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Development and evaluation of real-time RT-PCR using ear hair for specific detection of sheep persistently infected with border disease virus (BDV)

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ABSTRACT

The aim of this study was to develop an improved border disease virus (BDV) specific real time RT-PCR and to evaluate its performance on manually plucked hairs from sheep persistently infected with BDV that may act as a non-invasive alternate sample. The BDV real time RT-PCR assay reported here showed a high analytical sensitivity ($10^{0.6}$ TCID₅₀/ml), specificity (no reactivity with BVDV-1, BVDV-2, HoBi-like pestivirus and CSFV) and reproducibility. When the assay was validated on 210 samples from BDV-infected and uninfected sheep, it showed a 100% diagnostic sensitivity and specificity with virus isolation. Further evaluation of the assay on manually plucked hair follicles from ear (mid-lateral, mid-medial) and tail tip from sheep persistently infected with BDV showed that a minimum of 20 hair follicles need to be tested for correct diagnosis of BDV. The BDV load was comparatively higher in hairs from mid-medial ear than those from other tested locations. Evaluation on other samples from PI sheep demonstrated that the test performance was similar to that of pestivirus generic real-time RT-PCR, but improved than the currently available BDV specific real-time RT-PCR. Although more number of PI animals need to be evaluated, the results of the study showed that manually plucked hairs from mid-medial ear pinna is a suitable alternative sample in real-time RT-PCR for detection of BDV persistently infected sheep. Use of the non-invasive ear hair samples and the improved BDV specific real-time RT-PCR reported here may be useful for BDV surveillance in several sheep rearing countries.

1. Introduction

Border disease (BD) is primarily a viral disease of sheep, and occasionally of goats, cattle and wild ruminants. It causes significant economic losses in sheep farming and has a worldwide distribution (Nettleton et al., 1998). The economic losses are mainly due to losses associated with increased barrenness, abortion, still birth, weak lambs, lambs with tremor and abnormal fleece and neonatal death in the flock. Although originally BD was thought to be caused by border disease virus (BDV), it is now well known that BD can be caused by border disease virus (species *Pestivirus D*), bovine viral diarrhoea virus 1 (species *Pestivirus A*) and bovine viral diarrhoea virus 2 (species *Pestivirus B*) (Chappuis et al., 1984; Pratelli et al., 2001; Mishra et al., 2012), which belong to the genus *Pestivirus* in the family *Flaviviridae* (Smith et al., 2017). Besides, HoBi-like pestivirus (species *Pestivirus H*) can also infect and cause disease in sheep under experimental (Bauer mann et al., 2015; Decaro et al., 2015) and natural (Shi et al., 2016) conditions. Although mostly inapparent or mild, severe disease with high morbidity and

mortality has also been observed in outbreaks in sheep caused by BDV Aveyron strain (Chappuis et al., 1984; Vega et al., 2015) and in Pyrenean Chamois by BDV-4 strain (Arnal et al., 2004; Marco et al., 2007). There is considerable genetic heterogeneity among BDV strains, and so far eight genotypes (BDV-1 to BDV-8) have been identified apart from Tunisian and Turkish genetic groups (Becher et al., 2003; Dubois et al., 2008; Hornberg et al., 2009; Peterhans et al., 2010; Giammarioli et al., 2015; Mishra et al., 2016).

Similar to other pestiviruses, BDV genome consists of a single-stranded positive-sense RNA of about 12.3 kb in length. A single open reading frame codes for four structural proteins (C, E^{ns}, E1 and E2) and seven to eight non-structural proteins (N^{pro}, p7, NS2-3, NS4A, NS4B, NS5A, NS5B), and is flanked by 5'- and 3'-untranslated regions (UTR) (Meyers and Thiel, 1996). As 5'-UTR is highly conserved among the pestiviruses and partial 5'-UTR is efficiently amplified by RT-PCR from field samples, this region has been used widely for the development of nucleic acid based diagnostics.

Infection of pregnant ewes with BDV before the fetus becomes

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immunocompetent at about day 85 of pregnancy can lead to the birth of persistently infected (PI) animals that shed the virus throughout their lifetime and are the continuous source of infection within and among flocks (Nettleton et al., 1998). Since the main mode of BDV transmission is through close contact with PI animals and by vertical transmission from PI ewe, identification and removal of PI animals is crucial to prevent spread of infection and for control of BD. Identification of PI animals depends on the sensitivity and specificity of the diagnostic tests employed and the ease of sampling.

The most commonly used tests employed for diagnosis of BDV PI sheep are virus isolation, antigen ELISA or BDV specific real-time RT-PCR (OIE, 2017). However, virus isolation can be performed mostly in a reference laboratory, is laborious, time consuming and requires use of BDV specific mAbs or BDV specific RT-PCR for agent identification. On the other hand, due to lack of BDV Ag-ELISA kits, commercially available BVDV Ag-ELISA kits are being widely used for detection of BDV antigen, wherein low sensitivity and even failure of detection of BDV antigen in blood of sheep and even in some of the BDV isolates have been reported (Orr et al., 1993; Kittelberger and Pigott, 2008; Mishra et al., 2016). Moreover, due to cross reactivity between BDV, BVDV-1 and BVDV-2, pestivirus antigen positive sample requires further testing for differentiation and presence of colostral antibodies in young animals interfere with virus isolation and antigen ELISA. To overcome these problems, currently, BDV specific real-time RT-PCR assay is being used more commonly due to their high sensitivity, specificity and rapidity (Willoughby et al., 2006). However, moderate sensitivity of this assay in detection of BDV in blood of PI sheep has been observed in a recent study from India (Mishra et al., 2016), emphasizing the need for development of more sensitive BDV real time RT-PCR assay.

Although several sample matrices from PI animals may be used, whole blood and serum samples are more commonly used for identification of BDV in sheep. Ear notch samples from sheep are less commonly used for BDV testing due to the absence of ear tagging practice in most of the sheep rearing countries, unlike BVDV in cattle. However, both are invasive techniques and require involvement of a qualified veterinarian and willingness of the owner during sampling. Moreover, it is difficult to convince the livestock owners, especially in developing countries, the utility of blood sampling in apparently healthy PI sheep. A recent study has shown utility of manually plucked hair follicles from ear of PI calves in BVDV diagnosis (Singh et al., 2011) but no report is available regarding its usefulness for detection of BDV in sheep. BDV is prevalent in sheep in India (Mishra et al., 2016). Hence, the aim of this study was to develop a BDV specific real-time RT-PCR assay and to evaluate its performance on manually plucked ear hairs collected from a sheep persistently infected with BDV at different time intervals so that this non-invasive sample may be of practical significance for detection of BDV.

2. Materials and methods

2.1. Viruses and cells

Pestivirus strains [3 BDV strains of BDV-3 genotype, 12 BVDV-1 strains (7 strains of BVDV-1b genotype and 5 strains of BVDV-1c genotype), 3 BVDV-2 strains (2 strains of BVDV-2a genotype and 1 strain of BVDV-2b genotype), 4 strains of HoBiPeV (3 strains of HoBiPeV-c and 1 strain of HoBiPeV-d) and 6 CSFV strains (CSFV 1.1 genotype) were obtained from the pestivirus repository of ICAR-National Institute of High Security Animal Diseases, Anand Nagar, Bhopal, India (Table 1). BDV-3 isolate, Ind 830-09 (Mishra et al., 2016) was propagated on pestivirus free sheep foetal thymus (SFT-R) cells (Cell Culture Collection of Veterinary Medicine, Friedrich-Loeffler Institute, Island of Riems, Germany) using EMEM containing 10% horse serum as described earlier (Mishra et al., 2016) and titre of the virus stock was determined and stored at -80°C until use. Additionally, a plasmid

Table 1

Pestivirus strains and negative controls used in the study for specificity testing of BDV real time RT-PCR (this study).

S.No	Species	Virus name	Genotype	strain	Real time RT-PCR
1	Pestivirus A	BVDV-1	BVDV-1b	Ind S-17555	Negative
2			BVDV-1b	Ind S-1449	Negative
3			BVDV-1b	Ind S-1226	Negative
4			BVDV-1b	Ind S-11580	Negative
5			BVDV-1b	Ind S-11600	Negative
6			BVDV-1c	Ind S-10241	Negative
7			BVDV-1c	Ind S-18119	Negative
8			BVDV-1c	Ind A12	Negative
9			BVDV-1b	Ind S-15734	Negative
10			BVDV-1b	Ind S-15815	Negative
11			BVDV-1c	Ind KG27	Negative
12			BVDV-1c	Ind 390	Negative
13	Pestivirus B	BVDV-2	BVDV-2a	Ind 141353	Negative
14			BVDV-2a	Ind 3012339	Negative
15			BVDV-2b	Ind 51966	Negative
16	Pestivirus H	HoBiPeV	HoBiPeV-c	Ind ABII5385	Negative
17			HoBiPeV-d	Ind BHA5309	Negative
18			HoBiPeV-d	Ind NAR0116	Negative
19			HoBiPeV-d	Ind MDV18963	Negative
20	Pestivirus D	BDV	BDV-3	Ind830-09	Positive
21			BDV-3	Ind840-09	Positive
22			BDV-3	Ind850-09	Positive
23			BDV-4	BDV-4 5' UTR clone	Positive
24	Pestivirus C	CSFV	CSFV 1.1	Marigaon	Negative
25			CSFV 1.1	Kamrup	Negative
26			CSFV 1.1	Lokhra	Negative
27			CSFV 1.1	Khanpara	Negative
28			CSFV 1.1	Haflong	Negative
29			CSFV 1.1	Boko	Negative
30			Negative control	Negative control (MDBK cells)	Negative
31			Negative control	Negative control (SFT-R cells)	Negative
32			Negative control	Negative control (PBL)	Negative
33			Negative control	Negative control (Serum)	Negative
34			Negative control	Negative control (Ear hair follicles))	Negative

* In-vitro transcribed RNA was used for testing.

bearing 5'-UTR insert of genotype BDV-4 strain (gift from Ana L. Garcia Perez, Instituto Vasco de Investigación Agraria (NEIKER), 48,160 Derio, Vizcaya, Spain) (Table 1) was used to generate BDV-RNA by in vitro transcription as described earlier (Dubey et al., 2015).

2.2. Virus isolation and detection by IPMA

The SFT-R cells, tested free of pestivirus by real-time RT-PCR and maintained in Eagle's Minimum Essential Medium (EMEM; Sigma) containing 10% horse serum (Gibco-BRL Life Technologies) were used for virus isolation as described earlier (Mishra et al., 2016). All the samples (serum, leukocytes, nasal swab, oral swab, rectal swab and hair follicles) from sheep were inoculated onto sub-confluent monolayers of SFT-R cells in 6-well tissue culture plates. After 5 days, the cultures were frozen and thawed thrice and the clarified supernatant was subjected to immuno peroxidase monolayer assay (IPMA) using pan-pestivirus (WB103/105) and BDV specific (WS363) monoclonal antibodies as described earlier (Mishra et al., 2008).

2.3. Primers and probes

TaqMan probe for the BDV specific real-time RT-PCR was selected based on sequence alignment of the published sequences of the highly

conserved 5'-UTR of pestiviruses like BDV (X818-AF037405; Reindeer-AF144618; Ind 830-09-KT934377; Gifhorn-GQ902940; C121-DQ275625 and DQ273159; AV-EF693984; 90-F-6335-EF693990 I; 712/02-AJ829444), BVDV-1 (SD1-M96751, NADL-M31182), BVDV-2 (890-U18059), HoBi-like pestivirus (Th/04_Khonkaen-FJ040215), CSFV (Alfort-J04358), Tunisian sheep (SNIT-AY452484), Turkey sheep (Burdur/05-AM418428 and EU930015) and Giraffe (Giraffe-1-AF144617) using Clustal W program of DNASTAR computer program package (Lasergene Inc., Madison, USA) and designed using Primer Express software (Applied Biosystems). The specificity of the probe sequences were tested using BLAST searches (NCBI). Previously published primers 106 F and 179R (La Rocca and Sandvik, 2009) and newly designed BDV probe were synthesized commercially and used for development of BDV real-time RT-PCR. The sequences of the primers and probe are as follows: Forward primer (106 F): 5'-CCATRCCCDTGTAGGACTAGC-3' (position 96–117 nt of BDV reference strain X818); Reverse Primer (179R): 5'-GYGTYGAACTACTGACGACT-3' (position 179–198 nt of BDV strain X818); Probe (HSIND_BD): 5'-[6FAM]-ACTA GCCGTCGTGGTGAAATCCCTGAGTGG-[BHQ1]-3' (position 128–157 nt of BDV strain X818). Additionally, the primers and probes of previously reported BDV real-time RT-PCR (Willoughby et al., 2006) and pestivirus generic TaqMan real time RT-PCR (Hoffmann et al., 2006) were used for comparison in the study.

2.4. Extraction of viral RNA

Viral RNA was extracted from infected cell culture supernatants of pestivirus strains, serum and swabs or uninfected cell culture supernatants by QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) and from blood leukocytes using RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocols. The RNA was recovered in 30 μ l of RNase-free water and stored at -80°C until used. During optimization of real-time RT-PCR and determination of analytical sensitivity, RNA extracted from serial 10-fold dilutions of BDV-3 isolate Ind 830-09, of known titre ($10^{5.6}$ TCID₅₀/ml) was used.

RNA from hair follicles was extracted using RNeasy Mini kit (Qiagen) with minor modifications of a previously reported protocol (Singh et al., 2011). Briefly, each sample of hair follicles stored in 0.5 ml of RNA stabilization solution (RNA later, Ambion, USA) was grounded by vigorous vortexing for 2 min with 200 μ l of RLT buffer and sterile sand. RNA extraction was then carried out from 150 μ l of lysate as per the manufacturer's protocol with a final elution volume of 30 μ l and stored at -80°C until use.

2.5. Optimization of BDV specific real time RT-PCR

Optimization of BDV Real time RT-PCR assay was carried out using several combinations of primer and probe concentrations and RNA template volumes employing a commercial real time RT-PCR kit (SuperScript III Platinum one-step real time RT-PCR, Invitrogen) in Light Cycler 480 (Roche, USA). The assay was carried out in 25 μ l volume with the following thermal profile; one cycle of 50°C for 45 min, one cycle of 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 60 s with fluorescence acquisition. Each sample was tested in duplicate and appropriate positive and negative controls were included. The results were determined based on the analyses of Cp (crossing point) values.

2.6. Analytical sensitivity, specificity and reproducibility of the assay

To determine analytical sensitivity of the BDV real time RT-PCR, RNA extracted from serial 10-fold dilutions of BDV-3 isolate Ind 830-09, of known titre ($10^{5.6}$ TCID₅₀/ml) was used to construct the standard curve. The samples with same dilutions were also tested by virus isolation using SFT-R cells and panpestivirus specific real time RT-PCR (Hoffmann et al., 2006). The analytical specificity of the assay was

evaluated using RNA obtained from BVDV-1, BVDV-2, HoBiPeV, CSFV and BDV strains as described in Table 1.

The reproducibility of the assay was assessed by testing RNA from six serial ten-fold dilutions of BDV-3 isolate Ind 830-09 ($10^{5.6}$ TCID₅₀/ml) and performing assays in triplicate on six different days. The mean, standard deviation (S.D.) and coefficient of variation (C.V.) of Cp values were estimated using standard methods.

2.7. Diagnostic sensitivity and specificity of BDV real time RT-PCR

Diagnostic specificity of the assay was initially evaluated using 80 negative control samples (blood leukocytes, serum and nasal swab from sheep; uninfected cell culture supernatants) of known pestivirus negative status, verified earlier by virus isolation or RT-PCR.

Diagnostic validation of the assay was then performed on 210 samples (85 blood leukocytes, 96 sera and 29 tissues) from sheep and diagnostic sensitivity and specificity of the assay were determined. These samples were obtained either from archived collection of samples from BDV infected sheep and BDV uninfected sheep available at NIHSAD from our previous study or samples from sheep submitted to NIHSAD for pestivirus diagnosis.

2.8. Identification of sheep persistently infected with BDV (PI sheep), sample collection and processing

A six-month old male mutton breed sheep (No. 12) persistently infected with BDV (Ind-293299/12) was used in this study. Sheep belonging to a migratory sheep flock in Madhya Pradesh state in the year 2012, was initially found positive for BDV, by virus isolation and BDV specific real-time PCR described herein and negative for BDV antibodies by virus neutralization test (VNT) as described previously (Mishra et al., 2016). The sheep was found negative for BVDV-1 and BVDV-2 by real-time RT-PCR (Baxi et al., 2006). The PI status of the sheep was confirmed by collecting PBL and serum samples after one month interval and testing them employing the tests described above. The PI sheep was kept at an animal quarantine shed with appropriate veterinary care under standard management conditions and was used for sampling at various intervals, till completion of the study, as per the guidelines of Institutional Animal Ethics Committee (Approval no. 38/IAEC/HSADL/09) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. Molecular characterization of the BDV isolate from the PI sheep is subject of another study (Manuscript under review).

Various samples from the sheep (No. 12) persistently infected with BDV (Ind-293299/12) were collected aseptically which included whole blood with EDTA and without EDTA, nasal swab, oral swab, ocular swab, rectal swab and manually plucked hairs. After collection, the swabs (Himedia, India) were immediately submerged in 1 ml of sterile EMEM containing antibiotics in sterile tubes. For sampling of hairs, bunch of ear hair follicles (with root) were manually plucked aseptically from different locations of the ear (base, mid and tip of the ear pinna on both medial and lateral borders) and were placed in sterile tubes. Similarly, wool follicles from back of the body (loin region) and tip of the tail were also collected. Hairs without follicle from the same locations were also collected and all the samples were transported immediately to the laboratory by maintaining the cold chain at 4°C . Equivalent samples were also collected from three known pestivirus negative sheep. Sampling from the PI sheep was done thrice at an interval of 15 days.

Serum was separated from whole blood without EDTA, while peripheral blood leukocytes (PBL) were separated from EDTA blood as per standard protocol and stored at -80°C until use. All the swab samples were also stored at -80°C . Manually plucked hair follicles collected in tubes were transferred on sterile petri-plates and separated into four different groups, containing approximately 10, 20, 50 and 50–100 hair follicles of each. The hair samples were placed in tubes containing

0.5 ml of RNA stabilization solution (RNA later, Ambion, USA) for viral RNA extraction and stored at -80°C . Bunch of cut hair shafts (100) without follicles were also processed similarly.

2.9. Evaluation of BDV real time RT-PCR on samples from PI sheep

All the samples including manually plucked hairs collected at different intervals from PI sheep and negative controls were tested by BDV real time RT-PCR developed in this study. The same samples were also tested for BDV by panpestivirus real time RT-PCR (Hoffmann et al., 2006) and previously reported BDV real time RT-PCR (Willoughby et al., 2006) as described.

2.10. Statistical analysis

The relative diagnostic sensitivity and diagnostic specificity of BDV real time RT-PCR described herein were determined as compared with virus isolation, the gold standard test for BDV diagnosis. The concordance analyses (number of correct assessments/number of all assessments in percentage) were performed. Cohen's kappa (K) was used to estimate inter-assay concordance as suggested earlier (Landis and Koch, 1977). The viral load in various test samples including manually plucked hairs was assessed from the Cp values obtained by BDV real time RT-PCR from the standard curve.

3. Results

3.1. Analytical sensitivity of BDV real time RT-PCR

Following optimization, the assay was performed in a total volume of 25 μl containing 12.5 μl of 2X RT-PCR mastermix, 0.5 μl of Rox dye, 0.5 μl of Taq mix, 0.5 μl (400 nM) of forward primer 106 F, 0.5 μl (400 nM) of reverse primer 179R, 0.5 μl (200 nM) of probe HSIND_BD, 2.0 μl of template RNA and 8.0 μl of nuclease free water.

Analytical sensitivity of the BDV real time RT-PCR assay generated from ten-fold serial dilutions of a BDV-3 isolate of $10^{5.6}$ TCID₅₀/ml titre and linearity of the standard curve are shown in Fig. 1. The standard curve showed linear correlations ($R^2 = 0.99$) between the crossing point (Cp) value and \log_{10} TCID₅₀ with the average slope value of -3.003. (Fig. 1). The limit of detection of the assay was $10^{0.6}$ TCID₅₀. Based on Cp value data analyses of positive ($n = 20$) and negative ($n = 48$) controls, samples were considered positive for BDV, if the Cp values were 35.0 or less, while Cp values between 35.0–40.0 were

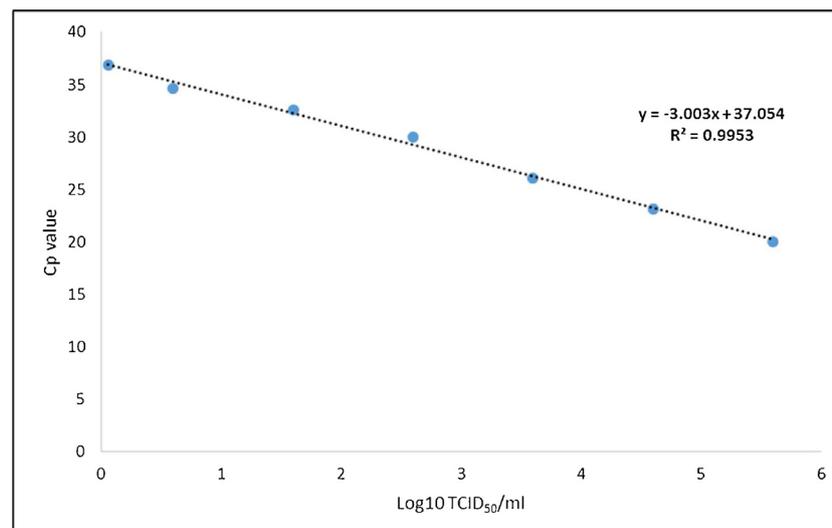


Fig. 1. Standard curve generated from 10-fold dilutions of BDV-3 isolate ($10^{5.6}$ TCID₅₀/ml to $10^{0.6}$ TCID₅₀/ml). The mean Cp values were plotted against virus titre in \log_{10} TCID₅₀.

Table 2

Analytical sensitivity between BDV real-time RT-PCR (this study) and virus isolation.

TCID ₅₀ /ml (BDV strain Ind 830-09)	Virus isolation	Real Time RT-PCR (Cp value)
$10^{5.6}$	Positive	20.06
$10^{4.6}$	Positive	23.15
$10^{3.6}$	Positive	26.05
$10^{2.6}$	Positive	29.98
$10^{1.6}$	Positive	32.59
$10^{0.6}$	Positive	34.66
$10^{0.06}$	Negative	36.85

considered doubtful, and Cp values > 40.0 was considered negative. When compared with virus isolation, the analytical sensitivity of the BDV real-time RT-PCR reported here was found to be $10^{0.6}$ TCID₅₀ (Table 2).

3.2. Analytical specificity of the assay

The BDV real time RT-PCR showed no cross reactivity with a panel of related pestivirus strains, comprising twelve BVDV-1 strains, three BVDV-2 strains, four HoBi-like pestivirus strains and six CSFV strains suggesting high analytical specificity of the assay (Table 1).

3.3. Reproducibility of the assay

The intra and inter plate assay variation was assessed on positive controls by performing BDV real time RT-PCR assays on six different days (Table 3). The coefficient of variation (CV) of intra plate assays was in the range of 0.52%–1.47%, while it was in the range of 0.5%–1.1% for the inter plate assays indicating good repeatability of the assay developed.

3.4. Diagnostic sensitivity and specificity

No positive results were obtained with any of the known negative ($n = 80$) samples, whereas all BDV strains or known BDV positive samples were found positive by the assay reported here showing diagnostic specificity of the assay.

The BDV real time RT-PCR was validated on clinical samples obtained from sheep. A total number of 210 samples (PBL, serum and tissues) were tested by both BDV real time RT-PCR and virus isolation.

Table 3
Mean Cp value of Intra- and Inter-plate assay.

TCID ₅₀ /ml	Inter-plate assay Mean Cp ± SD (CV%)	Intra-plate assay Mean Cp ± SD (CV%)
BDV 10 ^{5.6}	19.76 ± 0.179 (0.9)	19.89 ± 0.153 (0.77)
BDV 10 ^{4.6}	23.37 ± 0.134 (0.6)	23.446 ± 0.258 (1.14)
BDV 10 ^{3.6}	25.62 ± 0.288 (1.1)	25.556 ± 0.134 (0.52)
BDV 10 ^{2.6}	29.35 ± 0.233 (0.8)	29.102 ± 0.428 (1.47)
BDV 10 ^{1.6}	32.81 ± 0.164 (0.5)	32.932 ± 0.259 (0.78)
BDV 10 ^{0.6}	34.57 ± 0.129 (0.7)	34.49 ± 0.300 (0.84)
BDV 10 ^{0.06}	36.85 ± 0.138 (0.7)	36.59 ± 0.139 (0.95)

Cp-Crossing point, SD-Standard deviation, CV-Coefficient of variation.

Table 4
Diagnostic sensitivity and specificity of BDV specific real time RT-PCR (this study):

a. Comparison of BDV real time RT-PCR and virus isolation in field samples			
Type of sample	Number of sample	Real time RT-PCR Positive	VI positive
PBL	85	10	10
Serum	96	10	10
Tissues	29	0	0
Total	210	20	20

b. Diagnostic sensitivity and specificity of BDV real time RT-PCR and virus isolation			
	Virus isolation		
	Positive	Negative	Total
Real time RT-PCR Positive	20	0	20
Negative	0	190	190
Total	20	190	210
Relative sensitivity	100% (83.16% to 100.00%)		
Relative specificity	100% (98.08% to 100.00%)		

The results showed that of the 210 samples tested, 20 samples were found positive by both BDV specific real time RT-PCR and virus isolation indicating absolute correlation of results between these two tests (Table 4). Compared to virus isolation, BDV real time RT-PCR showed a diagnostic sensitivity of 100% and diagnostic specificity of 100% indicating a strong agreement.

3.5. BDV viraemia status and excretions in PI sheep

The PI sheep was found viraemic for BDV during the entire period of study, based on the results of virus isolation and BDV real time RT-PCR on blood leukocytes and serum collected thrice at 15 days intervals (Table 5). The Cp values in BDV real time RT-PCR ranged between 22.95 and 25.36 exhibiting a higher viral load (10^{4.14} TCID₅₀) in serum than PBL. VNT results revealed absence of BDV specific antibodies or cross reactive BVDV antibodies in the PI sheep for the same period. Compatible with the PI status, excretion of BDV was evident in all secretions tested, as all the nasal swabs, oral swabs, ocular swabs and rectal swabs were found positive for BDV RNA by BDV real time RT-PCR assay reported in this study (Table 5) (Fig. 2 and 3). The mean Cp values of different swabs were in the range of 24.60–30.83, lowest in nasal swab and highest in rectal swab indicating highest viral load (10^{4.14} TCID₅₀) in nasal swab of PI sheep. Comparable results were found between this new BDV specific real time RT-PCR and panpestivirus real time RT-PCR, whereas high Cp values (difference of 2–11 cycles) and some discrepancy in results was observed when tested by earlier reported BDV real time RT-PCR (Willoughby et al., 2006).

3.6. BDV real time RT-PCR test results of manually plucked hairs from PI sheep

The utility of manually plucked hairs from different parts of ear and

Table 5
Detection of BDV in clinical samples of PI sheep by BDV specific real time RT-PCR (this study) and comparison with earlier reported real time RT-PCR assays and virus isolation.

Sample type	1 st collection (Cp value)			2 nd collection (Cp value)			3 rd collection (Cp value)			*Viral load (log ₁₀ TCID ₅₀ /ml)			
	Real time RT-PCR			Real time RT-PCR			Real time RT-PCR						
	Pan-pesti	BDV real time (This Study)	BDV real time (Willoughby)	Pan-pesti	BDV real time (This Study)	BDV real time (Willoughby)	Pan-pesti	BDV real time (This Study)	BDV real time (Willoughby)		Virus isolation	Pan-pesti	BDV real time (This Study)
Nasal swab	P (25.02)	P (25.98)	P (32.65)	P (24.83)	P (25.01)	P (31.32)	P (21.94)	P (22.80)	P (30.60)	P	P (23.93)	24.60	4.14
Ocular swab	P (25.16)	P (25.35)	P (32.64)	P (24.34)	P (25.16)	P (31.03)	P (24.10)	P (25.32)	P (31.21)	P	24.53	25.28	3.91
Oral swab	P (24.26)	P (25.09)	P (33.14)	P (26.16)	P (26.98)	P (32.95)	P (24.65)	P (25.09)	P (31.64)	P	25.02	25.72	3.77
Rectal swab	P (30.11)	P (31.65)	P (34.40)	P (29.84)	P (30.01)	P (32.68)	No Cp	No Cp	No Cp	N	29.98	30.83	2.08
Urine	P (26.36)	P (25.68)	D (36.47)	P (26.12)	P (27.68)	D (35.34)	P (25.20)	P (26.13)	P (34.85)	*cyto-toxicity	25.89	26.50	3.51
Body wool (loin)	P (24.51)	P (25.38)	P (34.35)	P (26.96)	P (27.68)	D (36.35)	P (25.94)	P (27.53)	D (36.41)	P	25.80	26.86	3.39
PBL	P (23.16)	P (24.56)	P (33.83)	P (23.69)	P (24.35)	P (33.49)	P (24.79)	P (25.12)	P (32.83)	P	23.88	24.68	4.11
Serum	P (22.09)	P (22.95)	P (32.56)	P (24.64)	P (25.36)	P (34.56)	P (24.84)	P (25.04)	P (33.40)	P	23.86	24.45	4.19

P-Positive; N- Negative and D- Doubtful.

* Viral load corresponding to mean Cp value of real time RT-PCR (this study).

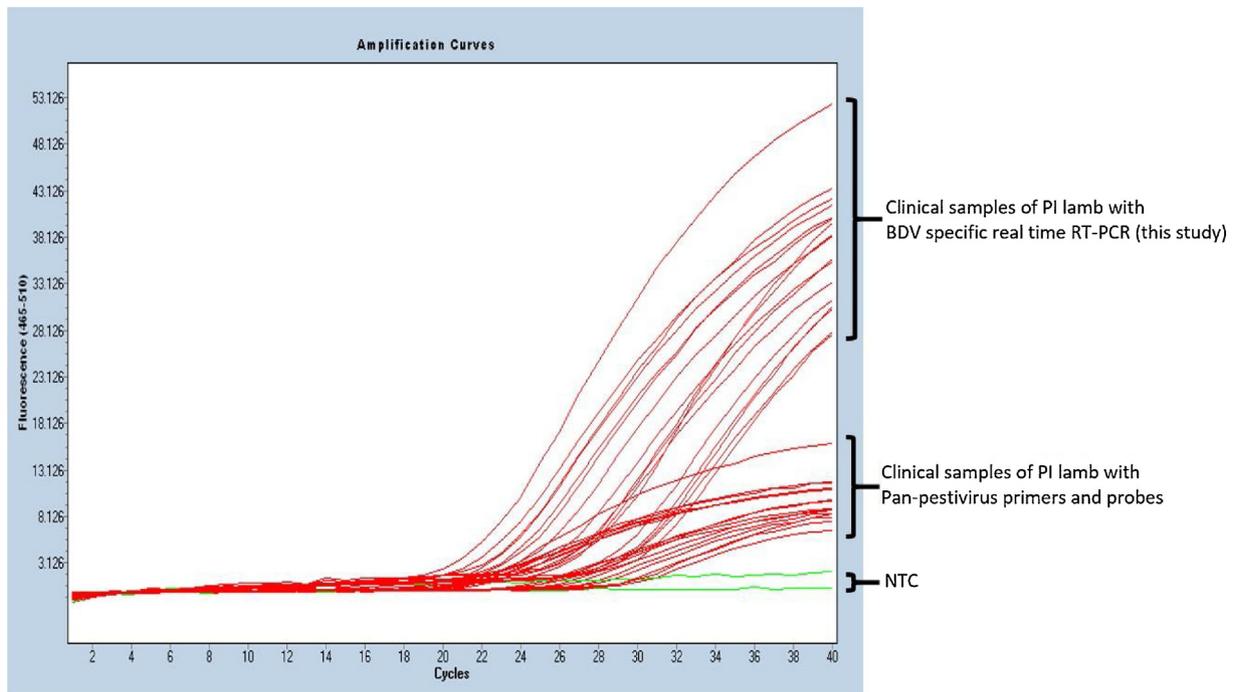


Fig. 2. Amplification curve for clinical samples of PI lamb tested by BDV specific real time RT-PCR (this study) and pan-pestivirus real time RT-PCR.

the tail tip of PI sheep as substitute samples was ascertained by the newly developed BDV real time RT-PCR and the results are shown in Table 6. The results showed that pools of hair follicles of different quantities (10, 20, 50 and 100) obtained from mid-lateral ear, mid-medial ear and tail tip were found positive for BDV by the newly developed BDV real time RT-PCR. When pools consisting of < 10 plucked hairs were tested, the results were either negative or doubtful (data not shown). Although Cp value of < 35 was obtained for the sample pools of 10 hair follicles or more, the viral load estimation indicated that a minimum of 20 hair follicles is suitable for detection of BDV in PI sheep by the newly developed real time RT-PCR (Table 6). Testing of 50 and 100 hair follicles showed that there was not much difference in Cp

values except for tail hairs (2 cycles difference). BDV testing of hair follicles from various regions of ear pinna and tail of PI sheep revealed that viral load was comparatively higher in manually plucked hairs from mid-medial location of ear than those from other tested locations displaying better suitability of hairs from mid-medial ear for BDV testing. The mean Cp values of the hair sample pools varied between 24.85 and 32.15 but the values obtained for each pool remained almost constant during the 1st, 2nd and 3rd collection. The hairs without follicles were tested negative by the BDV specific real time RT-PCR.

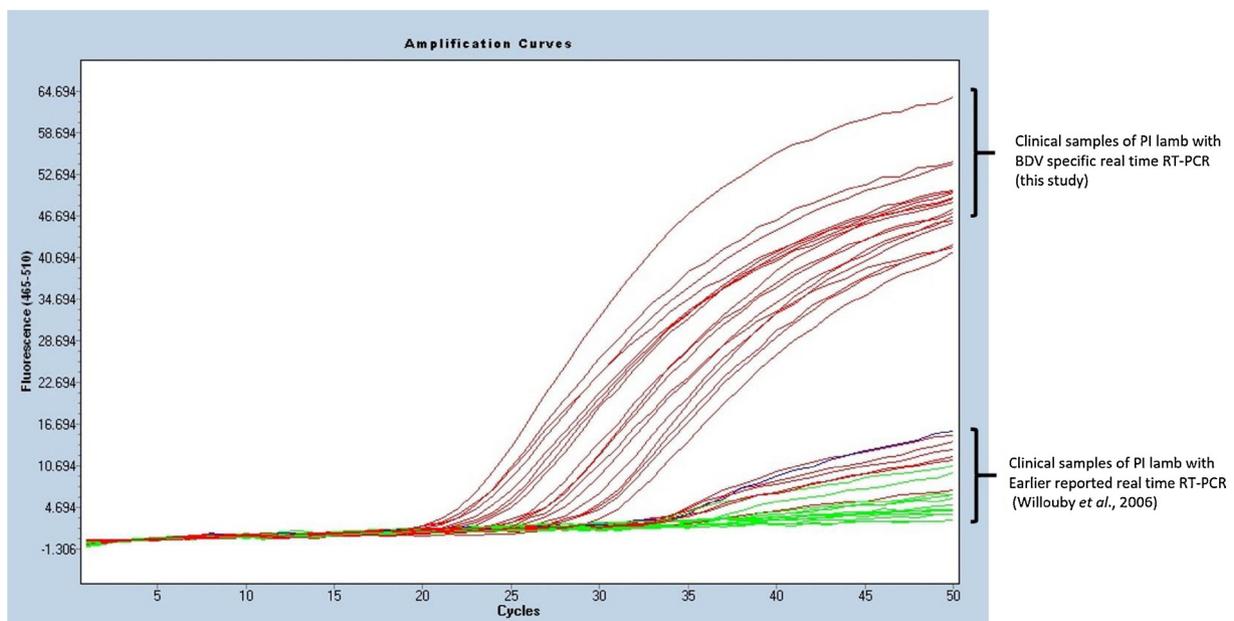


Fig. 3. Amplification curve for clinical samples of PI lamb tested by BDV specific real time RT-PCR (this study) and earlier reported real time RT-PCR (Willoughby et al., 2006).

Table 6
Analytical sensitivity comparison of BDV specific real time RT-PCR (this study) and earlier reported real time RT-PCR (Willoughby et al., 2006) with various quantity of hair follicles.

Samples from PI Lamb	1 st collection (Cp value)		2 nd collection (Cp value)		3 rd collection (Cp value)		BDV real time (This Study) BDV real time (This Study)	Viral load (log10 TCID ₅₀ /ml)
	BDV real time (Willoughby)	BDV real time (This Study)	BDV real time (Willoughby)	BDV real time (This Study)	BDV real time (Willoughby)	BDV real time (This Study)		
Ear hair mid -lateral (10 hair follicles)	P (32.15)	N	P (30.38)	N	N	N	31.26	1.93
Ear hair mid -lateral (20 hair follicles)	P (29.65)	N	P (28.62)	N	P (28.35)	N	28.87	2.72
Ear hair mid -lateral (50 hair follicles)	P (28.85)	D (37.65)	P (27.65)	D (37.39)	P (27.45)	D (37.79)	27.98	3.02
Ear hair mid -lateral (~100 hair follicles)	P (28.09)	D (36.05)	P (27.02)	D (36.72)	P (27.11)	D (36.08)	27.41	3.21
Ear hair mid -medial (10 hair follicles)	P (31.08)	N	P (29.72)	N	P (30.85)	N	30.55	2.17
Ear hair mid -medial (20 hair follicles)	P (27.58)	D (37.65)	P (26.78)	D (36.38)	P (26.46)	D (37.49)	26.94	3.37
Ear hair mid -medial (50 hair follicles)	P (26.44)	P (34.45)	P (25.15)	P (33.87)	P (25.19)	P (34.63)	25.59	3.82
Ear hair mid -medial (~100 hair follicles)	P (25.63)	P (34.05)	P (24.85)	P (32.11)	P (24.69)	P (33.15)	25.06	3.99
Tail (tip) hair (10 hair follicles)	P (32.05)	N	P (30.00)	N	P (30.59)	N	30.88	2.06
Tail (tip) hair (20 hair follicles)	P (29.16)	D (36.49)	P (28.59)	D (37.42)	P (28.35)	D (36.69)	28.70	2.78
Tail (tip) hair (50 hair follicles)	P (28.15)	D (35.85)	P (27.93)	D (35.91)	P (28.02)	D (36.42)	28.03	3.01
Tail (tip) hair (~100 hair follicles)	P (26.23)	P (34.19)	P (25.58)	P (33.41)	P (25.36)	P (33.16)	25.72	3.77

P-Positive; N- Negative and D- Doubtful.

* Viral load corresponding to mean Cp value of real time RT-PCR (this study).

3.7. Comparison of results of hair samples with earlier reported BDV real time RT-PCR

Comparison of BDV test results of manually plucked hairs by the newly developed BDV real time RT-PCR and the previously reported real time RT-PCR (Willoughby et al., 2006) showed less sensitivity of the later test (Table 6), as out of 12 different pools of hair samples, only three (25%) samples were found positive, while four were negative and five were doubtful for BDV. Moreover, Cp values in these positive samples were almost approaching the cut-off value set for declaring the sample as doubtful for BDV.

4. Discussion

Reports of BDV infection in cattle from sheep in several countries and its potential interference with BVDV eradication plans in cattle, negative effects on sheep production and high mortality in Pyrenean chamois wildlife population have enhanced renewed interest in BDV and its control (Braun et al., 2015; Krametter-Froetscher et al., 2010; Vega et al., 2015). Similar to BVD in cattle, Identification and removal of sheep persistently infected with BDV remains the hallmark of BDV control. However, the success is mostly dependent on the sensitivity and specificity of the BDV diagnostic test being applied and rely heavily on testing cost, ease of sampling and shipping, especially in less developed countries. Due to many limitations of antigen ELISA and virus isolation and problems of cross contamination in conventional RT-PCR, Taqman real time RT-PCR is being widely applied for detection of BDV. Several sample matrices including whole blood, serum, and ear notch samples involving invasive techniques are used for routine diagnosis of BDV PI sheep, but invasive methods are not very practicable in the field due to resistance from sheep farmers. To alleviate these problems, here we report the development of a BDV specific real time RT-PCR and its diagnostic evaluation on manually plucked hairs as a non-invasive alternate sample for detection of sheep persistently infected with BDV.

Since BD in sheep can be caused by BDV, BVDV-1 and BVDV-2 and with recent reports of HoBi-like pestivirus infection in sheep, there is a need to develop rapid and sensitive BDV specific molecular tests. Although several real time RT-PCR assays have been reported for detection of pestiviruses such as BVDV and CSFV, only a few reports are available for development and use of BDV specific one step real time RT-PCR (Willoughby et al., 2006; La Rocca and Sandvik, 2009). Willoughby et al., 2006 developed a BDV real time RT-PCR using MGB probe, but MGB probe has been reported to be less tolerant to sequence mismatches than longer standard probes (Whiley and Sloots, 2006). We observed a moderate sensitivity of this test in detecting BDV in field samples and also sequence mismatches in the BDV probe binding region (Mishra et al., 2016; data not shown). The duplex real time RT-PCR employed by La Rocca and Sandvik, 2009 showed cross reactivity of BDV probe with CSFV. Moreover, earlier reported BDV probes (Willoughby et al., 2006; La Rocca and Sandvik, 2009) showed certain sequence mismatches with BDV strains from Spain (Hurtado et al., 2009) indicating a need of improvement in BDV specific real time RT-PCR.

For BDV real time RT-PCR assay development, we used newly designed BDV probe and the published primers (La Rocca and Sandvik, 2009) targeting 5'-UTR of BDV genome. These redesigned primers based on extended 5'-UTR sequence data have been shown to detect majority of BDV strains by eliminating three mismatches (La Rocca and Sandvik, 2009). BDV real time RT-PCR reported here was not only highly sensitive and specific but also provided improvement over earlier BDV real time assays, as it could detect BDV as low as 0.6 TCID₅₀ without any cross reactivity with BVDV-1, BVDV-2, HoBiPeV and CSFV. Moreover, when evaluated on 210 field samples, the diagnostic sensitivity and specificity of the assay was in perfect agreement with the virus isolation. Although it would have been ideal to test BDV strains representing all the genotypes of BDV, it is not always practically

feasible.

Similar to that observed earlier in PI sheep, positive results of BDV isolation, panpestivirus real time RT-PCR and BDV real time RT-PCR developed in this study was found in blood, serum and in all most all excretion routes. Urine samples were positive for BDV RNA but negative for virus isolation due to cytotoxicity, whereas rectal swab was negative for BDV in the third sampling. High viral load in serum and nasal swab found in this study has also been observed earlier (Cabezón et al., 2010). However, when the results were compared with previously reported BDV real time RT-PCR (Willoughby et al., 2006), the new BDV real time RT-PCR assay was found more sensitive in all of the samples tested from the PI sheep and some of the positive samples produced doubtful results by the previously reported real time RT-PCR demonstrating superiority of the new test.

Plucked hair or wool has been used mostly for direct examinations for surface parasites such as mites and lice, fungal and bacterial infections but rarely for viral infections (OIE, 2018). However, there is a lone report of utility of manually plucked ear hairs using a real time RT-PCR assay in detection of cattle persistently infected with BVDV (Singh et al., 2011). BDV has been shown to exist in almost all organs and excretion occurs through most of the secretions in sheep having persistent infection with BDV resulting in virus transmission to susceptible animals (Loken, 1995). However, no report exists regarding detection of BDV in plucked hairs from sheep. Here we report detection of BDV in manually plucked hairs of BDV PI sheep by developing a new BDV real time RT-PCR.

Collection of blood and tissue (ear notch) sample from sheep requires invasive methods which harm the animal during sample collection indicating importance and practicability of non-invasive method. A previous study has shown that samples containing at least 30 manually plucked ear hairs can be used as an alternate sample in BVDV real time RT-PCR for detection of BVDV PI cattle (Singh et al., 2011). Our results showed that using the newly developed BDV real time RT-PCR, samples of at least 20 manually plucked hairs from different regions of ear and tail tip may be useful as an alternative to blood or serum samples for detection of sheep PI with BDV and indicating high sensitivity of the test. However, more numbers of PI animals need to be tested to support the requirement of minimum number of 20 hair follicles for use in the improved test. While selecting best hair samples, plucked hairs from mid-medial region of ear from PI sheep were found more suitable for BDV testing due to higher viral load. Conversely to results of the new BDV real time RT-PCR, earlier reported BDV real time RT-PCR (Willoughby et al., 2006) failed to detect BDV RNA or produced doubtful results in nine out of the twelve hair sample pools in all the three collections during the entire period of study. Although BDV antigen and RNA has been demonstrated in skin and BDV antigen in keratinocytes and hair follicles (Terpstra, 1978; Thur et al., 1997; Cabezón et al., 2010), and highest viral load has been found in skin by pestivirus generic real time RT-PCR (Hurtado et al., 2009), the exact reasons of variation in viral RNA load in hairs from different regions found in this study are not known and may be subject of future studies.

The use of manually plucked hairs from ear and a sensitive BDV specific real time RT-PCR in detection of BDV PI sheep has advantages. Being noninvasive, hairs can be sampled from apparently healthy sheep easily without any objection by the farmers, while the improved BDV specific assay may be useful in identification of BDV PI sheep in control and eradication programs.

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