Diagnosis of bovine virus diarrhoea by two enzyme-linked immunosorbent assays


Summary: Two enzyme-linked immunosorbent assays (ELISAs) for the diagnosis of bovine virus diarrhoea (BVD) are described: CHEKIT-BVD-VIRUS and CHEKIT-BVD-SERO. The first test detects virus antigen in leucocytes, resulting in identification of persistently-infected animals, while the second detects antibodies to BVD virus (BVDV). It is well known that even persistently-infected animals may have antibodies to heterologous BVDV strains. These animals are still negative to the CHEKIT-BVD-SERO test because of immunotolerance to conserved virus antigens. Data on these two tests are summarised and a scheme is presented for the diagnosis of BVD using a combination of these two tests.


INTRODUCTION

Infection by bovine virus diarrhoea (BVD) virus in cattle may lead to “shipping fever” (bovine respiratory disease) or diarrhoea in calves. Deaths following infection by BVD virus (BVDV) are mainly a result of secondary bacterial infections. If pregnant cows are infected, this may result in the birth of persistently-infected calves, which shed virus throughout life. A number of months or years later, mucosal disease (MD) may develop. Other outcomes of foetal infection are death, abortion or malformation. Infection of the immunocompetent foetus is associated with a normal immune response.

Prevalence of persistently-infected animals ranges between 0.1% and 1% (1, 9). Persistently-infected animals are a key factor in the epidemiology of BVD-MD. As they shed virus throughout life, the elimination of these animals is necessary for BVD-MD control. To date, systematic testing has been limited, as virus isolation had to be performed in cell culture. This procedure is expensive and time-consuming.

Development of enzyme-linked immunosorbent assays (ELISAs) greatly facilitates diagnosis of BVD-MD. Two ELISAs are described: CHEKIT-BVD-SERO and CHEKIT-BVD-VIRUS. The former, CHEKIT-BVD-SERO, detects antibodies...
against pestiviruses while CHEKIT-BVD-VIRUS detects viral antigen in blood samples. This paper provides a general description of these two tests and how they may be used in diagnosis.

**CHEKIT-BVD-VIRUS**

CHEKIT-BVD-VIRUS detects the viral non-structural protein (nsp) 125/80 in blood specimens. A monoclonal antibody, BVD/C16 (specific for nsp 125/80) (10), is coated to test plates (Fig. 1). This monoclonal antibody binds antigen from the sample. Bound antigen is detected by an anti-BVDV peroxidase conjugate. BVD/C16 is pestivirus-specific and detects all strains of BVDV (3).

![Schematic representation of an enzyme-linked immunosorbent assay for bovine virus diarrhoea (BVD) virus:](image)

**Fig. 1**

Wells are coated with monoclonal antibody BVD/C16 (a). The monoclonal antibody binds non-structural protein 125/80 from the sample (b) and finally the bound antigen is detected by anti-BVDV peroxidase (PO) conjugated antibodies (c).

Anti-coagulated blood samples serve as probes. As viraemia is associated with leucocytes, these are separated from the sample. This can be performed easily by haemolysis of red blood cells. Erythrocytes are haemolysed using haemolysis buffer (HLB) and leucocytes are then pelleted by centrifugation. The pellet is washed with HLB and lysed with leucocyte lysis buffer (LLB). After lysis of leucocytes, nsp 125/80 is released and the sample is ready for use in the ELISA.

This procedure has two advantages:

- viraemia in BVDV infection is cell-associated, and the high concentration of viral antigen in leucocytes results in a good resolution between positive and negative samples (see below)
- viral antigen is not masked by maternal antibodies.

Samples may be stored for seven days at room temperature without serious loss of sensitivity. However, after lysis of leucocytes, it is advisable to store prepared samples at −20°C. Minimum blood volume for sample preparation should be 5 ml. A technique requiring a reduced volume of blood may be available soon.
The washing of microplates is vital for the successful performance of the test. Microplates must be washed three times, each time allowing incubation for three minutes between washes. After each wash, plates must be tapped dry.

The distinction between positive and negative samples is clearcut and results may therefore be read visually without magnification. Figure 2 summarises results obtained with CHEKIT-BVD-VIRUS. Results are given as percentages of the positive control, which is defined as 100% (corresponding to an optical density of 0.6-1.2). Samples testing negative by virus isolation in cell culture with amplification give percentage positivity values lower than 2.5%, while positive samples give values higher than 100%.

![Graph](image)

**Optical density as a percentage of the positive control**

**Fig. 2**

**Results from 137 blood samples tested for bovine virus diarrhoea (BVD) virus by an enzyme-linked immunosorbent assay:**

**CHEKIT-BVD-VIRUS**

In one study, two samples gave values of 35% due to leucopenia, as measured by cytofluorometry. Samples from these animals which were retested fourteen days later gave values exceeding 100%. Clear resolution between positive and negative results defined the cut-off at 30%. Comparison of this ELISA with virus isolation from leucocytes in cell culture with amplification showed a specificity of 97% and a sensitivity of 99.1% (5, 6). Specificity might have been even higher, as three animals were slaughtered or sold before retesting. Transient viraemic animals were negative to the CHEKIT-BVD-VIRUS test.

**CHEKIT-BVD-SERO**

CHEKIT-BVD-SERO is an indirect ELISA which detects antibodies directed against pestiviruses. Viral and control antigen are coated to test plates, and samples are diluted ten-fold. After incubation with samples, the plates are washed and incubation
with anti-ruminant immunoglobulin (Ig)G peroxidase conjugate follows. The reaction is shown by colour development of CHEKIT-chromogen. The difference in optical densities between viral and control antigen (net extinction) is the basis for interpretation of results. Serum, plasma and milk may be used as samples.

Figure 3 demonstrates the results obtained with CHEKIT-BVD-SERO compared to a sero-neutralisation assay, using BVDV Grub 313/83.

![Graph](image)

**Fig. 3**

**Comparison of an enzyme-linked immunosorbent assay (CHEKIT-BVD-SERO) and a neutralisation assay for the detection of bovine virus diarrhoea virus**

Of 313 sera, six tested negative by seroneutralisation and positive by ELISA. This may be due to the use of a conserved antigen in CHEKIT-BVD-SERO and the broader reactivity of ELISA in general (8). However, five sera tested negative by ELISA and positive by seroneutralisation, with titres of 1/2 or 1/4. The latter observation seems to be a minor problem since it is known that persistently-infected animals may have low neutralising antibody titres (see below).

Prevalence of antibodies directed against BVDV is approximately 75% (7). In animals older than two years, prevalence exceeds 80% (4).

Persistently-infected animals are immunotolerant (they do not have antibodies to the homologous, persisting BVDV strain), but it is known that even persistently-infected animals may have neutralising antibodies to BVDV after superinfection by a heterologous BVDV, or after vaccination (11, 2). These persistently-infected animals are still immunotolerant, but are able to recognise small differences between surface antigens of different BVDV strains when they become superinfected.

The aim of this study was to develop a test which distinguishes seropositive, non-viraemic animals from those which are seronegative and non-viraemic, and those which are persistently viraemic.
This problem was resolved by the use of a conserved, or common viral antigen. Since this viral antigen is associated with all strains of BVDV, immunotolerant animals should not have antibodies to this protein. To demonstrate this, sera of two persistently-infected animals, superinfected by a heterologous virus, were tested by a neutralisation assay and with CHEKIT-BVD-SERO. These sera had neutralising titres of 1/4,000 when tested with the heterologous virus used for immunisation, but remained negative when tested with the persisting or homologous virus. As expected, these sera gave negative results by CHEKIT-BVD-SERO. With regard to these results, two points should be considered:

- such high neutralisation titres in persistently-infected animals are more the exception than the rule
- frequency of persistently-infected animals with neutralising antibodies is low.

Figure 4 shows a schematic comparison between seroneutralisation and CHEKIT-BVD-BERO. Some persistently-infected animals test positive to seroneutralisation while such animals always give negative results by CHEKIT-BDV-SERO, regardless of whether or not they have neutralising antibodies.

![Diagram](attachment:fig4.png)

**Fig. 4**

Prevalence of bovine virus diarrhea virus-specific antibodies and distribution of persistently-infected animals in seropositive and seronegative cattle as measured by neutralisation (a), or enzyme-linked immunosorbent assay (ELISA) (b)

It is evident that only animals which test negative by CHEKIT-BVD-SERO need to be tested for virus. This reduces the amount of work involved and the costs of testing.

However, persistently-infected calves younger than six months will have maternal antibodies, which will be detected by ELISA. Testing for antibodies in calves is not recommended. Calves should be directly tested for virus.
SCHEME FOR BVD DIAGNOSIS

Figure 5 shows a possible scheme for BVD diagnosis using a combination of CHEKIT-BVD-SERO and CHEKIT-BVD-VIRUS. On the first day, samples arrive and are divided into groups, according to the age of the animal. In view of the presence of maternal antibodies, samples from calves younger than six months are tested directly for virus. All other samples are first tested for antibodies. Samples which give negative results by CHEKIT-BVD-SERO are tested for virus together with samples from animals younger than six months. The following day, information on the serological and virological status of a herd is available. Non-immune animals may then be vaccinated if necessary.

**Fig. 5**

Scheme for diagnosis of bovine virus diarrhoea (BVD)-mucosal disease based on two enzyme-linked immunosorbent assays (CHEKIT-BVD-SERO and CHEKIT-BVD-VIRUS)

Thus far, the authors have recommended retesting of animals yielding positive results by CHEKIT-BVD-VIRUS, because virus persistence has yet to be proved. However, there is some evidence that transient viraemic animals test negative by the CHEKIT-BVD-VIRUS test. To date, no transient viraemic animal has been detected with CHEKIT-BVD-VIRUS. However, four transient viraemic animals tested negative by this test (6).
Figure 6 gives an example where a herd of thirty-eight animals was tested for antibodies. Except for three animals, all tested positive by CHEKIT-BVD-SERO. All animals were tested for BVDV with CHEKIT-BVD-VIRUS. The three animals which tested negative by CHEKIT-BVD-SERO gave positive results with CHEKIT-BVD-VIRUS. Early slaughter of such animals could reduce economic losses.

![Graph showing optical density of samples tested by CHEKIT-BVD-SERO and CHEKIT-BVD-VIRUS.](image)

* Samples are presented in ascending order of optical density as measured by CHEKIT-BVD-SERO

**Example of the combined use of two enzyme-linked immunosorbent assays for the diagnosis of bovine virus diarrhoea (BVD) virus (CHEKIT-BVD-SERO and CHEKIT-BVD-VIRUS) in a herd of thirty-eight animals**

Three animals were persistently-infected with BVD virus (left), while all the other animals had antibodies to BVD virus.

---

**Résumé :** Les auteurs décrivent deux techniques enzyme-linked immunosorbent assay (ELISA) appliquées au diagnostic de la diarrhée virale bovine (bovine virus diarrhoea : BVD) : CHEKIT-BVD-VIRUS et CHEKIT-BVD-SERO. Le premier test détecte l'antigène viral dans les leucocytes, permettant ainsi d'identifier des animaux à infection persistante, tandis que le second repère les anticorps du virus de la diarrhée virale bovine. Il est bien
connu que même les animaux à infection persistante peuvent présenter des anticorps dirigés contre des souches hétérologues du virus de la BVD. Les réactions de ces animaux restent négatives au test CHEKIT-BVD-SERO en raison d'une immunotolérance à l'égard des antigènes viraux conservés. Les auteurs font le bilan des données relatives à ces deux tests et présentent un protocole les utilisant de façon combinée en vue du diagnostic de la BVD.


*  
  

Resumen: Los autores describen dos técnicas inmunoenzimáticas ELISA aplicadas al diagnóstico de la diarrea viral bovina: CHEKIT-BVD-VIRUS y CHEKIT-BVD-SERO. La primera de estas pruebas detecta el antígeno viral en los leucocitos, permitiendo identificar animales con infección persistente; la segunda, detecta los anticuerpos del virus de la diarrea viral bovina. Es sabido que aun los animales con infección persistente pueden presentar anticuerpos correspondientes a cepas heterólogas del virus de la diarrea viral bovina. Las reacciones de estos animales siguen siendo negativas al CHEKIT-BVD-SERO debido a una inmunotolerancia respecto de los antígenos virales conservados. Los autores concluyen con un balance de los datos obtenidos en una y otra prueba y proponen un programa basado sobre la asociación de ambas pruebas para el diagnóstico de la diarrea viral bovina.


*  
  
REFERENCES


