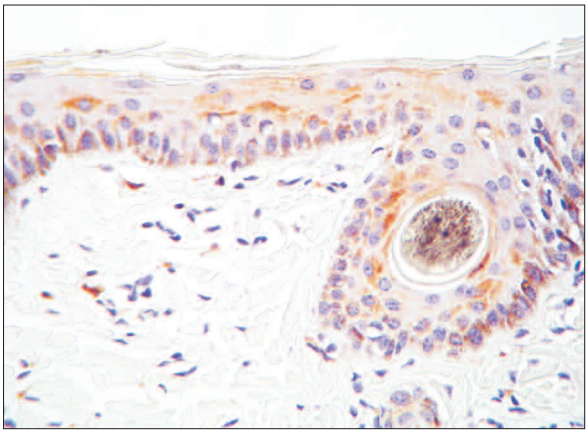

FIGURE 1 - *Immunostaining of BVDV antiserum in the skin biopsy. Kearatinocytes of the deep and superficial layers off the epidermis sowed a clear granular cytoplasmic signal. Counterstained with haematoxylin. X 40.*

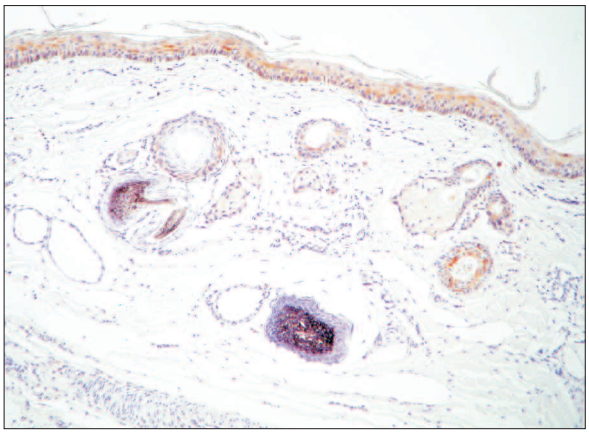

FIGURE 2 - *Immunostaining of BVDV antiserum in the skin biopsy. Positive signal was clearly evident in the sebaceous and apocrine glands and hair follicles epithelium, well distinguishable from dark melanine granules. Counterstained with haematoxylin. X 10.*

stain. IHC staining was always strong in the keratinocytes of the deep and superficial layers of the epidermis and adnexa. Positive cells showed a finely granular cytoplasmic brown staining, different from coarse regular spheroidal and elliptical regular melanin granules (Fig. 1).
Also hair follicles epithelium frequently had a positive immunoreaction especially in the external layers. Mild to moderate staining was also detected in the sebaceous and apocrine gland cells. Rarely IHC signals were detected in the smooth muscle cells of the arterioles and interstitial dermal cells such as fibroblasts or dendritic cells (Fig. 2).

DISCUSSION

In this study, IHC on skin biopsies for PI animals identification has been used as a parallel test to antigen and antibody ELISAs in a voluntary control program. This diagnostic tool is confirmed as a valid approach for PI animal detection. Indeed, high performance values were found, even if both sensitivity and negative predictive values resulted slightly lower than those reported by other studies (Njaa *et al.*, 2000; Grooms and Keilen, 2002; Brodersen, 2004).
Concerning the 2 false negative results obtained by IHC, it seems unlikely that the viral strains were not recognized by the monoclonal antibody 15C5 as this antibody recognizes an epitope of envelope glycoprotein E^{rns}, conserved in different viral strains of both genotypes of BVDV (Silva-Krott *et al.*, 1994). In addition, in the same herd of one false negative animal other PI subjects were correctly identified by IHC. We believe instead that this slight lack of sensitivity of IHC could be referred to a prolonged conservation in formalin solution of the 2 specimens. In fact an extended fixation period results in reducing staining intensity due to a blanking of the target epitope (Ramos-Vara and Beissenherz, 2000).
Nevertheless in this study, some biopsy samples showed a clearly detectable positive signal after a prolonged fixation period up 7 months. Such a result may be explained with the high virus amount and distribution in skin tissue. Even if a limited fixation period has to be applied to improve IHC performances, in case of retrospective survey or in any other logistic circumstances or environmental conditions that do not guarantee blood samples conservation, IHC skin biopsies could be considered.
The present findings of IHC reliability in specimens of young calves must be evaluated in the light of the BVDV control perspective. In fact early identification and removal of PI calves shortens the time of virus spreading in the herd, enhancing the chances to control BVDV infections. Moreover, the auricular biopsy proved an

easy and fast sampling method compared with blood collection in this age class.

In conclusion, IHC on skin biopsies resulted, a reliable test for identification of PI animals, providing an alternative and/or complementary method to VI and antigen ELISA, particularly in neonatal calves, where the sensitivity of the latter tests can be hampered by the presence of maternal antibodies. In addition fixed tissues do not present the inconvenience of laboratory virus contamination. Provided that prolonged fixation is avoided, IHC is a inexpensive, sensitive, specific and safe diagnostic test to identify persistently infected cattle.

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