

Immunohistochemical diagnosis of persistent infection with Bovine Viral Diarrhea Virus (BVDV) on skin biopsies

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Summary

Detection of persistent infection with Bovine Viral Diarrhea Virus (BVDV) is essential for both epidemiological and clinical reasons. In addition to the classical virological methods such as virus isolation in tissue culture, ELISA and RT-PCR, immunohistochemistry of skin biopsies has become a useful and reliable tool. Assuming that the presence of BVDV antigen in skin structures is restricted to persistent infection, this method could differentiate from transient infection. In order to answer this question, 6 calves were experimentally infected orally with a non-cytopathic genotype 1 BVDV strain belonging to the subtype k. The calves developed fever, mucopurulent nasal discharge, coughing and leucopenia with relative lymphopenia. Immunohistochemistry of skin biopsies taken daily up to day 13-post infection did not reveal any evidence of BVDV infection. BVDV was, however, isolated from blood samples on cell cultures. Anti-NS3-antibody-ELISA and serum neutralization tests showed that all six calves seroconverted. We conclude that in acute BVDV infections, with genotype 1 and the subtypes found in Switzerland (b, e, h and k) viral antigen is not found in epidermal structures of the skin. In contrast, persistently infected animals test positive for BVD viral antigen by immunohistochemistry of the skin.

Keywords: bovine viral diarrhea virus, ELISA, experimental infection, immunohistology, RT-PCR

Immunhistologischer Nachweis von persistenten BVDV (Bovines Virus Diarrhoe Virus)-Infektionen anhand von Hautbiopsien

Aus epidemiologischer und klinischer Sicht ist der Nachweis einer persistenten BVDV-Infektion von grosser Bedeutung. Zusätzlich zu den klassischen virologischen Methoden wie die Virusisolation anhand von Zellkulturen, ELISA und RT-PCR, hat sich die Immunhistologie an Hautbiopsien als ein nützliches und zuverlässiges Werkzeug bewährt. In der Annahme, dass BVDV-Antigen nur bei persistenten, nicht aber bei transienten Infektionen in epidermalen Hautstrukturen vorkommt, sollte man mit der Immunhistologie eine persistente von einer akuten Infektion unterscheiden können. Um diese Frage zu beantworten, wurden 6 Kälber oral mit einem nicht zytopathogenem BVDV des Genotyps 1, Stamm k experimentell infiziert. Die Kälber zeigten Fieber, mukopurulenten Nasenausfluss, Husten und eine Leukopenie mit einer relativen Lymphopenie. Bis zum 13 Tag post infektionem täglich entnommene Hautbiopsien waren immunhistologisch stets negativ auf BVDV-Antigen. Das BVDV wurde aber aus dem Blut mittels Zellkulturen isoliert und eine Serokonversion wurde bei allen 6 Kälbern mittels dem anti-NS3-Antikörper-ELISA und dem Serumneutralisationstest festgestellt. Aus diesen Ergebnissen schliessen wir, dass bei einer akuten BVDV-Infektion mit dem Genotyp 1 und den in der Schweiz gefundenen Stämmen (b, e, h und k) immunhistologisch kein Virusantigen in epidermalen Hautstrukturen nachgewiesen werden kann und dass daher ein positiver immunhistologischer Nachweis in Hautbiopsien auf eine persistente Infektion schliessen lässt.

Schlüsselwörter: Bovines Virus Diarrhoe Virus, ELISA, experimentelle Infektion, Immunhistologie, RT-PCR

Introduction

Bovine viral diarrhea virus (BVDV), the etiological agent of BVD/Mucosal Disease, belongs to the genus pestivirus within the flaviviridae family, together with hog cholera virus of pigs and Border

disease virus of sheep (Francki and Fauquet, 1991; Moennig and Plagemann, 1992). BVD viruses are divided into non-cytopathic (ncp) and cytopathic (cp) biotypes, depending on their effect on cell cul-

tures (Underdahl et al., 1957; Gillespie et al., 1960). BVDV infections occur worldwide and cause considerable economic losses (Houe, 1995; Bundesamt für Veterinärwesen, 2005). Infections with BVDV induce either acute (transient) or persistent infections. Transient infections in immunocompetent animals usually cause rather non-specific clinical symptoms such as a short-term fever, reduction of food intake, sometimes respiratory problems, i.e. coughing and transient viremia (Brownlie, 1991; Moennig and Plagemann, 1992; Sandvik et al., 1997). Rarely, animals may exhibit more severe intestinal and respiratory signs (Bolin et al., 1985; Baker, 1995). More serious forms of acute infection have been reported, such as severe thrombocytopenia and haemorrhagic syndrome (Rebhun et al., 1989; Corapi et al., 1990; Bolin and Ridpath, 1992). BVDV genotype 2 has been identified as a cause of this syndrome. In rare instances hemorrhagic syndromes have also been observed in association with BVDV genotype 1 (Ehrensperger and Meier, 1992). Infection of pregnant cows may result in abortion or malformation, or birth of small and weak calves, depending on the stage of gestation at the point of infection with BVDV (Weiss et al., 1994). Transplacental infection of the fetus with ncp BVDV between the 40th and 120th day of gestation induces immunotolerance and persistent infection (Lies, 1985; Baker, 1987). Persistently infected calves shed virus throughout their lives. They may be undersized and weak and sometimes show tiny erosions in the muzzle, tongue and gingiva as well as elevated body temperature and diarrhoea (Weiss et al., 1994; Braun and Thür, 1996). As a result of mutation or recombination of the persisting ncp strain of BVDV to a cp biotype, or super infection with a cp BVDV, these animals may develop the lethal mucosal disease (Done et al., 1980; Duffell and Harkness, 1985; Brownlie, 1991). Persistently infected animals are epidemiologically important due to the lifelong shedding of virus (Duffell and Harkness, 1985). The prevalence of persistently infected animals in Switzerland is estimated around 1% (Schönmann, 1997; Rüfenacht et al., 2000).

Several methods are available for virus detection, including antigen-ELISA, virus isolation, RT-PCR and immunohistochemistry (Dubovi, 1996; Thür et al., 1996; Thür et al., 1997; Sandvik, 1999; Hilbe et al., 2007). In a recent survey on BVDV diagnostic methods applied during the years 2004 and 2005 in the US (Driskell and Ridpath, 2006) it was shown that 77% of the labs offer various methods. Among 445'648 tests performed, ear notch testing was most frequently run (44%), either by ELISA (22%) or by immunohistochemistry (22%) in equal proportions, followed by ELISA of serum (14%) and virus isolation (6.5%) and RT-PCR (4.5%). In Europe, for routine diagnosis, mostly antigen-capture-ELISA on EDTA blood for

the detection of the non-structural viral protein NS23 and E^{rns}-ag-ELISA on serum and skin are being used. These methods are relatively cheap, fast and have a good sensitivity, but may miss persistently infected young calves, due to masking by colostral antibodies („diagnostic gap“). Virus isolation serves as the „gold standard“, but is costly and time consuming (Nettleton and Entrican, 1995; Brodersen, 2004).

RT-PCR is a highly sensitive and specific method, but with a certain risk of false positive results due to contamination (Heinrich, 1991; Persing, 1991) or transient infections (Hilbe et al., 2007). Real time RT-PCR is an alternative, which permits the processing of multiple samples with a smaller risk of contamination. Circulating antibodies appear not to interfere with RT-PCR or real time RT-PCR (Kaiser, 2001). The immunohistochemical detection of the BVD-antigen in skin biopsies is an alternative method for BVDV detection in individual animals (Bielefeldt Ohmann, 1982; Duffell and Harkness, 1985; Bielefeldt Ohmann, 1988; Wilhelmssen, 1991; Haines et al., 1992; Thür et al., 1996). Both cryostat sections or paraffin-embedded skin biopsies can be used and BVD-antigen is found in epidermal cells, hair root sheaths and blood vessel walls of persistently infected animals. Colostral antibodies do not interfere, which makes this method suitable for detection of infection in young animals (personal observation) (Nettleton and Entrican, 1995; Brodersen, 2004).

The aim of this study was to test the hypothesis, that virus antigen does not appear in skin biopsies of calves during experimental acute BVDV infection, meaning that the presence of BVDV antigen in skin structures is conclusive for persistent infection.

Animals, Material and Methods

Animals

The study was performed on six fattening calves of different breeds over a two-month period. The animals were between three and six months of age and negative for BVDV antigen as well as BVD antibodies. During the experiment they were kept in an isolation unit and fed ad libitum with milk powder (UFA 200, calf fattening milk powder, Sursee, Switzerland), hay and straw. During an adaptation period of 4 weeks, skin biopsies and blood samples were taken once a week as preinfection controls. EDTA-blood and serum were tested for antigen and antibody, respectively, another EDTA blood sample was taken for hematological analysis (total leucocyte and absolute lymphocyte count).

Experimental infection

On day 0, the animals were infected by spraying into the oral cavity 5×10^7 tissue culture infectious dosis (TCID₅₀) of an ncp field strain of BVDV genotype 1 known as 110/39 and belonging to the subtype k. Skin biopsies, blood samples (EDTA blood and serum) and nasal swabs were taken daily up to day 13 post infection (p.i.), and three times a week from days 15 to 30 p.i. The animals were daily examined clinically.

Antigen detection in skin biopsies

The skin biopsies were taken from the shoulder region under local anesthesia (2 ml lidocain s.c.) with a 6mm (Ø) punch (Stiefel Laboratories, CH-8400 Winterthur, Switzerland) and snap frozen in liquid nitrogen. Five to 8µm thick cryostat sections were cut and fixed for 10 min with acetone (-20°C) before incubation with H₂O₂ (3% H₂O₂, 0,2% NaN₃) for 10 min at room temperature (RT). The following monoclonal antibodies were used for primary incubation (1 h at 37°C): Ca3/34-C42 (dilution 1:100, specificity: E2/gp55 and E^{rns}/gp48. Dr. Bommeli AG, CH-3097 Bern-Liebefeld, Switzerland; NBWU 102) and C16 (dilution 1:100, specificity: NS2-3/nsp125/80. Dr. Bommeli AG, CH-3097 Bern-Liebefeld, Switzerland; NBMU 322). This incubation step was followed by the EnVision reagent (DAKO®) and AEC (Amino-Ethyl-Carbazole) chromogen (Bolin et al., 1988; Moennig et al., 1987). Between every step the slides were washed with PBS (pH 8). A positive and negative control slide was included with each batch of specimens analyzed.

After processing the snap frozen skin biopsies for cryostat sections they were thawed and fixed in 4%-buffered formalin for 20 h and then embedded in paraffin. After sectioning, the slides were deparaffinated and counterstained with hemalaun for 4 min before incubation with 0,05% pronase (DAKO Cytomation, Pronase, S 2013, Zug, Switzerland) for 10 min at RT. The slides were then incubated with two different monoclonal antibodies 15c5 (specific for E^{rns}/gp48, dilution 1:10'000. E. Dubovi, New York State College of Veterinary Medicine, Cornell University, USA) or C42 (also specific for E^{rns}/gp48, dilution 1:400. Prof. Moennig, Institute of Virology, Hannover, Germany) over night at RT, and the reaction was visualized by the EnVision method (DAKO Cytomation, EnVision+™, peroxidase, mouse, K 4001, Zug, Switzerland) and AEC (Amino-Ethyl-Carbazole, 00-2007 Substrate Kit, Zymed Laboratories, CA 94080 San Francisco, USA) as chromogen (Haines et al., 1992).

Antigen detection in blood and nasal swabs

The antigen-capture-ELISA with white blood cells was performed according to the method previously described (Strasser et al., 1994). This test detects the non-structural viral protein NS23. The detection of E^{rns}-antigen in serum samples was performed with the HerdChek® Bovine Diarrhea Virus Test Kit (Herd-Chek® Bovine Diarrhea Virus Test Kit, Idexx Laboratories, USA).

Virus isolation from EDTA blood

After the preparation of leucocyte pellets from EDTA blood the cells were resuspended in 1.5 ml of cell culture medium (MEM (Earle's Minimal Essential Medium), GIBCO BRL®, F4315, Paisley, Scotland). Subsequently, the cell culture medium was discarded and 0.3 ml of the leucocyte pellets were distributed into 2 cell culture tubes with BT (bovine turbinate cells from 3 to 5 month old fetuses, slaughterhouse, Berne) or MDBK (Madin-Darby-Bovine Kidney cells, American Type Culture Collection) cells and incubated at 37°C for 60 min. 2 ml cell culture medium were added and the tubes were incubated for 5 to 7 days at 37°C and then frozen at -20°C. The results were evaluated after immunoperoxidase staining under the microscope using cells transferred to 96 well micro titer plates and incubated with CO₂ for 3 to 4 days at 37°C.

Virus isolation from nasal swabs

Nasal swabs were introduced in medium (MEM) and stored at -80°C. Subsequently, the samples were sterile filtrated and used to inoculate BT cells. After 5 days the cells were transferred to micro titer plates and the results evaluated after a further 4 to 5 days incubation using immunoperoxidase staining.

Antibody detection

Serum was tested for antibodies using the anti-NS3-antibody-ELISA and examined as described by Canal (Canal et al., 1998).

Serum neutralization test (SNT)

The sera were diluted 1:10 in medium (MEM) and heat-inactivated. Serial dilutions were incubated with 100 TCID₅₀ for 1 h at 37°C in a CO₂ incubator. 100µl of the serum virus mix was transferred to a micro titer well (96 well plate, four wells per dilution) and incubated for 4 to 5 days at 37°C in the CO₂ incubator. The results were evaluated after immunoperoxidase staining.

Results

Clinical and hematological findings

Body temperature transiently rose to 41°C between days 2 and 10 p.i., and leucocyte count decreased to among 2000 and 4000/mm³ between days 2 and 8 p.i.. The relative lymphocyte number decreased between the 3rd and the 7th day p.i., in three animals even below 45% (Figs. 1–3). Between days 2 and 15 p.i., the animals had mucopurulent nasal discharge and dry cough. Calves #4 and 5 had diarrhea on the 17th day p.i. After day 17 p.i. the animals showed no signs of infection.

Antigen detection in skin biopsies (cryostat and paraffin-embedded skin biopsies)

All structures of the skin, especially cells of the stratum basale of the epidermis, hair root sheaths and blood vessel walls were evaluated and all skin biopsies were clearly negative in all animals at any time (Fig. 4).

Antigen detection by antigen-capture-ELISA with EDTA blood

In cells of the buffy coat NS23 antigen was not detected in any of the animals at any time.

Antigen detection by E^{rns}-antigen-ELISA in the serum

The membrane protein E^{rns} was detected only around day 7 p.i. in one animal, the levels of the other animals remained below the cut-off value of 30%.

Antigen detection by virus isolation (EDTA blood and nasal swabs)

From the buffy coat cells (EDTA blood) virus was isolated in animals #1, 2, 4 and 6 between days 4 and 9 days p.i. No virus was isolated from animals #3 and 5. Virus was isolated from the nasal swabs of animals #1, 2, 4, 5 and 6. Animal #3 remained negative.

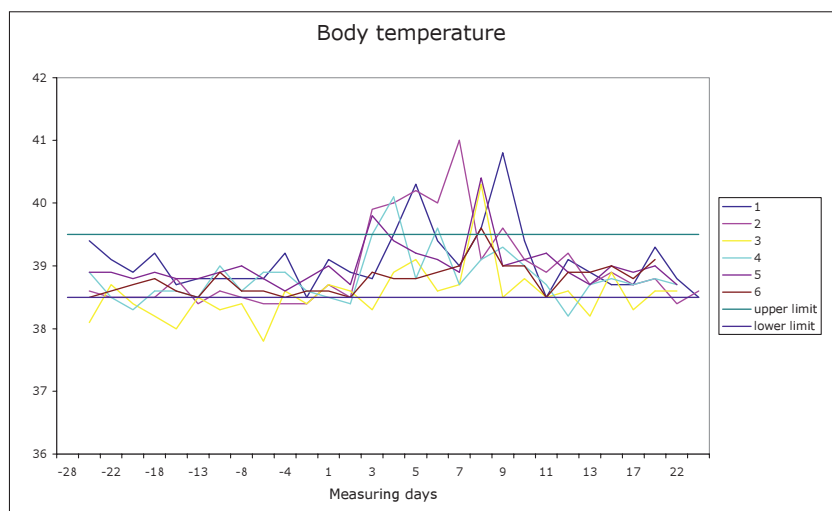


Figure 1: Body temperature (°C) of the 6 experimentally infected animals. Lower limit: 38.5°C. Upper limit: 39.5°C.

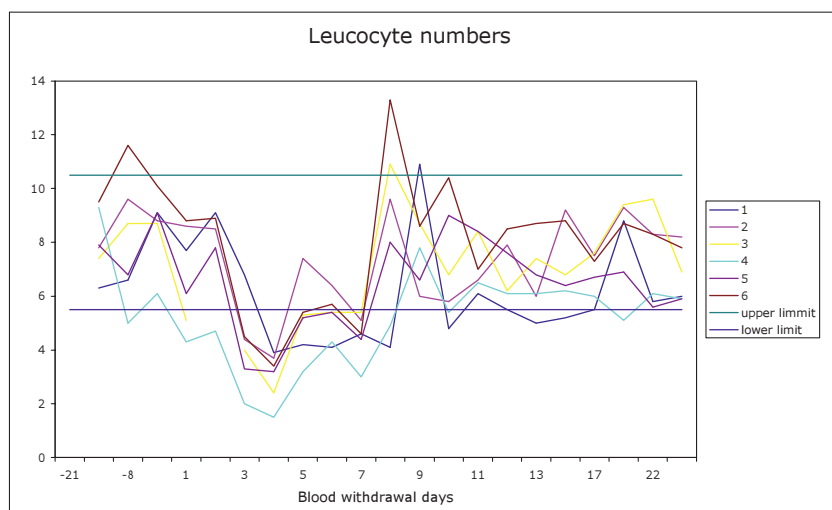


Figure 2: Leucocyte numbers (10³/mm³) of the 6 experimentally infected animals. Lower limit: 5.3. Upper limit: 10.3.

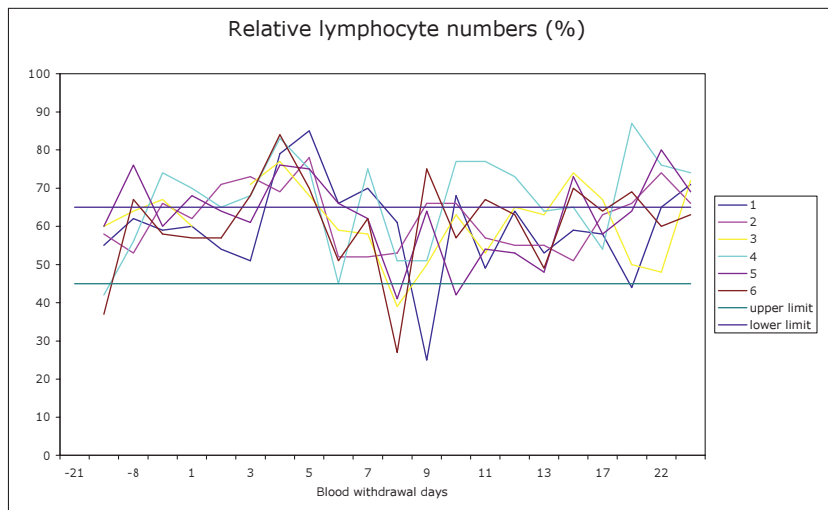


Figure 3: Relative lymphocyte numbers (%) of the 6 experimentally infected animals. Lower limit: 45%. Upper limit: 65%.

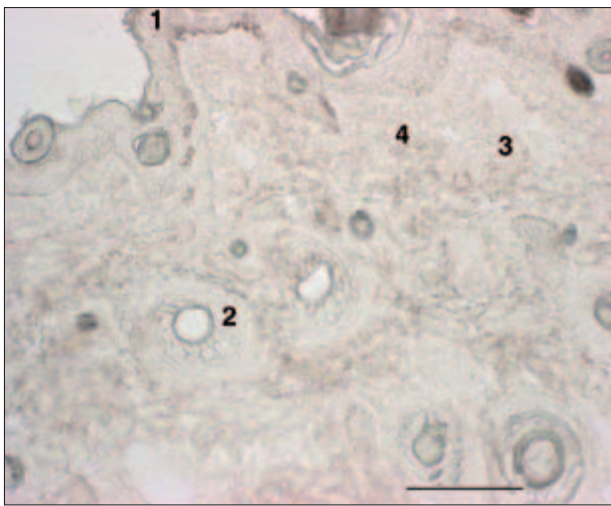


Figure 4: Immunohistochemistry of a BVDV-antigen negative skin biopsy of animal 4 at day 9. This animal was transiently infected. Virus could be detected by RT-PCR at day 9 in the buffy coat and nasal swab. Note that the epithelial cells of the epidermis (1) and the hair shaft (2), the media cell of the vessels (3) and the fibrocytes (4) are negative. Peroxidase technique, AEC chromogen, monoclonal antibody C16. Bar = 100µm.

Antibody detection in the blood

Based on ELISA results, animals #1, 2, 4, 5 and 6 seroconverted between the 17th and 22th day p.i., whereas calf #3 seroconverted on day 28 p.i. By serum neutralization test, all 6 calves produced neutralizing antibodies starting from day 14 p.i.

Discussion

The aim of this study was to evaluate if BVDV antigen can be detected by immunohistochemistry in skin biopsies during acute infection. Virus antigen was not detected in snap frozen or paraffin-embedded skin biopsies at any time point in 6 animals experimentally infected with an ncp BVDV field strain. A similar in-

vestigation, in which 16 calves with acute BVDV infection were tested for virus in the blood and in skin biopsies by means of RT-PCR and immunohistology, also showed that skin biopsies remained negative for viral antigen during transient infection (Ridpath et al., 2002).

In contrast, using calves that had been infected intranasally with 10^8 TCID₅₀ ncp BVDV, Njaa and colleagues (2000) found virus antigen in skin biopsies of 4 of 10 calves between days 10 and 14 p.i. They demonstrated BVDV antigen in paraffin sections, mainly focally in the stratum spinosum extending slightly into the follicular ostia (Njaa et al., 2000). The discrepancy with our results might well be due to the fact that the Canadian study was performed with a 2-fold higher infectious dose than in our study, and that a genotype 2 BVDV with possibly higher virulence was used. The virus antigen distribution is not exactly consistent with what we consider to be typical in persistently infected animals, where, in addition to epithelial structures, the smooth muscle cells of blood vessel also are positive and the distribution is diffuse and not multifocal (Brodersen, 2004). A study published more recently, Cornish et al. (2005), found 8 acutely infected calves of 559 calves tested for BVDV by different methods. Four of these 8 cases were positive by ear notch immunohistology with an intensity and distribution of BVD antigen similar to that found in p.i. calves. Virus was not isolated from these calves. This might be explained by the fact that in that survey most viruses belonged to genotype 1 and the genetic group a (Cornish et al., 2005). In Switzerland all BVDV isolated belong to genotype 1 but most of the genetic groups isolated are e, h, k and e decreasing frequency (Stalder et al., 2005).

The viral dose used in this study to infect the animals is assumed to exceed that required for natural infection. Based on the fact that the virus distribution in the body depends on the amount of virus, we postulate that in an acute, natural BVDV infection no virus

antigen is detectable in the skin and that therefore BVDV detection in the skin by means of immunohistochemistry is proof of persistent infection with the virus genotype 1 and the strains found in Switzerland (Fig. 5). In the last few years we tested about 3000 skin biopsies with immunohistochemistry of which 400 were BVDV-positive. All these BVDV-positive skin biopsies had a diffuse virus distribution with virus presence in epithelial structures and smooth muscle cells of blood vessels indicating a persistent infection (unpublished data). A recent study comparing five diagnostic methods for detecting BVDV has shown that immunohistochemistry in 258 skin biopsies identified only persistently infected animals (Hilbe et al., 2007). Another practical aspect of the immunohistological approach to detect BVDV in the skin is based on our observation that it does not interfere with colostral antibodies. Therefore, it can be used to detect BVDV in very young calves that were infected in utero (Hilbe et al., 2007).

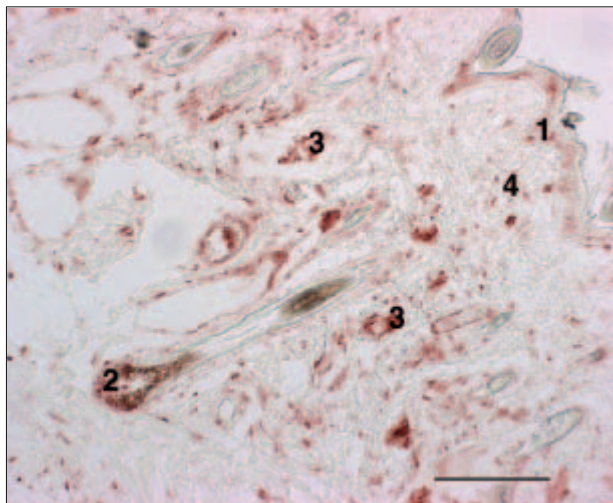


Figure 5: Immunohistochemistry of a BVDV-antigen positive skin biopsy of a persistently infected animal. Note that the basal cells of the epidermis (1), the epithelial cells of the hair root sheath (2), the media cell of the vessel (3) and fibrocytes (4) are positive. Peroxidase technique, AEC chromogen, monoclonal antibody C16. Bar = 100µm.

In the experiment described here, we demonstrated a seroconversion with rising antibody titers by means of antibody-ELISA and SNT. This and the leucopenia as well as the temperature elevation indicated that infection had been established in all six calves. By means of the E^{rns}-ag-ELISA the envelope protein was only detected in the serum of one animal on day 7 p.i. Earlier reports suggest that the antigen-capture-ELISA could discriminate between a persistent and a transient viremia. The non-structural protein NS23 may be present in low concentrations in transiently viremic animals, which may explain why the animals remained negative for NS23 in our experiment (Gottschalk et al., 1992; Strasser et al., 1994). In 4 animals of our study, virus was isolated in cell culture from the buffy coat (EDTA blood) and in five animals from nasal swabs between the 4th and the 9th day p.i. These results underline the necessity to separate animals vaccinated with an attenuated virus from pregnant animals. Although our experiment was carried out with only a small number of animals it can be concluded that a transient acute BVDV infection does not interfere with the immunohistological detection of persistent BVDV infection by means of skin biopsies. Based on our routine diagnostic experience, this is true for the genotype 1, in particular for the strains known in Switzerland, like b, e, h or k.

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Mise en évidence immunohistologique de la persistance du virus de la diarrhée virale bovine (BVD) au moyen de biopsies cutanées

D'un point de vue épidémiologique et clinique, la mise en évidence d'une infection à BVD persistante est d'une grande importance. En complément aux méthodes virologiques classiques, telles que l'isolation du virus sur culture de cellule ou les tests Elisa et RT-PCR, l'immunohistologie sur biopsie cutanée s'est révélée un instrument utile et fiable. Partant de l'idée que l'antigène BVD ne se trouve dans les structures épidermiques que lors d'infections persistantes et non lors d'infections transitoires, il devrait être possible de différencier par immunohistologie une infection persistante d'une infection aiguë. Pour répondre à cette question, on a infecté expérimentalement 6 veaux par voie orale au moyen d'un virus de BVD non cytopathogène

Individuazione immunostologica di un'infezione persistente da BVDV (virus della diarrea virale bovina) sulla base di biopsie cutanee

Sotto l'aspetto epidemiologico e clinico l'individuazione di un'infezione persistente da BVDV è di grande importanza. Oltre ai metodi virologici classici come l'isolamento del virus grazie a culture di cellule, ELISA e RTV-PCR, l'immunostologia ha dimostrato che le biopsie cutanee sono uno strumento utile e fidato. L'ipotesi era di differenziare, riferendosi all'immunostologia, un'infezione persistente da una acuta, poiché l'antigene BVDV si ritrova sulle strutture epidermiche solo nel caso di infezione persistente e non transiente. Per rispondere a questa domanda sono stati infettati in via sperimentale 6 vitelli per via orale con un BVDV di genotipo 1 ceppo k non citopatogeno. I vitelli hanno mostrato febbre, scolo nasale mucopuru-

du génotype 1 souche k. Les veaux ont présentés de la fièvre, un écoulement nasal mucopurulent, de la toux ainsi qu'une leucopénie avec une lymphopénie relative. Les biopsies cutanées prélevées quotidiennement jusqu'au 13ème jour après l'infection sont restées négatives à l'immunohistologie quant au virus de la BVD. Le virus a par contre pu être isolé dans le sang, sur des cultures cellulaires et une séroconversion a été constatée chez les 6 veaux au moyen d'un test Elisa anti anticorps NS3 et d'un test de séroneutralisation. On peut en conclure qu'il n'est pas possible, lors d'une infection aiguë due au virus BVD du génotype 1 et des souches trouvées en Suisse (b, e, h et k), de mettre en évidence d'antigène viral dans les structure épidermiques et que donc, une découverte immunohistologique positive dans des biopsies de peau permet de conclure à une infection persistante.

lento, tosse e una leucopenia con relativa linfopenia. Quotidianamente, per un periodo di 13 giorni dopo l'infezione, sono state effettuate biopsie cutanee che risultavano sempre immunohistologicamente negative all'antigene BVDV. Il BVDV è stato isolato dal sangue grazie a culture di cellule ed è stata constatata, in tutti i 6 vitelli tramite il test ELISA dell'anticorpo anti NS3 e il test di sieroneutralizzazione, una sierconversione. Dai risultati deduciamo che non si può provare la presenza di un antigene del virus nelle strutture epidermiche sotto il profilo immunohistologico nel caso di un'infezione acuta da BVDV di genotipo 1 e dai ceppi ritrovati in Svizzera (b, e, h, k). Perciò: una positiva individuazione immunohistologica nelle biopsie cutanee implica la presenza di un'infezione persistente.

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