

Comparison of Four Diagnostic Techniques for Detecting Bovine Virus Diarrhoea Virus (BVDV) in Buffy Coat Samples after Long-Term Storage

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Received: 19.04.2001

Abstract: In this paper, four diagnostic techniques were compared in terms of their ability to detect BVD virus in buffy coats frozen for more than one year. The samples were selected according to their initial test results. Forty-five BVDV positive and 15 BVDV negative buffy coats were used. The techniques used were the virus isolation-immunoperoxidase test (IPX), immunoperoxidase monolayer assay (IPMA), indirect immunoperoxidase monolayer assay (IIPMA) and reverse transcription-polymerase chain reaction (RT-PCR). All BVDV negative samples from previous studies were also negative on the tests used in this study. Out of 45 BVDV-positive samples, 42 (93.3%) were found to be positive with RT-PCR, 41 (91.1%) with virus isolation-IPX, 40 (88.8%) with IIPMA and 37 (82.2%) with IPMA. The results obtained indicated that RT-PCR is more suitable for the detection of BVD virus in frozen buffy coats stored for more than one year when compared to other techniques, and IIPMA can be used for the routine diagnosis of BVDV infection as an alternative technique to virus isolation-IPX.

Key Words: BVDV, diagnosis, immunoperoxidase, RT-PCR

Uzun Süreli Saklanan Kan Örneklerinde Bovine Virus Diarrhea Virus (BVDV) Tespitinde Kullanılan Dört Tekniğin Karşılaştırılması

Özet: Bu çalışmada, en az bir yıl süre ile dondurulan kan örneklerinde BVD virusunun tespiti amacıyla kullanılabilecek 4 adet teknik karşılaştırıldı. Numuneler daha önce elde edilen test sonuçları doğrultusunda seçildi. Çalışmada 45 adet BVDV pozitif ve 15 adet BVDV negatif kan örneği olmak üzere toplam 60 örnek test edildi. Araştırmada virus izolasyon-immunoperoxidaz testi (IPX), immunoperoxidaz monolayer testi (IPMA), indirekt immunoperoxidaz monolayer testi (IIPMA) ve reverz transkriptaz-polimeraz zincir reaksiyonu (RT-PZR) kullanıldı. Daha önce BVDV negatif olarak tanısı konan tüm örnekler bu çalışmada kullanılan tekniklerin tamamı ile negatif olarak tespit edildi. 45 BVDV-pozitif kan örneğinin 42 adedi (% 93,3) RT-PZR, 41 tanesi (% 91,1) virus izolasyon-IPX, 40 adedi (% 88,8) IIPMA ve 37 adedi (% 82,2) ise IPMA tekniği ile pozitif olarak bulundu. Bu çalışmadan elde edilen bulgular, bir yıldan uzun süre ile donuk olarak saklanan kan örneklerindeki BVDV virusunun tespiti için diğer tekniklere oranla RT-PZR tekniğinin daha duyarlı olduğunu ve IIPMA tekniğinin BVDV enfeksiyonunun rutin tanısı amacıyla virus izolasyon-IPX tekniğine bir alternatif olarak kullanılabileceğini ortaya koydu.

Anahtar Sözcükler: BVDV, tanı, immunoperoxidaz, RT-PZR

Introduction

Bovine Virus Diarrhoea Virus (BVDV) as an important member of the Pestivirus genus is a very common agent affecting livestock production throughout the world. Serological studies have shown that the presence of antibodies to BVDV in cattle is 60-90% (1-4). Economic losses are directly related to multiple clinical forms of the infection that vary from subtle enteric infection to fatal mucosal disease caused by a combination of cytopathic (cp) and non-cytopathic (ncp) biotypes of the virus (5-7). The other consequence with epidemiological importance

is the birth of persistently infected (PI) calves. PI calves are born into herds as a result of intrauterine infection with ncp biotype at early gestation. PI animals serve as a virus source by shedding the virus life along with various body excretions and are therefore responsible for the presence of the virus within herds (8). For epidemiological purposes, it is important to detect the viremic status of animals in addition to their serological status. As a general approach for distinguishing PI animals from those infected transiently (acute), the most common way is to repeatedly isolate the virus in at least

two-week intervals from the suspected animal (9). Although numerous techniques have been reported in order to diagnose the viremic status of animals (10-14), the cultural inoculation of buffy coat samples is considered the most preferred technique for this purpose by many diagnostic laboratories (15). However, the efficacy of cultural isolation and subsequent identification steps need to be proved, particularly in retrospective research, for the materials collected for various purposes and stored over long periods.

In this study, the reliability of three test systems, namely the immunoperoxidase monolayer assay (IPMA), indirect immunoperoxidase monolayer assay (IIPMA) and reverse transcription-polymerase chain reaction (RT-PCR) to the conventional virus isolation-immunoperoxidase test (IPX), were compared in the detection of bovine pestivirus in buffy coat samples frozen for more than one year.

Materials and Methods

Cell Line, Virus and Monoclonal Antibodies (MAbs)

BVDV-free Madin Darby Bovine Kidney (MDBK) was used. Cells were grown in Dullbecco's Modified Essential Medium (DMEM) supplemented with 5% Fetal Calf Serum. A non-cytopathic German isolate (0712/80/Hannover) of BVDV was used as a reference virus, and it was serially passaged and plaque purified in MDBK cells. The Institute for Virology, Hannover Veterinary School, Germany, kindly supplied MAbs C16 (p80/125 specific) and CA34 (gp53 specific), which were used in this research. Equal volumes of both MoAbs were mixed before the tests and were used in IIPMA.

Test Materials

A total of 60 frozen buffy coat samples were selected from archive samples submitted to the laboratory between May 1997 and July 1999 and were stored at -20°C for further investigations. The selection of test materials was performed according to their initial test results. Forty-five of them were previously recorded as being positive for BVD virus presence by conventional virus isolation-IPX test. The remaining buffy coat samples ($n = 15$) were used in the research as BVDV-free negative controls.

Isolation of BVD Virus (Isolation –IPX)

After seeding culture tubes with MDBK cell culture, they were inoculated with the test materials. The culture medium was changed at 24 h postinoculation and cells were maintained for a further 4 days to obtain efficient viral growth. At the end of the period, the culture drums were frozen and thawed twice and culture fluids were subsequently inoculated onto MDBK cells grown in 24-well plates. Isolations were proved by immunoperoxidase (IPX) staining as described in the section "IPMA".

Infecting Cell Monolayers

The 24-well plates were seeded with MDBK cells at a concentration of 75,000 cell/ml and were incubated for 24 h in CO_2 incubator. After cells reached confluence, they were directly inoculated with buffy coat samples in duplicate in two separated plates. The medium was changed 24 h postinoculation and the cells were maintained for a further 2 days under the same conditions. At 72 h postinfection, culture medium was collected for future use and the cell surface was washed several times with PBS. The cells were then fixed by heat at 80°C for 4 h.

Immunoperoxidase Monolayer Assay (IPMA)

The first series of cells fixed were incubated with HRPO-conjugated polyclonal pig anti-BVDV antibody for 1 h at 37°C . After the removal of excess conjugate, the cells were intensively washed with PBS containing Tween 20 several times. Intracytoplasmic viral accumulation was seen as brown-reddish aggregates by adding dimethylformamide (Sigma, Germany) and aminoethylcarbasole (Sigma, Germany) as a colorogenic substrate. The test was evaluated by checking the plate under a cell culture microscope.

Indirect Immunoperoxidase Monolayer Assay (IIPMA)

After fixing the infected cells, the second series of cells were incubated with pooled MAbs (C16 and CA34) for 1 h in a humidified chamber. Biotinylated anti-mouse antibody (from sheep, Cat. Nr. RPN1001, Amersham, UK) was added as a second antibody to the test and the system was incubated for a further 1 h under the same conditions. After incubation with peroxidase labeled streptavidin-biotin complex (Amersham, UK), the test was visualized by adding colorogenic substrate as mentioned in section "IPMA". The test was evaluated by checking reddish-brown colored intracellular aggregates under a cell culture microscope.

Detection of BVD Virus RNA Using RT-PCR

The method for the detection of BVD virus RNA was a modification of the method as described by Greiser-Wilke et al. (16). BVD virus RNA was extracted using an acid guanidium-phenol-chloroform-isoamyl alcohol mixture as described by Chomczynski and Sacchi (17). An RNA pellet was dissolved in 20 ml sterile DEPC-treated water and was used for cDNA synthesis. Complementary DNA strand synthesis was performed in a total volume of 20 ml containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM DTT, 3 mM MgCl, 0.5 mM each dNTP (MBI, Fermentas, Lithuania), 40 U RNase inhibitor (MBI, Fermentas, Lithuania), random hexamere (50 ng/ml) (MBI, Fermentas, Lithuania), and 200 U M-MLV reverse transcriptase (MBI, Fermentas, Lithuania). The reaction mixture was incubated first at 37 °C for 60 min for synthesis of cDNA and then at 70 °C for 10 min for inactivation of MMLV-RT.

PCR was carried out with routine protocol in a mixture of 50 µl volume consisting of 50 mM Tris-HCl (pH 8.3), 30 mM KCl, 2 mM MgCl, 0.2 mM of each dNTP (MBI, Fermentas, Lithuania), 15 pmol each primer, and 2 U Taq DNA Pol I (MBI, Fermentas, Lithuania). The primer set used in PCR was as follows:

Primer-1 (Forward) --- 5'-GCA GAT TTT GAA GAA AGA CAC TA- 3' (Position 4937-4960)

Primer-2 (Reverse) --- 5'-TTG GTG TGT GTA AGC CCA- 3' (Position 5339-5321)

Initial denaturation was performed at 94 °C for 6 min. The mixtures were then cycled 35 times at 94 °C for 45 s, 55 °C for 1 min and 72 °C for 1 min. The final 72 °C step was prolonged for 10 min. The PCR products were analyzed by electrophoresis after ethidium bromide staining.

Results

In immunocytochemical diagnosis, while IPMA detected BVDV in 37 (82.2%) buffy coat samples, IIPMA detected three more BVD viruses (40 of isolates) among the same samples (Table). The staining of positive samples with indirect IPMA revealed more intense, highly efficient and lower background staining when compared to IPMA. In the IPMA test, although there was no specific cellular staining in some wells (e.g. C1 and D1 in panel A, Figure 1), background staining caused false evaluation by the naked eye while there was one stained plaque in C1 and no staining in D1 of panel B with the same samples (e.g. panel B, Figure 1). Moreover, immunostaining with HRPO conjugated pig anti-BVDV Ig's (IPMA test) revealed more faded results in comparison to the IIPMA test. The difference in the quality of staining can be seen in disseminated (e.g. C5D5, Figure 1) or restricted (e.g. C4D4 or A6B6, Figure 1) infected areas in both staining techniques.

During the cultural incubation none of the samples produced a cytopathic effect on MDBK cells, as recorded during initial testing. After one year of freezing 21 (91.1%) of 45 initially BVDV-positive buffy coat samples were detected to be BVDV-positive by routine virus isolation-IPX technique.

In the RT-PCR technique 42 (93.3%) of previously BVDV-positive frozen buffy coats gave the desired amplification. The analysis of amplification products indicated that amplified DNA from all 42 samples was about 402 bp product, as expected (Figure 2).

The number of BVDV positive buffy coats (n = 37) obtained by IPMA were also positive in the other tests, and similarly, 3 negative samples detected by RT-PCR were common for the other three techniques.

Table. The sensitivity of tests used in terms of percentage of detection of BVD virus in samples stored frozen for long periods.

	Virus isol.-IPX		IPMA		Indirect IPMA		RT-PCR	
	+	-	+	-	+	-	+	-
Positive (n = 45)	41(91.1) [§]	4	37(82.2)	8	40(88.8)	5	42(93.3)	3
Negative (n = 15)	0	15	0	15	0	15	0	15

[§] Numbers in brackets indicate sensitivity of the techniques in percentage terms.

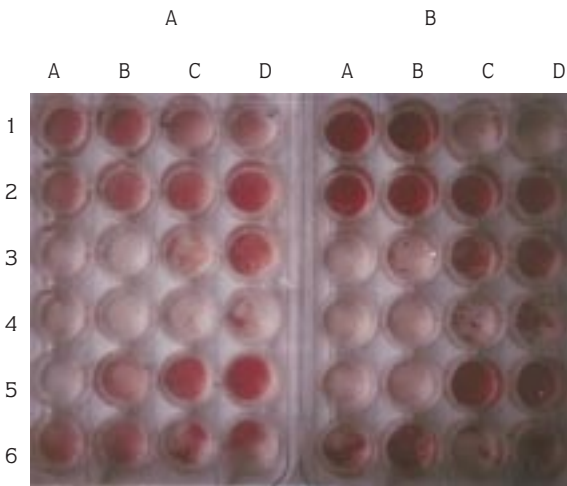


Figure 1. Comparison of IPMA and IIPMA results after inoculation of MDBK cells in duplicate in separated 24-well plates. Panel-A: Polyclonal antibody based technique IPMA. Panel-B: Monoclonal antibody based technique IIPMA. Inoculation protocols were identical for each plate, and the samples were inoculated in duplicate (e.g. buffy coat sample inoculated into wells A1B1 in panel A were the same in wells A1B1 in panel B).

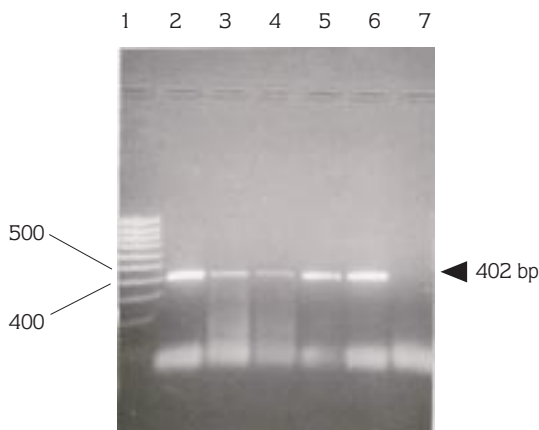


Figure 2. Agarose gel electrophoresis of reverse transcription-polymerase chain reaction (RT-PCR) products derived from the NS3 region of BVDV from frozen buffy coats. Lanes: 1:100 bp DNA ladder (MBI, Fermentas, Lithuania), 2-6: Frozen buffy coat samples, 7: Negative control.

The techniques revealed relative sensitivities in descending order of 93.3%, 91.1%, 88.8% and 82.2%. Buffy coats involved in the study as non-BVDV carrier blood samples gave negative results by means of the test systems used here. Thus, specificity was calculated to be 100% for all techniques.

Discussion

In this study, the ability of detecting the BVD virus by means of virus isolation-IPX, direct IPMA, indirect IPMA and RT-PCR techniques in buffy coat samples after long-term storage at -20°C was investigated. Freezing for more than one year at -20°C led to the decreasing viability of BVD viruses in samples at the end of the research. While 42 of the previous 45 BVDV positive buffy coats were found to be positive by RT-PCR, 41 of them were positive with virus isolation-IPX, 40 of them with IIPMA and 37 with IPMA. The techniques revealed relative sensitivities of 93.3%, 91.1%, 88.8% and 82.2%, respectively. Specificity was calculated to be 100% for all techniques.

In the research, RT-PCR gave better results than the other techniques. This result most probably arose from the destructive effect of freezing on the viability of viruses. The RT-PCR method had the advantage of ascertaining BVDV nucleic acid sequences in samples in which the virus had been inactivated, e.g. during transport or storage or due to the presence of neutralizing antibodies (13,18). On the other hand, the lower immunodetection sensitivity of viruses compared to RT-PCR might be related to either the poor growth of the virus or to the presence of toxic elements in the sample released after cell lysis during freezing and thawing that might have been repeated several times. The same elements such as various proteases could have destroyed BVDV RNA in some of the buffy coats ($n=3$) used and this might influence the rate of sensitivity of the RT-PCR technique for frozen samples. The other possibility behind missing BVDV-positive buffy coats by RT-PCR might be related to matching problems of the primers used. Although primers were selected from a non-structural protein coding region (p125) of the BVDV gene (16), some single base changes in the annealing sites of primers could be responsible for a failure in amplification. On the other hand, since the three samples that gave no amplification by the RT-PCR test also tested negative with the other techniques used, the first approach (degradation of RNA by proteases) seems more logical than the second (13).

This research demonstrated that virus isolation-IPX is more efficient than either of the immunostaining systems with short incubation periods but less efficient than RT-PCR. Beyond the toxic effect caused by the substances of cells lysed during storage, the high efficiency of the

technique on immunostaining procedures is believed to be dependent completely on the pre-amplification of viruses low in number from stored samples before immunostaining. This point agrees with the results reported before by Horner et al. (12).

Two kinds of immunostaining systems with short periods of incubation (three days) after the direct inoculation of test material were also compared with each other in terms of their capability in virus detection. The monoclonal antibody based immunoperoxidase monolayer assay (IIPMA) detected three more BVDV positive buffy coat samples over the polyclonal antibody based immunoperoxidase monolayer assay (IPMA). The possible reason for this variation could be non-specific properties of polyclonal serum used in reacting with certain antigenic variants of BVDV. Moreover, anti-NS3 and anti-E2 monoclonal antibodies (C16 and CA34) used in this research initially were proved to react with nearly all (19) or most (20) of the field isolates of BVDV from Germany and all of the field isolates in Turkey (unpublished data), respectively. This data suggests that the prior screening of reaction patterns between a panel of monoclonal antibodies and BVDV field isolates would be useful in selecting the best combination in order to increase the sensitivity of IIPMA by means of capturing the most common epitopes of isolates. Therefore, the greatest potential disadvantage of the use of MAb as a diagnostic reagent, monospecificity to a single epitope, could be avoided.

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The second advantage of IIPMA compared to IPMA is the high specificity in staining that eliminates background reactions. The results of this study are in full agreement with those obtained by Deregt and Prins (21).

In conclusion, the results obtained in this study indicated that immunostaining techniques (IPMA and IIPMA) are less sensitive than RT-PCR and virus isolation-IPX. Among the immunostaining procedures with short-term incubation periods, IIPMA has shown greater advantages in terms of sensitivity and staining quality than IPMA. Although there is a 2% (only one sample) difference in the detection ability of BVDV between RT-PCR and virus isolation-IPX, it might be postulated that the two techniques have close sensitivity. However, it is obvious that RT-PCR has an advantage since it is faster and less laborious than the virus isolation-IPX technique. The IIPMA technique can be used as a time saving alternative for the routine diagnosis of BVD virus infection in laboratories without RT-PCR technique facilities. However, it should be remembered that the MAbs used in the test should have the broadest range of reactivity. In this way, an important disadvantage of MAbs, e.g. reaction with a single epitope, might be avoided in testing.

Acknowledgments

The authors wish to thank Dr. V. Moennig from the Institute for Virology, Hannover Veterinary School, for providing the MAbs and pig anti-BVDV hyperimmune serum used in this research.

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