Control of bovine virus diarrhoea-mucosal disease in cattle: examples of the combined use of serological screening, viral antigen detection and vaccination

J.-C. THIBAULT *, D. CREVAT * and G. CHAPPUIS **

Summary: As part of the preparatory phase of a disease control programme in three herds infected with bovine virus diarrhoea-mucosal disease (BVD-MD) virus (demonstrated by virus isolation), initial serological screening was performed on all livestock older than six months (351 animals) by blocking enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody recognising a common epitope to the different strains of BVD-MD virus.

The presence of immunotolerant, persistently-infected animals was strongly suspected, as a high percentage (334 = 95.2%) of cattle showed positive serological reactions, while the other members of the herd (17 = 4.8%) continued to give negative results, even after vaccination with a live vaccine. Whole blood samples from all cattle were then tested individually for viral antigen by an ELISA technique which had previously been tested successfully. As a result, a total of nine viraemic animals were identified in the three herds. A confirmatory test was performed by the reference amplification method on cell culture with virus identification using specific fluorescein isothiocyanate-labelled monoclonal antibodies.

The identification and elimination of the persistently-infected animals led to the recovery of a negative serological status for the herds. It was therefore recommended that protective measures should be taken to avoid the reappearance of viraemic animals, involving vaccination and systematic viral testing before introducing any new animal into the herd. It was advisable that these measures should be maintained until all the potential reservoirs and vectors of BVD-MD virus are better known.


INTRODUCTION

Infection by bovine virus diarrhoea-mucosal disease (BVD-MD) virus in an adult immunocompetent animal is generally subclinical, underdeveloped and accompanied by rapid seroconversion (1).
However, transplacental infection by a non-cytopathogenic strain can provoke abortion, embryo resorption and malformations, or cause the birth of persistently-infected calves which are immunotolerant to the virus. Immunotolerant, persistently-infected (IPI) animals do not produce antibodies to the viral strain with which they were infected in the uterus, and such animals are permanent excretors of the virus (1, 2).

Clinically non-identifiable, IPI animals are responsible for persistence of infection in a herd and are the origin of sporadic manifestations of BVD before developing MD (in the case of a mutation or superinfection by a cytopathogenic strain with the same antigenic characteristics as the initial strain), which is always fatal (2, 3).

The control of BVD-MD thus depends essentially on the identification and elimination of IPI animals. This has always been difficult due to the complexity of the diagnostic techniques, notably those necessitating virus isolation from individual animals of the herd, which is too complex and time-consuming for routine use.

The development of new diagnostic tools has introduced the possibility of applying a new testing protocol to allow more efficient and wider control of the disease in an infected environment.

MATERIALS AND METHODS

MATERIALS

The application of the disease control protocol discussed below in herds infected by the BVD-MD virus requires specific diagnostic tools developed and validated by the research laboratories of Rhône Mérieux. All tools cited hereafter are commercially available, unless stated otherwise (reference technique).

Serology (antibody detection)

The serological technique employed for the detection of anti-BVD-MD virus antibodies was a blocking ELISA using a commercially-available kit.

After duplicate distribution of the positive and negative controls, samples are added in single wells, diluted at 1:10 in the diluent provided in the kit. After incubation overnight at 4°C, the plates are washed four times and the conjugate, reconstituted to its final dilution, is added to each well. A second incubation for 1 h at 4°C is followed by a new series of four washes. The ready-to-use substrate is added to the wells, which are then incubated in a dark place for 30 min at laboratory temperature (20 ± 5°C). The reaction is stopped and the reading is performed bichromatically at 450 and 630 nm.

Virology (viral antigen detection)

The presence of the virus is revealed by a commercially-available indirect immunoenzymatic technique. A non-structural viral protein antigen (p80/120) is detected in the blood of IPI animals (5). The manipulation, performed strictly according to the kit instructions, includes four principal steps:

- 50 µl of blood collected on anticoagulant are added to wells containing 50 µl of lysis buffer. This is designed to liberate p80/120 antigen present in white blood cells. Incubation for 2 h at 37°C allows the p80/120 in the sample to fix to the monoclonal antibodies (MAbs) which coat the bottom of the wells.
– After a series of washes, a rabbit anti-p80/120 antiserum is added to each well. During a second incubation for 1 h at 37°C, the antibodies fix to the p80/120 antigen captured in the first incubation.

– A further washing step is followed by the addition of a goat anti-rabbit immunoglobulin (Ig)G conjugate coupled to peroxidase. Fixation to the rabbit Ig takes place during a third incubation for 1 h at 37°C.

– The presence (or absence) of the complex MAb-antigen-antibody-labelled antibody is revealed by adding a ready-to-use tetramethylbenzidine substrate. After incubation for 30 min at laboratory temperature, the reaction is stopped by applying an acid solution. The reading is performed bichromatically at 450 and 630 nm.

**Cell culture (reference technique)**

In parallel to the viral antigen detection, blood samples were screened for virus isolation as follows: 100 µl of blood are added to 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 fluid from frozen and thawed infected cell cultures is used to infect fresh IPB3 cells. After 3 days, cells are fixed in acetone and the virus present is stained using a mixture of anti-BVD virus MAbs and fluorescein-labelled goat anti-mouse IgG.

**SELECTION OF HERDS**

Candidate herds were selected by practising veterinarians on the basis of clinical suspicion – symptoms suggestive of BVD-MD in young calves (diarrhoea, ulceration, slow growth, weakness and/or respiratory difficulties) or adults (return to oestrus, abortion) possibly accompanied by episodes of hyperthermia – or after confirmation of clinical cases of MD (virus isolation by cell culture, or evidence of virus provided by immunofluorescence [IF] on frozen organ sections from dead animals).

Double blood sampling was performed on the entire herd using dry tubes and tubes containing heparin or added ethylenediamine tetra-acetic acid (EDTA). The blood samples were refrigerated and then sent to a diagnostic laboratory (Rhône Mérieux Diagnostics) very rapidly (in 1 to 3 days).

**LABORATORY-APPLIED PROTOCOL**

All blood samples from cattle over six months old were subjected to the following analyses:

– serological diagnosis (antibody detection) by blocking ELISA

– p80/120 antigen detection by indirect ELISA on whole blood

– virus isolation by cell culture.

Following the identification of viraemic animals, confirmed after a second control sampling, it was advised that these animals be eliminated from the herd.

Further tests were performed after six and twelve months (still in progress in certain herds) on the new generations (animals aged from six to twelve months).
PRELIMINARY RESULTS

Three herds were subjected to the complete protocol as described above. A fourth herd was subjected to a reduced serological survey and is currently being investigated further. The results obtained are described in Table I.

**Table I**

*Results of serological and virological surveys for bovine virus diarrhoea-mucosal disease conducted in four herds of cattle*

<table>
<thead>
<tr>
<th>Herd</th>
<th>No., age and sex of animals</th>
<th>Serology</th>
<th>Virology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Anti-p80 blocking ELISA</td>
<td>Antigenaemia (p80 antigen)</td>
</tr>
<tr>
<td>Herd 1</td>
<td>88 animals</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1 animal</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Herd 2</td>
<td>Calves &gt; 6 months</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>6 animals</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>1 animal</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Heifers 12-30 months</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>41 animals</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4 animals</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Bulls</td>
<td>30 animals</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>58 animals</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2 animals</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Herd 3</td>
<td>Calves 12-18 months*</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>28 animals</td>
<td>-</td>
<td>+</td>
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<tr>
<td></td>
<td>4 animals</td>
<td>-</td>
<td>+</td>
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<tr>
<td></td>
<td>Bulls 18-24 months</td>
<td>+</td>
<td>-</td>
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<tr>
<td></td>
<td>18 animals</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1 animal</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Cows &gt; 2 years</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>65 animals</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>4 animals</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Herd 4</td>
<td>Calves &gt; 6 months</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>6 animals</td>
<td>+ (low)</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>2 animals</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>Calves 8-10 months</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>6 animals</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>Heifers 2 years</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>5 animals</td>
<td>(+ low)</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>1 animal</td>
<td>-</td>
<td>NT</td>
</tr>
</tbody>
</table>

* no calves aged 6-12 months were present in herd 3
ELISA: enzyme-linked immunosorbent assay
NT: not tested
One of the herds was suspected of harbouring BVD-MD, without any previous virological confirmation (herd 1), but there had clearly been a recent introduction of animals into the herd followed by the occurrence of BVD-like clinical symptoms in the livestock. One viraemic individual was subsequently identified.

Two other herds (herds 2 and 3) had experienced episodes of chronic BVD for several months and some animals presenting classical symptoms of MD had died. Virus isolation and/or virus identification by IF on frozen organ sections provided confirmation of active circulation of BVD-MD virus. Residual IPI animals were identified in both herds.

The fourth herd displayed various symptoms suggesting the circulation of BVD-MD virus. A serological survey on different age-groups in the herd demonstrated a very low overall prevalence of antibodies to BVD-MD (3/20 = 15%), together with a weak antibody response in animals which tested positive (50-60% competition for a positive threshold fixed at 40% in the kit used).

For the first three herds tested, which were subjected to the complete protocol and in which IPI animals were isolated, the initial results indicate the following:

- a high prevalence (334 of 351 animals = 95.2%) of seropositivity
- clear identification of nine IPI animals (each IPI animal identified by the antigen detection method was confirmed as positive by the reference method, including after a second sampling)
- perfect concordance of the serological results obtained with these animals: IPI cattle were all found to be seronegative by the blocking ELISA.

**DISCUSSION**

Recent publications have described the major economic consequences which can accompany BVD-MD in contaminated livestock (6, 7, 9, 11); this concerns both the chronic form (BVD with high morbidity but low mortality) and the acute form (MD with low morbidity but high mortality). Having developed mathematical models, Houe and colleagues (6) estimated annual losses caused by the disease in Denmark at approximately DKK 100M (US$ 16.2M) in a livestock population of less than two million milking cows. In Norway, a country relatively spared by BVD-MD (20% average prevalence), annual losses could rise to over NOK 50M (US$ 7.3M) (7). In certain countries, eradication schemes have commenced in recent years.

**LIMITATIONS OF CLASSICAL CONTROL PROCEDURES**

Control of BVD-MD requires the elimination of animals which are permanently excreting virus, and several procedures have been proposed in the past in order to attain this goal. Of these procedures, the systematic vaccination of suspect herds (to prevent virus circulation) combined with the hope of a natural elimination of IPI animals (average life span generally considered to be two to three years) has been widely applied in the past few years. Unfortunately, the effort required for long-term vaccination (minimum five years), as well as the survival of certain IPI animals well beyond two to three years, have led to failures (relapses) which are discouraging for cattle breeders as well as veterinarians.
An improvement in this procedure was proposed when simplification of antibody diagnostic techniques enabled preliminary serological screening of animals. This must be followed by virological identification of animals testing serologically negative but capable of being viraemic (7). (However it should be noted that such animals might never have been in contact with the virus.) This procedure, applied in certain countries, could rapidly reach the limit of its usefulness, especially in countries where vaccination is authorised, due to the fact that a second viral passage (wild virus or vaccinal strain) can interfere with the classical serological techniques by rendering IPI animals partly antibody-positive (a limitation of the indirect ELISA or seroneutralisation technique in antibody identification).

Additionally, the routine application of long and costly virological techniques for confirmation in a large number of animals remains problematic, and consequently such techniques are not applicable in wider disease control.

The newly-available tools (serological techniques using a blocking ELISA combined with an antigen ELISA enabling the detection of a viral marker) improve identification and therefore elimination of IPI animals, as these tests can be performed on large numbers of animals at a cost which makes the use of such tools feasible in livestock management.

**Choice of techniques**

*Serology*

Antibody detection by blocking ELISA is of undeniable theoretical interest, as this technique uses a MAb conjugate marked with peroxidase and directed against a shared epitope common to all strains of BVD-MD. This marker enters into competition with any antibodies present in an immunocompetent animal which has undergone seroconversion after contact with a wild virus strain or vaccinal strain. On the contrary, all IPI animals are incapable of developing a serological reaction against this epitope and will continue to test negative; this is true even in the case of an IPI animal subjected to superinfection by a second viral strain different from the strain with which it was infected *in utero*.

This blocking ELISA was thus preferred to classical ELISA techniques (e.g. indirect ELISA) or seroneutralisation, due to the higher specificity of the results obtained.

This theoretical consideration was effectively verified in the results obtained on the nine IPI animals identified in herds 1, 2 and 3 (Table I), and also on a large number of these animals (over thirty) still being studied at present. Conversely, no IPI animals were found among the 334 individuals which tested seropositive using the anti-p80 system.

Consequently, this type of serological test is highly secure and represents a potential improvement on the classic protocol (based on serology followed by a virological search performed on seronegative individuals). However, the laborious application and indirect approach of blocking ELISA may still constitute a practical disadvantage because of its two-step procedure.

*Antigenaemia detection*

Parallel to the above approach aimed at improving the existing procedure, it was possible to demonstrate the value of direct utilisation of an antigen detection test for the p80/120 non-structural protein (8, 10), notably in the case of intervention in a herd in
which virus circulation has already been confirmed: demonstration of virus presence by cell culture, IF, antigen detection in diseased or dead animals, clinical suspicion of MD (herds 2 and 3), or seroconversion observed in one or several serum samples.

The results obtained on the 351 animals tested in herds 1, 2 and 3 (Table I) showed that this strategy enabled early and direct demonstration of the presence of nine IPI individuals.

Animals experiencing transitory viraemia have hitherto not been detected in this test. This has also been reported by several authors (10; P. Nettleton, P.D. Kirkland, personal communication) using similar techniques. This fact can be considered as advantageous for a strategy which requires the elimination of IPI animals alone (often desired for control of this disease).

Direct and individual antigen diagnosis using ELISA technology on whole blood therefore offers new prospects for the control of BVD, partly due to the easy application (performed in less than 5 hours on 50 µl heparinised or EDTA blood samples) and favourable cost of this test (particularly when used on large numbers of animals).

The value of serology as a preliminary to antigen detection

Serology remains a particularly valuable tool in determining possible or even definite (by seroconversion) virus circulation in a suspected herd, especially in the case of long-lasting infections, covering different age classes (young animals in particular).

The value of instigating a limited serological survey prior to antigen detection is illustrated by the results obtained in herd 4 (Table I). Although the clinical signs were suggestive of BVD, they were not sufficiently specific to justify the expense of performing a viral search on the entire herd.

This strategy was confirmed by the serological results obtained in herd 4, showing a low prevalence of anti-BVD antibodies, which were confined to young animals (possibly due to colostral antibodies). This suggests that there was probably no active virus circulation and thus leads to the conclusion that a systematic antigen diagnostic search is not justified in this herd at present.

Above all, in cases where doubt persists and the onset of infection is suspected, it is possible to repeat the serological survey a few months later, on animals which previously tested seronegative, or to widen the survey to include other age classes. This enables surveillance of the onset of any seroconversion translated by an active virus circulation and thus justifying the application of measures designed to identify the IPI animals.

Proposal for a disease control procedure in a suspect herd

Consequently, a procedure designed to control the disease in infected or suspected livestock can be used, bringing together in an appropriate manner serological survey techniques, virus identification and protection measures, such as vaccination in healthy herds. The proposed procedure comprises three fundamental steps:

a) The probability of infection can be evaluated by confirmation of virus circulation.

This involves demonstrating the presence of IPI animals:

- in diseased animals (MD) by detection of the virus in the blood
- in dead animals by demonstrating the virus (IF applied to organ sections upon the death of an animal showing clinical symptoms suggestive of MD)
- in the course of a transaction (sale of animals) accompanied by the antigenic detection of BVD-MD

- by observation of seroconversion in one or several animals in the herd.

Virus circulation can be confirmed by a serological survey on a sample of (young) non-vaccinated animals which are in contact with the rest of the herd. However, testing of a herd at two- to three-month intervals should be preferred. This strategy will be recommended, prior to any antigen diagnosis in a herd simply suspected of BVD, and will provide economic justification for following an identification protocol for IPI animals.

b) Identification of permanent excretors of the virus involves individual and systematic antigen diagnosis of the entire herd, immediately after confirmation or strong presumption of virus circulation. This can be performed easily with new, commercially-available antigen detection techniques (p80/120), notably as described above. IPI animals can be detected and subsequently eliminated.

A new set of samples from young calves aged less than six months should be taken six months later, to detect any IPI animals in the new generations. Serological surveillance will then be instituted for young non-vaccinated animals in contact with the herd, to confirm that this generation is serologically negative, thus leading to the assumption that the virus is not present.

c) The herd should be protected, through vaccination, against the risk of a new infection generating IPI animals.

The identification and elimination of IPI animals in a herd previously infected with BVD-MD virus leads to rapid exhaustion of the principal sources of the virus, and consequently renders the herd serologically negative. However, the virus can renew its circulation in the herd as a result of primary infection, notably in one of the following ways:

- introduction into the herd of excretor animals, even from other species

- contact with an excretor animal during grouping of livestock (contests, fairs, etc.)

- action of animate or inanimate vectors capable of transporting the virus.

To diminish the risks of a new virus circulation capable of regenerating the birth of IPI calves in a herd, it will be necessary to protect the females (cows and heifers) by vaccination before the reproduction phase. This protection should be maintained by an annual booster, performed three to five weeks before calving, which will strengthen the immunity of cows and provide colostral protection for newborn calves against the risks of BVD.

In order to avoid introducing infected excretor animals into the herd, it is also recommended that:

- IPI animals should be identified by performing a p80 antigen test with every transaction

- animals should be isolated before introduction into the herd if these animals might be transitory excretors following a recent infection in the herd of origin.
CONCLUSIONS

Routine application of conventional procedures for identifying IPI animals (serology, possibly preceded by vaccination and accompanied by traditional virus isolation [cell culture] for confirmation) has proved difficult due to the cost and complexity (numerous interventions and difficult analyses) of these procedures.

Direct and individual antigen diagnosis using an indirect ELISA on whole blood offers new prospects for controlling BVD, due to the ease of utilisation (performed in less than five hours on blood samples drawn on anticoagulant) and favourable cost-effectiveness of this technique.

Serological diagnosis can be used to demonstrate virus circulation in a suspect herd, prior to any antigenic determination. A serological survey may be performed on young non-vaccinated animals (6-18 months) which are in contact with the rest of the herd, and preferably repeated at intervals of 3-4 months if there is a high level of seropositivity.

* * *

RÉSÉUMÉ: Dans le cadre de la phase préparatoire d'un programme de prévention portant sur trois élevages infectés par le virus de la diarrhée virale bovine-maladie des muqueuses (bovine virus diarrhoea-mucosal disease : BVD-MD), infection confirmée par isolement du virus, un premier dépistage sérologique a été réalisé sur tous les sujets de plus de six mois (351 animaux) à l'aide d'un test enzyme-linked immunosorbent assay (ELISA) de compétition utilisant un anticorps monoclonal reconnaissant un épitope commun aux différentes souches de virus BVD-MD.

La présence d'animaux immunotolérants à infection persistante a été fortement suspectée : on a observé un pourcentage élevé (334 = 95,2 %) de réactions sérologiques positives, tandis que les résultats sont demeurés négatifs sur le reste du troupeau (17 = 4,8 %), même après immunisation avec un vaccin vivant. Les prélèvements de sang complet de l'ensemble du troupeau ont alors été analysés, individuellement, pour déceler l'antigène viral au moyen d'une technique ELISA qui avait fait ses preuves précédemment. Cette technique a permis d'identifier neuf animaux qui présentent une virémie dans les trois élevages. Le résultat a été confirmé par la méthode d'amplification de référence sur culture cellulaire avec un procédé de reconnaissance du virus utilisant des anticorps monoclonaux spécifiques marqués à l'isothiocyanate de fluorescéine.

L'identification et l'élimination des animaux à infection persistante ont ainsi permis d'aboutir à l'élimination de toute réaction sérologique positive dans les troupeaux. Il a, par conséquent, été recommandé de prendre des mesures préventives pour éviter la réapparition de sujets avec virémie notamment par la vaccination et la recherche systématique de ces virus, avant d'introduire de nouveaux animaux dans ces troupeaux. Il a, d'ailleurs, été conseillé de...
mener ces mesures tant que l'on n'aurait pas une connaissance plus précise de tous les réservoirs et vecteurs potentiels du virus BVD-MD.


Resumen: Como parte de la etapa preparatoria de un programa de control dirigido a rebaños de bovinos infectados por el virus de la diarrea viral bovina-complejo de enfermedad mucosa (bovine virus diarrhoea-mucosal disease: BVD-MD), infección confirmada por el aislamiento del virus, un primer examen serológico se realizó en todos los animales mayores de seis meses (351 animales) mediante la prueba inmunoenzimática de competición ELISA, con un anticuerpo monoclonal que reconoce un epitopio común a las diferentes cepas del virus BVD-MD.

La presencia de animales inmunotolerantes con infección persistente se pudo sospechar en gran medida: se registró, en efecto, un alto porcentaje de reacciones seropositivas (334 = 95,2%), mientras que los resultados fueron negativos para el resto del rebaño (17 = 4,8%), aun después de una vacunación con vacuna viva. Se investigó entonces individualmente en las muestras de sangre entera del conjunto de los bovinos para detectar el antígeno viral por medio de una técnica ELISA que había mostrado anteriormente su eficacia, y se identificaron así nueve animales con viremia en los tres rebaños. Este resultado pudo ser confirmado luego por medio del método de amplificación de referencia en cultivo celular, con un procedimiento de identificación del virus en que se usaron anticuerpos monoclonales específicos con un marcador de isotiocianato fluorescente.

La identificación y la eliminación de animales con infección persistente permitieron así llegar a resultados seronegativos para el conjunto de los rebaños. Como consecuencia de lo cual se recomendó tomar medidas preventivas para evitar la reaparición de individuos infectados por el virus, tales como vacunación y pruebas diagnósticas sistemáticas antes de introducir nuevos animales en el rebaño. Se estableció, por último, que sería conveniente mantener estas medidas hasta que puedan conocerse con mayor precisión el conjunto de los reservorios y vectores potenciales del virus BVD-MD.

REFERENCES


