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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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

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-
mmercial kits are available, most based on sand-
wich 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-poly-
merase 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 opti-
mised by choosing the appropriate sampling strat-
egy 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 devel-
oped. 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 for-
malin 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 investi-
gation 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 vol-
untary 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 divid-
ed 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 sodi-
um 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 sam-
ple 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 manu-
facturer’s instructions. The antigen ELISA 
detects E\textsuperscript{\textdagger} 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 neutraliza-
tion (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 mono-
clonal 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 

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% H₂O₂ 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.

<table>
<thead>
<tr>
<th>BVDV animal categories</th>
<th>Blood samples</th>
<th>skin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>ELISA Ag 1st - 2nd</td>
</tr>
<tr>
<td>uninfected</td>
<td>75</td>
<td>neg - neg</td>
</tr>
<tr>
<td>uninfected</td>
<td>2</td>
<td>pos - neg</td>
</tr>
<tr>
<td>uninfected</td>
<td>1</td>
<td>neg - inc.</td>
</tr>
<tr>
<td>uninfected</td>
<td>1</td>
<td>neg - neg</td>
</tr>
<tr>
<td>PI</td>
<td>29</td>
<td>pos - pos</td>
</tr>
<tr>
<td>PI</td>
<td>2</td>
<td>pos - pos</td>
</tr>
</tbody>
</table>

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.
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, 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 an inexpensive, sensitive, specific and safe diagnostic test to identify persistently infected cattle.

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REFERENCES


