Five hours to identify immunotolerant cattle, persistently infected with bovine virus diarrhoea virus

D. CREVAT *, D. VANDENBERGH **, G. CHAPPUIS *, C. LECOMTE ** and A. RENARD **

Summary: Detection of animals which are persistently-infected with bovine virus diarrhoea virus (BVDV) is of prime importance in the control of pestivirus infections in cattle, as these animals constitute the main reservoir of the virus.

Identification of such animals can be readily performed using crude whole blood samples with a sandwich enzyme-linked immunosorbent assay (ELISA) requiring only approximately five hours. This ELISA uses a combination of monoclonal antibodies as the capture agent and an immunological amplification step of the specific signal for detecting the non-structural 80/120 kDa protein of BVDV. The degree of correlation between this ELISA and virus isolation as the reference method is 100% for animals older than six months.

KEYWORDS: Bovine virus diarrhoea virus – Cattle – Crude whole blood samples – ELISA – Monoclonal antibodies – Persistent infection – Pestivirus.

INTRODUCTION

The genus Pestivirus includes three serologically-related viruses: hog cholera virus (HCV) in pigs, Border disease virus (BDV) in sheep and bovine virus diarrhoea virus (BVDV) in cattle (4).

BVDV induces a wide variety of foetal and post-natal disorders in cattle and causes significant economic losses in the livestock industry. Pre-natal infection can lead to the generation of immunotolerant, persistently-infected (IPI) animals which continually excrete large amounts of virus throughout life (3) and constitute the main reservoir of BVDV.

The prevention and control of BVDV infections entail the identification and removal of IPI animals and the judicious use of vaccination programmes (2). Until recently, virus isolation after culture amplification and immunofluorescence was the method most widely available for the detection of such animals (7). Other diagnostic technologies based on enzyme-linked immunosorbent assays (ELISAs) (8, 13, 15), flow cytofluorometry (14) and viral genomic detection after polymerase chain reaction (9, 12) have also been used successfully. However, all of these methods have significant drawbacks:
they are time-consuming, because of the necessity for sample preparation (lymphocytes and/or genome extractions)

they can be expensive (specialised equipment and trained laboratory personnel are required).

Control plans require a sensitive, easy-to-perform, rapid and inexpensive assay to screen large numbers of samples for persistent infection.

The non-structural 80/120 kDa protein (p80) of BVDV is a good target for a sandwich ELISA; this protein shares specific antigenic determinants which are highly-conserved in all pestivirus strains and is produced in large amounts during the virus multiplication stage in IPI animals (5, 6).

A sandwich ELISA was optimised using crude whole blood samples, and performance was compared with that of the virus isolation used as the reference method.

MATERIALS AND METHODS

Animals

Six cattle persistently-infected with BVDV were available, with ages ranging from 1 to 2.5 years. These animals were the progeny of cattle which had been infected in early pregnancy with a non-cytopathogenic strain of BVDV. Three of the progeny had been vaccinated against bovine virus diarrhoea-mucosal disease (BVD-MD) with an inactivated vaccine. Two of the progeny had been vaccinated with a modified live anti-BVD-MD vaccine. The remaining IPI animal was not vaccinated and was used as a control.

In addition, two one-year-old cattle from BVD-MD-free herds were challenged intranasally and intravenously ($10^3.7$ CCID$_{50}$/animal) with the New York strain of BVDV.

Another 180 cattle with ages ranging from six months to seven years were used. These animals originated from seventeen different BVDV-infected herds in different areas of France. The majority had been previously vaccinated with a modified live vaccine.

Blood samples (5 ml) were collected in glass tubes containing 50 units of heparin and used to test for the presence of BVDV in cell culture and p80 antigen by ELISA. Blood samples collected in glass tubes without anticoagulant were used to prepare serum samples by centrifugation for 15 min at 800 g. These serum samples were then employed to test for the presence of anti-p80 antibodies using a blocking ELISA.

ELISA reagents

A set of mouse monoclonal antibodies (MAbs) was prepared and characterised by indirect immunofluorescence and radio-immunoprecipitation as previously described (10). Three MAbs (227H3, 227C6 and 202D12) were selected for their specificity and high relative affinity to the p80 antigen using a panel of twenty-three BVDV, BDV and HCV strains or field isolates. A combination of these MAbs was selected so that the ELISA provided the greatest signal:background ratio using blood samples from one IPI animal and one anti-p80 positive non-IPI animal. Optimisation was also carried out using titration of a crude p80 recombinant antigen solution diluted in a TNT buffer (see below).
Characterisation and production of the p80 recombinant antigen is described elsewhere (11).

An anti-p80 hyperimmune serum was prepared from rabbits after eight injections (at one-month intervals) of 50 µg of the recombinant p80 antigen in 0.5 ml of 0.025 M tris buffer, pH 10, emulsified in 0.5 ml of Freund’s complete adjuvant. Protein concentrations were determined with bicinchoninic acid (BCA reagent). Purity of the monoclonal antibodies and recombinant p80 antigen solutions was assessed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis and gel scanning, which attained 98% and 10% of the total proteins respectively.

**ELISA procedure**

Microtitre plate wells were coated with 100 µl of the MAb mixture in 0.050 M carbonate buffer (pH 9.6) and incubated overnight at 4°C. After washing, plates were blocked overnight at 4°C with a TNT buffer (0.010 M tris hydrochloride, 0.150 M sodium chloride, 0.1% Tween 20 and 3% [v/v] horse serum inactivated for 30 min at 56°C, pH 7.4).

Fifty µl of blood samples collected on anticoagulant were diluted directly into the coated wells with 50 µl of the TNT buffer containing 1% Nonidet P-40 (v/v).

One hundred µl of a positive control were included in a well on each plate. This consisted of a solution of the p80 recombinant antigen at 0.5 µg protein/ml in the TNT buffer.

After incubation for 2 h at 37°C and subsequent washing, 100 µl of rabbit anti-p80 serum diluted in the TNT buffer were added and incubated for 1 h at 37°C. After further washing, 100 µl of a goat anti-rabbit immunoglobulin (Ig)G horseradish peroxidase conjugate, diluted in the TNT buffer, were added to each well for 1 h incubation at 37°C. The enzymatic reaction was carried out by adding 100 µl of substrate buffer (0.25 mM tetramethylbenzidine in citrate-phosphate buffer [pH 5.0] containing 0.1 µl/ml of 30% H₂O₂) and reacting for 30 min in a dark place at 20°C. The reaction was subsequently stopped using 50 µl of 2N H₂SO₄.

Optical densities (OD) were measured on a microplate reader with 450 and 630 nm filters. Results are expressed as % sample:

\[
\% \text{ sample} = \frac{\text{OD sample}}{\text{OD positive control}} \times 100
\]

**Virus isolation**

Samples were screened for the presence of BVDV by indirect immunofluorescence following the method described by Waxweiler and colleagues (17) with some modifications. One hundred µl of blood samples were added onto IPB3 cell monolayers (a continuous cell-line of bovine lung cells: IFFA Poumon Bovin 3) in four microplate wells. After incubation for three days, supernatant from frozen and thawed infected cell cultures was used to infect fresh IPB3 cells. After a period of incubation lasting three days, cells were fixed in acetone and stained by an indirect immunofluorescence test using a mixture of anti-BVDV MAbs and fluorescein-labelled goat anti-mouse IgG.

Virus isolation and ELISA were conducted independently by two different workers.
Anti-p80 antibody detection

Serum samples were tested using a blocking ELISA to detect anti-p80 antibodies. Briefly, microtitre plates were coated with 100 µl of the p80 recombinant antigen using a capture monoclonal antibody. One hundred µl of a 1:10 dilution of serum samples were incubated overnight at 4°C in each coated well. After washing, 100 µl of an anti-p80 group pestivirus-specific monoclonal antibody labelled with horseradish peroxidase were added and incubated for 1 h at 4°C. One hundred µl of substrate buffer were then added to each well and incubated for 30 min in a dark place at 20°C. The reaction was stopped by adding 50 µl of 2 N H$_2$SO$_4$ and optical densities were measured at 450 and 630 nm. ODs obtained with samples were compared with “cut-off” OD values calculated from positive and negative control sera.

RESULTS

ELISA optimisation

Capture efficiency of different mixtures of MAbs coated onto the solid phase was explored in two successive assays (Table I). The first assay demonstrated that MAb 202D12 was essential but not sufficient by itself to generate a high signal:background ratio. Equal quantities of each MAb in the mixture displayed the best result. In the second assay, the ratio was clearly increased by association of the three MAbs in a definite proportion: a capture solution containing one, four and eight volumes of 1 mg/ml purified MAbs 227H3, 227C6 and 202D12 respectively, achieved the optimal specific signal using both blood samples from IPI cattle and dilutions of the p80 recombinant antigen solution.

This combination was used in further experiments for the calibration of the rabbit anti-p80 serum and the goat anti-rabbit IgG peroxidase conjugate (data not shown).

Comparison between ELISA and virus isolation

A total of 188 blood samples were screened in double blind assays. ELISA results were expressed as % sample, using as positive control a solution of p80 recombinant antigen containing 0.5 µg/ml of total proteins. Three categories of animals were isolated (Table II).

Blood samples from 117 anti-p80 antibody-positive (naturally-infected and/or vaccinated) and 38 anti-p80 antibody-negative cattle were negative for virus isolation. These samples consistently displayed a % sample below 50%, with no significant differences between the ELISA values of the two groups (mean values and standard deviations: 28.9 and 29.1, and 7.1 and 6.7, respectively).

Thirty cattle of at least six months of age tested clearly positive by the reference method as well as by ELISA, with a % sample which ranged from 140% to 220%. This population included twenty-four animals from seventeen different herds and the six IPI cattle (see “Materials and Methods”).

The persistent BVDV infection status of these animals was confirmed with new blood samples, taken at least one month later.

A third group included three cattle with transient p80 antigenaemia observed during the onset of natural or experimental BVDV infections: one blood sample had been
**TABLE I**

Optimisation of the mixture of monoclonal antibodies (MAbs) acting as the capture agent in an enzyme-linked immunosorbent assay for the detection of immunotolerant cattle, persistently infected with bovine virus diarrhoea virus

<table>
<thead>
<tr>
<th>Mab (a)</th>
<th>Composition of the mixture (volume/volume) First assay</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>227H3</td>
<td>1 0 0 1 0 1 1</td>
<td></td>
</tr>
<tr>
<td>227C6</td>
<td>0 1 0 1 1 0 1</td>
<td></td>
</tr>
<tr>
<td>202D12</td>
<td>0 0 1 0 1 1 1</td>
<td></td>
</tr>
<tr>
<td>Signal: background ratio (b)</td>
<td>1.6 1.9 2.8 1.5 2.7 2.4 4.1</td>
<td></td>
</tr>
<tr>
<td>p80 recombinant antigen concentration (c)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.160 (d) 0.239 0.420 0.208 0.439 0.409 0.792</td>
<td></td>
</tr>
<tr>
<td>0.75</td>
<td>0.086 0.109 0.219 0.101 0.326 0.231 0.406</td>
<td></td>
</tr>
<tr>
<td>0.19</td>
<td>0.079 0.097 0.103 0.089 0.120 0.099 0.184</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.068 0.093 0.072 0.090 0.084 0.084 0.106</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Mab (a)</th>
<th>Composition of the mixture (volume/volume) Second assay</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>227H3</td>
<td>1 1 1 1 1 1 1</td>
<td></td>
</tr>
<tr>
<td>227C6</td>
<td>1 1 1 1 2 2 4</td>
<td></td>
</tr>
<tr>
<td>202D12</td>
<td>1 2 4 8 2 4 8</td>
<td></td>
</tr>
<tr>
<td>Signal: background ratio (b)</td>
<td>4.0 4.6 5.1 5.3 5.0 5.7 6.0 6.9</td>
<td></td>
</tr>
<tr>
<td>p80 recombinant antigen concentration (c)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.765 (e) 0.812 0.804 0.841 0.813 0.960 0.917 0.981 1.232</td>
<td></td>
</tr>
<tr>
<td>0.75</td>
<td>0.400 0.410 0.407 0.392 0.394 0.426 0.408 0.493 0.692</td>
<td></td>
</tr>
<tr>
<td>0.19</td>
<td>0.192 0.183 0.172 0.176 0.192 0.199 0.190 0.251 0.366</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.101 0.099 0.098 0.094 0.106 0.128 0.141 0.148 0.151</td>
<td></td>
</tr>
</tbody>
</table>

a) ion exchange chromatography purified MAbs adjusted to a concentration of 1 mg/ml

b) signal:background ratio = \(\frac{OD \text{ blood sample from one IPI animal}}{OD \text{ blood sample from a non-IPI animal}}\)

c) concentrations of p80 antigen crude solution are expressed in µg protein/ml

d) OD (450/630 nm)

IPI: immunotolerant, persistently-infected
TABLE II

Comparative evaluation of enzyme-linked immunosorbent assay (ELISA) and virus isolation for the detection of immunotolerant, persistently-infected (IPI) cattle with bovine virus diarrhea virus (BVDV)

<table>
<thead>
<tr>
<th>ELISA % sample</th>
<th>Virus isolation reference method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>≥100%</td>
<td>30 (a)</td>
</tr>
<tr>
<td>50-100%</td>
<td>2 (b)</td>
</tr>
<tr>
<td>&lt; 50%</td>
<td>1 (c)</td>
</tr>
</tbody>
</table>

a) blood samples from IPI cattle confirmed by two blood assays with an interval of more than one month
b) blood samples from two cattle challenged with the New York strain of BVDV (10^3 CCID50); blood sampling at seven days post-infection, during the transient viraemic stage
c) blood sample from one animal with transient antigenaemia; antibodies observed in serum fifteen days later

collected from an animal in a non-vaccinated herd where 90% of the animals were positive for anti-p80 antibodies indicative of pestivirus circulation in this herd; the other two samples were taken from the challenged cattle seven days after an experimental infection. These samples displayed ELISA results of 50-80% sample and were classified as doubtful or negative, but tested positive by virus isolation. Blood samples taken fifteen days later from the same animals showed negative results with the reference method, and the presence of anti-p80 antibodies in the sera was demonstrated using the blocking ELISA.

Persistence of p80 antigenaemia in IPI cattle

Table III illustrates the absence of variation in p80 antigenaemia in six IPI cattle older than six months, after either two vaccinations with inactivated vaccine or one injection of modified live vaccine.

Blood samples taken at days 0, 21, 49, 63, 85 and 106 did not show significant decreases of p80 antigenaemia after these antigenic stimulations. Furthermore, no anti-p80 antibodies were detected in the blocking ELISA.

DISCUSSION

Previous reports (8, 13, 15) have described ELISAs to identify cattle or sheep persistently-infected with pestivirus. These tests consistently include a sample preparation step, usually leukocyte isolation, in order to enhance the protein concentration of BVDV or BDV before testing. Indeed, pestiviruses have a known affinity for cells of the immune system, particularly the peripheral blood mononuclear cells (1). However, such treatment tends to be unfeasible for large-scale screening purposes.
Table III

Persistence of p80 antigenaemia after vaccination in immunotolerant cattle persistently infected with bovine virus diarrhoea virus

<table>
<thead>
<tr>
<th>Vaccination</th>
<th>Animal No.</th>
<th>Day 0</th>
<th>Day 21</th>
<th>Day 49</th>
<th>Day 63</th>
<th>Day 85</th>
<th>Day 106</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inactivated vaccine</td>
<td>124</td>
<td>175</td>
<td>191</td>
<td>189</td>
<td>171</td>
<td>176</td>
<td>198</td>
</tr>
<tr>
<td>(days 5 and 24)</td>
<td>125</td>
<td>200</td>
<td>232</td>
<td>211</td>
<td>224</td>
<td>221</td>
<td>218</td>
</tr>
<tr>
<td></td>
<td>438</td>
<td>213</td>
<td>210</td>
<td>214</td>
<td>208</td>
<td>208</td>
<td>204</td>
</tr>
<tr>
<td>Live vaccine (day 0)</td>
<td>121</td>
<td>170</td>
<td>171</td>
<td>160</td>
<td>154</td>
<td>168</td>
<td>172</td>
</tr>
<tr>
<td></td>
<td>122</td>
<td>201</td>
<td>200</td>
<td>189</td>
<td>192</td>
<td>178</td>
<td>195</td>
</tr>
<tr>
<td>Not vaccinated</td>
<td>439</td>
<td>186</td>
<td>163</td>
<td>177</td>
<td>181</td>
<td>192</td>
<td>187</td>
</tr>
</tbody>
</table>

* % sample = \( \frac{\text{OD sample}}{\text{OD positive control}} \times 100 \)

OD: optical density

In this study, anticoagulant crude whole blood was used, in an effort to avoid lengthy preparation steps. The test was successfully developed, taking into account these time constraints, and the result was a procedure lasting only approximately five hours. Problems due to proportionally low concentrations of viral components in such samples were overcome by the following factors:

- the ELISA targeted the BVDV p80 non-structural protein which shares common antigenic determinants in all pestivirus strains and is expressed with relatively high levels in IPI animals (5, 6)
- the dilution of blood samples in a lysis buffer exposed the viral proteins from the leukocytes
- immunological amplification of the specific signal used two successive reagents for revelation
- the judicious combination of three monoclonal antibodies acting as capture agent significantly improved the signal/background ratios.

The association of several MAbs for signal amplification purposes in ELISA technology has previously been described. Using MAbs specific to different epitopes on hepatitis B virus surface antigen, Wands and colleagues (16) increased the sensitivity of an ELISA intended for the identification in humans of chronic hepatitis B virus carriers. Similar results were obtained in this study, indicating that an amount estimated to be approximately 50 ng/ml of pure p80 recombinant protein (10% of the crude solution) could be easily detected. This concentration was chosen as the cut-off value in the present ELISA.

Under these conditions and for animals older than six months, IPI cattle can be clearly identified from a population of vaccinated, convalescent or healthy cattle. This ELISA on crude whole blood samples exhibits complete agreement with the reference method. Discrepant results may be related to either the transient viraemic period at the onset of the BVDV infection or the presence of colostral antibodies in young calves.
In both cases, appearance or persistence of anti-p80 antibodies in serum can mask the antigen which cannot be subsequently captured onto the solid phase. In addition, consistent with virus excretion, the level of p80 antigenaemia is probably lower during the transient viraemic stage than in true IPI cattle. Application of this ELISA in calves younger than three to six months requires an easily-performed sample preparation: after lysis of red blood cells using an ammonium chloride solution, leukocytes are pelleted by centrifugation at 1,000 g for 15 min and the supernatant containing the colostral antibodies discarded. The leukocyte pellet is resuspended in the TNT lysis buffer (as before) and 100 µl of the resulting suspension used in the ELISA procedure. This procedure has been used with success and such treatment avoids the interference of colostral antibodies and increases the test sensitivity by a factor of between 5 and 10.

In IPI cattle older than six months, p80 antigenaemia seems to be quite constant, even though the animals are submitted to anti-BVD-MD vaccinations as reported here. Similar results have been obtained with samples from animals in the field before and after vaccination with live vaccine.

The present test has been validated on samples from a total of thirty persistently-infected cattle from seventeen herds located in various areas in France, which would be expected to cover a diverse range of pestivirus isolates. Field testing of more than 500 crude blood samples confirmed total agreement with virus isolation.

Shannon and colleagues (15) recently reported that blood clots and tissues (especially lymph nodes, tonsils and spleen) can be successfully used to identify IPI cattle. Further studies are in progress for the testing of such samples with the present ELISA and for the validation of this test in goats and sheep.

ACKNOWLEDGEMENTS

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Résumé : Le repérage des animaux atteints d'une infection chronique par le virus de la diarrhée virale bovine (BVDV) est de toute première importance pour le contrôle des infections à pestivirus chez les bovins, d'autant plus que ceux-ci constituent le réservoir principal du virus.

Ce repérage peut être aisément réalisé à partir d'échantillons de sang total grâce à un test immuno-enzymatique (ELISA) de type « sandwich ». Cette épreuve ELISA entièrement réalisée en cinq heures détecte la protéine non structurale du BVDV de 80/120 kDa et utilise plusieurs anticorps monoclonaux comme agent de capture et une étape d'amplification du signal. Une corrélation parfaite (100 %) existe entre les résultats de cette épreuve ELISA et la méthode de référence par isolement viral, dans le cas des animaux âgés de plus de six mois.

* * *


Resumen: La identificación de animales afectados por una infección crónica debida al virus de la diarrea viral bovina (BVDV) es una tarea de la mayor importancia para el control de las infecciones por pestivirus en bovinos, ya que estos animales constituyen la reserva principal del virus.

Esta identificación puede llevarse a cabo cómodamente a partir de muestras de sangre total (sin adición) gracias a una prueba inmunoenzimática (ELISA) de tipo «sandwich». La prueba ELISA realizada íntegramente en cinco horas detecta la proteína no estructural del BVDV de 80/120 kDa y se vale de varios anticuerpos monoclonales como agente de captura así como incluye una etapa de amplificación de la señal. En el caso de los animales de más de seis meses de edad, se da una correlación perfecta (100%) entre los resultados de esta prueba ELISA y el método de referencia por aislamiento viral.


* * *

REFERENCES


