Journal of Virological Methods 198 (2014) 56–63

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Rapid detection of neutralizing antibodies against bovine viral diarrhoea virus using quantitative high-content screening

Michael Eschbaumer*, Sampson Law, Cristina Solis, Adam Chernick, Frank van der Meer, Markus Czub

Faculty of Veterinary Medicine, University of Calgary, 3280 Hospital Drive NW, Calgary, AB T2N 4N1, Canada

Article history:
Received 19 September 2013
Received in revised form
26 November 2013
Accepted 17 December 2013
Available online 2 January 2014

Keywords:
BVDV
Neutralization assay
IN Cell Analyzer
High-throughput
Dose–response curves
Nonlinear regression

A B S T R A C T

Bovine viral diarrhoea virus (BVDV) is an important cause of morbidity, mortality and economic losses in cattle worldwide. Humoral immunity to BVDV plays a major role in the protection against infection and disease. In vitro serum neutralization tests can quantify humoral responses, but standard protocols are time-consuming and labour-intensive. The objective of this study was to develop a highly sensitive assay based on high-content cell-by-cell screening that is faster and less subjective than the conventional protocols. It can detect a neutralizing antibody response within the first week after infection of an animal, takes less than 24 h to complete and excludes operator bias by automated data acquisition and analysis.

1. Introduction

Bovine viral diarrhoea virus (BVDV) is an important cause of morbidity, mortality and economic losses in dairy and beef cattle worldwide (Maclachlan and Dubovi, 2010). BVDV is an umbrella term for two distinct virus species, BVDV1 and BVDV2, within the family *Flaviviridae*, genus *Pestivirus* (Ridpath, 2008). Both species are divided further into several subgroups based on nucleotide sequence similarities (Flores et al., 2002; Vilček et al., 2004). With reference to their behaviour in cell culture, BVDV strains are separated into cytopathic (cp) and non-cytopathic (ncp) biotypes (Ridpath, 2008). Independent of the biotype or strain, acute BVDV infection can lead to enteric, respiratory or reproductive disease and is accompanied by immune suppression and secondary infections. Ncp strains can also establish persistent infections with immune tolerance in unborn calves. In persistently infected (PI) animals, ncp BVDV can mutate into antigenically identical cp BVDV and cause fatal mucosal disease (Ridpath, 2008).

Humoral immunity plays a major role in protection against BVDV. Presence of neutralizing antibodies in blood correlates with protection afforded by BVDV vaccination (Saliki and Dubovi, 2004). This protection can be conferred to immunologically naïve animals through passive transfer of maternal antibodies (Howard et al., 1989; Shope et al., 1976). The main target of neutralizing antibodies (NA) is the highly immunogenic BVDV glycoprotein E2, which shows considerable variation among strains (Deregt et al., 1998).

Serum NA titres are a useful indicator of vaccine efficacy (Saliki and Dubovi, 2004). Neutralization assays provide a means of assessing this protective response in an in vitro test (Onions, 1983). The Manual of Diagnostic Tests and Vaccines of the World Organisation for Animal Health (OIE) sets the standard for a BVDV microneutralization test (OIE, 2012), and similar protocols have been in use for decades (Gillespie et al., 1961). The test is straightforward: a known quantity of virus is incubated with a serial dilution of the diagnostic specimen and then brought in contact with susceptible indicator cells. If virus replication causes visible damage to the cells, the test can be read directly; otherwise, viral antigen in infected cells must be visualized by a specific immune stain (OIE, 2012).

Abbreviations: BVDV, bovine viral diarrhoea virus; CCD, charge-coupled device; cp, cytopathic; DAPI, 4′,6-diamidino-2-phenylindole; ELISA, enzyme-linked immunosorbent assay; FDA, Food and Drug Administration; FITC, fluorescein isothiocyanate; HCS, high-content screening; ID, infectious dose; mAb, monoclonal antibody; MDBK, Madin-Darby bovine kidney; NA, neutralizing antibody-bodies; ncp, non-cytopathic; ND, neutralizing dose; OIE, World Organisation for Animal Health; PBS, phosphate-buffered saline; PI, persistently infected; RT-PC, Rreverse transcription polymerase chain reaction; SD, standard deviation; TCID, tissue culture infectious dose.

* Corresponding author. Present address: Foreign Animal Disease Research Unit, USDA/ARS Plum Island Animal Disease Center, P.O. Box 848, Greenport NY 11944, United States. Tel.: +1 631 323 3368.

E-mail addresses: meschbau@ucalgary.ca, michael.eschbaumer@ars.usda.gov (M. Eschbaumer).

http://dx.doi.org/10.1016/j.jviromet.2013.12.017
0166-0934/

Commonly, reading a serum neutralization test requires manual microscopic examination, which is time-consuming and labour-intensive (Huang et al., 2010). Manual test evaluation based on visual readout is subjective, requires considerable skill and puts practical limits on the number of events that can be counted per sample (Abai et al., 2007; Johnson et al., 2008). Another drawback of conventional protocols is the long turnaround time. It often takes a week from the arrival of a sample in the laboratory until the test results are available. This is due largely to the long incubation following the addition of the indicator cells – for example, the OIE protocol recommends 4–5 days (OIE, 2012).

The objective of this study was to develop an improved BVDV serum neutralization test using a high-content screening (HCS) system, where that incubation time is reduced drastically and manual test evaluation is replaced by computerized image analysis and data processing.

2. Materials and methods

2.1. Serum samples

Fifteen 6-month-old BVDV-free beef calves were inoculated intranasally with 1.6×10^7 median tissue culture infectious doses ($TCID_{50}$) of BVDV2 subgenotype 2a strain "1373", which had been isolated from an outbreak in Ontario, Canada, in the 1990s (Carman et al., 1998; Stoffregen et al., 2000). The initial BVDV antigen- and antibody-negative status of all calves as well as the success of the infection was confirmed by real-time quantitative RT-PCR (VetMAX™-Gold BVDV Detection Kit, catalogue no. 4413938, life technologies, Carlsbad, CA, USA) and ELISA (BVDV Total Ab Test, 99-44000, IDEXX, Westbrook, ME, USA) (data not shown). The calves originated from a closed herd that has been free of BVDV and bovine herpesvirus for generations. Ten other animals (calves N1–N10) from the same herd were used as negative controls.

For five infected calves (calves 1–5), blood samples were collected 7 days before (day −7) as well as 1, 3, 5, 7, 9, 11, 14 and 30 days after infection. The other ten infected calves (calves 6–15) were bled on days −7, 7, 9 and 14 only. All samples from all infected calves were tested in the HCS assay, but only samples taken on days −7, 7, 9 and 14 were tested with the standard OIE protocol. The ten negative control animals were bled once and tested in both assays. All animal procedures were approved by the University of Calgary's Animal Care and Use Committee (permit no. AC12-143).

For further validation, six field samples (from farms in Alberta, Canada; permit no. SHC10R-16) that had been positive in the BVDV antibody ELISA (BVDV Total Ab Test, IDEXX; data not shown) were also tested in both neutralization assays. All sera were inactivated by incubation at 56 °C for 1 h and stored at −20 °C until testing.

2.2. Virus stocks and titrations

For the in-vitro tests, BVDV2a 1373 was grown in Madin-Darby bovine kidney (MDBK) cells (CCL-22, American Type Culture Collection, Manassas, VA, USA), then aliquoted and stored at −80 °C. The infectivity of the frozen virus stock was determined before use in the neutralization tests. Different dose finding methods were used for the standard OIE protocol and the HCS assay and are described in detail later on. Briefly, serial ten-fold dilutions of the virus stock were prepared in triplicate on 96-well tissue culture plates (353075, BD Biosciences, Mississauga, ON, Canada), MDBK cells were added and the plates were incubated for 96 h (standard OIE protocol) or 16 h (HCS assay). For the standard OIE protocol, the median tissue culture infectious dose ($TCID_{50/96h}$) was estimated with the Spearman–Kaerber formula (Spearman, 1908; Kärber, 1931), corresponding to the reciprocal of the virus dilution that leads to infection in 50% of replicate wells after a 96-h incubation. For the HCS assay, the 90% infectious dose ($ID_{90/16h}$), i.e. the reciprocal of the virus dilution that leads to 90% infected cells per well at 16 h, was estimated by nonlinear regression (see Section 2.6).

2.3. Serum neutralization tests

All neutralization tests were performed in triplicate on 96-well tissue culture plates. The plate layout was identical for the standard OIE protocol and the HCS assay; they differed only in the amounts of virus and cells that were used.

Serial fourfold dilutions of the serum samples were made in tissue culture media (Dulbecco's modified Eagle medium supplemented with 5% horse serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B; 11995, 16050-130, 15240-062, life technologies). From an initial dilution of 1/2 (0.5×4^0), the sera were diluted in seven steps to 1/32768 (0.5×4^{-7}), in a final volume of 75 μL. An identical volume of BVDV2 1373 diluted from the original stock was added to each serum dilution. Each neutralization plate also contained 12 wells with virus but without serum (no-serum controls) and 12 wells with neither virus nor serum (no-virus controls).

The virus dose added to the serum dilutions differed between the standard OIE protocol and the HCS assay. As suggested in the OIE manual, the standard protocol used 100 $TCID_{50/96h}$, estimated from a virus titration that had been incubated for 96 h. The HCS assay, on the other hand, used a higher dose, 1 $ID_{90/16h}$, estimated from a 16-h titration, corresponding to the shorter incubation time in the assay itself. After serum and virus had been mixed, the plates were left at 37 °C for 1 h before cells were added. In the standard OIE protocol, 1×10^4 cells per well were added and the plates were incubated for another 96 h. The HCS assay used 4×10^4 cells per well and a 16-h incubation.

Sixteen or 96 h after the cells had been added, the plates were washed once with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) and fixed for 30 min at room temperature in 4% (w/v) paraformaldehyde (158127, Sigma–Aldrich, Oakville, ON, Canada) in PBS. After another wash with PBS, all wells were filled with 100 μL PBS and the plates were stored at 4 °C until staining.

2.4. Immunostaining

Fixed cells were incubated for 1 h at 37 °C with a monoclonal antibody (mAb) against the viral E2 glycoprotein (348, VMRD, Pullman, WA, USA) (Deregt et al., 1998), diluted 1/1000 in PBS with 0.1% (w/v) saponin (47036, Sigma–Aldrich) and 0.1% (w/v) bovine serum albumin (A7906, Sigma–Aldrich). This was followed by incubation with a secondary antibody conjugated to a fluorescent dye (Alexa Fluor® 488 goat anti-mouse IgG; A11019, life technologies) under the same conditions. Cells were washed three times with PBS with 0.1% (v/v) Polysorbate 20 (TWEEN® 20; P1379, Sigma–Aldrich) between steps. Finally, cell nuclei were stained with 600 nM 4′,6-diamidino-2-phenylindole (DAPI; D3571, life technologies) in PBS for 5 min at room temperature in the dark. The DAPI overlay was removed, wells were filled with 100 μL PBS and plates were stored at 4 °C until image acquisition.

2.5. Image acquisition and analysis

The plates were read by an IN Cell Analyzer 2000 automated microscope (GE Healthcare, Little Chalfont, United Kingdom). Using a high-performance CCD camera (1040 × 1392 pixels, 6.4 μm × 6.4 μm pixel size) and a Nikon 4×/0.20 plan apochromat objective, one centred field of view covering 12.5% of the total