Nucleic Acid sequence-based amplification to detect bovine viral diarrhoea virus on individual and pooled plasma, sera and ear notch samples

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SUMMARY

A nucleic acid sequence-based amplification (NASBA®) test was designed to detect bovine viral diarrhoea virus (BVDV) in calf sera, bovine plasmas, and was also shown to be applicable for bovine ear notches. The NASBA method was compared to the antigen-capture enzyme-linked immunosorbent assay (ELISA) for 36 BVDV-positive calf sera, 6 BVDV-positive and 82-negative bovine plasma samples. Comparison of the two methods showed 100% correlation for positive calf sera, and NASBA detected 2 additional positive bovine plasmas. NASBA was also compared to an in-house RT-PCR for 53 positive and 136 negative additional bovine plasmas and it detected 51 positive and 138 negative results (the positive and negative percent agreements were 92.5% and 98.5%, respectively). Each sample tested individually was also evaluated in simulated pools of 10 samples, and the overall percent agreement between individual and pooled samples was 100%. Additionally, among 201 ear notches tested individually from BVDV-unknown status cows, the NASBA method only detected one positive sample. When ear notches were tested as real pools of 10 samples (n = 48), the total agreement between individual and pooled samples was 100%. Consequently, the use of NASBA performed on the EasyQ® Analyzer, coupled to the EasyMAG® extraction is an automated, accurate and rapid tool for screening the BVDV status from individual and pooled samples.

Keywords: Bovine viral diarrhoea virus, cattle, NASBA, EasyMAG, ELISA, reverse transcription-polymerase chain reaction, plasma, serum, ear notch, pooled sample.

RÉSUMÉ

Test d’amplification NASBA (nucleic acid sequence based amplification) pour la détection du virus de la diarrhée bovine dans les plasmas, sera et biopsies auriculaires, testés individuellement ou par pool

Le test d’amplification NASBA® (nucleic acid sequence-based amplification) a été mis au point pour détecter le virus de la diarrhée bovine (BVDV) à partir de sera de veaux et de plasma bovin. Ce test est aussi applicable pour détecter le virus à partir de biopsies auriculaires bovines. Ce test NASBA a été comparé avec la méthode ELISA (antigen-capture enzyme-linked immunosorbent assay) sur 36 sera de veaux BVDV-positifs, 6 plasma bovins BVDV-positifs, et 82 plasma bovins BVDV-négatifs. Les résultats montrent une corrélation de 100% pour les sera de veaux positifs, et de plus le test NASBA détecte 2 plasmas supplémentaires ayant un statut BVDV-positif. Le test NASBA a également été comparé à un test d’amplification RT-PCR expérimentale sur 53 autres plasmas positifs et 136 négatifs. Le test NASBA a détecté 51 plasma positifs et 138 plasma négatifs (les pourcentages d’agrément positifs et négatifs étaient respectivement de 92.5% et 98.5%). Chaque échantillon testé individuellement a également été testé en pools simulés de 10 échantillons, le pourcentage total d’agrément entre échantillons individuels et pools étant alors de 100%. De plus, à partir de 201 biopsies auriculaires de vaches ayant un statut BVDV inconnu, le test NASBA a détecté un seul échantillon positif. Lorsque chaque biopsie auriculaire a été testée en pools réels de 10 échantillons (n = 48), le pourcentage total d’agrément était de 100%. En conséquence, l’utilisation du test NASBA et de l’analyseur EasyQ®, couplés à l’extraction sur EasyMAG® est un outil automatisé, précis et rapide pour le dépistage du statut BVDV à partir d’échantillons, qu’ils soient testés individuellement en pools.

Mots clés : Virus de la diarrhée bovine, bétail, NASBA, EasyMAG, ELISA, reverse transcription-polymerase chain reaction, plasma, sérum, biopsie auriculaire, pools d’échantillons.

Introduction

Bovine viral diarrhoea virus (BVDV) is one of the major infections present in cattle herds. It causes atypical clinical symptoms, reproductive disorders, mucosal disease and contributes to bovine respiratory disease complex [1, 6]. One cause of infection is the foetal transmission of the virus in the first trimester of gestation which leads to persistently infected (PI) animals [3, 10, 11]. PI animals are the major source of virus spread and it is thus very important to identify and isolate them from cattle herds [13]. In Europe, it has been proposed that a systematic approach was required to reduce the prevalence and the incidence of BVDV [12]. Currently, the BVDV antigen enzyme-linked immunosorbent assay (ELISA) method although inexpensive was compared to recent molecular techniques of detection [9], but the method was an inaccurate way to detect the presence of PI animals because of the lack

main reservoir of virus and increases the disease incidence causing acute infection in cattle and great economical damages [3, 10, 11]. PI animals are the major source of virus spread and it is thus very important to identify and isolate them from cattle herds [13]. In Europe, it has been proposed that a systematic approach was required to reduce the prevalence and the incidence of BVDV [12]. Currently, the BVDV antigen enzyme-linked immunosorbent assay (ELISA) method although inexpensive was compared to recent molecular techniques of detection [9], but the method was an inaccurate way to detect the presence of PI animals because of the lack

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of sensitivity [14]. Nucleic acid amplification technologies such as reverse transcription-polymerase chain reaction (RT-PCR) in bulk milk [10], RT-PCR in pooled ear notches [8, 9], quantitative-polymerase chain reaction (qPCR) in follicular fluids [15], RT-PCR and qPCR in pooled serum [17] improved PI animal detection in cattle herds because of a higher sensitivity.

In the present study, the bioMérieux laboratory developed an accurate and robust detection tool coupled to a rapid sample preparation and an automated purification system to detect PI animals present in the cattle herd. This method is based on the use of the Boom® nucleic acid extraction [2] and the nucleic acid sequenced-based amplification (NASBA) technique [4, 5, 20]. NASBA is a sensitive, isothermal, transcription-based amplification system specifically designed for the detection in real-time of ribonucleic acid (RNA) targets. Prior to NASBA, the isolation of nucleic acids by the Boom method is based on the denaturing properties of guanidine isothiocyanate and binding of nucleic acids to silica particles. The whole process is controlled by the use of a synthetic RNA internal control (IC). The presence of an IC co-extracted and co-amplified with each sample is determinant to verify the validity of the test and therefore, to interpret the presence or not of a BVDV contaminated animal. If the IC gives a non-valid result, the sample should be re-extracted. This assay was developed for use with calf serum and bovine plasma samples tested individually and as pools of 10 samples. Moreover, this assay was also adapted to be used with bovine ear notches specimens, tested individually or as pools of 10 samples, each reducing test cost price per specimen and allowing a higher throughput. Testing bovine ear notches has an important benefit since sampling is easier to perform and is less constraining for the animals. When bovines are tagged for identity, the piece of ear removed can be preserved in a sterile tube and tested directly for BVDV in a veterinary laboratory.

Materials and Methods

SAMPLES (FIGURE 1)

A total of 36 BVDV-positive calf sera were obtained from the Austrian Agency for Health and Food Safety, the BVDV-positive status being previously established by ELISA (Herd Check BVDV Ag/Serum Plus, IDEXX).

A total of 277 bovine plasmas was obtained from Fribourg agro-alimentary laboratory: the status of the specimen was established by either ELISA (Herd Check BVDV Ag/Serum Plus, IDEXX) in a dedicated veterinary laboratory for 88 of them, or by an in-house RT-PCR (Macherey-Nagel viral RNA extraction coupled with in-house RT-PCR performed on a Bio-Rad instrument) in another laboratory for the 189 remaining bovine plasmas. Among them, 59 bovine plasmas were BVDV-positive and 218 plasmas were negative.

In addition, 201 bovine ears were obtained from the BELL slaughter-house in Switzerland. A grip Allflex was employed to remove ear notches with a diameter of 3 mm. Status of these specimen was unknown. All samples were stored at -20°C before use.

In this study, all specimens were tested individually and each of them was also tested in pools of 10 samples.

NUCLEIC ACID PURIFICATION

Purification was performed with the EasyMAG instrument (bioMérieux SA). The principle of the “Generic Protocol” extraction is based on the lysis of samples and stabilization of nucleic acids in 2 mL of lysis buffer which is a chaotropic guanidinium isothiocyanate buffer at 4.8 M. Silica particles mixed with the IC (synthetic RNA internal control) are added to each lysate and incubated for 10 minutes at room temperature to bind the nucleic acids. After 2 washing steps of the magnetic pellet in 2 different washing buffers, purified nucleic acids are released from silica particles in 25 µL of the elution buffer at 60°C for 5 minutes.

Sera and plasma extractions were performed with 100 µL of each specimen when tested individually. Due to low volume of samples available, the pooling of 10 specimens was simulated with a solution of Basematrix 53 (SeraCare). This is a processed, dialysed and defibrinated human plasma product sharing same properties as bovine sera and plasmas. Simulated pools of 10 specimens consisted to extract 100 µL of one real sample diluted in 900 µL of Basematrix 53.

When tested individually, bovine ear notches were disrupted 10 minutes in 900 µL of lysis buffer at room temperature before the nucleic acid extraction. Then, the extraction was performed with 100 µL of supernatant. When tested as real pools of 10 samples, 10 ear notches were spiked in one tube of 900 µL of lysis buffer and incubated 10 minutes at room temperature, and then the extraction was performed with 100 µL of supernatant.

NASBA METHOD

The BVDV genome is a positive sense single-stranded RNA of approximately 12.5 kb length [16]. As the virus belongs to the pestiviruses genus, the 5’-untranslated region (UTR) is highly-conserved [19], and consequently NASBA
primers and molecular beacon were designed in this specific region for the BVDV detection. An in silico study was performed to check the specificity of the primers. Sequence of the RNA synthetic internal control (IC) was 5'-CAG-UGG-UA GUU-CGU-UGG-AUA-AGG-AAG-GCA-GCA-GCA-GCA-GCA-GA-CCC-TAT-GCC-Dabsyl-3' and a molecular beacon MB-IC designed to bind to the RNA synthetic internal control (IC) was 5'-CAG-UGG-UA GUU-CGU-UGG-AUA-AGG-GCA-GCA-GCA-GCA-GA-CCC-TAT-GCC-Dabsyl-3'. A molecular beacon MB-WT designed to bind to the RNA wild type BVDV target was 5'-FAM-CC-TCG-agaaGaTg-aC-GAG-GG-aC-GA-CCC-TAT-GCC-Dabsyl-3' (a = 2'-O-methyl-A; g = 2'-O-methyl-G).

Results

The sensitivity of the NASBA method was 100.0% [90.3%; 100.0%] in agreement with the ELISA reference method for the 36 individual BVDV positive calf sera (Table I). Identical sensitivity was obtained when the same calf sera with a BVDV-positive status were tested independently in 36 simulated pools of 10 samples (data not shown). No calf serum with a BVDV-negative status was tested. Consequently, the PPA of the NASBA results between individual and pooled positive samples detected was 100.0% [90.3%; 100.0%].

Eighty-eight bovine plasmas which BVDV status was known by the ELISA reference method (6 positive and 82 negative) were also tested by the NASBA method (Table II). When tested individually, the 6 ELISA-positive plasmas were all detected as positive with the NASBA method, and sensitivity was 100.0% [54.1%; 100.0%] in agreement with the reference method. On the other hand, 80 plasmas among the 82 ELISA-negative plasmas were detected as negative and 2 as positive by the NASBA method, and specificity was 97.6% [91.5%; 99.7%] in agreement with ELISA. When all these individual plasma were tested independently in a total of 88 simulated pools of 10 samples, the NASBA results between individual and pooled samples were completely concordant: the PPA for the 8 positive plasma was 100.0% [63.1%; 100.0%] and the NPA for the 80 negative plasma was 100.0% [95.5%; 100.0%] (data not shown). Consequently, the PPA of the NASBA on individual specimens and pooled bovine plasma samples was 100.0% [95.9%; 100.0%].

The 189 bovine plasmas tested by the in-house RT-PCR method were also tested by the NASBA method (Table III). Among the 53 individual in house RT-PCR positive plasmas, 49 were detected as positive and 4 as negative with the NASBA test, leading to a PPA of 92.5% [81.8%; 97.9%] between the 2 methods. Among the 136 individual in house RT-PCR negative plasmas, 134 were detected as negative and only 2 as positive with the NASBA test and the NPA was 98.5% [94.8%; 99.8%] in accordance with the RT-PCR results. Consequently, the OPA between NASBA and the in-house RT-PCR methods for individual plasma samples detected was 96.8% [93.2%; 98.8%]. When all the individual plasmas were tested independently in a total of 189 simulated pools of 10 samples, NASBA results were absolutely concordant with the individual tests: the PPA for the 51 positive plasmas was 100.0% [93.0%; 100.0%] and the NPA for the 138 negative plasma was 100.0% [97.4%; 100.0%] (data not shown). Consequently, the OPA of the NASBA on pooled plasma samples and individual specimens was 100.0% [98.1%; 100.0%].

The NASBA method detected only one positive bovine ear notch among the 201 notches with BVDV unknown status.
when tested individually. A total of 48 real pools (composed of a random mix of 10 ear notches using 192 out of the 201 specimens) which 2 included the NASBA positive specimen were assembled and were investigated by the NASBA method (Table IV). The 2 real pools containing the positive specimen were detected as positive, leading to a PPA between individual and pool results of 100% [15.8%; 100.0%]. In parallel, all the 46 remaining pools without the NASBA positive specimen gave negative results, leading to a NPA of 100% [92.3%; 100.0%]. Consequently, the OPA of the NASBA method for bovine ear notches tested individually or as pools of 10 samples was 100.0% [92.6%; 100.0%].

**Discussion**

Performing NASBA to detect BVDV in calf sera and bovine plasmas tested individually is a sensitive and specific method of screening cattle herd for PI animals. Furthermore, results obtained with pooled samples are very encouraging for the detection of the BVDV. When tested individually, the ability of the NASBA method and the ELISA reference method to detect the BVDV-positive calf sera and bovine plasmas were highly correlated. Sensitivity reached 100% agreement between the two methods for both the 36 BVDV-positive calf sera and the 6 BVDV positive bovine plasmas tested. Nevertheless, the number of BVDV-positive bovine plasmas has to be increased in order to claim a significant statistical sensitivity analysis. The ability of NASBA to correctly detect negative plasmas was also high: specificity reached 97.6% for the 82 samples tested: the NASBA method has detected 2 additional positive samples in comparison with the ELISA test. According to the *in silico* study, NASBA primers were highly specific to the BVDV. These two BVDV positive results could correspond to another source of PI animals (e.g. transiently infected animals, vaccinated animals or PI animals that shed low amount of the virus) not detected by the reference method [8] which is also less sensitive [14].

<table>
<thead>
<tr>
<th>ELISA status</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
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<td>NASBA status</td>
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<td></td>
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<td>36</td>
</tr>
<tr>
<td>Negative</td>
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<td>0</td>
</tr>
<tr>
<td>Total</td>
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<td>0</td>
<td>36</td>
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</tbody>
</table>

**TABLE I:** Comparison of the NASBA and ELISA methods applied to the calf sera (n = 36) tested individually.

<table>
<thead>
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<th>ELISA status</th>
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<th>Negative</th>
<th>Total</th>
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<tbody>
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<td></td>
</tr>
<tr>
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<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Negative</td>
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<td>80</td>
<td>80</td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td>82</td>
<td>88</td>
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**TABLE II:** Comparison of the NASBA and ELISA methods applied to bovine plasmas (n = 88) tested individually.

<table>
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</tr>
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<td>Positive</td>
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<td>2</td>
<td>51</td>
</tr>
<tr>
<td>Negative</td>
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<td>134</td>
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<tr>
<td>Total</td>
<td>53</td>
<td>136</td>
<td>189</td>
</tr>
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</table>

**TABLE III:** Comparison of the NASBA and the in-house RT-PCR methods applied to bovine plasmas (n = 189) tested individually.

<table>
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<tr>
<th>ELISA status</th>
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<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>NASBA status</td>
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<td></td>
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</tr>
<tr>
<td>Positive</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>46</td>
<td>46</td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
<td>46</td>
<td>48</td>
</tr>
</tbody>
</table>

**TABLE IV:** NASBA results from bovine ear notches with unknown BVDV status tested individually or as real pools of 10 samples (48 real pools containing each 10 ear notches were obtained by randomly mixing the initial ear specimens and 2 of them have included the unique NASBA-positive ear notch).
When comparing the two molecular techniques of amplification, the amount of positive plasmas tested individually and detected by the in-house RT-PCR method (53 plasmas) was higher than with the NASBA method (51 plasmas) resulting to a PPA of 92.5%. One possible explanation for this discrepancy may be due to the degradation of the samples following several freeze and thaw cycles before being evaluated in NASBA. Indeed, it was described that this treatment could decrease the amplification signal [18]. Another possible cause is the use of an internal control (IC) co-extracted and co-amplified with each sample tested. The major advantage of the IC is to validate the whole process from extraction to amplification and to highlight that no inhibitor impacted the results. As the IC used the same primers as natural target in the present study, the consequent competition may decrease the sensitivity of the method for low positive samples. Each individual specimen tested was diluted with an equivalent of 9 other specimens simulated by the use of Basematrix 53. Properties of Basematrix 53 are similar to sera and plasma. In the present study, all individual BVDV-positive calf sera and bovine plasmas tested by NASBA were systematically detected in simulated pools of 10 samples. In the same way as individual specimen testing, an IC was used for each simulated pool to validate the results. As the prevalence of the disease is estimated around 1-2% [7], only a few BVDV-positive samples are usually encountered. Consequently, the pooling method reduced significantly the number of tests and the cost price per specimen. The volume required for purification of individual specimen is only 100 µL, therefore when a pool is detected as BVDV-positive the tracking of each specimen can be easily conducted again with such a low amount of sample within the same day. The whole process from extraction to amplification results is carried out on half a day of sample within the same day. The whole process from extraction to amplification results is carried out on half a day of sample within the same day. The whole process from extraction to amplification results is carried out on half a day of sample within the same day. The whole process from extraction to amplification results is carried out on half a day of sample within the same day.

In addition to plasma and serum analysis, NASBA is also a relevant method in the detection of BVDV in bovine ear notches, tested both individually, and as pools of 10 samples. Moreover, ear notches contained a large amount of inhibitors (e.g. mud, hair, bacteria, etc.), and these inhibitors were efficiently removed during the EasyMAG purification as indicated by the internal control data (not shown) for the 201 bovine ear notches tested individually, and especially for the 48 real pools of 10 samples which contained ten times more inhibitors. The eluates amplified in NASBA were in agreement between pools of 10 samples which contained ten times more inhibitors. Purified eluates of 1 mL in each EasyMAG disposable well. Purified eluates in the desired elution volume are then directly amplified by NASBA.

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Moreover, ear notches contained a large amount of inhibitors especially for the 48 real pools of 10 samples. Consequently, the pooling method reduced significantly the number of tests and the cost price per specimen. The volume required for purification of individual specimen is only 100 µL, therefore when a pool is detected as BVDV-positive the tracking of each specimen can be easily conducted again with such a low amount of sample within the same day. The whole process from extraction to amplification results is carried out on half a day and it is automated: no sample preparation is required and the 10 specimens can be directly pooled up to a total volume of 1 mL in each EasyMAG disposable well. Purified eluates in the desired elution volume are then directly amplified by NASBA.

In the present study, all individual BVDV-positive calf sera and bovine plasmas tested by NASBA were systematically detected by the in-house RT-PCR method for purification of nucleic acids. J. Clin. Microbiol., 1990, 28, 495-503. As the prevalence of the disease is estimated around 1-2% [7], only a few BVDV-positive samples are usually encountered. Consequently, the pooling method reduced significantly the number of tests and the cost price per specimen. The volume required for purification of individual specimen is only 100 µL, therefore when a pool is detected as BVDV-positive the tracking of each specimen can be easily conducted again with such a low amount of sample within the same day. The whole process from extraction to amplification results is carried out on half a day and it is automated: no sample preparation is required and the 10 specimens can be directly pooled up to a total volume of 1 mL in each EasyMAG disposable well. Purified eluates in the desired elution volume are then directly amplified by NASBA.

References


