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Genotyping of Pestivirus A (Bovine Viral Diarrhea Virus 1) detected in faeces and in other specimens of domestic and wild ruminants at the wildlife-livestock interface

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ABSTRACT

Pestiviruses are widespread in the world among ungulates and infect both domestic and wild animals causing severe economic losses in livestock. *Bovine Viral Diarrhea Virus* type 1 (BVDV-1), now re-designated as *Pestivirus A*, causes diseases mainly in cattle, while few data are available about infection in wild ruminants and about the role of these animals in viral maintenance and spread. In order to investigate BVDV-1 infection in domestic and wild ruminants, especially at the wildlife/livestock interface, bulk tank milk from dairy cattle and sheep and spleen from red deer, roe deer and fallow deer were analysed. Furthermore, faecal samples from Apennine chamois and from wild deer were evaluated as a suitable sample for detecting and genotyping pestiviruses. BVDV-1 RNA was found in all animal species tested but not sheep. Genotyping based on partial 5′UTR and N^{pro} sequences detected BVDV-1a in samples from Apennine chamois, red deer, roe deer and pasture-raised cattle, while BVDV-1c was found in a faecal sample from Apennine chamois and in a spleen sample from roe deer. For the first time BVDV-1 RNA was found and genotyped from faecal samples of wild ruminants and of cattle. BVDV-1a detection in Apennine chamois, red deer, roe deer and pasture-raised cattle suggests that the eventuality of viral transmission at the wildlife/livestock interface should be carefully evaluated. BVDV subgenotype 1c was found for the first time in roe deer and Apennine chamois in Central Italy, therefore the epidemiological role of these animals and the viral ecology should be further investigated.

1. Introduction

Pestiviruses are globally distributed members of the family *Flaviviridae* that cause severe economic losses in livestock (Smith et al., 2017; Yeşilbağ et al., 2017). The most common species of the genus *Pestivirus* are *Bovine Viral Diarrhea Virus* type 1 (BVDV-1) and type 2 (BVDV-2), *Classical Swine Fever Virus* (CSFV) and *Border Disease Virus* (BDV). On the basis of the most recent release of the International Committee on Taxonomy of Viruses (ICTV) in 2018, these pestiviruses have been re-designated as *Pestivirus A* (original designation BVDV-1), *Pestivirus B* (BVDV-2), *Pestivirus C* (CSFV) and *Pestivirus D* (BDV) (talk.ictvonline.org/taxonomy/, last access 3rd February 2019). Genetic changes in pestivirus genomes are very frequent and different genomic regions have been used for classification and typing purposes. For example, partial 5′UTR and N^{pro} sequences have been most frequently used for phylogenetic analyses and genotyping of BVDV isolates, allowing detection of at least 21 different BVDV-1 subtypes named from BVDV-1a to BVDV-1 u (Yeşilbağ et al., 2017).

Pestiviruses have a very broad host range, are widespread among ungulates and infect both domestic and wild swine and ruminants, mostly in the families *Bovidae* and *Cervidae* (Ridpath and Neill, 2016). Epidemiological data of *Pestivirus* infections in European wild ruminants are limited. Severe outbreaks in the Southern chamois (*Rupicapra pyrenaica*) caused by BDV have been reported in Spain starting from 2001 (Marco et al., 2007, 2015). Serological investigations in Europe showed that *Pestivirus* infections in wild ruminants are sporadic (Olde Riekerink et al., 2005; Fernández-Sirera et al., 2011; Casaubon et al., 2012; Fernández-Aguilar et al., 2016). Only a few cases of BVDV-1 RNA detection in European wild ruminants are described (Vilček and Nettleton, 2006; Casaubon et al., 2012; Fernández-Aguilar et al., 2016).

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In order to detect and genotype BVDV, blood samples are usually analysed, but viral sequences have been obtained also from bulk milk specimens (Luzzago et al., 2014; Vilček et al., 2001) and from spleen of dead animals (Nelson et al., 2016; Passler et al., 2009).

In Central Italy, the wild ruminants' population is represented mainly by roe deer (*Capreolus capreolus*), fallow deer (*Dama dama*), and sporadically by red deer (*Cervus elaphus*) and Apennine chamois (*Rupicapra pyrenaica ornata*) (Carnevali et al., 2009). The Apennine chamois is a subspecies of chamois currently living only in limited areas of Central Italy and it is classified as "vulnerable" in the red list of threatened species drawn up by the International Union for Conservation of Nature (IUCN, 2018). The Apennine chamois is a protected species under Italian and international laws (L.157/92, CITES Appendix II) and reintroduction projects have been carried out in several protected areas in Central Italy, according to the Italian Action Plan for the Apennine chamois. Starting from 2008, Apennine chamois has been reintroduced in the Monti Sibillini National Park, with further support from the European project LIFE Coornata - development of coordinated protection measures for Apennine chamois LIFE09 NAT/IT/000183 (years 2010–2014) (Rossetti et al., 2015). In the Monti Sibillini National Park breeding of cattle, sheep and goats often relies on traditional practice based on small farms and extensive farming systems, where animals graze freely on mountain pastures. Vaccination rate for pestiviruses in livestock in this context is usually low and the risk of viral transmission at the wildlife/livestock interface should be contemplated. Considering that the Monti Sibillini National Park is a protected area where hunting is prohibited and that capture for collecting blood is rare, expensive and stressful for wild ruminants, alternative sampling methods should be evaluated.

The aims of this work were i) to evaluate whether faeces can be used to detect *Pestivirus* RNA in wild ruminants and ii) to investigate and characterise genetically pestiviruses infecting domestic and wild ruminants, with special attention to the wildlife/livestock interface.

2. Materials and methods

2.1. Study design and sampling

Samples were obtained from different domestic and wild ruminants living in different areas of Marche Region (Central Italy), between 2014 and 2016 (Tables 1 and 2, Fig. 1). Different specimens were taken from different groups of animals, preferring specimens that can be easily sampled. Samples were collected and grouped as follows (Table 3): faeces from wild ruminants (red deer and Apennine chamois) living in the protected area of the Monti Sibillini National Park, where animals can not be hunted (group A); spleen or faeces from wild ruminants (roe deer, fallow deer, red deer) living in areas not located in the protected Park, where animals are killed following hunting calendars (group B); faeces from two beef cattle farms (group C), bulk tank milk from dairy cattle (group D) and sheep (group E) raised from early Summer to Autumn in pastures close or overlapping to the areas where wild animals of groups A and B were sampled. Spleen and bulk tank milk samples were the preferred specimens for collection but, when they were not available, faecal samples were collected on the basis of encouraging results obtained in a preliminary study on faeces of beef cattle belonging to a BVDV-positive farm (data not shown). The animal species origin of each faecal sample from wild ruminants (groups A and B) collected from the environment was identified on the basis of its location and its morphological aspect (Mustoni et al., 2002).

2.2. RNA extraction

RNA extraction methods from faecal samples and from bulk tank milk samples were first validated on samples experimentally infected with BVDV strain TVM-2 (kindly provided by Prof. C. Valente) and then were performed with a commercial kit based on silica-membranes (RecoverAll Total Nucleic Acid Isolation Kit, Ambion, Life Technologies). All procedures were carried out in a biosafety cabinet with an ultraviolet lamp; a specific laboratory cleaner (DNA cleaner, Diatech pharmacogenetics) was used to provide effective cleaning from DNA and RNA. To avoid any contamination, no more than five samples were processed at the same time, and mono use plastic tools were used. A Pestivirus-negative bovine faecal sample was used as negative control during each RNA extraction and RNA retrotranscription run.

2.2.1. Faecal samples preparation

Six millilitres of RNAse-free PBS were added to 1.5 *g* of each faecal sample. After mechanical disruption and mixing, samples were centrifuged at 500 x g for 10 min at 4 °C; 1 ml of the supernatant was transferred to a new tube and was centrifuged at 13,000 x g for 15 min at 4 °C. The final supernatant (100 µl) was used for RNA extraction. In case of pool samples (groups A and C), 200 µl of the final supernatant of each sample were mixed with the other samples included in the pool and 100 µl of this mixture were used for RNA extraction.

2.2.2. Bulk tank milk samples

Aliquots of 45 ml of each bovine or ovine bulk tank milk sample were centrifuged at 500 x g for 20 min at 10 °C. All cream and the upper part of the liquid were discarded. The remaining whey was transferred to a new tube and 2 ml were put back in the original tube together with the pellet. After mixing, each sample was transferred in a new tube and centrifuged at 13,000 x g for 10 min. Once all the supernatant was discarded, the pellet was washed with RNAse-free PBS three times and used for RNA extraction (Supplementary file S1).

2.2.3. Spleen samples

Spleen samples were collected from hunters and were stored at −20 °C up to 1 month or at 4 °C up to 1 week before being brought to the laboratory. Aliquots of 50 mg of spleen were used for RNA extraction by using 1 ml Trizol RNA Reagent (Thermo Fisher Scientific, Massachussets, USA) following the manufacturer's instructions. After drying, the RNA pellet was resuspended in 20 µl RNAse-free water.

2.3. RNA retrotranscription

Two microlitres of each RNA sample were retrotranscripted (PrimeScript RT reagent Kit, Takara Bio Inc.) in 20 µl final volume. The cDNA obtained was immediately used for PCR or stored at −20 °C.

2.4. Real-time PCR

SYBR Green Real Time PCR was carried out with primers specific for amplification of a 106 bp sequence of *Pestivirus* sp. (Baxi et al., 2006). The PCR mixture included 10 µl 2X SYBR Premix Ex Taq II (Tli RNase H Plus), 0.4 µl 50X ROX Reference Dye (Takara Bio Inc.), 4 pmol each primer Pesti-FW (5′- CTA GCC ATG CCC TTA GTA G -3′) and Pesti-RS (5′- CGT CGA ACC AGT GAC GAC T -3′), 2 µl cDNA and PCR grade water up to 20 µl final volume. PCR conditions were 95 °C for 30 s, 40 cycles of 94 °C for 15 s and 60 °C for 60 s. The final melt curve was obtained by ramping from 65 °C to 95 °C and acquiring data every 0.3 °C of temperature increment. PCR products were visualized in 1.5% agarose gel, as described below. PCR-grade water instead of cDNA was used as negative control and cDNA obtained from the cytopathic TVM-2 strain was used as positive control. All the samples and controls were run in duplicate.

2.5. PCR and sequencing

All Real-Time PCR positive samples were used for PCR amplification and sequencing of the 5′ untranslated region (UTR) and of the N^{pro} gene, similarly as reported in previous studies (Becher et al., 1997, 1998; Vilček et al., 2001; Giammarioli et al., 2008; Luzzago et al., 2014;

Table 1
samples collected from wildlife animals and results of PCR and genotyping.

Samplecode	Group	Animal species	Sample	Included in the Park	N. animals tested (in pool)	RealTime PCR	BVDV typing
15F5	A	Red deer	faeces	yes	4	pos	1a
15F6	A	Red deer	faeces	yes	4	pos	1
15F7	A	Red deer	faeces	yes	4	pos	1
15F8	A	Red deer	faeces	yes	2	pos	1a
15F9	A	Apennine chamois	faeces	yes	4	–	
15F10	A	Apennine chamois	faeces	yes	3	pos	1a
15F11	A	Apennine chamois	faeces	yes	3	pos	1
15F12	A	Apennine chamois	faeces	yes	3	pos	1c
15F13	A	Red deer	faeces	yes	2	–	
16M40	B	Roe deer	spleen	no	1	–	
16M41	B	Roe deer	spleen	no	1	pos	1
16M45	B	Roe deer	spleen	no	1	pos	1a
16M46	B	Roe deer	spleen	no	1	–	
16M49	B	Roe deer	spleen	no	1	pos	1c
16M52	B	Roe deer	spleen	no	1	–	
16M53	B	Roe deer	spleen	no	1	pos	1a
16M54	B	Roe deer	spleen	no	1	–	
16M55	B	Roe deer	spleen	no	1	–	
16M56	B	Roe deer	spleen	no	1	–	
16M57	B	Roe deer	spleen	no	1	–	
16M58	B	Roe deer	spleen	no	1	–	
16M59	B	Roe deer	spleen	no	1	–	
16M64	B	Fallow deer	spleen	no	1	–	
16M65	B	Roe deer	spleen	no	1	–	
16M66	B	Red deer	spleen	no	1	–	
16M67	B	Roe deer	spleen	no	1	–	
16M68	B	Fallow deer	spleen	no	1	–	
16M69	B	Roe deer	spleen	no	1	–	
16M70	B	Roe deer	spleen	no	1	–	
16M71	B	Red deer	spleen	no	1	pos	1a
16M72	B	Red deer	spleen	no	1	–	
16M73	B	Roe deer	spleen	no	1	–	
16M74	B	Roe deer	spleen	no	1	–	
17M1	B	Roe deer	faeces	no	1	–	
17M2	B	Roe deer	faeces	no	1	–	
17M3	B	Roe deer	faeces	no	1	–	
17M4	B	Roe deer	faeces	no	1	–	
17M5	B	Fallow deer	faeces	no	1	–	
17M6	B	Fallow deer	faeces	no	1	–	
17M7	B	Fallow deer	faeces	no	1	–	
17M8	B	Fallow deer	faeces	no	1	–	
17M9	B	Fallow deer	faeces	no	1	pos	1

Legend: A are wild ruminants living in the protected Monti Sibillini National Park, where animals can not be hunted; B are wild ruminants living in areas not located in the protected National Park, where animals are killed.

Decaro et al., 2016).

PCR mixture for amplification of a 288 bp sequence of the 5′ UTR included 50 µl 2X Taq PCR Master Mix (Qiagen), 25 pmol each primer (324-F: ATG CCC WTA GTA GGA CTA GCA; 326-R: TCA ACT CCA TGT GCC ATG TAC; Vilček et al., 1994), 2 µl cDNA, and PCR grade water up to 50 µl final volume. Amplification conditions were 94 °C for 5 min, 45 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 7 min followed by refrigeration at 4 °C.

Considering that preliminary single PCR tests to amplify the N^{pro} gene showed too low sensitivity, a nested PCR protocol was used to obtain 428 bp products. First round PCR mixture included 50 µl 2X Taq PCR Master Mix (Qiagen), 25 pmol each primer (OI100-F CAT GCC CWY AGT AGG ACT AGC and 1400-R ACC AGT TGC ACC AAC CAT G; Becher et al., 1997, 1998), 2 µl cDNA, and PCR grade water up to 50 µl final volume. Second round PCR included 50 µl 2X Taq PCR Master Mix (Qiagen), 25 pmol each primer (BD1-F: TCT CTG CTG TAC ATG GCA CAT G and BD3-R:CCA TCT ATR CAC ACA TAA ATG TGG T; Vilček et al., 2001), 2 µl of the product of the first round PCR and PCR grade water up to 50 µl final volume. Amplification conditions of both first and second round PCRs were 94 °C for 5 min, 35 cycles at 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 7 min followed by refrigeration at 4 °C.

PCR products (10 µl each) were visualized in 1.5% agarose gel and positive samples were submitted to an external laboratory for sequencing (BMR Genomics, Padua, Italy). Selected nucleotide squences of the 5′UTR and of the N^{pro} were deposited in GenBank (accession numbers MK622855-MK622867).

2.6. Sequence analysis

The obtained sequences were evaluated by BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and they were aligned with sequences of at least one of each BVDV-1 subtype known so far (from BVDV-1a to BVDV-1 u). Alignment was carried out by MUSCLE (EMBL-EBI, https://www.ebi.ac.uk/Tools/msa/muscle/), manual editing was performed with BioEdit software version 7.0.5 (freely available at http://www.mbio.ncsu.edu/bioedit/bioedit.html), and phylogenetic trees were inferred with the software MEGA 7.0.21. The estimated best-fitting nucleotide substitution model was Kimura 2-parameter with gamma-distributed rates among sites and with bootstrap values based on 1000 repetitions. Pairwise distances between sequences of samples and sequences of reference strains belonging to different subtypes were calculated by MEGA 7.0.21 with the p-distance model.

Table 2
samples collected from bovine and results of PCR and genotyping. Bulk tank milk samples were collected also from 18 ovine farms; they are not reported in the table because all were negative for pestiviruses by Real Time PCR.

Sample code	Group	Animal species	Sample	Included in the Park	BVDV vaccination	Total animals (n =)	Animals tested (n =)	Grazing	Real Time PCR	BVDV typing
15F1	C	Bovine	faeces	yes	no	40	10	yes	pos	1a
15F2		Bovine					10		–	
15F3		Bovine					10		pos	1a
15F4		Bovine					10		pos	1a
15F14	C	Bovine	faeces	yes	no	21	10	yes	–	
15F15		Bovine					10		–	
14T1	D	Bovine	b. t. milk	no	no	64	24	no	pos	1a
14T2	D	Bovine	b. t. milk	no	no	40	25	yes	pos	1a
14T3	D	Bovine	b. t. milk	yes	no	140	50	yes	pos	1a
14T4	D	Bovine	b. t. milk	yes	no	30	18	no	–	
14T5	D	Bovine	b. t. milk	yes	no	20	10	yes	–	
14T6	D	Bovine	b. t. milk	yes	no	78	50	yes	pos	1a
14T7	D	Bovine	b. t. milk	yes	no	50	30	yes	pos	1
14T8	D	Bovine	b. t. milk	yes	no	40	20	yes	pos	1a
14T9	D	Bovine	b. t. milk	no	no	46	33	yes	pos	1a
14T10	D	Bovine	b. t. milk	no	no	88	27	yes	pos	1a
14T11	D	Bovine	b. t. milk	no	no	160	65	no	–	
14T12	D	Bovine	b. t. milk	no	yes	75	42	no	–	
14T13	D	Bovine	b. t. milk	no	no	70	30	no	–	
14T14	D	Bovine	b. t. milk	no	yes	237	110	no	–	
14T15	D	Bovine	b. t. milk	no	no	75	33	no	pos	1
14T16	D	Bovine	b. t. milk	no	yes	231	138	no	–	
14T17	D	Bovine	b. t. milk	no	no	60	36	no	pos	1
14T18	D	Bovine	b. t. milk	no	yes	83	60	no	–	

Legend: b. t. milk is bulk tank milk; C are beef cattle pasture-raised some months close to the areas where wild animals included in the study were living; D are dairy cattle, some of which are pasture-raised for months close to the areas where wild animals included in the study were living.

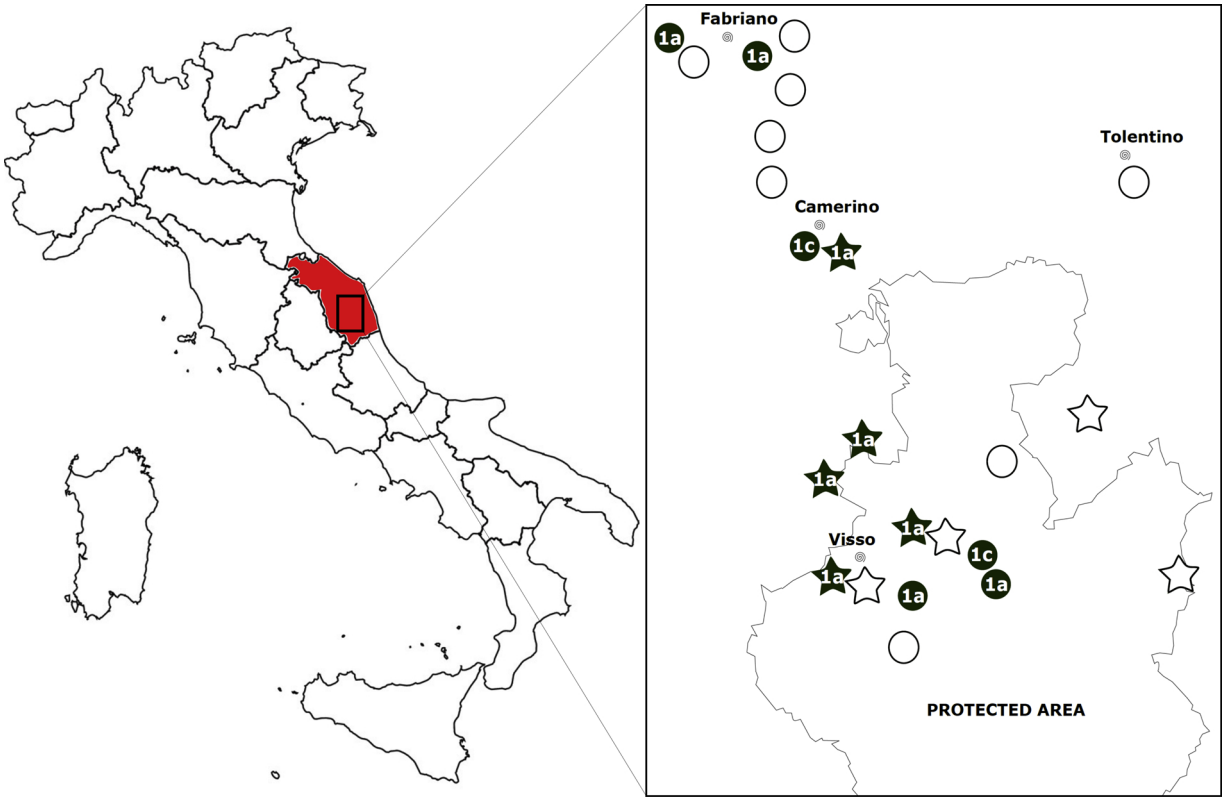

Fig. 1. The map shows the locations where samples were collected from wild ruminants (circles) and cattle (stars). The border of the protected area of the Monti Sibillini National Park is shown. Stars and circles correspond to the towns where one or more samples were collected. Black stars and black circles are PCR positive sample, and the detected subtypes are reported in the center in white letters. White stars and circles are PCR negative samples.

Table 3
groups of animals and specimens collected in each area.

Group	Area	Animals	Specimens	
A	Protected area	Wild ruminants	Faeces *n = 5 red deer n = 4 Apennine chamois*	
B	Hunting area	Wild ruminants	Spleen *n = 19 roe deer n = 2 fallow deer n = 3 red deer*	Faeces *n = 4 roe deer n = 5 fallow deer*
C	Protected area	Beef cattle	Faeces *n = 6*	
D	Areas close to areas A or B	Dairy cattle	Bulk tank milk *n = 18*	
E		Dairy sheep	Bulk tank milk *n = 18*	

following hunting calendars.
repetitions. Only a few samples with identical sequences have been included in the final tree.

3. Results

A total of 26 samples resulted positive for *Pestivirus* spp. by Real Time PCR (Tables 1 and 2), showing a Melt Temperature of 83.1 °C. In particular, 3 out of 4 pools of faecal samples from cattle of a farm (group C) and 10 out of 18 bovine bulk tank milk samples (group D) were positive. Four out of 5 pools of faecal samples from red deer and 3 out of 4 pools of faecal samples from Apennine chamois collected in the National Park (group A) were positive, while 1 out of 5 faecal samples from fallow deer and none of the 4 faecal samples from roe deer collected outside the National Park (group B) were positive. Positive results were obtained in 5 out of 24 spleen samples (group B), one from red deer and 4 from roe deer. All samples collected from sheep (group E) were negative.

Sequences suitable for analysis were obtained from 18 samples. BLAST analysis showed that all samples had the highest homology with BVDV-1. Phylogenetic analysis showed that most BVDV-1 samples (n = 16) belonged to the subtype 1a and were closer to the reference strain NADL. Two faecal samples obtained from Apennine chamois and roe deer resulted infected by BVDV-1 subtype 1c (Figs. 2 and 3). Calculation of pairwise distances among sequences (Supplementary file S2) showed that N^{pro} sequences obtained from these latter samples had the highest identity with strains Deer-NZ (U80903), Bega (AF049221), Shitara/01/05 (AB359926) and Astur2-36 (AY182162). Eight samples positive for *Pestivirus* spp. by Real Time PCR, from which editable 5′UTR or N^{pro} sequences were not obtained, resulted positive for BVDV type 1 by nested PCR (Decaro et al., 2016).

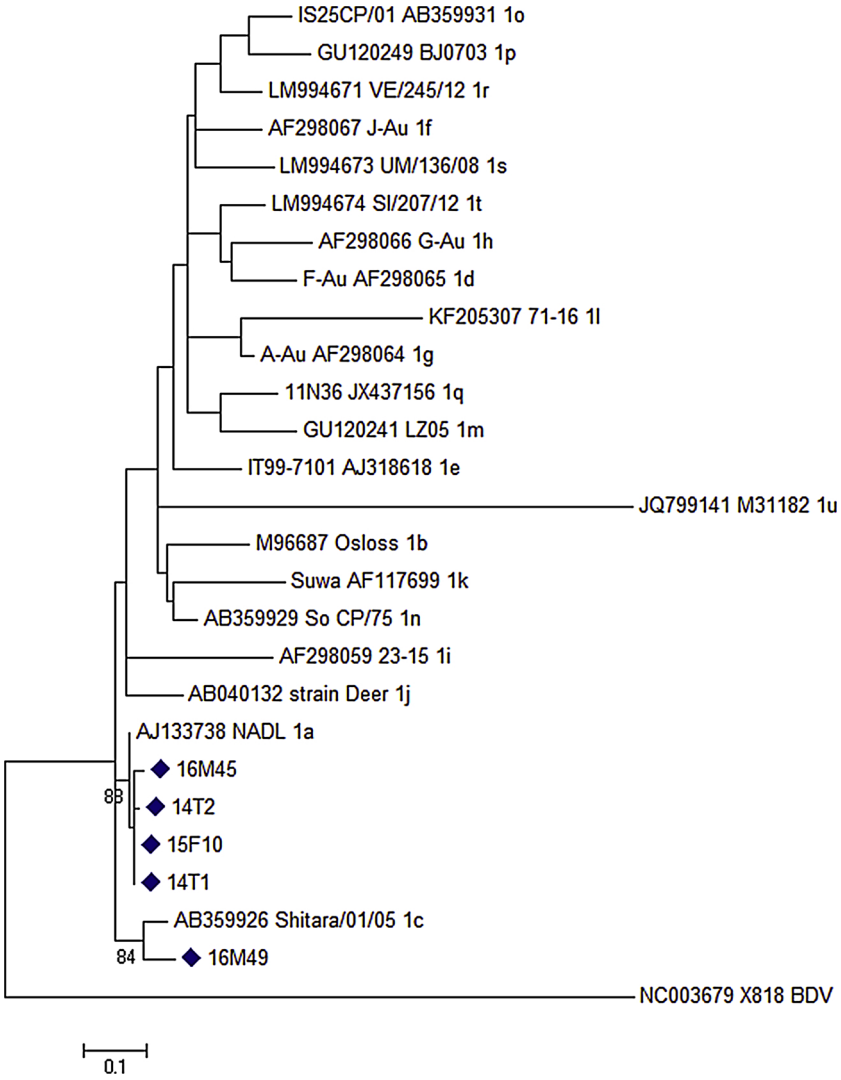

Fig. 2. Phylogenetic analysis of the 5′ UTR sequences of different subtypes of BVDV-1 obtained from GenBank and of those obtained from samples (GenBank accession numbers MK622855-MK622859). Reference sequences are indicated with their accession number, name of the strain and their assigned subgenotype. Samples are indicated with their laboratory code and a diamond (◆). BDV sequence NC003679 was used as outgroup. The phylogenetic analysis was performed with a maximum likelihood (ML) method using the Kimura 2-parameter model with a gamma distribution and with bootstrap values based on 1000 repetitions. Only a few samples with identical sequences have been included in the final tree (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

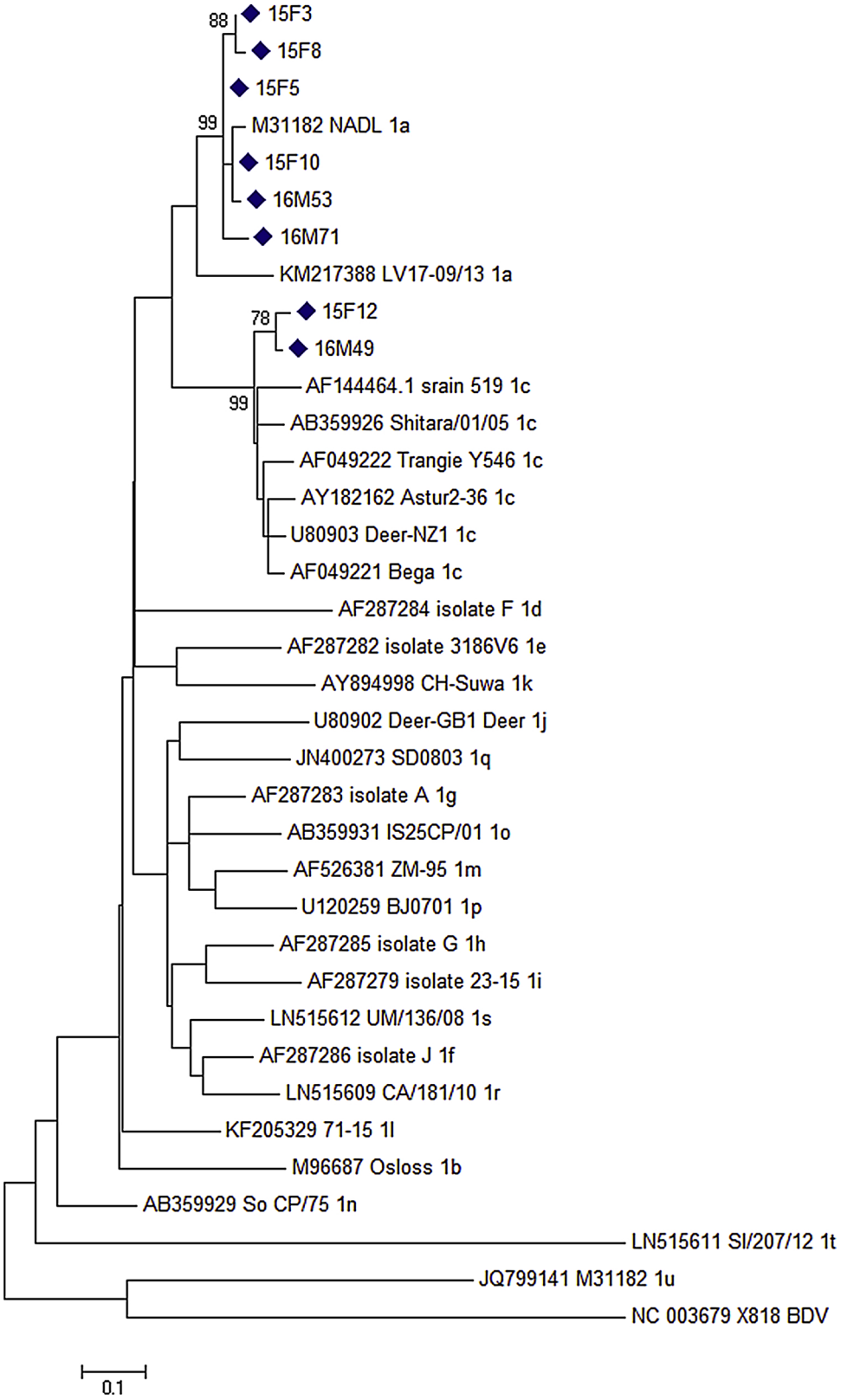

Fig. 3. Phylogenetic analysis of the N^{pro} sequences of different subtypes of BVDV-1 obtained from GenBank and of those obtained from samples (GenBank accession numbers MK622860-MK622867). Reference sequences are indicated with their accession number, name of the strain and their assigned subgenotype. Samples are indicated with their laboratory code and a diamond (◆). BDV sequence NC003679 was used as outgroup. The phylogenetic analysis was performed with a maximum likelihood (ML) method using the Kimura 2-parameter model with a gamma distribution and with bootstrap values based on 1000 (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

4. Discussion

In this study *Pestivirus* infection was investigated in red deer, roe deer, fallow deer, Apennine chamois and in domestic sheep and cattle pasture-raised close to the areas where samples from wild animals were collected. In different animal species only BVDV-1 was detected and, interestingly, good quality RNA of BVDV-1 was obtained from faecal samples collected both from the rectum and from the environment. Although further evaluations are necessary, these findings suggest that faecal samples are suitable for monitoring BVDV spread in animals and in the environment, especially when capture of wild animals is difficult or too stressful.

Analysis of the partial 5′UTR and N^{pro} nucleotide sequences obtained from our samples detected subgenotype BVDV-1a in both wild ruminants and cattle, and subgenotype BVDV-1c only in wild ruminants living in two separated geographic areas of the Marche region. BVDV-1a was found in cattle and in red deer, roe deer and Apennine chamois. Although the predominant subgenotype worldwide is BVDV-1b, BVDV-1a is the second most frequent genotype in the world and is the predominant one in South Africa (Yeşilbağ et al., 2017). In Europe, BVDV-1a is mainly spread in Ireland and in the UK, where it is also the most common subgenotype (Booth et al., 2013; O'Brien et al., 2017; Yeşilbağ et al., 2017). BVDV-1c is reported as the predominant subtype in Australia (Ambrose et al., 2018), it is the second most frequent BVDV-1 subtype in Japan and South Africa, and only a few cases have been reported in Europe, precisely in Germany, Spain and Italy (Becher et al., 1999; Luzzago et al., 2014; Giammarioli et al., 2015; Yeşilbağ et al., 2017). In Italy, a recent analysis of 371 sequences of BVDV obtained between 1995 and 2013 from cattle showed that BVDV-1b and BVDV-1e were predominant, while BVDV-1a and BVDV-1c were respectively only 2.4% and 0.3% of all BVDV subtypes (Luzzago et al., 2014). BVDV-1a strains have been found in Northern, Southern and Central Italy, including also the Marche region and some neighbouring regions (Luzzago et al., 2014; Giammarioli et al., 2015). A BVDV-1c sequence was detected in Southern Italy, far from the Marche region (Luzzago et al., 2014), although in a subsequent study another BVDV-1c strain was found in livestock in the Umbria region, which is neighbouring the Marche region (Giammarioli et al., 2015). All BVDV-1c viruses detected so far in Italy were in livestock, while we detected them only in wild ruminants. The role of BVDV-1c in the pathogenesis of the disease is not clear. *In vitro* cytopathic effect and mucosal disease have been reported for the German BVDV-1c strain 93–519 and for the Spanish BVDV-1c strain Astur2-36, and persistent infection was reported for the Spanish BVDV-1c strain León-34 (Becher et al., 1999; Arias et al., 2003), while no detailed data are available about the Italian BVDV-1c strains. Experimental infection studies carried out in Australian cattle by using 5 BVDV-1c isolates (4 non-cytopathic and 1 cytopathic) showed mild disease and very low risk of viral transmission by nasal secretions, suggesting a low pathogenicity of the subgenotype 1c isolates used (Ambrose et al., 2018). In our study, BVDV-1c was found in the faeces of Apennine chamois and in the spleen of a roe deer. In the first case, the chamois was not examined for signs of disease; however these animals are frequently monitored for species conservation purposes and severe diseases were not observed in the months following the faecal sampling. Similarly, no signs of disease were observed in the roe deer infected by BVDV-1c.

Although there is evidence of susceptibility to BVDV infection in more than 50 species of the order *Artiodactyla* (Passler and Walz, 2010; Nelson et al., 2016), only a few studies are focused on genotyping BVDV infecting deer or chamois in Europe. BVDV-1b has been found in red deer populations in proximity of extensively raised and BVDV infected cattle in South-Central Spain (Rodríguez-Prieto et al., 2016), while BVDV-1 h has been found for the first time in an Alpine chamois in Switzerland (Casaubon et al., 2012).

Recent serological and molecular investigations on the wildlife-livestock interface in the distribution area of chamois in the Cantabrian Mountains (North-western Spain) showed 10.8% seroprevalence in red deer, but not in roe deer and in Cantabrian chamois; in addition, BVDV RNA was not found in these animal species, suggesting that *Pestivirus* infections in wild ruminants were sporadic and most probably dependent on a domestic source (Fernández-Aguilar et al., 2016). The same conclusion was suggested by other authors, which found seropositivity by ELISA and by virus neutralisation test only in 1 out of 892 red deer in southern Spain (Paniagua et al., 2016). Very low seroprevalence for BVDV (1.5%) was found also in Irish free-ranging deer, where three fallow deer, a red deer and a sika deer resulted seropositive by using a commercial ELISA kit (Graham et al., 2017). These low seroprevalence results were concordant with previous surveys for BVDV antibodies in wild ruminants in Europe (reviewed by Vilček and Nettleton, 2006). On the contrary, in south-central Spain a higher seroprevalence (19.5%) was detected by commercial ELISA tests and a high apparent prevalence (22.5%) was observed by Taqman RT-PCR assay in red deer. Furthermore BVDV-1b was found both in red deer and in cattle, suggesting circulation of this viral subgenotype between cattle and red deer populations living in proximity (Rodríguez-Prieto et al., 2016). In northern Italy 25.5% and 5.9% seroprevalence were found respectively in chamois and in red deer by a commercial ELISA test, although viral RNA was not detected in positive sera (Olde Riekerink et al., 2005). In the present study a high rate of BVDV-1 RNA detection was found, especially in faecal samples of wild ruminants collected in the protected area. These data should be carefully interpreted, since the sampling methods used were not aimed at obtaining prevalence data, but at maximising the possibility to detect and genotype pestiviruses. To this purpose, the samples had initially been pooled and unfortunately they were not available to repeat the analysis individually. However, it should be considered that in the protected area the farming system is very traditional, and vaccination for BVDV and voluntary eradication programs are not usually carried out. Seroprevalence for BVDV in unvaccinated cattle in these areas is about 28% (data not published), suggesting an active circulation of BVDV and presence of persistently infected cattle. Furthermore, we could hypothesize that since in the protected areas wild ruminants are not hunted, infected animals could survive and spread the virus more easily. Further studies should be aimed at verifying this hypothesis and at evaluating the real prevalence of pestiviruses infection in wild ruminants in this region.

Only a few wild ruminant species have been proven to become persistently infected with the virus and act as a significant transmission source to other susceptible species (Nelson et al., 2016). Limited data about BVDV transmission among wildlife and livestock are available. BVDV-1a transmission from acutely and experimentally infected white-tailed deer (*Odocoileus virginianus*) fawns to colostrum-deprived calves has been described (Negrón et al., 2012). Furthermore, BVDV-1b transmission from persistently infected cattle to cohabitant pregnant white-tailed deer using a model of natural challenge has been demonstrated (Passler et al., 2009). Monitoring for BVDV in Austrian farmed and free-living red deer (*Cervus elaphus*) failed in detecting the viral RNA in ear-notch samples and suggested a limited role of these wild animals in viral spread (Glawischnig et al., 2010). In our work, viral RNA has been found in different wild deer species, suggesting that their role in viral spread and environmental contamination should be further investigated. In particular, BVDV-1a was found both in wildlife (chamois and deer) and in pasture-raised cattle, thus wildlife/livestock transmission can be suspected. On the contrary, BVDV-1c was found in chamois but not in domestic cattle and the source of infection still remains unknown.

In conclusion, this study shows for the first time that faeces of wild ruminants can be used to evaluate *Pestivirus* infection and for molecular epidemiology purposes. BVDV-1a strains were found in cattle, red deer, roe deer, and Apennine chamois, and BVDV-1c was found only in roe deer and Apennine chamois for the first time in Italy. Although the transmission of BVDV-1 at the wildlife/livestock interface could not be demonstrated, the same subgenotype 1a has been found both in cattle and in wild ruminants, therefore the risk of viral transmission and its effect on wildlife health should be further investigated, especially where cattle are not vaccinated and are pasture-raised.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.07.002.

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