A microsphere-based immunoassay for rapid and sensitive detection of bovine viral diarrhoea virus antibodies

Hongyan Xia [a,b], Lihong Liu [b,*], Ann Nordengrahn [c], István Kiss [b], Malik Merza [c], Ronnie Eriksson [d], Jonas Blomberg [d], Sándor Belák [a,b]

[a] Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, SE-751 89 Uppsala, Sweden
[b] Joint R&D Division in Virology, The National Veterinary Institute & The Swedish University of Agricultural Sciences, SE-751 89 Uppsala, Sweden
[c] Svanova Biotech AB, SE-751 83 Uppsala, Sweden
[d] Laboratory Section of Virology, Department of Medical Sciences, SE-751 85 Uppsala, Sweden

ABSTRACT

This study describes a novel blocking microsphere-based immunoassay for highly sensitive and specific detection of antibodies against bovine viral diarrhoea virus (BVDV). The intra- and inter-assay variability are 4.9% and less than 7%, respectively, and variability of bead conjugations is less than 6.6%. The diagnostic performance of the assay was evaluated by testing a total of 509 serum samples. Based on a negative/positive cut-off value of 30.3%, the assay has a sensitivity of 99.4% and a specificity of 98.3% relative to ELISA. The new microsphere immunoassay provides an alternative to conventional ELISA systems and can be used for high-throughput screening in the BVD control programmes.

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1. Introduction

The genus *Pestivirus* of the family *Flaviviridae* consists of four approved species: *Bovine viral diarrhoea virus* 1 (BVDV-1), *Bovine viral diarrhoea virus* 2 (BVDV-2), *Classical swine fever virus* (CSFV), *Border disease virus* (BDV); and a tentative species Pestivirus of giraffe (Thiel et al., 2005). BVDV infections are usually very mild or inapparent clinically (Baker, 1987), but severe hemorrhagic syndrome is also observed in acute infection (Perdrizet et al., 1987; Corapi et al., 1989; Ridpath et al., 2006). Diaplacental infection can lead to birth of persistently infected animals that serve as a reservoir for further spreading of the virus (Sandvik, 2005). BVD is considered as one of the major diseases with a worldwide economic impact in the cattle industry. For example, the cost of BVDV infection has been estimated about $25 per cow in dairy herd in New Zealand (Reichel et al., 2008), and may exceed $400 per cow when infected with a high virulent strain or co-infected with other pathogens (Pritchard et al., 1989; Carman et al., 1998; Houe, 2003). Sweden is one of the first countries to implement national BVDV-eradication programme since 1993 (Lindberg and Alenius, 1999),

and now the country is almost free of BVDV (Ståhl et al., 2005; Lindberg et al., 2006).

Serological tests, including virus neutralization and ELISA, have been used widely for detection of BVDV antibodies. The recent advance in microsphere-based flow cytometric technology has provided the possibility to develop multiplex diagnostic assays on a single platform. These assays can be designed more sensitive than conventional immunoassays due to the uses of small beads (5 μm) that leads to better reaction kinetics approaching liquid-phase conditions, and of chromophore phycoerythrin, an exceptionally bright reporter dye (Krishhan et al., 2009). The microspheres used in Luminex xMAP technology (Luminex Corp., Austin, TX) are coded with unique combinations of fluorescent dyes, and can be immobilized with capture molecules, e.g. antibody. Immunoassays can be developed in a similar way as ELISA, but signals are detected and processed by Luminex analyzer. By using xMAP technology, a range of diagnostic assays has been developed in the recent years for the improved serological detection of viruses, e.g. respiratory syncytial virus that causes maladies (Jones et al., 2002), human immunodeficiency virus (Faucher et al., 2004), human papillomaviruses (Dias et al., 2005), equine arteritis virus (Go et al., 2008), and avian influenza virus (Watson et al., 2009). The objectives of this study were to develop a blocking microsphere-based immunoassay (bMIA) for detection of antibodies against BVDV, and to compare the performance of the assay with a commercial ELISA kit.

* Corresponding author at: Department of Virology, Immunobiology and Parasitology (VIP), The National Veterinary Institute (SVA), Travvaegen 22, SE-751 89 Uppsala, Sweden. Tel.: +46 18 674689; fax: +46 18 674669.

E-mail address: Lihong.Liu@sva.se (L. Liu).

2. Materials and methods

2.1. Bovine serum samples

A total of 509 serum samples were evaluated in this study. These included 476 clinical samples from Sweden, where only BVDV-1 is present; and 33 samples (including sera against BVDV-2) from Svanova Biotech AB, Uppsala, Sweden.

2.2. Preparation of viral antigen

Bovine Turbinate (TB) cells were maintained in F-DMEM with 10% foetal calf serum, 1% L-glutamine, and 1% antibiotics. Cells at 80% confluence were infected with Oregon C24V (kindly provided by Prof. Martin Beer, Institute of Diagnostic Virology, Friedrich-Loeffler-Institut (FLI), Greifswald-Insel Riems, Germany). At 48 h post-infection, cultures were frozen at −20 °C. After thawing, 0.1% Nonidet P-40 (NP-40) was added to the cell lysate and incubated at 37 °C for 1 h. Following centrifugation at $1000 \times g$ for 10 min, the supernatant was taken as viral antigen.

2.3. Coupling of monoclonal antibody to microspheres

MicroPlex microspheres were purchased from Luminex Corp (Austin, TX). The coupling reaction was performed according to the manufacturer's instructions. Briefly, 5 million microspheres were resuspended in 80 μl of activation buffer (100 mM monobasic sodium phosphate, pH 6.2). The microspheres were activated by 10 μl of 50 mg/ml of N-hydroxysulfosuccinate (Sulfo-NHS, Pierce, Rockford, IL), followed by 10 μl of 50 mg/ml 1-ethyl-3-3-dimethylaminopropyl carbodiimide (EDC, Pierce, Rockford, IL). After incubation at room temperature on an end-over-end rotator for 20 min, the microspheres were washed twice with 250 μl of 50 mM morpholineethanesulfonic acid (MES, pH 5.0) and resuspended in 500 μl of 50 mM MES. Two mAbs WB112 and WB103, which were described previously in blocking ELISA (Paton et al., 1991; Kramps et al., 1999), were added to the activated microspheres, respectively. After a 2-h incubation, the microspheres were washed twice by PBS-TBN (PBS, 0.1% BSA, 0.02% Tween-20, 0.05% Azide, pH7.4) and resuspended in 500 μl of the buffer PBS-TBN and stored at 4 °C in dark. The coupling reaction was confirmed by measuring fluorescent intensity on a Luminex 200 analyzer after incubation of 5000 microspheres with twofold serial dilutions (0.065-4 μg/ml) of R-phycoerythrin conjugated anti-mouse IgG (Sigma-Aldrich Co, St. Louis, MO) for 30 min.

2.4. Biotinylation of mAbs

Biotin labelling reaction was performed according to the manufacturer's instruction (Pierce, Rockford, IL). Briefly, 250 μg of WB112 or WB103 was incubated on ice with 12 μl of 10 mM Sulfo-NHS-LC-Biotin for 2 h. After removal of excess biotin using a desalting column, the biotinylated mAbs were stored at −20 °C until use.

2.5. Development of bMIA

The WB103-conjugated microspheres (250 microspheres/μl), viral antigen, and diluted serum samples were mixed in equal volume (20 μl) and incubated for 30 min on a plate shaker in dark. After addition of 20 μl of the biotinylated mAb WB112 and incubation for another 30 min, 20 μl of 40 μg/ml of R-phycoerythrin-conjugated streptavidin (Prozyme, San Leandro, CA) were added and followed by a further incubation for 30 min. Each sample was tested in duplicate if not indicated specifically. Fluorescence intensity of each reaction was measure on the Luminex 200 analyzer,

and median fluorescence intensity (MFI) was calculated based on the measurement of 100 beads per sample. The results were expressed as inhibition percentage and calculated as the following: $100 \times (\mathrm{MFI}_{\mathrm{negative\ control}} - \mathrm{MFI}_{\mathrm{sample}})/\mathrm{MFI}_{\mathrm{negative\ control}}$.

2.6. Reproducibility of bMIA

The reproducibility of the assay was assessed by determining the level of both intra-assay and inter-assay variations. Intra-assay variation within a plate was calculated as the mean percentage of coefficient of variation (CV%) for 12 samples determined in triplicate. Inter-assay variation was assessed by testing a panel of 31 samples (15 positive sera and 16 negative sera) in three separate runs.

2.7. ELISA procedure

ELISA was performed with a p80 blocking ELISA kit (Svanova Biotech AB, Uppsala, Sweden), according to the manufacturer's instruction. The kit had a sensitivity of 91.6% and specificity of 98.2% compared with virus neutralization test (Svanova Biotech AB, Uppsala, Sweden).

2.8. Statistical analysis

Receiver operating characteristics (ROC) curve was generated to assess the diagnostic performance of the bMIA. An ROC curve is a plot of a test's true positive fractions (TPF) versus false-positive fractions (FPF) for each possible cut-off value of the test and could achieve the best relationship between diagnostic sensitivity and specificity (Detilleux et al., 1999; Greiner and Gardner, 2000). Sensitivity and specificity were calculated as the following formula: sensitivity = [true positives/(true positives + false negatives)] × 100; specificity = true negatives/(true negatives + false positives) × 100. The analysis was performed by software Medcalc (MedCalc Software, Mariakerke, Belgium).

3. Results

3.1. Selection of coupling mAbs to microsphere

The coupling efficiency of each mAb to microspheres was compared at different amount of mAbs in coupling reaction with dilutions of a phycoerythrin-labelled anti-mouse IgG (PE-IgG). As shown in Fig. 1, both WB112 and WB103 had a very low MFI value when 1 μg of mAb was used in coupling reaction. At 4 μg/ml of PE-IgG, 5 μg of WB112 gave an MFI value of 5000, whereas WB103 gave an MFI value of 14 000. This indicated that, for the same amount of mAb, WB103 had a higher coupling efficiency than WB112. Further increasing WB103 from 5 μg to 25 μg had less effect on the MFI values. Therefore, WB103 (10 μg) was selected finally as the capture antibody for coupling to microspheres, and WB112 was used as detection antibody in this study.

### 3.2. Optimization of bMIA

Two key factors in the bMIA were determined in a sandwich immunoassay: optimal serum dilution factor and the amount of detection antibody. A twofold dilution of serum sample gave the maximum difference in MFI values between positive and negative sera (Fig. 2). Titration of biotinylated detection antibody (WB112-bio) showed that the MFI values reached the plateau at about 7 μg/ml of WB112-bio (Fig. 3). To maximize the sensitivity of the assay, 5 μg/ml of WB112 were used in the assay, which corresponds to 70% of the maximum plateau MFI value.

Table 1
Diagnostic performance of the bMIA compared with an ELISA by testing 509 samples.

bMIA	ELISA		Total
	Positive	Negative	
Positive	154	6	160
Negative	1	348	349
Total	155	354	509

### 3.3. Comparison of bMIA and ELISA

Sensitivity and specificity of the bMIA were compared with a commercial blocking ELISA. A total of 509 samples were tested in parallel by the two assays, and the results are presented in Table 1. Based on the nonparametric ROC analysis of all 509 samples, the cut-off value (percentage of inhibition) of the bMIA was determined as 30.3%. Under this cut-off value, the bMIA classified 160 samples as positive (including 26 BVDV-2 serum samples) and 349 samples as negative (Table 1). Comparing to blocking ELISA, the bMIA had a sensitivity of 99.4% and a specificity of 98.3%.
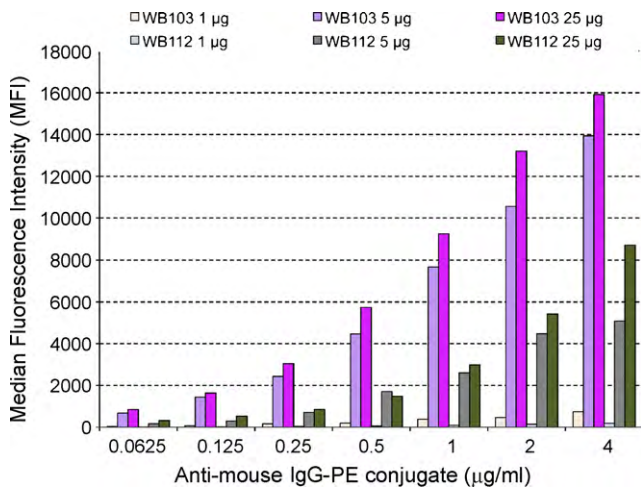
Fig. 1. Selection of capture antibody according to its coupling efficiency. Two mAbs Wb103 and WB112 at indicated amounts were coupled to 5 million microspheres in a 500-μl reaction volume, respectively. The median florescence intensity (MFI) was determined at various dilutions of anti-mouse IgG-PE conjugate.
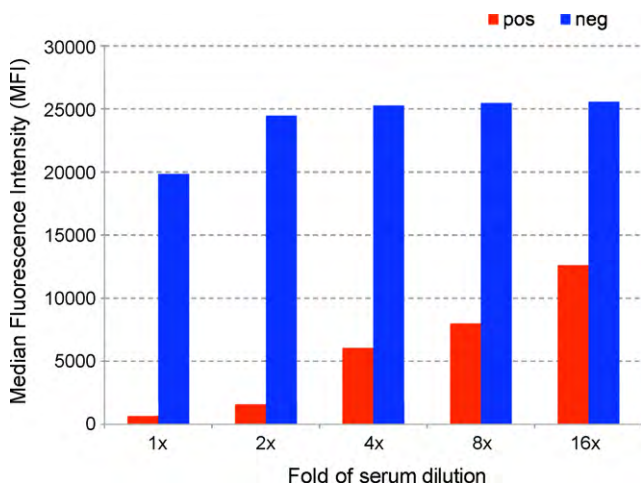
Fig. 2. Determination of serum dilution factor according to mean MFI values of four positive and four negative sera.
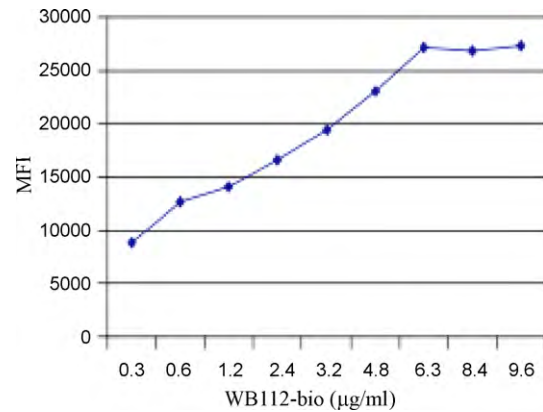
Fig. 3. MFI values at various concentrations of biotinylated mAb WB112 (WB112-bio).

3.4. Reproducibility

The reproducibility of the bMIA was evaluated by intra-and inter-assay variability, and variations in different preparations of conjugated microspheres. The mean CV% of 12 samples tested in triplicate in one plate was 4.9%. Inter-assay variation was determined by testing 31 samples in three separate runs. The mean CV% was below 7%. The reproducibility of the different preparations of conjugated microspheres was also investigated. The percentage of coefficient of variation (%CV) within three preparations of beads was less than 6.6%.

4. Discussion

This study describes the development and evaluation of a microsphere-based immunoassay for rapid and sensitive detection of bovine viral diarrhoea virus antibodies. The diagnostic performance of the new bMIA was compared to that of a commercial blocking ELISA system, by testing a large panel of 509 bovine sera. In general, the two assays worked consistently for detection of BVDV antibodies in 502 samples. Six samples were positive in bMIA but negative in ELISA. These samples had a percentage of inhibition value between 5% and 42% in blocking ELISA. One reason for this discrepancy could be that these sera blocked binding of the viral antigen to the detection antibody in the bMIA, leading to false-positive results. But more likely it was due to a slight higher sensitivity of the MIA than ELISA. Only one sample was negative by the bMIA but positive by ELISA with a percentage of inhibition value of 47.5% (just above the cut-off value of 45%). The observed discrepancies are in line with another comparison of ELISA with a Luminex assay for detection of human papillomavirus 16: six out of 215 samples reacted differently in the two assays (Faust et al., in press).

The bMIA utilises two mAbs against highly conserved NS3 protein of BVDV. As demonstrated, the assay is capable of detection antibodies against both BVDV-1 and BVDV-2. It would be very interesting to detect antibodies, when available, against novel pestiviruses Th/04_KhonKaen (Liu et al., 2009a) and SVA/cont-08 (Liu et al., 2009b), which have been proposed as a new species BVDV-3 (Liu et al., 2009c). Another direction would be to extend the assay for detection of antibodies against other viruses, e.g. bovine herpesvirus type 1, bovine respiratory syncytial virus, bovine coronavirus, bovine parainfluenza-3 virus, thus allowing a robust diagnosis of bovine respiratory diseases in a multiplex format. The power of a Luminex assay lies in its multiplex capacity. Khan et al. (2006) reported a multiplex microbead immunoassay for simultaneous detection of antibodies against six nonhuman-primate

viruses. Multiplexing can bring down the high cost of the bMIA dramatically by reducing the number of ELISA kit for each pathogen, labour input and hands-on time. This will, in turn increase the applicability of the assay.

In summary, the bMIA described above is an alternative to ELISA for detection of antibodies against BVDV. The assay is reliable and sensitive, providing a powerful tool for the BVD surveillance programmes. In addition, this assay can be extended to a multiplex format allowing a robust, high-throughput diagnosis of bovine respiratory diseases.

Acknowledgements

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