High-Sensitive Isolation of the BVD Virus from Bovine Plasma Pools

This application note describes the isolation and detection of the bovine viral diarrhea virus using the InviMag Virus DNA/RNA Mini Kit/KFmL (Invitek) and the Thermo Scientific KingFisher mL magnetic particle processor (Thermo Fisher Scientific) combined with real-time RT-PCR detection. In this experiment sample pools taken from a maximum of 50 animals were produced from cattle blood and up to 15 samples were processed on KingFisher mL in hands-free operation. The results show that very low amounts of viral RNA can be detected using this method.

By Dr. Hermann Nieper (LUA Sachsen, Standort Leipzig, Germany), Dr. Andrea Ockhardt (Invitek GmbH, Berlin, Germany), Dr. Arja Lamberg (Thermo Fisher Scientific, Vantaa, Finland)

Introduction

The detection and quantification of viruses in veterinary medicine allow fast and reliable identification of animal epidemics, e.g. avian influenza. Particularly with regard to diagnostic purposes these methods require extremely high efficiency and sensitivity for isolation and detection of nucleic acids in a large amount of samples or pooled samples.

The bovine viral diarrhea virus - mucosal disease (BVD-MD), also known as BVDV, is highly common in beef cattle and one of the most important causes for economic losses in the cattle industry. Due to the complex pathogenesis and insidious nature of BVDV infections, the laboratory diagnosis is an essential component of developing measures for the control and prevention of BVDV infections. The virus belongs to the Flaviviridae family, classified as belonging to the genus Pestivirus, and is closely related to the hog cholera virus in pigs and the border disease virus in sheep. Acute infection of immunocompetent animals with BVDV can result in a wide range of clinical syndromes, e.g. performance depression, diarrhea (diarrhea virus), respiratory and reproductive disorders (abortion and birth defects) as well as fatal haemorrhagic syndrome. Furthermore, immune suppressive effects also lead to opportunistic infections and cause problems with immunization. Infected animals shed the virus in nasal and oral secretions, faeces, urine and semen. The disease is primarily spread by cattle persistently infected (PI) with BVDV. PI animals originate from intrauterine infection in the first third of gestation before immunocompetence is being established in the foetus. In consequence, the animals remain infected

for life without any reaction of their immune system and sustain high virus concentrations.
Wolf (1997) claims that the economic loss amounts to € 12,000 as a result of fatal infections in a stock of 100 animals. The Government of the Federal Republic of Germany estimates, within its guidelines, the loss per cow at approximately € 160, if PI animals are not eliminated (BMELF 1998). Since November 2004, BVDV has been a reportable animal disease. With the passing of a BVDV protection ordinance in Germany (expected in 2007), it will become obligatory that all cattle have to be examined to combat the disease. The first aim of fighting against BVD is the detection and removal of PI animals, whose percentage among the total cattle population is actually between 0.1 – 2.0%. In accordance with this goal, an economical, quick, sensible and safe method of detection is needed. Besides virus cultivation, which may not be suitable for examining large numbers of animals, and the antigen ELISA, where individual animal examination is cost-intensive, RT-PCR offers a means of serum pool examination of up to 50 animals.
Since 2002, the German State of Saxony has implemented a voluntary program using this method to combat BVD. Due to the wide acceptance of this program and a vastly increasing number of samples, the semiautomatic purification of the serum pools with a Thermo Scientific KingFisher mL, from the company Thermo Fisher Scientific, in combination with the InviMag Virus DNA/RNA Mini Kit/KFmL, from the company Invitek, were implemented in 2004. Compared to using fully automatic instruments, this system provides a cost-effective step towards the standardization of labor-intensive nucleic acid purification.

Material and Methods

Isolation of viral RNA

Viral RNA of BVDV is purified in 45 min from serum and plasma pools using the commercial InviMag® Virus DNA/RNA Mini Kit/KFmL. The serum and plasma samples are obtained from cattle blood collected in farms. The quality of the incoming samples widely differs (differences in health of the donor, age, sample storage and transportation). Only 100 µl of serum or plasma sample per animal of a maximum of 50 animals are used to produce manually or automatically 5 ml pools for BVDV diagnostics.
From each pool an aliquot of only 200 µl serum or plasma is used for viral RNA isolation. The sample is transferred to the Extraction Tube, provided with the kit, and carefully mixed with 200 µl of ddH2O. The Extraction Tube already contains preformulated solid lysis reagents (non chaotropic lysis buffer, proteinase K), carrier nucleic acids and a precisely calibrated amount of internal RNA/DNA extraction controls, coated on the tube wall. The internal controls are copurified with the target sequences and allow to assess the extraction efficiency, and PCR amplification, respectively. This monitors the quality of the purified virus RNA and/or DNA and excludes false negative results. After mixing, all samples are placed into a Thermomixer (Eppendorf) and incubated while continuously shaking for 15 min at 65°C to support proteinase activity for lysis of the virus capsid and protein digestion. Incubation for 10 min at 95°C is recommended to remove residual proteins from virus nucleic acids by means of detergents. This step is necessary especially for virus nucleic acid-protein complexes of high stability.
During lysisan appropriate number of KingFisher mL tube strips needed for the samples (one tube strip per sample) are placed into the tube strip tray. The tube strips B to E are filled with buffers supplied by the kit according to Table 1. After lysis the samples are transferred into KingFisher mL tube strips (Tube A) and 400 µl of Binding Solution and 20 µl MAP Solution A is added and mixed with each lysate to adjust the binding conditions.

Note! It is important to mix the bottle with MAP Solution A carefully by vigorously shaking or vortexing.

Under these conditions viral RNA is bound to the magnetic particles quantitatively. During the process the beads are transferred through Wash Buffer R1 and R2 to remove all impurities, such as proteins, nucleases, PCR inhibitors, etc. After removal of ethanol from the beads, the viral RNA is eluted in Elution Buffer R and is ready for use.

Tube	Content	Sample/ Reagent volume
A	Lysed sample	200 µl
	MAP Solution A *	20 µl
	Binding Solution	400 µl
B	Wash Buffer R1	800 µl
C	Wash Buffer R2	800 µl
D	Wash Buffer R2	800 µl
E	Elution Buffer R	120 µl

Table 1 : Pipetting instructions for King Fisher mL and InviMag Virus DNA/RNA Mini Kit/KFmL

After filling the tube strips, the tray is placed in the instrument and the tip combs are inserted into the slots. The front lid is closed and the samples are processed using the "InviMAG_Virus_KFmL" purification protocol. After the program is completed, the tube strip is removed tray from the instrument and the eluates are stored for further use.

BVDV detection

Routinely, a 5 µl aliquot of each eluate (1/24) is finally used for the detection of BVDV from pools taken from 50 animals. Primers and conditions of the real-time one-step RT-PCR are described by Gaede et al. for RT-PCR, a QuantiTect™ sample RT-PCR Kit (Qiagen) used on a Rotorgene (Corbett). The detection system allows the secure detection of down to 10 genomic copies per reaction and is in the range of sensitivities required by the government. This corresponds approximately to a dilution of a persistently infected animal (PI) sample of at least 1:1000 (Figure 1). The quantitative assay below was performed to ensure sufficient efficiency of virus detection from diluted pools as a basis for the following routine diagnostic.

Detection of the internal control RNA

In a second step, the coated, internal control RNA (Extraction Tube) is detected using 5 µl of each eluate in a specific real-time RT-PCR (assay from AJ Roboscreen). The assay contains all reaction components, primers and probes. It enables the detection of errors during the extraction procedure of virus nucleic acids or of inhibitors during amplification (Figure 2). By means of this experiment false negative results are prevented and extraction, transcription and amplification quality are monitored. Basically the assay allows qualitative detection.

Results

The shown extraction method (InviMag® Virus DNA/RNA Mini Kit/KFmL) can realize highly efficient purification of viral RNA even from small copy numbers (see Figure 1). The quality of the extraction is monitored by an internal control system (see Figure 2). The extracted BVDV RNA and the controls were quantified by real-time RT-PCR. Viral RNA was detected down to a dilution of 1:1000 in the serum of two permanently infected animals. As a standard, an artificial nucleic acid was used to quantify genomic copies (Figure 1). Eight pools of sera from up to 50 animals were used for BVDV detection. All pools containing positive animals were detected. All extractions and amplifications were monitored by means of the positive control assay. All samples showed correct extraction and amplification procedures (Figure 2).

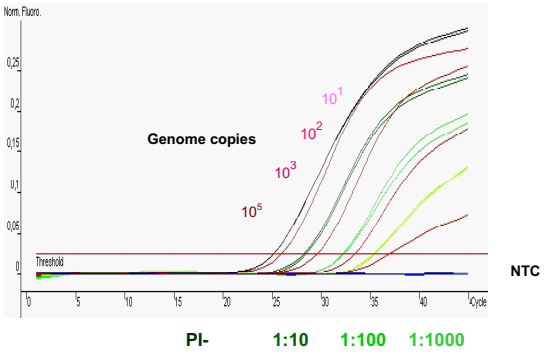

Figure 1: Amplification of dilution series of a control nucleic acid (red; from left to right 10,000/1,000/100 /10 copies in serum samples) and parallel dilution series from 2 PI (persistently infected animals, green; undiluted serum sample/1:10/1:100/1:1000 diluted serum sample) were amplified by real-time RT-PCR.

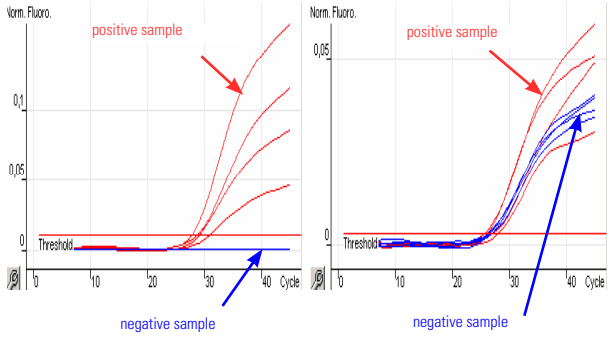

Figure 2:
(A) BVDV detection of 4 positive (red) animal serum samples from different serum pools and of 4 negative (blue) animal serum pools; all positive samples show a virus product
(B) Detection of internal control RNA in the eluates of 4 positive (red) and 4 negative (blue) serum pools; all samples show a detectable product from the control-RNA, which provides the evidence for successful extraction and amplification

Thermo SCIENTIFIC

Conclusion

The combined system for viral RNA extraction and detection shows sensitivity to viral dilutions down to at least 1:1000. According to future legal conditions, a system has to detect down to 50 – 100 copies/ml per real-time RT-PCR. The procedure described above fulfils the required criteria with respect to the BVDV protection ordinance for the detection of PI animals. Since the enforcement of the protection ordinance, nearly 200,000 animals have been examined with the described virus extraction and detection system in Saxony alone.

The InviMag Virus DNA/RNA Mini Kit/KFmL, which is designed for sensitive virus detection, provides simultaneous rapid and economical purification of viral DNA and RNA from different sources. In combination with the KingFisher mL, it has also been successfully used in this laboratory for other RNA viruses (Influenza A virus, Porcine Enterovirus, Avian Paramyxovirus 1) as well as for DNA viruses (Porcine Circovirus 2) from different basic materials, such as swabs, homogenized tissue samples and excrements.

References

Gaede et al. (2005). Berl. Münch. Tierärztl. Wschr. 118, 113-120

Wolf (1997). ITB-Schriftreihe, Bd. 1, 87-98

BMELF (1998). Leitlinien für den Schutz von Rinderbeständen vorm Virus der BVD/MD

This application note has been made in cooperation with Invitek GmbH

Invitek
Gesellschaft für Biotechnik & Biodesign mbH

Thermo SCIENTIFIC

North America: USA/Canada +1 866 984 3766
Europe: Austria +43 1 801 40 0, Belgium +32 2 482 30 30, Finland +358 9 329 100, France +33 2 2803 2000, Germany national toll free 08001-536 376, Germany international +49 6184 90 6940, Italy +39 02 95059 1, Netherlands +31 76 571 4440,
Russia/CIS +7 095 225 11 15, Spain/Portugal +34 93 223 3154, Switzerland +41 44 454 12 12, UK/Ireland +44 870 609 9203
Asia: China +86 21 6865 4588 or +86 10 5850 3588, India +91 22 5542 9494, Japan +81 45 453 9220, Other Asian countries +852 2885 4613
Countries not listed: +49 6184 90 6940 or +33 2 2803 2000
www.thermo.com/kingfisher

Lit no: AP-MIB-KF01-0407