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Comparison of the performance of five different immunoassays to detect specific antibodies against emerging atypical bovine pestivirus

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A B S T R A C T

Bovine pestiviruses represent a considerably variable group. In addition to the two accepted species BVDV-1 and BVDV-2, a number of atypical bovine pestiviruses have been detected both in foetal calf sera and in field samples. The sera collected during the initial six weeks of experimental infection of calves with atypical pestivirus, BVDV-1 and a combination of both viruses have been examined by routine and new diagnostic tests to validate their robustness and sensitivity. As expected, virus neutralization tests using homologous virus were able to differentiate the two groups infected by BVDV-1 or atypical pestivirus, whereas the animals inoculated with a mixture of these two viruses had a reaction pattern very similar to the homologous virus alone. It was found that immunoassays using whole virus and polyclonal antibodies are the most robust, but all tests examined were able to detect antibodies also from cattle infected with atypical pestivirus a few weeks after infection. The detection, however, was at a lower level and slightly delayed. Statistical validation of the threshold suggested by the manufacturer showed that in some cases the reduction of the cut-off values would improve the test sensitivity.

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1. Introduction

Atypical bovine pestiviruses are a group of viruses which together with two bovine viral diarrhoea virus species (BVDV-1 and BVDV-2), border disease virus (BDV) and classical swine fever virus (CSFV) form the *Pestivirus* genus of *Flaviviridae* family. The group was referred to previously as Hobi-like pestiviruses from the precursor strain isolated from Brazilian foetal bovine serum (FBS) (Schirrmeier et al., 2004). Phylogenetic and antigenic relationship of atypical bovine pestiviruses with BVDV and increasing evidence of further intercontinental spread of the viruses among cattle (Decaro et al., 2011; Xia et al., 2011) raised a discussion over the emergence of a new BVDV species (Liu et al., 2009). Detection of atypical pestiviruses requires introduction of new testing protocols (Liu et al., 2008), since RT-PCR assays used commonly (Vilček et al., 1994) fail to detect them (Stahl et al., 2009). Information on the use of serological tests designed to detect BVDV-1 and BVDV-2 for the detection of those atypical pestiviruses is limited (Decaro et al., 2012; Kampa et al., 2007; Larska et al., 2012; Schirrmeier et al., 2004). Virus neutralization test and enzyme-linked immunosorbent assay (ELISA) (Schrijver and Kramps, 1998) are used most commonly for routine serological diagnosis of BVDV. The presence of specific antibodies is considered a good indicator of natural infection in tested herds, both on the individual-level (serum or milk sample) and herd-level (bulk milk), or for estimation of the protective immunity after vaccination. The envelope glycoprotein E2 is considered the main neutralizing antigen which is relatively conserved provides cross-neutralization between all pestiviruses (Schirrmeier et al., 2004). Other viral proteins, such as: the envelope RNase, E^{rns} and non-structural protein NS3 (previously known as p80/p125) are also used as targets for pestivirus specific antibody responses. Virus neutralization allows quantitation of the antibodies and is considered as the ‘gold standard’ method, however it requires well-equipped facilities, contamination control and skilled personnel. Commercial ELISAs provide a good alternative for virus neutralization test, and are chosen more frequently as they allow fast and specific screening of large numbers of samples. Modern technologies, such as microsphere immunoassay (MIA), are introduced in BVDV diagnosis in response to a demand for rapid and efficient tests (Xia et al., 2010).

The aim of this study was to evaluate the ability of current diagnostic methods to detect antibodies against atypical bovine pestiviruses. A panel of sera collected from naive calves infected experimentally with an atypical bovine pestivirus, BVDV-1 and the

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mixture of BVDV-1 and atypical pestivirus were used. Increasing risk of the spread of atypical BVDVs prompts the search for the efficient tools supporting testing and control programs.

2. Materials and methods

2.1. Experimental design

Fifteen healthy calves, negative for the presence of BVDV and specific antibodies were divided into three groups of five animals each, and were inoculated with the same dose of atypical bovine pestivirus strain Th/04_KhonKaen (Group A); BVDV-1a strain Horton 916 (Ho916) (Group B); and a mixture of Ho916 and Th/04_KhonKaen (Group C). The procedure has been previously described in detail (Larska et al., 2012). A fourth group of 5 calves (control group) was mock-inoculated. Blood samples were collected from all animals before the inoculation and at 7, 14, 21, 28, 35 and 42 day post-inoculation (dpi). The animals were handled according to the protocol approved by the Ethics Committee for Animal Experiments by the Ministry of Science and Higher Education in Poland (No. 79/2010).

2.2. Virus neutralization test

To detect and quantify neutralizing antibodies against homo- and heterologous BVDV strains, all sera were tested against BVDV-1 strain DK258 (used as the reference strain in Danish BVDV control scheme) in Madin–Darby bovine kidney (MDBK) cells (Uttenthal et al., 2005) and Th/04_KhonKaen strain in bovine turbinate (BT) cells. The test was performed according to the protocol described previously (Larska et al., 2012). The neutralizing titres were calculated using the Spearman–Kärber method. The inhibitory effect of serum dilution above 1:10 was regarded as positive and as an indicator of seroconversion.

2.3. Indirect enzyme-linked immunosorbent assays (ELISAs)

2.3.1. i-ELISA-1

The Svanovir BVDV-Ab ELISA kit (Svanova Biotech, Uppsala, Sweden) is designed for detection of BVDV specific antibodies (IgG_1) in bovine serum or milk samples. The sensitivity and specificity relative to virus neutralization test provided by the manufacturer is 100% and 98.2%, respectively. The assay was performed according to the manufacturer's protocol with 1-h incubation of the serum under test in the coated plate. Prior to the test, serum samples were diluted 1:25. The results were calculated first by subtracting optical density (OD) value of the reference control sample from OD value of the tested sample (corrected OD; COD) and presented as percentage positivity (PP) by dividing the sample COD value by positive reference sample COD value. The cut-off value was set at 15 PP as recommended by the manufacturer of the test.

2.3.2. i-ELISA-2

The HerdChek BVDV Antibody Test Kit (IDEXX, Liebefeld-Bern, Switzerland) was the second indirect ELISA-based assay evaluated. The assay is characterized by high specificity and high sensitivity for detecting anti-BVDV-1 and anti-BVDV-2 antibodies. The ELISA was performed according to the manufacturer's protocol using 1:5 serum dilution and 1.5 h-incubation time of serum with antigen-coated plate. The results were expressed as corrected optical density (COD) values calculated by subtracting COD of the test positive control from the COD value of the sample tested. The COD $\geq$ 0.3 was interpreted as positive.

2.4. Blocking ELISAs

2.4.1. b-ELISA-1

The Svanovir BVDV p80-Ab ELISA (Svanova Biotech, Uppsala, Sweden) is a multispecies (bovine, ovine and caprine) blocking assay which consists of microplates coated with BVD NS3 (p80) protein and a peroxidase labelled specific to NS3 monoclonal antibody. According to the manufacturer the test detects antibodies against BVDV p80 (NS3) protein of BVDV-1, BVDV-2 and atypical pestivirus in bovine, caprine and ovine samples. The manufacturer's protocol was followed. The serum samples were diluted 1:10 in phosphate buffered saline (PBS) and incubated for 1 h in the antigen-coated plate. The results are shown as percent inhibition (PI) and the 45 PI was implemented as the cut-off value as recommended by the kit manufacturer.

2.4.2. b-ELISA-2

A liquid phase blocking ELISA for BVDV was performed (Rønsholt et al., 1997). The test is based on: (1) swine anti-BVDV capture IgG produced by infection of pigs initially with BVDV-1 and inoculated subsequently with CSFV Brescia; (2) rabbit-anti-BVDV produced by immunization of rabbits with a BVDV antigen adapted to primary rabbit cells; (3) crude BVDV-1 antigen produced in cell cultures. Briefly, the plates were coated by swine anti-BVDV capture IgG and blocked. The bovine serum sample was added shortly before the addition of an equal volume of BVDV antigen and incubated overnight. The next day plates were washed and bound BVDV antigen was detected by rabbit anti-BVDV and swine anti-rabbit antibodies. Negative and positive serum control wells were used to calculate the percentage inhibition (PI) and values below 50 PI were considered as positive. The sensitivity of the test was 96–97%, whereas the specificity was 94–98% compared to the virus neutralization test, according to the manufacturer.

2.5. Microsphere immunoassay (MIA)

A microsphere immunoassay developed recently using recombinant E^{rns} protein of Th/04_KhonKaen strain as the antigen was used for detection of antibodies (Vijayaraghavan et al., 2012). Briefly, five-fold diluted serum samples were incubated with the E^{rns} antigen-coupled-microspheres at 37 °C for 30 min on a plate shaker. After washing, a biotinylated anti-bovine IgG (Jackson ImmunoResearch, West Grove, USA) was added and incubated for another 30 min. After subsequent washing, a streptavidin-R-phycoerythrin conjugate (ProZyme, Hayward, Canada) was added and incubated at 37 °C for 30 min on a plate shaker. After washing twice, the microspheres were resuspended in 100 μl of PBS with 1% BSA and analyzed by a Luminex 200 analyzer. The median fluorescence intensity (MFI) was calculated based on the measurement of 100 beads per sample. The cut-off value of the test was calculated using Receiver operating curve analysis (ROC) with the Medcalc software (MedCalc Software, Mariakerke, Belgium).

2.6. Statistical analysis

All statistical analyses were performed using STATA software version 11 (StataCorp, College Station, Texas, USA). Statistical significance between animal groups within each immunoassay result sets was analyzed using ANOVA followed by Tukey's HSD (Honestly Significant Difference) and Dunnett's test. The results from individual sampling days were analyzed as repeated measures. Tukey's test was used to calculate a critical value to evaluate the significance of the difference between two pairs of means, which was then compared to the Tukey critical value, while Dunnett's test compared inoculated groups with the control

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