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Development of a TaqMan assay for sensitive detection of all pestiviruses infecting cattle, including the emerging HoBi-like strains

Michele Losurdo a, Viviana Mari a, Maria Stella Lucente a, Maria Loredana Colaianni b, Iolanda Padalino b, Nicola Cavaliere b, Canio Buonavoglia a,b, Nicola Decaro a,*

a Department of Veterinary Medicine, University of Bari, Strada per Casamassima Km 3, Valenzano, 70010 Bari, Italy
b Istituto Zooprofilattico Sperimentale di Puglia e Basilicata, via Manfredonia 20, 71100 Foggia, Italy

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A real-time RT-PCR assay based on the TaqMan technology was developed for rapid and sensitive detection of pestiviruses infecting cattle, i.e., bovine viral diarrhea virus (BVDV) 1, BVDV-2, and the emerging HoBi-like pestiviruses. The assay was linear and reproducible, being able to detect as few as 10 copies of viral RNA. By real-time RT-PCR analysis of 986 biological samples collected from cattle herd with clinical signs suggestive of pestivirus infection and from animals recruited in a pestivirus surveillance programme, 165 pestivirus positive samples were detected, including 6 specimens, 2 nasal swabs, and 4 EDTA–blood samples, that tested negative by a gel-based RT-PCR assay targeting the 5 UTR. The developed TaqMan assay represents a new reliable and effective tool for rapid and sensitive diagnosis of infections caused by all pestiviruses circulating in cattle, thus being useful for extensive surveillance programs in geographic areas where HoBi-like pestiviruses are co-circulating with BVDV-1 and BVDV-2.

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1. Introduction

Bovine viral diarrhea virus 1 and 2 (BVDV–1 and BVDV–2) belong to the genus Pestivirus of the Flaviviridae family (Simmonds et al., 2011), together with Classical swine fever virus (CSFV), and Border disease virus (BDV). Pestiviruses are enveloped single-stranded positive RNA viruses with a genome composed by a single open reading frame (ORF), flanked by 5′- and 3′-untranslated regions (UTRs). Transcription of the single ORF leads to the synthesis of a polypeptide that gives origin to 12 smaller proteins through cleavage by cellular and viral proteases: \( \text{N}^{\text{pro}} \), C, \( \text{E}^{\text{ms}} \), E1, E2, p7, NS2/NS3, NS4A, NS4B, NS5A, NS5B (Simmonds et al., 2011). The 5′UTR, \( \text{N}^{\text{pro}} \), and E2 regions are widely used for sequence comparison and phylogenetic analysis (Ridpath et al., 1994; Harasawa and Giangaspero, 1998; Schirrmeier et al., 2004; Liu et al., 2009). Two BVDV biotypes have been identified, namely cytopathogenic (cp) and non-cytopathogenic (noncp), both involved in the pathogenesis of mucosal disease (MD), a fatal form of BVDV infection in persistently infected (PI) calves. Acute BVDV infections can cause different clinical pictures, including transient pyrexia, leucopenia, oculonasal discharge, respiratory distress, diarrhea, and reproductive failure (Brownlie et al., 1984; Bolin et al., 1985).

An atypical pestivirus, named D32/00′HoBi′, was described as a contaminant of a fetal calf serum (FCS) batch of Brazilian origin (Schirrmeier et al., 2004) and proposed as prototype of a new pestivirus species, BVDV-3 (Liu et al., 2009). HoBi-like pestiviruses were later detected worldwide in FCS batches of southern American origin (Stalder et al., 2005; Stahl et al., 2010) and associated with subclínical natural infection (Stalder et al., 2005; Stahl et al., 2007).

HoBi-like strains were first associated to overt disease in Italy (Decaro et al., 2011, 2012a,c), but more recently this group of pestiviruses was responsible for clinical signs in cattle herds in southern America (Weber et al., 2014) and Asia (Kampa et al., 2010; Haider et al., 2014; Mishra et al., 2014).

Due to the economical impact of pestivirus infection in cattle herd, the main goal of diagnostic is a rapid and reliable detection of animals with acute or persistent infection. Several molecular assays have been developed for detection of bovine pestiviruses. A 5′UTR RT-PCR assay, largely used for pestivirus molecular screening (Vilcèk et al., 1994), was not able to detect the novel HoBi-like pestivirus, due to a nucleotide mismatch at the 3′ end of the forward primer (Decaro et al., 2012b). Whether the real-time RT-PCR assays developed for detection of pestiviruses (Young et al., 2006; La Rocca and Sandvik, 2009; Gaede et al., 2005) are able to detect HoBi-like pestiviruses has not been assessed. An assay was able to detect the prototype strain D32/00′HoBi′ (Hoffmann et al., 2006) and two additional HoBi-like viruses contaminating FCS batches.
Table 1
Oligonucleotides used in the present study for pestivirus detection and characterization.

| Assay           | Reference                  | Primer/probe       | Sequence 5′–3′ | Sense | Position | Amplicon size (bp) |
|-----------------|----------------------------|--------------------|----------------|-------|----------|--------------------|
| Real-time RT-PCR| This study                 | Pesti-qF           | CATGCCATGGAGAAGGAGCCTGCAGTGATGAGG   | +     | 128–147, 131–150, 125–144 | 195, 191, 198 |
|                 |                            | BVDVgen-R          | TATGGTGGTATGAAAGTGTTCCGGGCGG   | +     | 302–322, 303–323 | 267–287, 268–288 |
|                 |                            | BVDVgen-Pb         | FAM-CTGGCAGTGACAGTGGCGGACATGGTG   | +     | 267–287 | 287–288 |
| RT-PCR          | Vílique et al. (1994)      | 324                 | ATCCCGCTAGTCCGCTGGAGACCTGGG   | +     | 7–27   | 287–288 |
| Nested PCR      | Decaro et al. (2012b)      | PanBVDVpcRF        | CTCCTGTTGATGAGGCTGCACTAGTGGG   | +     | 368–388 | 1013 |
|                 |                            | PanBVDVpcRC        | CGTCCGACGACGACGGACTGCACTAGTGGG   | +     | 1364–1383 | 501 |
|                 |                            | BVDV-1 ncpF        | TTACACGCTGTCGCTAGGG   | +     | 879–897 | 829 |
|                 |                            | BVDV-2 ncpF        | ATCTGACGATGACGAGTGG   | +     | 551–571 | 210 |
|                 |                            | BVDV-3 ncpF        | TCCTGTTGGACACGCTGGAGACCTGGG   | +     | 1173–1192 | 210 |

* Oligonucleotide position is referred to the genomic sequence of BVDV-1 strain NAD (GenBank accession no. M31182).

Oligonucleotide position is referred to the sequence of BVDV-2 strain NewYork’93 (GenBank accession no.: AF502399.1).

Oligonucleotide position is referred to the sequence of atypical pestivirus strain Italy1/10-1 (GenBank accession no.: HQ231763.1).

Fig. 1. Nucleotide alignment of reference bovine pestivirus showing the binding region of oligonucleotides used in the panpestivirus real-time RT-PCR.

(Stähl et al., 2010), but it was not validated methodically with field samples. In addition, a nucleotide mismatch at the 3′ end of the probe with respect to the European HoBi-like reference strains might severely affect the assay sensitivity. A real-time RT-PCR assay for specific detection of HoBi-like viruses has been developed in recent years, but this assay does not detect simultaneously BVDV-1 and BVDV-2 and cross-reaction may occur for high-titer BVDV-2 samples (Decaro et al., 2012b, 2013b). In addition, two commercially available real-time RT-PCR assays proved to detect HoBi-like viruses, but with different efficiency on the basis of the sample typology (Bauermann et al., 2014).

In this study, to overcome the limitations of the existing methods, a pan-pestivirus TaqMan assay was developed for sensitive detection of bovine pestiviruses, including the novel HoBi-like viruses.

2. Materials and methods

2.1. Primers and probe set

Conserved genomic regions were selected to design the primers and the probe by aligning full-length genomes of reference strains of BVDV-1, BVDV-2, and HoBi-like pestivirus obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html). Sequence alignment was carried out using the BioEdit software package (Hall, 1999).

Oligonucleotides able to amplify a 193, 192, and 198-bp fragment of the 5′UTR region of BVDV-1, BVDV-2, and HoBi-like pestivirus (Table 1), respectively, were designed using Beacon Designer Software, Version 2.06 (Premier Biosoft International, Palo Alto, CA, USA). Primers and probe were synthesized by Eurofins Genomics (Ebersberg, Germany).

2.2. Pestivirus reference strains and biological samples

BVDV-1 strain NADL (courtesy of Dr Ferrari, Istituto Zooprofilattico Sperimentale di Lombardia e Emilia Romagna, Brescia, Italy), BVDV-2 strain 232/02 (Decaro et al., 2004a) and HoBi-like strain 1/10-1 Italy (Decaro et al., 2011), propagated in Madin–Darby bovine kidney (MDBK) cells grown in Dulbecco’s minimal essential medium, were used as reference bovine pestiviruses.

A total of 986 bovine biological samples were analyzed, consisting of 69 tissue samples from aborted fetuses, 95 nasal swabs, 186 fecal samples, and 636 EDTA–blood samples. The samples had
been collected from cattle herds with clinical signs suggestive of pestivirus infection and from herds included in a surveillance plan for the detection of persistently infected (PI) animals in Southern Italy (unpublished data).

RNA was extracted from reference strains and from biological samples using the QIAamp® cador®Pathogen Mini Kit (Qiagen S.p.A.), according to the manufacturer’s instructions.

2.3. Standard RNA for absolute quantification

Standard RNA for absolute quantification was obtained amplifying a 683-bp fragment of the 5′-UTR region of the BVDV-1 reference strain NADL using primer pair 324 (Vilcek et al., 1994) and BVDV-690R (5′-TCTATGCACATATAATTGTA-3′). The RT-PCR product was cloned into TOPO® XL PCR Cloning vector (Life Technologies Italia, Monza, Italy) and transcribed with RiboMAX™ Large Scale RNA Production System-T7 (Promega Italia, Milan, Italy) from the T7 promoter, according to the manufacturer's guidelines. After DNase treatment, the transcripts were purified using the QIAamp® RNA Easy kit (Qiagen S.p.A., Milan, Italy) and quantified by spectrophotometric analysis. Ten-fold dilutions of the RNA transcripts, representing 10⁹–10¹ copies RNA μl⁻¹ of template, were made in a mixed fecal/nasal swab suspension from a calf negative for pestivirus by RT-PCR (Vilcek et al., 1994). Aliquots of each dilution were frozen at −70°C and used only once.

2.4. Real-time RT-PCR

Reverse transcription was carried out in a 20-μl reaction mix consisting of PCR buffer 1× (KCl 50 mM, Tris–HCl 10 mM, pH 8.3), MgCl₂ 5 mM, 1 mM of each deoxynucleotide (dATP, dCTP, dGTP, dTTP), RNase Inhibitor 1 U, MuLV reverse transcriptase 2.5 U, random hexamers 2.5 U (MuLV reverse transcriptase kit, Applied Biosystems, Life Technologies, Milan, Italy), and 1 μl of standard dilutions or RNA extracts. Each standard or sample RNA was run in duplicates. Reverse-transcription was carried out at 42°C for 30 min, followed by a denaturation step at 99°C for 5 min.

Real-time PCR was performed on a CFX96™ Real-Time System (Bio-Rad Laboratories Srl, Milan, Italy) in a 25-μl reaction mixture containing 12.5 μl of iTaq™ Universal Probes Supermix (Bio-Rad Laboratories Srl), 600 nM of primers Pesti-qF and BVDgen-R, 200 nM of probes BVDgen-Pb and 10 μl of c-DNA. The thermal protocol consisted of activation of iTaq DNA polymerase at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 48°C for 30 s and extension at 60°C for 1 min. The detection of the increasing fluorescent signal was carried out during the extension step of the reaction and the data was analysed with the appropriate sequence detector software (Bio–Rad CFX Manager v. 3.1, Bio-Rad Laboratories Srl).

2.5. Internal control

An internal control (IC) was used to verify the efficacy of the extraction procedure and the absence of DNA-polymerase inhibition. The IC consisted of an RNA synthetic transcript containing the M gene of canine coronavirus (CCoV) type II (Decaro et al., 2005), which was added to the lysis buffer (AVL buffer, QIAGEN S.p.A.) at a concentration of 10,000 RNA copies ml⁻¹ of buffer before nucleic acid extraction. The fixed amount of the IC added to each sample had been calculated to give a mean Ct value in a genotype-specific real-time RT-PCR assay (Decaro et al., 2005) of 34.18 with a S.D. of 0.65 as calculated by 50 separate runs. Samples in which the Ct value for the IC was > 35.48 (average plus 2 S.D.) were excluded from the analysis.

2.6. Assay specificity, sensitivity, and reproducibility

To assess the assay specificity, pestivirus reference strains and other viral pathogens, including bovine respiratory syncytial virus (Decaro et al., 2014), bovine calcivirus (Di Martino et al., 2014),
bovine coronavirus (Decaro et al., 2008), bovine rotaviruses (Pratelli et al., 1999), bovine herpesviruses 1 (Thiry et al., 2006) and 4 (Buonavoglia et al., 1984), and ovine herpesvirus 2 (Decaro et al., 2003) were included in the analysis. Nasal swabs, and fecal specimens and EDTA–blood samples that tested negative for pestiviruses by gel-based RT-PCR (Vilcek et al., 1994) and distilled water were used as negative controls and blanks, respectively.

To evaluate the ability of the assay to detect the RNA of all bovine pestiviruses, BVDV-1, BVDV-2, and HoBi-like pestivirus reference isolates were also tested.

Serial ten-fold dilutions of standard RNA, containing from 10^6 to 10^9 copies of RNA transcripts, and the corresponding Ct values, were used to plot the standard curve for pestivirus RNA absolute quantification.

To evaluate the detection limit of the real-time PCR assay, ten-fold dilutions of an EDTA–blood sample containing 4.37 × 10^7, 2.18 × 10^6, and 4.56 × 10^5 RNA copies of BVDV-1, BVDV-2, and HoBi-like pestivirus, respectively, were made in an EDTA–blood sample from a pestivirus-negative calf and then tested.

To evaluate the reproducibility of the assay, biological samples containing pestivirus RNA titers spanning the whole range covered by real-time RT-PCR, were tested repeatedly, as described previously (Decaro et al., 2004b, 2005). Intra-assay and inter-assay coefficients of variation (CVs) were calculated dividing the standard deviation of each tested sample by its mean and multiplying that result by 100. Intra-assay CVs were evaluated by testing 10 times the same samples in one experiment, whereas the interassay reproducibility was assessed by repeating the experiment 10 times.

2.7. Gel-based RT-PCR for pestivirus detection

The detection of pestivirus RNA in all biological samples, reference viruses and RNA transcript dilutions was carried out by a panpestivirus gel-based RT-PCR (Vilcek et al., 1994), using SuperScript™ One-Step RT-PCR for Long Templates (Life Technologies Italia) and primers 324 and 326 that bind to the 5'UTR region of the pestivirus genome. The following thermal protocol was used: reverse transcription at 50 °C for 30 min, inactivation of Superscript II RT at 94 °C for 2 min, 45 cycles of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 30 s, with a final extension at 68 °C for 10 min. The PCR products were detected by electrophoresis in 1.5% agarose gels and visualized under UV light after ethidium bromide staining. The position and sequence of the primers used for conventional amplification are reported in Table 1.

2.8. Nested PCR for pestivirus characterization

Biological samples testing positive for pestivirus by real-time RT-PCR and/or by gel-based RT-PCR were submitted to a nested PCR (nPCR) protocol previously developed for the characterization of bovine pestiviruses (Decaro et al., 2012b). RT-PCR was carried out using SuperScript™ One-Step RT-PCR for Long Templates (Life Technologies Italia) and the following thermal protocol: reverse transcription at 50 °C for 30 min, inactivation of Superscript II RT at 94 °C for 2 min, 45 cycles of 94 °C for 30 s, 50 °C for 30 s, 68 °C for 1 min, with a final extension at 68 °C for 10 min. Nested PCR was performed using AmpliTaq Gold (Life Technologies Italia). The thermal conditions consisted of activation of AmpliTaq Gold polymerase at 94 °C for 10 min and 25 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and polymerization at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The PCR products were detected as described for the diagnostic gel-based RT-PCR. The position and sequence of the primers used for conventional amplification are reported in Table 1.

3. Results

3.1. Specificity, sensitivity, and reproducibility of the TaqMan assay

As expected on the basis of the oligonucleotide design, all reference strains, i.e., BVDV-1, BVDV-2, and HoBi-like pestivirus, were correctly detected by the panpestivirus TaqMan assay. All the other bovine viral pathogens, the pestivirus negative samples and the blanks tested constantly negative. All reference strains were also detected by the panpestivirus 5'UTR RT-PCR assay.

Ten-fold dilutions of BVDV-1 standard RNA were used to construct a standard curve that spanned nine orders of magnitude, from 10^1 to 10^9 copies of standard RNA, and showed linearity over the entire quantitation range, providing an accurate measurement over a very large range of starting target amounts. The coefficient of linear regression (R^2) was equal to 0.998 and the PCR efficiency ranged around 99%, with a slope of −3.682 (Fig. 2).

The detection limit of the assay was 4.37 × 10^1, 2.18 × 10^1, and 4.56 × 10^1 RNA copies for BVDV-1, BVDV-2, and HoBi-like pestivirus, respectively. By converse, the gel-based RT-PCR assay was able to detect as few as 4.37 × 10^0, 2.18 × 10^0, and 4.56 × 10^0 RNA copies for BVDV-1, BVDV-2, and HoBi-like pestivirus, respectively.
For BVDV-1, intra-assay CVs ranged from 4.9% (sample containing 7.15 × 10^5 RNA copies μL^−1) to 23.11% (4.33 × 10^2 RNA copies μL^−1) and the inter-assay CVs varied from 5.03% (3.98 × 10^2 RNA copies μL^−1) to 56.1% (2.05 × 10^1 RNA copies μL^−1). For BVDV-2, intra-assay CVs were between 3.10% (3.79 × 10^2 RNA copies μL^−1) and 34.2% (2.87 × 10^1 RNA copies μL^−1), while the inter-assay CVs were between 7.73% (3.79 × 10^2 RNA copies μL^−1) and 61.76% (3.52 × 10^1 RNA copies μL^−1). HoBi-like pestivirus intra-assay CVs ranged from 4.43% (2.76 × 10^6 RNA copies μL^−1) to 40.6% (5.08 × 10^2 RNA copies μL^−1) and inter-assay CVs were between 6.93% (1.04 × 10^7 RNA copies μL^−1), and 53.66% (6.82 × 10^1 RNA copies μL^−1).

3.2. Internal control detection

The IC was detected in all but three of the examined samples, with Ct values below the threshold value of 35.48, thus ruling out relevant RNA losses during nucleic acid extraction or DNA polymerase inhibition during real-time PCR amplification. The three IC negative samples were EDTA bloods and retesting 1:100 dilutions of these samples yielded positive results with Ct values below the established cutoff. However, even when diluted, these samples were pestivirus negative by both gel-based and real-time RT-PCR.

3.3. Analysis of biological samples

The results of the analysis of 986 biological samples collected from cattle herds with suspected pestivirus infection and during the pestivirus surveillance plan are summarized in Fig. 3. By using the panpestivirus gel-based RT-PCR, a total of 165 pestivirus positive samples were detected, which included 28/69 tissues from aborted fetuses, 24/95 nasal swabs, 22/186 feces, and 91/636 EDTA–blood samples. All these samples were confirmed to be pestivirus-positive by the developed real-time RT-PCR assay. Additionally, 6 specimens were detected by the real-time RT-PCR assay only, i.e., 2 nasal swabs and 4 EDTA–blood samples. In total, 980 samples yielded concordant results with the two assays (165 positive and 815 negative samples). The pestivirus-positive samples contained a wide range of pestivirus RNA copies per microliter of template, ranging from 3.09 × 10^2 to 7.35 × 10^7 (fetuses), from 1.51 × 10^3 to 6.69 × 10^6 (nasal swabs), from 4.80 × 10^2 to 7.85 × 10^6 (feces), and from 2.55 × 10^2 to 3.92 × 10^8 (EDTA–blood samples).

By the typing nPCR assay, all the samples positive by real-time RT-PCR but 12 specimens could be genotyped correctly. None of the 6 strains that tested positive by real-time RT-PCR and negative by the 5′UTR RT-PCR assay could be genotyped by the nPCR assay. One hundred and three strains were typed as BVDV-1, 15 strains as BVDV-2 and 41 specimens as Hobi-like viruses. All the 41 samples had been collected from two different cattle herds in Southern Italy.

4. Discussion

Pestivirus infections represent one of the main causes of economic losses for cattle herds worldwide. BVDV infection in cattle has a different outcome based on the age, and the immunological and reproductive status of infected animals. This includes subclinical infections, immunosuppression, acute diarrhea, respiratory disease, reproductive disorders, and mucosal disease in persistently infected calves (Baker, 1995; Ridpath, 2010). While BVDV-1 and BVDV-2 have been known to circulate in cattle for some decades, HoBi-like pestivirus emerged as bovine pathogen only a few years ago (Bauermann et al., 2013). Reports on HoBi-like pestivirus infections and related diseases are progressively increasing (Cortez et al., 2006; Kampa et al., 2010; Decaro et al., 2011, 2012a,c, 2013a, 2014; Weber et al., 2014; Haider et al., 2014; Mishra et al., 2014), and it is not clear if the existing diagnostic assays are able to detect efficiently this emerging group of pestiviruses (Schirrmeyer et al., 2004; Decaro et al., 2010). Although there are several molecular methods for diagnosis of pestivirus infection (Bludevi and Weinstock, 2003; Young et al., 2006; La Rocca and Sandvik, 2009; Yan et al., 2011; Fan et al., 2012; Zhang et al., 2014), only some assays have been demonstrated to be able to detect HoBi-like pestiviruses (Bauermann et al., 2013, 2014).

In this paper, we developed a panpestivirus real-time RT-PCR assay, based on the TaqMan chemistry, which was able to detect with the same efficiency all bovine pestiviruses, including the emerging HoBi-like group. As all the tested HoBi-like viruses belonged to the southern American lineage (Decaro et al., 2012b and unpublished data), other genetic lineages should be analysed to validate further the assay. However, based on the nucleotide alignment, other genetic lineages, including the Thai lineage, should be recognized by the primers and probes designed in this study (Fig. 1).

The assay was sensitive and reproducible, showing linearity over nine orders of magnitude. In comparison with a panpestivirus gel-based assay (Vilcek et al., 1994), the TaqMan assay was 1-log more sensitive. The two-step technique indicates a separate RT step followed by real-time PCR. This makes it possible sample cross-contamination when opening the tubes to aspirate cDNA. However, this risk is minimized as the copies of reverse-transcribed genomic RNA are much lower than the DNA molecules generated in PCR.

In addition, the real-time RT-PCR specificity is increased by the probe hybridization and considering that some steps are automated, the throughput could be very high. These advantages make the developed TaqMan assay an attractive tool for the laboratory diagnosis of pestivirus infection, mainly in extensive epidemiological surveys and in the eradication plans with the goal of identifying and slaughtering PI animals.

Using an IC allowed to rule out false-negative results and to identify three EDTA–blood samples where PCR amplification was inhibited. An exogenous IC was selected instead of an endogenous IC, such as glyceraldehydes-3-phosphate dehydrogenase, 185rRNA, or β-actin, since bovine RNA cannot be detected easily in cell-free samples (such as urines). However, even when not using any IC, dilution of extracts from samples rich in DNA, such as EDTA–blood, should prevent PCR inhibition.

An interesting finding of our study was that the Vilcek’s gel-based RT-PCR assay was able to detect all HoBi-like pestivirus positive specimens with the exception of two EDTA–blood samples. This protocol had been found to fail or to detect with low efficiency HoBi-like strains due to the presence of a mismatch at the 3′ end of primer 324 that prevents the correct primer annealing (Bauermann et al., 2013). Whether the higher efficiency observed in our study was due to a higher complementarity of the primer binding region of the Italian strains to primer 324 is unclear, although the HoBi-like strains circulating in Italy are strictly related to the Southern American viruses (Decaro et al., 2011, 2012a,c, 2013a,b).

In conclusion, the developed real-time RT-PCR assay represents a new reliable and effective tool for rapid and sensitive diagnosis of infections caused by all pestiviruses circulating in cattle, including the emerging group of HoBi-like viruses.

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