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Multiplex real-time RT-PCR assay for bovine viral diarrhea virus type 1, type 2 and HoBi-like pestivirus

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A B S T R A C T

HoBi-like pestiviruses are emerging pestiviruses that infect cattle causing clinical forms overlapping to those induced by bovine viral diarrhea virus (BVDV) 1 and 2. As a consequence of their widespread distribution reported in recent years, molecular tools for rapid discrimination among pestiviruses infecting cattle are needed. The aim of the present study was to develop a multiplex real-time RT-PCR assay, based on the TaqMan technology, for the rapid and unambiguous characterisation of all bovine pestiviruses, including the emerging HoBi-like strains. The assay was found to be sensitive, specific and repeatable, ensuring detection of as few as 10^0–10^1 viral RNA copies. No cross-reactions between different pestiviral species were observed even in samples artificially contaminated with more than one pestivirus. Analysis of field samples tested positive for BVDV-1, BVDV-2 or HoBi-like virus by a nested PCR protocol revealed that the developed TaqMan assay had equal or higher sensitivity and was able to discriminate correctly the viral species in all tested samples, whereas a real-time RT-PCR assay previously developed for HoBi-like pestivirus detection showed cross-reactivity with few high-titre BVDV-2 samples.

1. Introduction

The genus *Pestivirus* belongs to the *Flaviviridae* family and includes 4 recognised species: *Bovine viral diarrhea virus 1* and *2* (BVDV-1 and BVDV-2), *Classical swine fever virus* (CSFV), and *Border disease virus* (BDV) (Simmonds et al., 2011).

Pestiviruses have a positive single-stranded RNA genome, approximately 12.3 kb in length, composed by a single open reading frame (ORF), preceded and followed by 2 untranslated regions (5′ and 3′ UTR). The single ORF encodes for a polyprotein that gives origin to 12 smaller proteins by viral cleavage: N^{pro}, C, E^{rns}, E1, E2, p7, NS2/NS3, NS4A, NS4B, NS5A, NS5B (Simmonds et al., 2011). Among all the genomic regions, the 5′UTR, N^{pro} and E2 are widely used for comparison and phylogenetic analysis (Bauermann et al., 2013). On the basis of the capacity to cause a cytopathic effect (cpe) in cell cultures, two BVDV biotypes are known, cytopathogenic (cp) and non-cytopathogenic (ncp), both involved in the pathogenesis of mucosal disease (MD), a fatal outcome of BVDV infection in persistently infected (PI) calves (Brownlie et al., 1984; Bolin et al., 1985).

Pestivirus infection leads to significant economic losses worldwide showing a wide range of clinical signs, including mild upper respiratory signs, a transient decrease in circulating white blood cells, and a low-grade, short-term fever. However, there are virulent strains that cause severe respiratory disease, gastroenteric disorders, hemorrhagic syndrome, and pneumonia (Baker, 1995; Brownlie, 1990; Corapi et al., 1989). Reproductive disorders represent one of the most important consequences of the BVDV infection (Houe et al., 2006). Infection of pregnant cows during the first trimester of gestation with ncp BVDV strains, leads to failure of fertilization, return to estrus, abortion, congenital malformations, stillbirths, or the birth of PI animals which may appear normal or sometimes smaller and with congenital malformations. They are constantly viremic and BVDV seronegative, representing the main source of BVDV infection in the herd through their body fluids, so that eradication programs rely on the detection and slaughtering of these animals (Bauermann et al., 2013).

Because of their RNA nature, pestivirus genomes accumulate several mutations during replication that may lead to the emergence of new strains, lineages or species (Kirkland et al., 2007;

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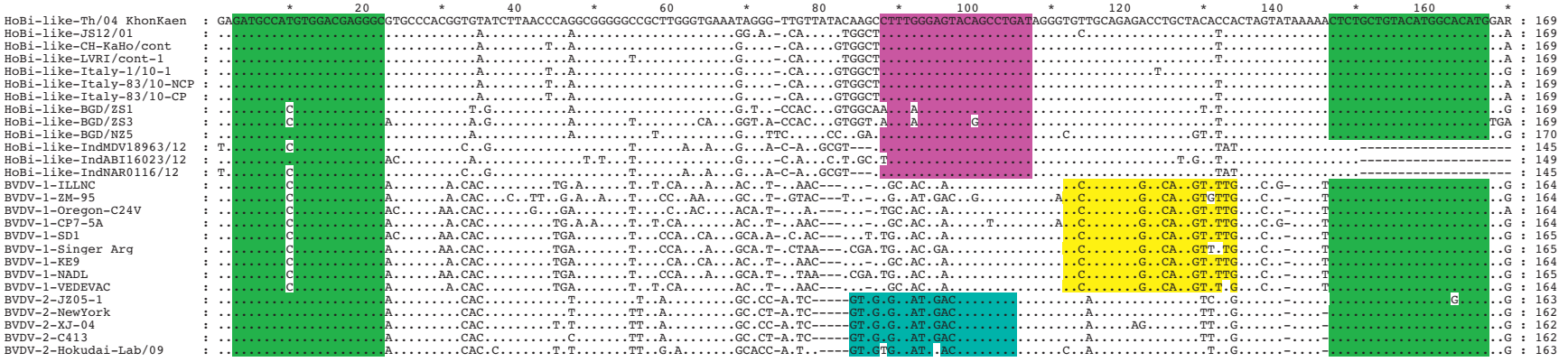

Fig. 1. Nucleotide alignment of reference pestiviruses showing the binding region of oligonucleotides used in the multiplex real-time RT-PCR assay.

Schirrmeier et al., 2004; Vilcek et al., 2005). Recently, additional four pestivirus species have been proposed within the genus *Pestivirus*: Pestivirus of giraffe, Pronghorn virus, Bungowannah virus, and HoBi-like pestivirus (Bauermann et al., 2013). The prototype strain (HoBi_D32/00) of this emerging group of pestiviruses, also known as BVDV-3 or atypical pestiviruses (Larska et al., 2012; Liu et al., 2009), was detected as contaminant of a batch of fetal bovine serum (FBS) imported from Brazil (Schirrmeier et al., 2004). Natural infections by HoBi-like pestiviruses have been reported in South America (Cortez et al., 2006; Weber et al., 2014), Asia (Kampa et al., 2010; Haider et al., 2014; Mishra et al., 2014) and Italy (Decaro et al., 2011, 2012a, 2012c, 2013a, 2013b, 2014). The virus has been associated to respiratory disease (Decaro et al., 2011, 2012c, 2013a) and abortions (Decaro et al., 2012a).

Several molecular methods are available for the detection and characterisation of pestiviruses. Most of them either do not detect HoBi-like pestiviruses at all or detect them with low efficiency (Schirrmeier et al., 2004; Ståhl et al., 2007, 2010). A recently developed real-time RT-PCR assay is able to detect HoBi-like pestiviruses without providing any simultaneous detection of BVDV-1 and BVDV-2 (Liu et al., 2008). However, the oligonucleotides used in this assay cross-react with high-titre BVDV-2 samples (Decaro et al., 2012b, 2013b). On the other hand, the nested PCR approach established by Sullivan and Akkina (1995) misclassifies HoBi-like viruses as BVDV-2 (Decaro et al., 2012b). In order to detect all pestiviruses infecting cattle using a single tool, a novel real-time RT-PCR assay has been recently developed (Losurdo et al., 2015). In addition, a nested PCR (nPCR) protocol has been set up for pestivirus typing (Decaro et al., 2012b). This assay has been proven to be specific as no cross-reactions between BVDV-1, BVDV-2 and HoBi-like pestiviruses were observed. However, the test is time consuming and labor intensive and can be exposed to a high risk of cross-contamination due to the post-PCR manipulations. To overcome these limitations, we have developed a multiplex real-time RT-PCR assay for simultaneous detection of the different species of bovine pestiviruses, including the emerging HoBi-like group, allowing a rapid, sensitive and specific diagnosis of pestivirus infection and characterisation of the viral species.

2. Materials and methods

2.1. Primers and probes design

The full-length genome of reference strains of BVDV-1, BVDV-2, HoBi-like pestivirus, BDV and CSFV were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html) and aligned using the BioEdit software package (Hall, 1999).

Forward and reverse primers were designed using Primer3 software, version 4.0 (http://frodo.wi.mit.edu/primer3/) to amplify a 164-nt fragment of the 5′UTR conserved region of all aligned pestiviruses. Three specific probes to detect BVDV-1, BVDV-2 and HoBi-like pestivirus were designed using Beacon Designer Software, version 2.06 (Premier Biosoft International, Palo Alto, CA, USA) (Fig. 1). Primers and probes were synthesised by Eurofins Genomics and are reported in Table 1.

2.2. Virus isolates and field samples

Pestivirus strains BVDV-1 NADL (courtesy of Dr Ferrari, Istituto Zooprofilattico Sperimentale di Lombardia ed Emilia Romagna, Brescia, Italy), BVDV-2 232/02 (Decaro et al., 2004), HoBi-like strain 1/10-1 Italy (Decaro et al., 2011), BDV BD91 (Buonavoglia et al., 1994), and an RNA extract of the CSFV lapinised Chinese vaccine (courtesy of Istituto Zooprofilattico Sperimentale di Lombardia ed Emilia Romagna, Brescia, Italy) were used to evaluate the specificity of the test. To rule out any cross-reactivity between bovine pestiviruses and other bovine viral pathogens, isolates of the following viruses were also tested: bovine coronavirus (Decaro et al., 2008), bovine rotaviruses (Pratelli et al., 1999), bovine respiratory syncytial virus (vaccine strain BRSV/375, CattleMaster 4, Zoetis Italia srl), bovine parainfluenza virus (vaccine strain TS RLB 103, CattleMaster 4, Zoetis Italia srl), and bovine herpesvirus types 1 (Thiry et al., 2006) and 4 (Tempesta et al., 1996).

A total of 159 pestivirus positive field samples were analysed. Ninety-eight samples were recruited from a previous study and had been already characterised by the nPCR assay (Decaro et al., 2012b), whereas additional 61 specimens were detected more recently during an epidemiological survey for HoBi-like pestivirus (unpublished data). The analysed samples included 28 tissue samples from aborted fetuses, 23 respiratory specimens from calves with respiratory disease, 19 fecal samples from calves with enteritis and 89 EDTA-blood samples from PI animals.

RNA was extracted from all samples using QIAamp®cador®Pathogen Mini Kit (Qiagen S.p.A., Milan, Italy), according to manufacturer's instructions.

2.3. Standard RNA for absolute quantification

RNA standards for BVDV-1, BVDV-2 and HoBi-like pestivirus were obtained amplifying a fragment of the 5′UTR region of reference strains BVDV-1 NADL, BVDV-2 232/06 and HoBi-like pestivirus Italy-1/10-1, using the common forward primer 324 (Vilcek et al., 1994) and three different pestivirus specific reverse primers (BVD1-690R: 5′-TCTATGCACACATAAATGTGGTA-3′, BVD2-657R: 5′-ACTACCGGTCACTCTGCCAACTCTCCTA-3′, BVD3-692R: 5′-TCGGTACACACATACATGTGATA-3′), designed using Primer3 software, version 0.4.0.

The RT-PCR products were cloned into TOPO® XL PCR Cloning vector (Invitrogen Srl, Milan, 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 QIAamp® RNA Easy kit (Qiagen S.p.A., Milan, Italy) and quantified by spectrophotometric analysis. Ten-fold dilutions of the RNA transcripts, representing 10^0 to 10^9 copies RNA μl^{-1} of template, were carried out in a mixed fecal/nasal swab suspension from a calf that tested PCR negative for pestivirus RNA (Sullivan and Akkina, 1995; Decaro et al., 2012b). Aliquots of each dilution were frozen at −70 °C and used only once.

2.4. Multiplex real-time RT-PCR

Reverse transcription of 1 μl of duplicates of the standard dilutions and RNA extracts was carried out using GeneAmp® RNA PCR kit (Life Technologies Italia Applera Italia, Monza, Italy) in a 20-μl reaction volume containing PCR buffer 1× (KCl 50 mM, Tris–HCl 10 mM, pH 8.3), $MgCl_2$ 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. Reverse-transcription was carried out at 42 °C for 30 min, followed by a denaturation step at 99 °C for 5 min.

The triplex real-time PCR targeting the 5′UTR gene of BVDV-1, BVDV-2 and HoBi-like pestivirus 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, Milan, Italy), 600 nM of primers Pesti-qF and Pesti-qR, 200 nM of probes BVD1-Pb and BVD3-Pb and 400 nM of probe BVD2-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 and annealing/extension at 60 °C for 1 min. The detection of the

Table 1
Primers and TaqMan probes used in the multiplex real-time RT-PCR assay for discrimination of bovine pestiviruses and other oligonucleotides used in the study.

Assay	Reference	Primer/probe	Sequence 5′-3′	Sense	Position	Amplicon size (bp)
Multiplex real-time RT-PCR	This study	Pesti-qF	GATGCCATGTGGACGAGGGC	+	229–248[a], 233–252[b], 116–135[c]	160[a], 156[b], 164[c]
		Pesti-qR	CATGTGCCATGTACAGCAGAG	–	368–388[a], 368–388[b], 259–279[c]	
		BVD1-Pb	FAM- CAATACAGTGGGCCTCTGCAGCA-TAMRA	–	341–363[a]	
		BVD2-Pb	VIC-GTGGCGTTATGGACACAGCCTG-BHQ2	+	307–328[b]	
		BVD3-Pb	TexasRed-ATCAGGCTGTACTCCCAAAG-BHQ2	–	200–219[c]	
Nested PCR	Decaro et al., 2012b	PanBVDVpcrF	CTCTGCTGTACATGGCACATG	+	368–388[a], 368–388[b], 259–279[c]	1016[a], 1016[b], 1013[c]
		PanBVDVpcrR	CGTCGAACCAGTGACGACT	–	1364–1383[a], 1364–1383[b], 1252–1271[c]	
		BVDV-1 npcrF	TTTCAAGCTGCTCHGAYAC	+	879–897[a]	505
		BVDV-2 npcrF	ATCCTGACCAATGCTAGGTCC	+	551–571[b]	833
		BVDV-3 npcrF	TCCTGTGGCAACCGGTAGGT	+	1061–1080[c]	211
HoBi-like pestivirus real-time RT-PCR	Liu et al., 2008	T134-F	GACTAGTGGTGGCAGTGAGC	+	27–46	87
		T220R	GAGGCATTCCTTGATGCGTC	–	94–113	
		T155r-P	FAM-ACTCGGGGCTTCGGTGATCCAGGG-BHQ1	–	48–71	
IC Real-time RT-PCR	Decaro et al., 2005	CCoV-For	TTGATCGTTTTTATAACGGTTCTACAA	+	6585–6611[d]	99
		CCoV-Rev	AATGGGCCATAATAGCCACATAAT	–	6660–6683[d]	
		CCoV-Pb	FAM-ACCTCAATTTAGCTGGTTCGTGTATGGCATT-TAMRA	+	6620–6650[d]	

IC, internal control.
[a] Oligonucleotide position is referred to the sequence of BVDV-1 strain NADL (GenBank accession no. M31182).
[b] Oligonucleotide position is referred to the sequence of BVDV-2 strain New York (GenBank accession no. AF502399).
[c] Oligonucleotide position is referred to the sequence of HoBi-like pestivirus strain Italy-1/10-1 (GenBank accession no. HQ231763).
[d] Oligonucleotide position is referred to the sequence of CCoV-II strain Insavc-1 (GenBank accession no. D13096).

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).

In order to verify the absence of RNA losses during the extraction step and the presence of RT-PCR inhibitors in the RNA templates, an internal control (IC), consisting of an RNA synthetic transcript containing the M gene of canine coronavirus (CCoV) type II (Decaro et al., 2005), was added to the lysis buffer (AVL buffer, QIAGEN S.p.A.) at a concentration of 10,000 RNA copies ml^{-1} of buffer prior to nucleic acid extraction. The fixed amount of the IC added to each sample had been calculated to give a mean C_T 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 C_T value for the IC was >35.48 (average plus 2 S.D.) were excluded from the analysis.

2.5. Specificity, sensitivity and repeatability of the multiplex real-time RT-PCR

Specificity of the assay was evaluated by testing pestivirus reference strains and other bovine viruses including bovine respiratory syncytial virus, bovine coronavirus, bovine rotavirus, bovine herpesvirus 1 and 4 and bovine parainfluenza virus.

Serial ten-fold dilutions of the BVDV-1, BVDV-2 and HoBi-like pestivirus standards containing from 10^0 to 10^9 copies of RNA transcripts and the correlate C_T values were used to set the standard curves for respective absolute quantifications.

Bovine nasal and faecal swabs and EDTA-blood samples that had tested negative for pestivirus and distilled water were used as negative controls and blank, respectively.

The sensitivity of the multiplex real-time RT-PCR assay was evaluated using 10-fold dilutions of EDTA-blood samples containing about 10^7, 10^6 and 10^7 copies of BVDV-1, BVDV-2 and HoBi-like pestivirus RNA, respectively, made in a EDTA-blood sample from a calf tested negative for BVDV. The same sample dilutions were submitted to nPCR (Decaro et al., 2012b) and to the HoBi-like TaqMan assay (Liu et al., 2008) for a comparison.

Intra-assay repeatability was evaluated testing 10 times the same samples in one experiment, and the inter-assay repeatability was verified repeating the experiment 10 times. Clinical samples containing virus amounts spanning the whole sensitivity limits of the multiplex real-time RT-PCR assay were selected for repeatability evaluation. Coefficients of variation (CVs) were calculated by dividing the standard deviation of each tested sample by its mean and multiplying that result by 100.

2.6. Nested PCR

The detection of pestivirus RNA in clinical samples and RNA transcript dilutions was carried out using a nPCR protocol previously developed for the characterisation of bovine pestiviruses (Decaro et al., 2012b). First- and second-step amplifications

were carried out using SuperScript™ One-Step RT-PCR for Long Templates (Life Technologies Italia) and AmpliTaq Gold (Life Technologies Italia), as previously described (Decaro et al., 2012b). Oligonucleotides are reported in Table 1.

2.7. HoBi-like pestivirus real-time RT-PCR

To compare the performance of the developed triplex assay with the only existing real-time RT-PCR assay claimed to detect specifically this group of viruses, all clinical samples were tested by means of the Liu's assay (Liu et al., 2008). Reverse transcription and real-time PCR were carried out as described for the triplex assay using oligonucleotides listed in Table 1.

3. Results

3.1. Performances of the multiplex real-time RT-PCR assay

No fluorescence signal was detected from either negative controls or distilled water and all of the other bovine pathogens, including the related pestiviruses BDV and CSFV, were not detected by the developed multiplex real-time RT-PCR. The assay was proven to be species specific since BVDV-1, BVDV-2 and HoBi-like pestivirus were correctly detected by the specific probes and no cross-reaction between the three pestiviruses was observed. The standard curves generated for each pestivirus using ten-fold dilutions of standard RNA covered a linear range of at least nine orders of magnitude (from $10^0/10^1$ to 10^9 copies of standard RNA) and linearity was observed over the entire quantification range (slopes of −3.294, −2.977 and −3.412 for BVDV-1, BVDV-2 and HoBi-like pestivirus, respectively). Coefficients of regression (R^2) were 0.996, 0.994 and 0.997 for BVDV-1, BVDV-2 and HoBi-like pestivirus, respectively.

The sensitivity of the assay was set at 10^0 RNA copies for BVDV-1 and at 10^1 RNA copies for BVDV-2 and HoBi-like pestivirus. Nested PCR had the same sensitivity in the case of BVDV-2 and HoBi-like pestivirus, but it was 1-log less sensitive than the TaqMan assay when BVDV-1 was processed. In addition, the specific TaqMan assay by Liu et al. (2008) was able to detect as few as 10^1 HoBi-like pestivirus RNA copies.

The repeatability was evaluated by calculating the intra- and interassay CVs. BVDV-1 CVs ranged from 9.48% (samples containing 4×10^2 copies RNA μl^{-1}) to 53.13% (1.8×10^3 copies RNA μl^{-1}); BVDV-2 CVs varied from 23.66% (1×10^5 copies RNA μl^{-1}) to 50.38% (4×10^3 copies RNA μl^{-1}); HoBi-like pestivirus CVs were between 9.01% (8×10^2 copies RNA μl^{-1}) and 21.50% (6×10^4 copies RNA μl^{-1}). Interassay CVs ranges were comprised between 24.89% (5×10^2 copies RNA μl^{-1}) and 47.65% (2×10^4 copies RNA μl^{-1}) for BVDV-1, between 16.63% 1×10^5 copies RNA μl^{-1}) and 49.68% (2×10^2 copies RNA μl^{-1}) for BVDV-2; between 13.18% (8×10^2 copies RNA μl^{-1}) and 16.73% (6×10^4 copies RNA μl^{-1}) for HoBi-like pestivirus.

The IC was detected in all the examined samples, with C_T values below the threshold value of 34.76, thus confirming the absence of RNA losses during nucleic acid extraction or DNA polymerase inhibition during real-time PCR.

In order to rule out any interference between the species-specific TaqMan probes contained in the same mix, a pestivirus negative EDTA-blood sample was also spiked with low (10^3 copies) and high (10^8 copies) concentrations of standard RNA of BVDV-1, BVDV-2 and HoBi-like pestivirus and the viral loads in the spiked samples were quantified using the developed assay. The RNA titres of the pestivirus species were calculated correctly, showing that no interference occurred during detection and quantitation of the different viruses contained in the same sample (data not shown).

3.2. Analysis of field samples

By analysis of 159 field samples tested positive by nPCR (Decaro et al., 2012b), there was a perfect agreement between gel-based and real-time RT-PCR assays. BVDV-1 and BVDV-2 were detected in 103 and 15 samples, respectively, whereas 41 specimens that had been collected from two different cattle herds in southern Italy tested positive for HoBi-like pestivirus.

The amount of RNA detected in the samples covered a wide range of copies per microlitre of template, ranging from 4.02×10^1 to 7.26×10^7 (BVDV-1), from 5.78×10^1 to 8.45×10^6 (BVDV-2) and from 3.72×10^1 to 2.75×10^7 (HoBi-like pestivirus).

The Liu assay (Liu et al., 2008) was able to type correctly all HoBi-like strains and did not recognise any of the BVDV-1 positive samples, but 3 out of 15 BVDV-2 strains were mistyped as HoBi-like viruses. The BVDV-2 titres of the mistyped samples were above 10^6 RNA copies μl^{-1} of template.

4. Discussion

Several PCR-based methods have been developed for sensitive and rapid detection of BVDV in clinical samples (Bhudevi and Weinstock, 2003; Young et al., 2006; La Rocca and Sandvik, 2009; Yan et al., 2011; Fan et al., 2012; Zhang et al., 2014; Losurdo et al., 2015), but only few methods are currently available for unambiguous discrimination between BVDV-1 and BVDV-2 (Sullivan and Akkina, 1995; Letellier and Kerkhofs, 2003; Baxi et al., 2006; LeBlanc et al., 2010). The emergence of HoBi-like pestivirus that causes clinical pictures overlapping those induced by BVDV-1 and BVDV-2 (Bauermann et al., 2012, 2013, 2014), posed several concerns as for the ability of existing diagnostic methods to efficiently detect this emerging group of pestiviruses (Schirrmeier et al., 2004). The panpestivirus RT-PCR developed by Vilcek et al. (1994), which is commonly used for BVDV molecular screening, fails to detect or detects 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. Other conventional and real-time RT-PCR protocols are able to detect the novel pestivirus but do not provide any virus characterisation, which is helpful to assess virus epidemiology (Elvander et al., 1998; Letellier et al., 1999; Gaede et al., 2005; Hoffmann et al., 2006; Losurdo et al., 2015). A TaqMan assay that was claimed to be specific for HoBi-like pestivirus was recently developed (Liu et al., 2008), but this assay could not discriminate simultaneously BVDV-1 and BVDV-2 and showed partial cross-reaction with high-titre BVDV-2 samples (Decaro et al., 2012b, 2013b). This cross-reaction was confirmed by the present study since 3 out 15 BVDV-2 positive samples reacted as HoBi-like strains when tested by the Liu's assay.

Recently, a nPCR assay was established for simultaneous discrimination of all pestiviruses infecting cattle, including HoBi-like pestivirus (Decaro et al., 2012b). This method was specific and reliable, but also cumbersome as it requires two separate steps, an RT-PCR followed by the nested amplification; in addition, it presents a certain risk of cross-contamination between positive and negative samples.

To overcome the limitations of existing methods, we have developed a triplex real-time RT-PCR assay, based on the TaqMan chemistry, which was able to differentiate efficiently BVDV-1, BVDV-2 and HoBi-like pestivirus. The assay was proven to be repeatable and linear over a range of at least 9 orders of magnitude, from $10^0/10^1$ to 10^9 RNA copies, thus ensuring an accurate measurement of pestivirus RNA loads in clinical samples. In comparison with nPCR, the real-time RT-PCR assay was equally or slightly more sensitive and less time-consuming. Labeling the species-specific probes with different fluorophores enables a correct

characterisation of the pestiviral strains contained in clinical samples. In addition, the 96-well format of the real-time PCR plates ensures a high throughput, with the chance to test simultaneously several samples, which is useful for large-scale epidemiological surveys. The developed assay is a closed system in which the tube is never opened post-amplification, and this reduces the possibility of cross-contamination of new samples with previously amplified products. A certain carryover may occur due to the separation between RT and fluorogenic PCR, but we preferred a two-step assay, since one-tube methods are less sensitive than a two-step RT-PCR procedure (Nakamura et al., 1993; Bustin, 2000) and the risk of RNA degradation is increased if analyses are performed over a long period of time (Postollec et al., 2011). In order to further reduce the risk of contamination, we have strictly separated the different working steps and carried out pipetting in different laminar flow hoods. In addition, the two-step assay requires more work, thus reducing the laboratory capacity. Another limitation of the study is that the analysed pestiviral strains were geographically homogeneous, so that theoretically less common subtypes or divergent viruses circulating in different countries could not be correctly characterised. However, sequence alignment of the oligonucleotide binding regions showed that they are conserved within the same viral species for BVDV-1 and BVDV-2. As for HoBi-like viruses, even the more divergent strains recently identified in Asia (Mishra et al., 2014; Haider et al., 2014) displayed only few mismatches, which should be tolerated by the oligonucleotides, including the TaqMan probe (Fig. 1). Anyway, the assay needs to be validated with those divergent strains that seem to be widespread in the Asian continent. The developed assay does not recognise BDV, which was recently detected in cattle (Strong et al., 2010; Braun et al., 2013). However, at the moment the epidemiological situation does not require including BDV in the diagnostic algorithm for pestivirus detection in cattle.

In conclusion, the triplex TaqMan assay can be used in extensive epidemiological surveys, thus helping assess the best prophylactic measures against pestiviruses infecting cattle and facilitating eradication programmes against these viruses, which are responsible for marked economic losses in cattle herds worldwide.

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