Validation of a commercial ELISA for the detection of bluetongue virus (BTV)-specific antibodies in individual milk samples of Dutch dairy cows

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Abstract

A recently developed indirect ELISA for the detection of bluetongue virus (BTV)-specific antibodies in bovine milk samples was compared to that of the routinely used competitive ELISA on serum samples.

During the bluetongue outbreak in the Netherlands in 2006, caused by BTV serotype 8, coupled serum and milk samples were obtained from 470 individual cows from 10 BTV-infected farms with an average seroprevalence of 57%. In addition, bulk milk samples of the same farms, and historically BT-negative samples were tested. Compared to the ELISA for sera, the relative specificity and sensitivity of the ELISA for milk samples is 96.5% and 98.9%, respectively when using a S/P% cut-off value of 50% as advised by the manufacturer. The optimal cut-off value was found at S/P% of 90% revealing an optimal specificity (99.0%) combined with an optimal sensitivity (98.1%). Titres in positive individual milk samples ranged from 1 to 2048 with a peak titre of 128. Bulk milk samples contained antibodies with titres ranging from 64 to 512.

The ELISA for milk samples was found to be a reliable and robust test. This diagnostic tool is very useful, and may replace the ELISA for serum samples as first choice in order to get insight into the status of lactating individual animals and therewith of the entire herd with respect to BTV infection.

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1. Introduction

Bluetongue is a vector-borne viral disease of ruminants, including sheep, goats and cattle, caused by bluetongue virus (BTV). Clinical signs are seen mainly in sheep whereas the infection in cattle often occurs asymptomatic (Verwoerd and Erasmus, 1994;
Gibbs and Greiner, 1994). BTV is a member of the Orbivirus genus of the family Reoviridae, and consists of 24 serotypes with considerable immunological cross-reaction (Gorman, 1990). Animals can become infected by BTV-infected midges of the genus Culicoides that have had a blood meal from viraemic animals (Erasmus, 1990).

In 2006, an outbreak of bluetongue occurred in the Netherlands, Belgium, Germany, France and Luxembourg by a BTV variant of serotype 8 (Wuijckhuise van et al., 2006; Mehlhorn et al., 2007). Routine laboratory diagnosis has successfully been performed in the Netherlands by an in-house real-time RT-PCR (reverse transcription and polymerase chain reaction) on EDTA-blood samples (manuscript in preparation) and by serology using a commercial competitive ELISA (ID.VET, Montpellier, France). Positive PCR results can be found in cattle a few days post-infection till 200 days post-infection or more. Using the ID.VET ELISA, seroconversion has been detected starting 9–14 days post-infection.

In accordance to EU legislation, control measures must be taken and disease monitoring and surveillance is of fundamental importance to assess the risk posed by animal movements. For dairy cattle, testing for antibodies in milk samples rather than in serum samples would be very cost-effective and acceptable because of the easy availability of the former sample type.

In this study, the performance of an indirect ELISA developed by ID.VET for the detection of BTV-specific antibodies in bovine milk samples (mELISA) was compared to that of the routinely used competitive ELISA on serum samples (sELISA).

2. Materials and methods

2.1. Serum and milk BTV antibody ELISAs

The commercially available competitive ELISA for serum (sELISA; ID.VET, Montpellier, France) was performed as described in the kit manual. Briefly, 50 µl of buffer and 50 µl of serum were added to the wells of a BTV-VP7-coated microtitre plate. After incubation for 45 min at room temperature (rT), 100 µl anti-VP7 peroxidase conjugate was added and incubated for 30 min at rT. After washing, wells were incubated for 15 min at rT with 100 µl TMB substrate. Colour development was stopped by the addition of 100 µl 0.5 M H2SO4. S/N ratios (ODsample/ODnegative control) were calculated using optical density values measured at 450 nm (OD450). S/N ratios <0.4 were considered as positive.

A recently developed, indirect ELISA for milk (mELISA) was performed according to instructions of the manufacturer (ID.VET). Briefly, 50 µl skimmed milk and 50 µl ‘wash solution’ (final sample dilution 1:2) were added to the wells of a BTV-VP7-coated microtitre plate. After incubation for 45 min at rT, plates were washed and incubated with 100 µl anti-ruminant peroxidase conjugate for 30 min at rT. After washing, wells were incubated for 15 min at rT with 100 µl TMB substrate. Colour development was stopped by the addition of 100 µl 0.5 M H2SO4. S/P% (ODsample/ODpositive control × 100%) was calculated using optical density values measured at 450 nm (OD450). S/P% ≥50% was considered as positive.

Milk samples were also tested in the mELISA after a previously prepared dilution of 1:4 in the kit wash solution (final sample dilution 1:8). Titres of individual milk samples were determined in the mELISA by testing twofold serial dilutions prepared in negative bulk milk. The titre of a milk sample was defined as the highest dilution that gave a positive response (S/P% ≥50%).

2.2. BTV-PCR

Viral dsRNA was automatically isolated from EDTA-blood (MagNA Pure isolation robot, Roche). First strand cDNA synthesis (RT), PCR, and real-time detection were performed in a one-tube, closed system (Light Cycler 2.0, Roche). The target for amplification is located on genome segment 10 encoding NS3, and real-time detection was performed by hydrolysis of a Taqman probe. A weak positive control served as cutoff for positivity for the PCR-test. This test is able to give a positive signal up to 7 days before antibodies can be detected after experimental infection (manuscript in preparation).

2.3. Test samples

In this study different types of samples were analysed:

1. Historically negative individual milk samples (n = 55) and bulk milk samples (n = 88) from Dutch dairy cows.

2. Coupled samples (blood, EDTA-blood and a milk sample) were taken simultaneously of each lactating cow from 10 dairy farms selected based on a high BTV seroprevalence (Table 1). In addition, from each of these 10 farms a bulk milk sample was taken.

Milk and bulk milk samples, containing Na-azide as preservative, were defatted by centrifugation and stored at −20 °C in aliquots to circumvent repeated freezing and thawing. Serum samples were stored at −20 °C before testing. EDTA-blood samples were stored at 4 °C for up to 2 days before processing and testing by PCR.

Bulk milk taken from two BT-negative farms, was used to make serial twofold dilutions of individual milk samples.

To test the effect of a preservative on the performance of the mELISA, either Na-azide or BSM (Broad Spectrum Microtabs, D&F Control Systems Inc., San Ramon, CA, USA) was added to this bulk milk.

2.4. Determination of relative sensitivity and specificity, statistical analysis

The relative sensitivity of the mELISA is defined as the proportion of seropositive animals giving a positive response by testing the corresponding individual milk samples:

\[
\text{seropositive animals with a positive milk sample} \quad \frac{\text{all seropositive animals}}{}
\]

The relative specificity of the mELISA is defined as the proportion of seronegative animals giving a negative response by testing the corresponding individual milk samples:

\[
\text{seronegative animals with a negative milk sample} \quad \frac{\text{all seronegative animals}}{}
\]

Relative sensitivities and relative specificities of the mELISA were calculated at different S/P% cut-off values. These cut-off values were used to classify milk samples as either positive or negative. Curves were constructed by plotting the relative specificity and the relative sensitivity against the corresponding S/P% cut-off values ranging from 0% to 240%.

Receiver operating characteristic (ROC) curves (Kraemer, 1992) of the mELISA were constructed by plotting the sensitivity on the ordinate as a function of the specificity at different cut-off values. The area under the ROC curve is a quantitative measure of the test’s performance. These curves were plotted to assess the relative performance of the mELISA when milk samples were tested undiluted or four times diluted.

Statistical analysis (sensitivity, specificity, confidence intervals and the agreement index kD) was performed using Win Episcope 2.0 (http://www.clive.ed.ac.uk/winepiscope/).

3. Results

Specificity of the mELISA was determined by testing 55 historically negative individual milk samples and 88 historically negative bulk milk samples. All samples tested negative with a mean S/P% ± S.D. of 11.0% ± 2.8% and 10.2% ± 0.8%, respectively. Based on the mean S/P% + 3S.D. of these negative samples the cut-off of the mELISA should be around an S/P% of 20%.

The relative sensitivity and relative specificity of the mELISA was determined using the sELISA as the gold standard test. Coupled serum and milk samples obtained from individual cows (n = 470, see Table 1) from 10 BTV-infected farms were analysed. The
overall results are shown in Table 2. The relative specificity of the mELISA is 96.5% (195/202 × 100%) with a 95% confidence interval (CI) between 94.0% and 99.1%. The seven animals with discordant results were negative in the BTV-PCR test.

The relative sensitivity amounts to 98.9% (265/268 × 100%; 95% CI: 97.6–100%). Two of the three animals with a discordant result showed a positive BTV-PCR response. The overall accuracy is 97.9% (460/470 × 100%; k = 0.96).

A previous 4× dilution of milk samples in wash solution revealed a somewhat higher specificity (97.5%; 95% CI: 95.4–99.7%) and a somewhat lower sensitivity (98.1%; 95% CI: 96.5–99.8%) at the prescribed cut-off SP% value of the mELISA of 50%.

To verify the positive/negative cut-off value of the mELISA when milk was tested undiluted, a frequency distribution diagram was constructed based on the results of the 470 milk samples from the 202 BT-antibody seronegative and 268 BT-antibody seropositive animals (see Fig. 1). In addition, relative sensitivity and relative specificity at different SP% cut-off values were calculated and plotted (see Fig. 2). Highest relative sensitivity (98.1%; 95% CI: 96.5–99.8%), combined with highest relative specificity (99.0%; 95% CI: 97.5–100%) was observed at a cut-off SP% value of 90% (see also Fig. 3). ROC curves were constructed to compare the performance of the mELISA when milk samples were tested either undiluted or 4× diluted in wash solution (Fig. 3). The test with undiluted milk samples shows a slightly greater area under the ROC curve compared with the test performed with 4× diluted samples indicating a somewhat better performance with undiluted milk samples.

To determine the BTV-specific antibody titres in the individual milk samples, twofold serial dilutions in negative bulk milk were tested in the mELISA. Typical dose–response curves of eight serially diluted milk samples (one mELISA negative and seven seropositive samples) are shown in Fig. 1.

mELISA positive samples) are depicted in Fig. 4. Similar curves were found when dilutions were made in bulk milk containing either azide or BSM as preservative. Fig. 5 depicts the frequency distribution diagram of the antibody titres of the 470 individual milk samples. Milk samples from seropositive animals showed a mean 2 log titre of 7. The seven positive milk samples from seronegative animals had 2 log titres of 0 \( (n=5) \) and 1 \( (n=2) \).

The bulk milk samples collected from the 10 BT-infected farms showed 2 log titres of 6 \( (n=5) \), 7 \( (n=4) \) and 9 \( (n=1) \) in the mELISA. The bulk milk sample with the highest titre \( (2^9) \) originated from the farm with the highest seroprevalence \( (95\%) \).

4. Discussion

The commercial ID.VET mELISA is a recently developed indirect ELISA able to detect BTV-VP7-specific antibodies in milk samples.
The performance of the mELISA was investigated using the ID.VET sELISA as the reference test. In a previously performed study, the ID.VET sELISA showed both a high specificity (100%) and a high sensitivity (98%) relative to BTV-PCR results on field samples obtained at the early stage (September and November 2006) of the BT outbreak in the Netherlands (manuscript in preparation). Moreover, the ID.VET sELISA showed a good performance in proficiency testing (Workshop on bluetongue diagnostics and epidemiology, Brussels, Belgium, 28–29 November 2006). In our laboratory (Central Veterinary Institute of Wageningen UR), the sELISA is an ISO17025 accredited test and used for routine serum testing of both cattle and sheep.

In the study described it was investigated whether the mELISA can be used as a tool to identify BT-seropositive dairy cows. Analysis of historical...
negative individual \((n = 55)\) and bulk milk samples \((n = 88)\) indicated a specificity of 100% in this small set of data.

Testing individual milk samples obtained from seropositive \((n = 268)\) and seronegative cattle \((n = 202)\) revealed a high sensitivity and a high specificity relative to serology testing. Of the 470 milk samples tested in the mELISA, only 10 discordant results \((= 2.1\%)\) with the sELISA were found at the prescribed positive/negative cut-off S/P\% value of 50%. From the results as shown in Fig. 2, it can be concluded that the test shows a high sensitivity and a high specificity that change minimally between cut-off values of 30% and 110%. Highest sensitivity combined with highest specificity revealed seven discordant results (five false negatives and two false positives) and was found at a cut-off S/P\% value of 90%. A \(4 \times\) dilution of milk samples before testing did not result in a better performance (Fig. 3): highest accuracy revealed nine discordant results (six false negatives and three false positives) at a cut-off value of 80%.

Samples investigated were from farms with a high seroprevalence ranging from 34% to 95%. These farms were not very recently infected (samples were taken probably more than 4 months after primary infection), and most seropositive animals have reached there maximum antibody levels resulting in a minimum number of false negative test results. In case of a recently infected farm, at least part of the sera taken from infected animals may contain relatively low antibody titres giving weaker responses in the ELISA. For this reason a positive/negative cut-off value should be chosen resulting in a test with an optimal sensitivity combined with a specificity producing an acceptable low number of false positives (high sensitivity combined with an acceptable good specificity). As can be deduced from the results of this study and presented in Figs. 1 and 2 using a S/P\% cut-off value of 50% resulted in three false negatives (sensitivity = 98.9\%), two of which were confirmed by a positive BTV-PCR and seven false positives (specificity = 96.5\%), all of which were confirmed by a negative BTV-PCR. In view of the impact of these false positives, one may decide to confirm (false) positive milk test results by serum antibody testing and/or by BTV-PCR. When a higher specificity is required, the cut-off value can be changed to a S/P\% of 90% with minimal effects on sensitivity.

Antibody levels in the individual milk samples were determined by testing twofold serial dilutions, prepared in negative milk, in the mELISA. Fig. 5 shows a titre distribution of positive milk samples between \(2^0\) and \(2^{11}\) with a peak at \(2^7\). Of the eight milk samples with the lowest titre \((2^0\) and \(2^1\)), seven showed discordant results with the serum test (positive milk samples from seronegative animals). This suggests that animals develop BTV-specific antibody mELISA titres of \(2^2\) or higher and that by bulk milk detection of a herd prevalence of 25% or lower seems to be feasible. BTV-specific antibody titres of the bulk milk samples of the 10 farms ranged from \(2^6\) to \(2^9\). A weak positive correlation was observed between the seroprevalence and the bulk milk titre showing the highest titre in the bulk milk sample obtained from the farm with the highest seroprevalence. The application of the mELISA for bulk milk testing is now being validated by testing bulk milk samples from BTV-infected farms with different percentages of seropositive lactating animals.

Due to immunological cross-reaction with BTV-VP7 (Gorman, 1990) it cannot be excluded that the presence of antibodies directed against BTV-related Orbiviruses like epizootic haemorrhagic disease virus (EHDV) may result in positive responses in the mELISA and/or sELISA. In case animals are tested positive in the mELISA one should consider to further investigate these animals by more specific serum tests and/or PCR. However, up to this moment EHDV never has been detected in this part of Europe.

We conclude that the mELISA using undiluted milk at cut-off S/P percentages between 30\% and 110\% is a very reliable diagnostic tool. Because milk samples are easy and cost-effective to obtain this could be the first choice of testing in order to get insight to the status of animals and therewith of the herd concerning bluetongue virus infection.

References


