Evidence for Tunisian-Like Pestiviruses Presence in Small Ruminants in Italy Since 2007

Evidence for Tunisian-like pestiviruses presence in small ruminants in Italy since 2007. Molecular typing of sheep and goat pestiviruses.

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Abstract

The genus *Pestivirus*, which belongs to the *Flaviviridae* family, includes ssRNA+ viruses responsible for infectious diseases in pigs, cattle, sheep, goats and other domestic and wild ruminants. Like most of the RNA viruses, pestivirus has high genome variability with practical consequences on disease epidemiology, diagnosis and control. In addition to the officially recognized species in the genus *Pestivirus*, such as BVDV- 1, BVDV-2, BDV and CSFV, other pestiviruses have been detected. Furthermore, most of the ruminant pestiviruses show low or absent species specificity observed in serological tests and are able to infect multiple species. Particularly, small ruminants are receptive hosts of the most heterogeneous group of pestiviruses. The aim of this study was to carry out the molecular characterization of pestiviruses isolated from sheep and goats in Sicily, Italy. Phylogenetic analysis of two viral genomic regions (a fragment of 5'-UTR and the whole N^{pro} regions) revealed the presence of different pestivirus genotypes in the analyzed goat and sheep herds. Two out of five viral isolates clustered with BVDV-1d viruses, a strain widespread in Italy, but never reported in Sicily. The other three isolates formed a distinct cluster with high similarity to Tunisian isolates, recently proposed as a new pestivirus species. This represents the first evidence for Tunisian-like pestivirus presence in small ruminants in Italy. Furthermore, one of the isolates was collected from a goat, representing the first isolation of Tunisian-like pestivirus from this species.

Keywords:

Pestivirus, phylogenetic analysis, Tunisian-like pestivirus, border disease virus, bovine viral diarrhea virus, classical swine fever virus, sheep, goats.

Introduction

Pestiviruses are a heterogeneous group of related viruses that differ in their antigenicity, cytopathogenicity and virulence. Pestiviruses have an ssRNA+ genome approximately 12.3 kb in length. It contains a single open reading frame coding for structural (C, E^{rns}, E1, E2) and non-structural proteins (N^{pro}, NS2, NS3, NS4A, NS4B, NS5A, NS5B) flanked by 5' and 3' untranslated regions (UTR) (Simmonds et al., 2012).

Genus *Pestivirus*, within the *Flaviviridae* family, currently comprises four recognized species: Bovine Viral Diarrhea Virus types 1 (BVDV-1), Bovine Viral Diarrhea Virus type 2 (BVDV-2), Border Disease Virus (BDV) and Classical Swine Fever Virus (CSFV). In addition to the recognized species, four additional pestivirus species have been proposed, but they remain officially unrecognized (Simmonds et al., 2012). These putative species include Giraffe-1 pestivirus, Pronghorn antelope pestivirus, Bungowannah virus and a group of viruses variously referred to as Atypical pestivirus or HoBi-like viruses for which has been proposed the name BVDV-3 (Simmonds et al., 2012; Bauermann et al., 2013). The species demarcation of pestiviruses is described mainly by genetic analysis, and the 5'-UTR and N-terminal autoprotease (N^{pro}) regions are the most commonly used for genetic classification (Vilcek et al., 2001; Giammarioli et al., 2011; Simmonds et al., 2012).

In contrast to what is observed in CSFV infections, ruminant pestiviruses show low species-specificity in serological tests (Vilcek et al., 1997; Krametter-Froetscher et al., 2009). In fact, BVDV-1, BVDV-2 and BDV are frequently detected either in cattle, sheep, goats and pigs. Moreover, ruminant pestiviruses are genetically classified into various subtypes. To date, the viruses included in the BVDV-1 species are differentiated into at least 20 subtypes, indicated as 1a-1t (Giammarioli et al 2015a), BVDV-2 consists of three subtypes named 2a-2c (Jenckel et al., 2014), and BDV includes at least eight subtypes designated BDV-1 to BDV-8 (Valdazo-Gonzáles et al., 2007; Dubois et al., 2008; Giammarioli et al., 2011; Peletto et al., 2016). Other pestiviruses have

been isolated from batches of contaminated sheep pox virus vaccine in Tunisia and from sheep and goats in Turkey and Tunisia and genetically characterized (Thabti et al., 2005; Oguzoglu et al., 2009). Although they are considered BDV, phylogenetic analysis places these strains slightly further outside all other BDV and closer to CSFV, indicating an evolutionary history separate from other BDV genotypes (Liu et al., 2009; Becher et al., 2012; Oguzoglu, 2012). Based on these studies, small ruminants are hosts of the most heterogeneous group of pestiviruses; this finding, together with the significant importance of sheep and goat farming in Italy has generated great interest in the analysis of strains circulating in the population of small ruminants in Italy. Pestivirus infection in Italy has been reported since the 1990s (Buonavoglia et al., 1994) and a survey showed a seroprevalence rate of 11.6% in Sicily (Iannizzotto et al., 1998). Several studies have shown that Italy is one of the countries with the highest reported diversity of pestiviruses in small ruminants (Pratelli et al., 2001; Rosamilia et al., 2014; Giammarioli et al., 2015b). However, no data are available on pestivirus strains circulating in small ruminants in Sicily, Italy.

This study deals with the genetic characterization of pestiviruses isolated from sheep and goats farmed in Sicily, Italy and it highlights the presence of various pestivirus species in the small ruminant population, including, viruses related to Tunisian isolates recently proposed as a novel pestivirus species named Tunisian sheep virus (TSV) (Liu et al., 2009).

2. Material and methods

2.1. Location, animals and viruses

The viral strains analyzed in this study were isolated from small ruminant specimens (Table 1): two viruses were isolated from ovine fetuses, one in 2007 (hybrid breed, strain 70282/2007/EN) and one

in 2012 (hybrid Valle del Belice x Comisana breed, strain 59434/2012/EN); three viruses were isolated from goats, one from an adult in 2007 (Saanen breed, strain 92019/2007/AG), and two from fetuses in 2011 (hybrid breed, strains 71982/2011/PA and 71982/2011/2PA). Samples were collected from four farms with reproductive problems and herds were serologically positive for pestivirus. Farms were located in Sicily region, Italy: one farm was in the province of Agrigento (AG), two farms in the province of Enna (EN) and one farm was in the province of Palermo (PA). Previous serological tests were carried out to determine the infectious status of the farm by a solid phase indirect enzyme linked immunosorbent assay (ELISA) developed to detect Border Disease Virus specific antibodies (IgG_1) in ovine and caprine serum and milk samples (SVANOVIR® BDV-Ab; Svanova Biotech, Uppsala, Sweden). Post-mortem examinations were conducted on aborted fetuses and the diseased goat.

To isolate the viruses, homogenized organs were prepared in Minimum Essential Medium (MEM; Gibco, Life Technologies, Paisley, UK) supplemented with antibiotic/antimycotic (1,000 U/mL penicillin, 1 mg/mL streptomycin, 2.5 μg/mL amphotericin B; Gibco, Life Technologies, Paisley, UK) and inoculated in cell lines. For this purpose, primary Ovine Fetal Kidney (OFK) and Madin Derby Bovine Kidney (MDBK) cell monolayers, previously tested as free from contamination with any endogenous pestivirus, were used. Cell culture medium (MEM; Gibco, Life Technologies) was supplemented with gamma irradiated fetal bovine serum, free of pestivirus antibodies, antigens and genome. The infected cells were incubated for 6-7 days at 37°C and checked daily. After three passages, cell lysates were tested by RT-PCR assay to confirm pestivirus isolation. For this purpose viral RNA was extracted from 200 μL of cell lysates using the High Pure RNA Isolation Kit (Roche, Mannheim, Germany) according to the instruction manual of the manufacturer. RT-PCR was performed essentially as previously described (Kim and Dubovi, 2003) and primers used are reported in Table 2. Briefly, the RT-PCR was performed using a commercial kit (AccessQuick™ RT-PCR System, Promega, Wisconsin, USA) in a total volume of 25 μL. The master mix consisted

of 12.5 µL of 2X AccessQuick™ master mix, 0.4 µM of each primer, 2.5 µl of template RNA, 0.5 µL of AMV Reverse Transcriptase, 0.12 U/µL of RNase Inhibitor and Nuclease-free PCR water. RT-PCR was conducted with the following thermal cycle: 45°C for 30 minutes to synthesize the first strand cDNA, 94°C for 10 minutes to inactivate the reverse transcriptase, and 35 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute. A final extension was performed at 72°C for 10 minutes.

Samples collected were also tested for the presence of other infectious agents such as *Brucella* spp., *Campylobacter* spp., *Salmonella* spp., *Escherichia coli*, *Listeria* spp., *Pasteurella* spp., *Mycoplasma* spp., *Corynebacterium* spp., *Clostridium* spp. The bacteriological screening and pathogen identification were conducted according to Office International Epizooties (OIE) standardized protocols (OIE, 2015). For *Corynebacterium* spp. and *Clostridium* spp. methods were adapted from Carter and Cole, (1990). *Chlamydophila* spp. presence was detected by direct PCR in biological samples (OIE, 2015). Furthermore, parasitological tests to detect *Cryptosporidium* spp., *Neospora caninum*, *Toxoplasma gondii*, *Theileria* spp., nematodes and cestodes were conducted (Euzeby, 1958). Direct molecular detection through PCR assays was conducted for *N. caninum*, *T. gondii*, *Theileria* spp. according to molecular assays as elsewhere described (Vitale et al., 2013; Office International Epizooties (OIE), 2015).

2.2. Genetic typing

For genetic typing of isolates viruses, viral RNA was extracted from cell culture supernatants with TRI REAGENT® (Molecular Research Center Inc., Ohio, USA) according to the manufacturer's instructions. The synthesis of cDNA was carried out using either random hexamers or pestivirus-specific primers and SuperScript III reverse transcriptase (Invitrogen, San Diego, CA, USA), following the conditions recommended by the manufacturer.

Viruses isolated in 2007 and 2011 were subjected to a nested PCR assay to identify and distinguish among BVDV-1, BVDV-2 and BDV genotypes (Sullivan and Akkina, 1995). Briefly, the first amplification step was conducted with primers P1 and P2 (Table 2) that amplify a 826 bp product of the E^{rns} region from any of the three pestivirus species, while the second round nested PCR was conducted with type-specific primers (TS1, TS2, TS3; Table 2) which yielded DNA products of unique size characteristic for each pestivirus genotype (BDV: 566 bp product; BVDV-2: 448 bp product; BVDV-1: 223 bp product). The reaction mixtures for the PCRs had a total volume of 25 µL and contained 2.5 µL of 5X PCR Buffer, 1.5 mM $MgCl_2$, 0.4 µM of each primer, 1 µl of cDNA, 2.5 units of Platinum Taq DNA polymerase (Invitrogen) and Nuclease-free water. The thermal cycle for nested PCR consisted of 95°C for 1 min and 25 cycles of 94°C for 1 min, 54°C (primers P1 and P2) or 50°C (primers TS1, TS2, TS3 and P2) for 45 sec and 72°C for 45 sec. A final extension was performed at 72°C for 7 min.

All viral strains isolated in the study were further amplified using a combination of primers described previously (Table 2) to obtain two fragments, including the 5'-UTR and N^{pro} regions for sequencing (Vilcek et al., 1997). The reaction mixtures and thermal cycles were conducted as described previously. PCR products were purified by High Pure PCR Product Purification (Roche) and used as templates in automated sequencing reactions (*ABI* PRISM Genetic Analyzer, Applied Biosystems Carlsbad, CA). Alignment and phylogenetic analysis were conducted on the 5'-UTR region fragment without the primers' region (246 bp) and on the complete sequence of the Npro region (501 bp). Nucleotide sequences were aligned and compared with sequences previously obtained from strains isolated from cattle (Ciulli et al., 2008a; Cannella et al., 2012) and with sequences available in GenBank (www.ncbi.nlm.nih.gov) using Clustal W in BioEdit software (http://bioedit.software.informer.com/). Percentage of similarity of pairwise distances was calculated with BioEdit software. Phylogenetic analysis of 5'-UTR and N^{pro} regions were performed with MEGA 5 software (www.megasoftware.net) using the neighbor-joining method

according to the Kimura two-parameter model. Bootstrap analysis was carried out on 1000 replicates.

3. Results

At post-mortem examinations the fetuses showed systemic lesions characterized by jelly and hemorrhagic subcutaneous edema and the presence of serum hemorrhagic fluid in serous cavities (pleural and peritoneal effusion). We also observed edematous and congested organs, catarrhal abomasitis, hemorrhagic enteritis and fibrinous pleuritis. The adult specimen showed dehydration, weight loss, increased lung volume, catarrhal bronchopneumonia, hemorrhagic-necrotic enteritis and a liver sprinkled with necrotic foci.

Viral strains isolated in this study showed no (70282/2007/EN, 92019/2007/AG, 59434/2012/EN) or mild (71982/2011/PA, 71982/2011/2PA) cytopathic effects in cell culture. However, RT-PCR analysis of cell lysates confirmed that pestiviruses were isolated from all organs investigated (brain, lung, heart, liver, spleen, intestine), demonstrating systemic infection. All viral isolates, in fact, were identified with the RT-PCR by the amplification of a specific product of 290 bp with primers DL1 and DL4 (Table 3).

The two goat fetuses were also positive for *Chlamydophila* spp., *Campylobacter* spp., *Salmonella* spp. and *E. coli* (71982/2011/PA, 71982/2011/2PA). The adult goat was positive for *Corynebacterium* spp. (92019/2007/AG).

All viral strains tested with the nested PCR (70282/2007/EN, 92019/2007/AG, 71982/2011/PA, 71982/2011/2PA) were identified as pestiviruses (by the amplification of a specific product of 826 bp with primers P1 and P2 (Table 3), but only two of them were further characterized by nested PCR. Strains 71982/2011/PA and 71982/2011/2PA gave a product of 223 bp as the result of the

amplification with primer TS3-P2 specific for BVDV-1 (Table 3). No products were obtained for strains 70282/2007/EN and 92019/2007/AG by nested PCR analysis (Table 3).

PCR sequences for the 5'-UTR region were obtained for all the isolates (70282/2007/EN, 92019/2007/AG, 71982/2011/PA, 71982/2011/2PA, 59434/2012/EN), while N^{pro} region sequences were obtained for one ovine isolate (70282/2007/EN) and three caprine isolates (71982/2011/PA, 71982/2011/2PA and 92019/2007/AG). The sequences have been deposited in GenBank (NCBI) with the accession numbers KU856551–KU856559.

An alignment for each analyzed region (5'-UTR and Npro) was built, including sequences obtained in this study, sequences previously obtained from strains isolated from cattle (Ciulli et al., 2008a; Cannella et al., 2012) and sequences representative for each pestivirus cluster retrieved from GenBank database. Alignment files were used for both phylogenetic analysis and percentage of similarity of pairwise distance calculation.

Phylogenetic analysis of the ovine and caprine viruses analyzed in this study assigned them to different viral groups inside the *Pestivirus* genus. On the basis of phylogenetic analysis of the 5'-UTR region, pestiviruses were clustered in the four official species recognized in the genus *Pestivirus* (BVDV-1, BVDV-2, BDV and CSFV); furthermore, ovine and caprine pestiviruses clustered in two further groups named Turkey and Tunisian isolates (Fig. 1). Three (70282/2007/EN, 92019/2007/AG, 59434/2012/EN) out of five of the Italian small ruminant pestiviruses isolated in this study clustered together with the Tunisian isolates (Fig. 1). Bootstrap analysis strongly supported these results (Fig. 1). The other two viruses isolated from goat fetuses in 2011 (71982/2011/PA and 71982/2011/2PA) clustered within the BVDV-1 genotype (Fig. 1). A further comparison of the 5'-UTR sequences of BVDV-1 isolated from goats in 2011 with sequences previously obtained from BVDV-1 strains isolated from cattle in Italy (Ciulli et al., 2008a; Cannella et al., 2012) and with sequences available in GenBank representing BVDV-1 subtypes showed that they clustered with subtype BVDV-1d (Fig. 2). The analysis of the complete

N^{pro} region confirmed the results obtained with 5'-UTR region analysis, clustering the new Italian small ruminant strains and Tunisian strains in a separate branch (Fig. 3).

No correlation was observed between pestiviruses isolated from sheep and goats in this study and strains previously isolated from small ruminants in Italy and classified into different subtypes of the BDV genotype (Rosamilia et al., 2014; Giammarioli et al 2015).

The percentage of similarity of pairwise distances confirmed the results obtained by phylogenetic analysis (Table 4). Strains 71982/2011/PA and 71982/2011/2PA showed the highest nucleotide identities with viruses of the BVDV-1 genotype (>86.8% and >79.9% for the 5'-UTR and N^{pro} region, respectively) compared with other genotypes (<75.3% and <69.4% for the 5'-UTR and N^{pro} region, respectively). On the other hand, the three new Italian isolates forming a distinct cluster (70282/2007/EN, 92019/2007/AG, 59434/2012/EN) showed the highest nucleotide identities with Tunisian strains (>88.4% and >81.9% for the 5'-UTR and N^{pro} region, respectively) with the highest value with the French isolate (91.7-92.1% and 81.9-82.9% for the 5'-UTR and N^{pro} region, respectively), followed by CSFV (83.0-84.2 and 74.4-76.9 for the 5'-UTR and N^{pro} region, respectively) and Turkish strains (81.3-82.5 and 71.0-72.6% for the 5'-UTR and N^{pro} region, respectively). The nucleotide identity of these strains with all BDV subtypes was always ≤82.1% and ≤72.8% for the 5'-UTR and N^{pro} region respectively.

4. Discussion

In this study, pestiviruses were isolated from four aborted fetuses and the internal organs of a sick goat collected from four seropositive farms located in Sicily, Italy. Other microorganisms were detected in these specimens, so the direct responsibility of the pestivirus in abortion or inducing the observed pathology in the goat could not be established. However, the role of pestivirus as an immunosuppressive agent, exacerbating the pathogenicity of co-infecting microorganisms, should

also be considered (Oguzoglu, 2012). In our study, co-infections with other microorganisms, such as *Chlamydophila* spp., *Campylobacter* spp., *Salmonella* spp., *E. coli* and *Corynebacterium* spp, were present in three of the five pestivirus infected subjects. This could be due to the typical leukopenia associated with pestivirus infection; in fact, it is well-known that the immunosuppressive activity of pestiviruses promotes the engraftment and virulence of other pathogens (Peterhans et al., 2003; Daly and Neiger, 2008).

Although the presence of pestiviruses in sheep has been reported in Sicily since 1994 (Buonavoglia et al., 1994) and pestivirus seroprevalence in Sicilian sheep and goat flocks has been previously reported (Iannizzotto et al., 1998), no information was available on viral strains detected in small ruminants. Previous investigations genetically characterized pestiviruses collected from cattle in Sicily, showing the circulation of BVDV-1b and BVDV Ie, i.e. subtypes with a wide temporal-spatial distribution in Italy (Cannella et al., 2012; Luzzago et al., 2014).

Traditionally, all pestiviruses isolated from goats and sheep have been referred to as BDV, but genetic characterization has shown that they can be infected with a wide range of pestiviruses including BDV, BVDV-1 and BVDV-2 (Pratelli et al., 2001, Giammarioli et al., 2015b). Equally, cases have reported of calves persistently infected with BDV (Krametter-Froetscher et al., 2009). Furthermore, recent studies showed that BDV strains have a genetic diversity greater than other pestivirus species (Giammarioli et al., 2011). Moreover, recent genetic and antigenic analyses of ovine and caprine pestiviruses showed the presence of two new and unique groups named Turkey and Tunisian isolates, both proposed as new species inside the *Pestivirus* genus (Liu et al., 2009; 2011; Becher et al., 2012; Oguzoglu, 2012). In consideration of these findings, to prevent the spread of disease, monitoring of the pestivirus status of all ruminants, particularly in mixed herds (cattle, sheep and goat) is needed. Furthermore, low cross-neutralization between different pestivirus species and the detection of new and atypical strains of pestiviruses raises the problem of using biomolecular investigations able to elucidate genetic characteristics of isolated viruses (Decaro et

al., 2012). However, even in the context of molecular analysis, it is essential to make an informed choice among the available methods (for example, in the choice of the target region and/or primers) because of rapid viral evolution, typical of RNA viruses, as it introduces variables in the gene sequence that can affect the interpretations of the results depending on the method applied (Battilani et al., 2003; Konig et al., 2003; Ciulli et al., 2008b; Luzzago et al., 2012).

In this study, the application of a method previously described to identify and distinguish among BVDV-1, BVDV-2 and BDV genotypes failed to identify two out of four strains on which it was applied (Sullivan and Akkina, 1995). Further analysis showed that the two unidentified viruses could not be included in one of the species identified by this method (BVDV-1, BVDV-2 and BDV). On the contrary, sequencing analysis of two regions considered the most useful to classify viruses of the *Pestivirus* genus, (Vilcek et al., 1997; Simmonds et al., 2012) allowed us to define the viral species or, at least, the relationship between the viruses isolated in this study and those previously known. Particularly, genetic characterization of 5'-UTR and N^{pro} regions allowed us to assign the isolated viruses to two groups within the *Pestivirus* genus. The two strains isolated in 2011 from goat fetuses were found to belong to the species BVDV-1, subtype BVDV-1d. Genetic characterization of pestiviruses isolated from bovines collected in the same region did not indicate the circulation of this subtype in cattle (Cannella et al., 2012); however, high heterogeneity was previously reported in Italian BVDV-1 isolates, including the presence of the BVDV-1d subtype (Luzzago et al., 2001; Falcone et al., 2003; Ciulli et al., 2008a; Giammarioli et al., 2008). A recent spatial reconstruction of BVDV-1 dispersion in Italy hypothesized that the BVDV-1 infection originated in larger animal populations in Northern Italy then diffused to smaller populations in other parts of Italy following the patterns of national commercial flow (Luzzago et al., 2012). On the basis of this study, Sicilian BVDV-1 strains were related to those of three localities, particularly Lombardy and Emilia Romagna, Umbria and Marche and Apulia, in two of which the BVDV 1d subtype has been frequently detected (Falcone et al., 2003; Ciulli et al., 2008a; Giammarioli et al.,

2008; Luzzago et al., 2012). Although the cited study referred to cattle, due to the lack of species barrier for BVDV infection, all ruminants have to be considered, in our opinion, as a unique receptive population for BVDV. This is true, at least, in some specific geographical areas, such as Sicily, where the practice to breed cattle and small ruminates together is common. In fact, four samples (92019/2007/AG, 71982/2011/PA, 71982/2011/2PA, 59434/2012/EN) out of five analyzed in this study were collected from farms with a mixed bovine/sheep/goat population.

Even more interesting were the results of the genotyping of the other three strains detected in one sheep and one goat in 2007 and in one sheep in 2012. The 5'-UTR region phylogenetic analysis definitively placed these strains outside of all species of the genus *Pestivirus* that have been officially recognized; in fact, they did not cluster with BVDV-1 and BVDV-2, nor with any of the BDV subtypes, and they seemed only distantly related to CSFV. On the contrary, they were strictly related to a group of Tunisian isolates, and formed a distinct cluster that originated from a branch common to Tunisian isolates (Thabti et al., 2005). N^{pro} region phylogenetic analysis and nucleotide identity analysis confirmed the relationship between the new small ruminant Italian strains and Tunisian isolates. This is the first evidence of Tunisian-like pestivirus presence in Italy. However, Tunisian-like pestivirus presence has been just detected in another European country, France (Dobois et al., 2008; Martin et al., 2015). Based on the date of sample collection, the first finding of a Tunisian-like pestivirus in an Italian sample was in 2007 and evidence for a Tunisian-like pestivirus in sheep in 2012 shows the persistence of this viral species in the same geographical region. However, a similar occurrence was reported in France, where Tunisian-like pestiviruses were detected in sheep samples collected in the PACA (Provence-Alpes-Côte d'Azur) region in 1991 and nineteen years later, in 2010 (Dubois et al., 2008; Martin et al., 2015).

No absolute data are available to establish the Tunisian-like strain way of introduction in Sicily, Italy, nor there is evidence for the association with the use of contaminated vaccines such as previously reported (Thabti et al., 2005). The finding of similar strains in several countries (France, Tunisia and Italy) might reflect the trade in small ruminants among these countries, and emphasizes

once more the importance of sanitary controls of the imported and exported animals as previously pointed out (Dobois et al., 2008).

Previous genetic and antigenic characterization classified Tunisian isolates within the BDV (Thabti et al., 2005), even if sequence similarity with CSFV and tree topology assigned them to CSFV. In this respect, the new Italian isolates also showed higher nucleotide identity of the 5'-UTR and N^{pro} regions with CFSV strains than with all the BDV strains (BDV1-BDV8). These results are in accordance with a comprehensive phylogenetic and evolutionary study of pestiviruses that definitely propose Tunisian-like pestivirus as new species independent of BDV (Liu et al., 2009).

This study reinforces the findings for previous works (Thabit et al., 2005; Liu et al., 2009; Martin et al., 2015) that Tunisian-like pestivirus probably had the CSFV's most recent ancestor rather than BDV.

Liu and coauthors (2009) proposed the name Tunisian sheep virus (TSV) for this new species, on the basis of previous isolation of these viruses from both Tunisian sheep and different batches of a contaminated Tunisian sheep pox vaccine (Thabti et al., 2005). Findings of Tunisian-like pestiviruses in France were also detected in sheep (Dubois et a., 2008; Martin et al., 2015). However, in the present study Tunisian-like pestiviruses were detected both in sheep and goat, representing the first evidence of involvement of this last species in Tunisian-like pestivirus infection. For this reason, we propose the classification for this atypical pestivirus as Tunisian Small Ruminant Virus (TSRV).

However, from an epidemiological point of view, the finding of similar viruses in both sheep and goats is not surprising since other pestiviruses have been frequently isolated from both sheep and goats, such as Turkey isolates (Postel et al., 2015) and BDV (Giammarioli et al., 2015b; Mao et al., 2015).

In conclusion, these results, show for the first time, the presence of Tunisian-like pestiviruses in Italy. Furthermore, Tunisian-like pestivirus infection was detected, for the first time, in goat, suggesting even for this viral species the ability to interspecies transmission. However, it will be necessary to investigate further to define the pathogenetic and epidemiological roles of these viruses not only in the small ruminant, but also in the bovine population and wild ruminants.

Conflict of interest

The authors declare no conflict of interest.

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Table 1. Details of pestivirus strains analyzed in this study.

Name	Host	Animal/specimen	Province/farm	Year	Genbank accession no.	
					5' UTR	N^{pro}
70282/2007/EN	sheep	fetus	Enna/farm 1	2007	KU856551	KU856556
92019/2007/AG	goat	adult, internal organs	Agrigento	2007	KU856552	KU856557
71982/2011/PA	goat	fetus	Palermo	2011	KU856553	KU856558
71982/2011/2PA	goat	fetus	Palermo	2011	KU856554	KU856559
59434/2012/EN	sheep	fetus	Enna/farm 2	2012	KU856555	nd

Table 2. Nucleotide sequences of the primers employed in the study.

Primers	Sequences (5'→3')	Genome Position[a]	Amplified DNA product (bp)	References
DL1	GCCATGCCCTTAGTAGGACTAGC	105-127	290	Kim and Dubovi, 2003
DL4	CAACTCCATGTGCCATGTACAGC	394-371		
324	ATGCCCTTAGTAGGACTAGCA	108-128	288	Vilcek et al., 1994
326	TCAACTCCATGTGCCATGTAC	395-375		
P1	AACAAACATGGTTGGTGCAACTGGT	1424-1449	826	
P2	CTTACACAGACATATTTGCCTAGGTTCCA	2221-2250		
TS1	TATATTATTTGGAGACAGTGAATGTAGTAGCT	1684-1716	566 (TS1 and P2)	Sullivan and Akkina, 1995
TS2	TGGTTAGGGAAGCAATTAGG	1802-1821	448 (TS2 and P2)	
TS3	GGGGGTCACTTGTCGGAGG	2027-2045	223 (TS3 and P2)	
BD1	TCTCTGCTGTACATGGCACATG	367-388	738	Vilcek et al., 1997
BD2	TTGTTRTGGTACARRCCGTC	1104-1085		

[a] The genome positions of each primer are based on the sequence of BVDV strain NADL (GenBank accession number M31182).

Table 3. RT-PCR (Kim and Dubovi, 2003 protocol) and RT-nested/PCR (Sullivan and Akkina, 1995 protocol) results.

PCR	70282/2007/EN	92019/2007/AG	71982/2011/PA	71982/2011/2PA	59434/2012/EN
DL1-DL4	+	+	+	+	+
P1-P2	+	+	+	+	nd
TS1- P2 (BDV)	-	-	-	-	nd
TS2- P2 (BVDV-2)	-	-	-	-	nd
TS3- P2 (BVDV-1)	-	-	+	+	nd

nd: not determined

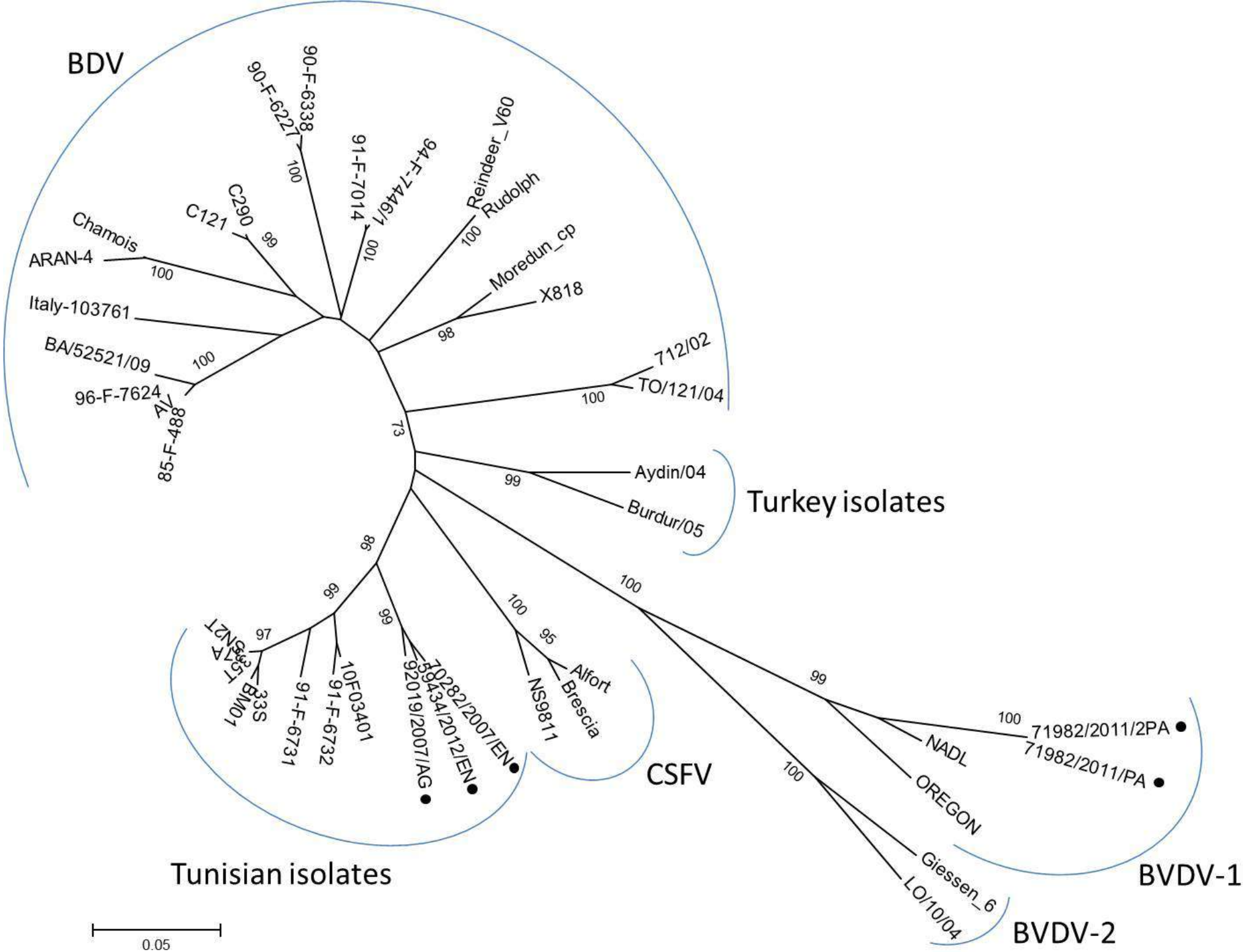
Table 4. Comparisons of nucleotide sequences of 5'UTR and N^{pro} regions of the small ruminants pestiviruses isolated in this study (70282/2007/EN; 92019/2007/AG; 71982/2011/PA; 71982/2011/2PA; 59434/2012/EN) and pestiviruses classified in other genotypes and subgroups. Pestivirus strains used for comparison are those used for generation of the phylogenetic trees and Genbank accession numbers are reported in relative captions. Percentage of similarity of pairwise distances for 5'UTR and N^{pro} (bold) regions of the most and least divergent isolates are shown.

	BVDV-1	BVDV-2	BDV1	BDV2	BDV3	BDV4	BDV5	BDV6	BDV7	BDV8	Turkey	Tunisian strains		CSFV
												French	Tunisian	
70282/2007/EN	73.1-73.2	70.2-70.9	79.2-80.8	78.0-78.4	78.3-79.1	78.8-81.3	77.2-78.8	79.6-80.0	78.6-79.0	77.9	81.3	90.0-91.7	88.4-89.6	83.0-83.8
	64.0-66.0	**65.0-68.4**	**71.6-73.4**	**69.8-70.4**	**70.0-72.8**	**68.2-69.8**	**72.0-72.2**	**70.0-71.0**	**71.8-72.0**	**70.2**	**71.6-72.6**	**82.7-84.1**	**82.5-84.1**	**74.4-75.0**
92019/2007/AG	73.5-73.6	69.8-70.5	80.4-82.1	78.8-79.2	79.5-80.4	78.4-80.9	78.0-79.6	80.8-81.2	80.6-81.0	78.7	82.1-82.5	90.4-92.1	88.8-90.0	84.2
	65.0-65.8	**65.2-68.6**	**70.2-71.2**	**71.0-71.6**	**70.2-71.2**	**68.2-69.2**	**70.0-70.2**	**71.0-71.2**	**70.6-70.8**	**70.8**	**71.0-71.4**	**81.9-82.7**	**82.3-83.1**	**74.4-76.9**
71982/2011/PA	86.8-91.8	74.0-75.3	68.6-69.1	68.2-68.6	69.9-70.7	67.0-68.8	67.4-69.1	68.1-68.4	68.2-69.4	69.6	70.8-72.0	67.0-70.7	67.0-68.5	68.2-69.1
71982/2011/2PA	**79.9-80.9**	**66.8-69.4**	**64.8-65.2**	**63.2-64.0**	**62.5**	**64.0-66.4**	**65.2-65.4**	**64.6-65.8**	**65.2-65.4**	**66.2**	**64.4-65.6**	**63.0-65.8**	**64.0-65.4**	**67.2-68.0**
59434/2012/EN	72.3-73.2	70.6-71.3	78.8-80.4	78.0-78.4	77.9-78.7	77.6-80.1	76.8-78.4	79.2-79.6	77.8-78.2	78.3	81.7	90.491.7	88.8-90.0	82.6-83.0

Fig. 1. Phylogenetic tree constructed with 5'-UTR sequences of the five pestivirus isolated in this study (70282/2007/EN, 92019/2007/AG, 71982/2011/PA, 71982/2011/2PA, 59434/2012/EN, labelled by the black dot symbol) and pestivirus sequences retrieved from Genbank. BVDV-1: NADL, Oregon, (M31182, AF091605); BVDV-2: Giessen_6, LO/10/04 (AY379547, AM749823); BDV-1: X818, Moredun CP (AF037405, U65022), BDV-2: Rudolf, Reindeer_V60 (AB122086, AF144618); BDV-3: 90-F-6227, 90-F-6338 (EF693989, EF693991); BDV-4: C121, C290, ARAN-4, Chamois (DQ275625, DQ275624, AM765803, AY738080); BDV-5: 96-F-7624, AV, 85-F-488, BA/52521/09 (EF693998, EF693984, EF693985, LM999988); BDV-6: 94-F-7446/1, 91-F-7014 (EF693996, EF693993); BDV-7: 712/02, TO/121/04 (AJ829444, AM900848); BDV-8: Italy-103761 (KT072634); Turkey: Aydin/04, Burdur/05 (NC_018713, KM408491); Tunisian: BM01, SN2T, 37A, 35T, 33S, 10F03401, 91-F-6731, 91-F-6732 (AY453630, AF461996, AF461999, AF462000, AF462002, KC859386, EF988632, EF988632); Alfort, Brescia, NS9811 (X87939, AF091661, AF521706).

Fig. 2. Phylogenetic tree constructed with 5'-UTR sequences of the two BVDV-1 isolated in this study (71982/2011/PA, 71982/2011/2PA, labelled by the black dot symbol) and BVDV-1 sequences previously obtained from strains isolated from cattle (Ciulli et al., 2008; Cannella et al., 2011) and sequences retrieved from Genbank. BVDV-1a: Oregon, (AF091605); BVDV-1b: IT99-5555, NY-1, IT99-5274, (AJ318600, FJ387232, AJ318612); BVDV-1d: 1/B/01, 3/Vr/95 (AY451339, AJ293595), BVDV-1e: 16/Lo/99, 17a/Cr/00, IT99-5279 (AJ293610, AJ293611, AJ318608); BVDV-1f: IT99-3690 (AJ318615); BVDV-1h: 5/Vr/96, G-Au, IT99-4292 (AJ293597, AF298066, AJ318609); BVDV-1j: M557A/90, M065B/93, Deer, 2/Vr/95 (U97449, U97409, AB040132, AJ293594); BVDV-2: 890 (L32886).

Fig. 3. Phylogenetic tree constructed with N^{pro} sequences of four pestiviruses isolated in this study (70282/2007/EN, 92019/2007/AG, 71982/2011/PA, 71982/2011/2PA, labelled by the black dot symbol) and pestivirus sequences retrieved from Genbank. BVDV-1: Osloss, NADL, Oregon (M96687, M31182, AF091605); BVDV-2: 230/98-K1 (Gi-4), 230/98-K3 (Gi-6), Giessen-1 (AF144468, AF144470; AF104030); BDV-1: X818, BD31 (AF037405, U70263), BDV-2: Chemnitz AZ79, Bison-1 (AY163652, AF144476); BDV-3: Gifhorn, CH-BD2 (AY163653, AY895009); BDV-4: C121, M3 (DQ273159, DQ273163); BDV-5: 96-F-7624, AV (EF693976, EF693962); BDV-6: 90-F-6335, 91-F-7014 (EF693968, EF693971); BDV-7: 712/02, TO/121/04 (AJ829444, AM900847); BDV-8: Italy-103761 (KT072635); Turkey: Aydin/04, Burdur/05 (NC_018713, KM408491); Tunisian: BM01, 33S, SN1T, 10F03401, 91-F-6731, 91-F-6732 (AY453629, AY452485, AY452484, KC859390, EF693982, EF693983); Alfort, Brescia, Schweinfurt (X87939, AF091661, AF144472).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Burdur/05
Turkey isolates
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Brescia
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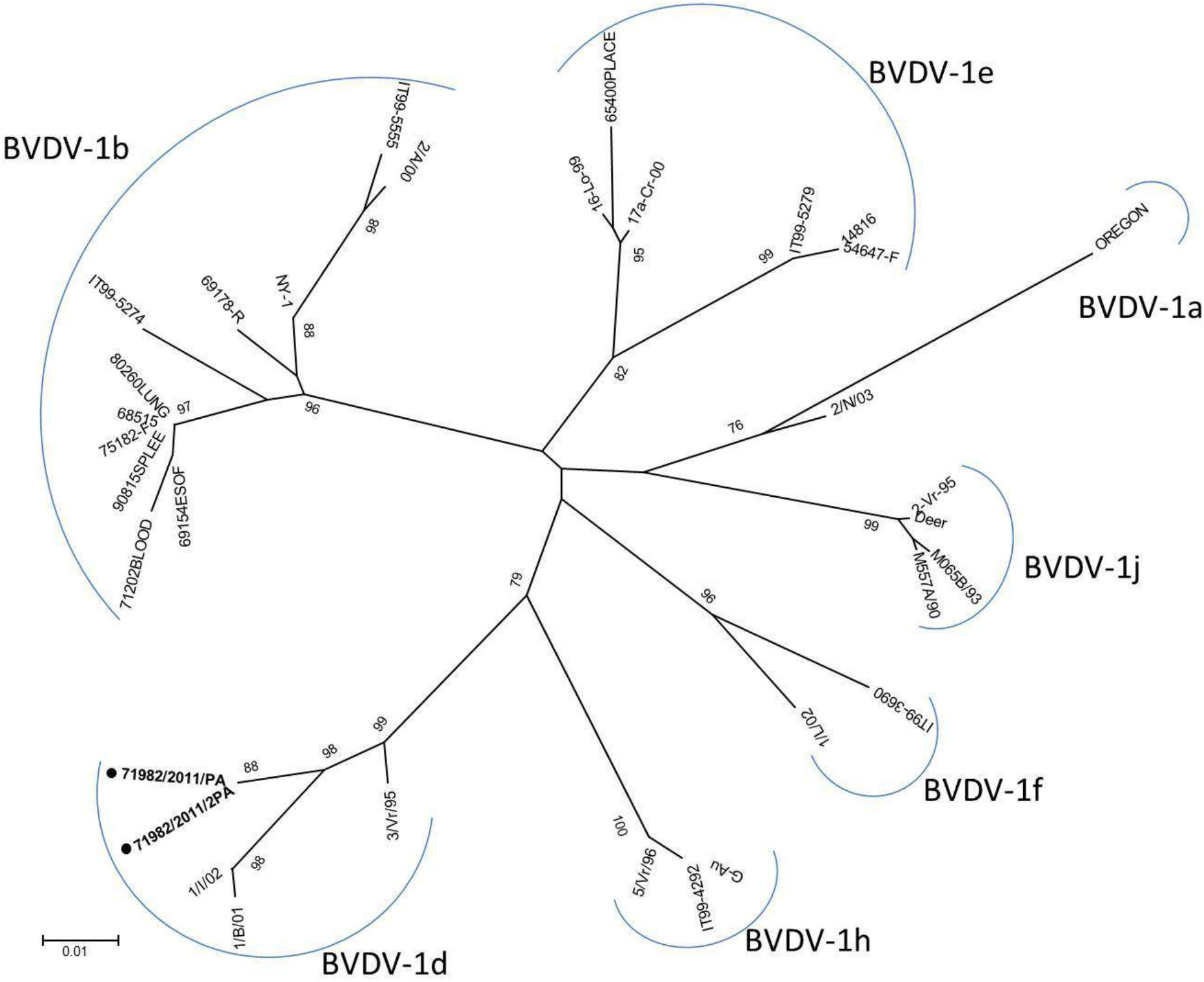
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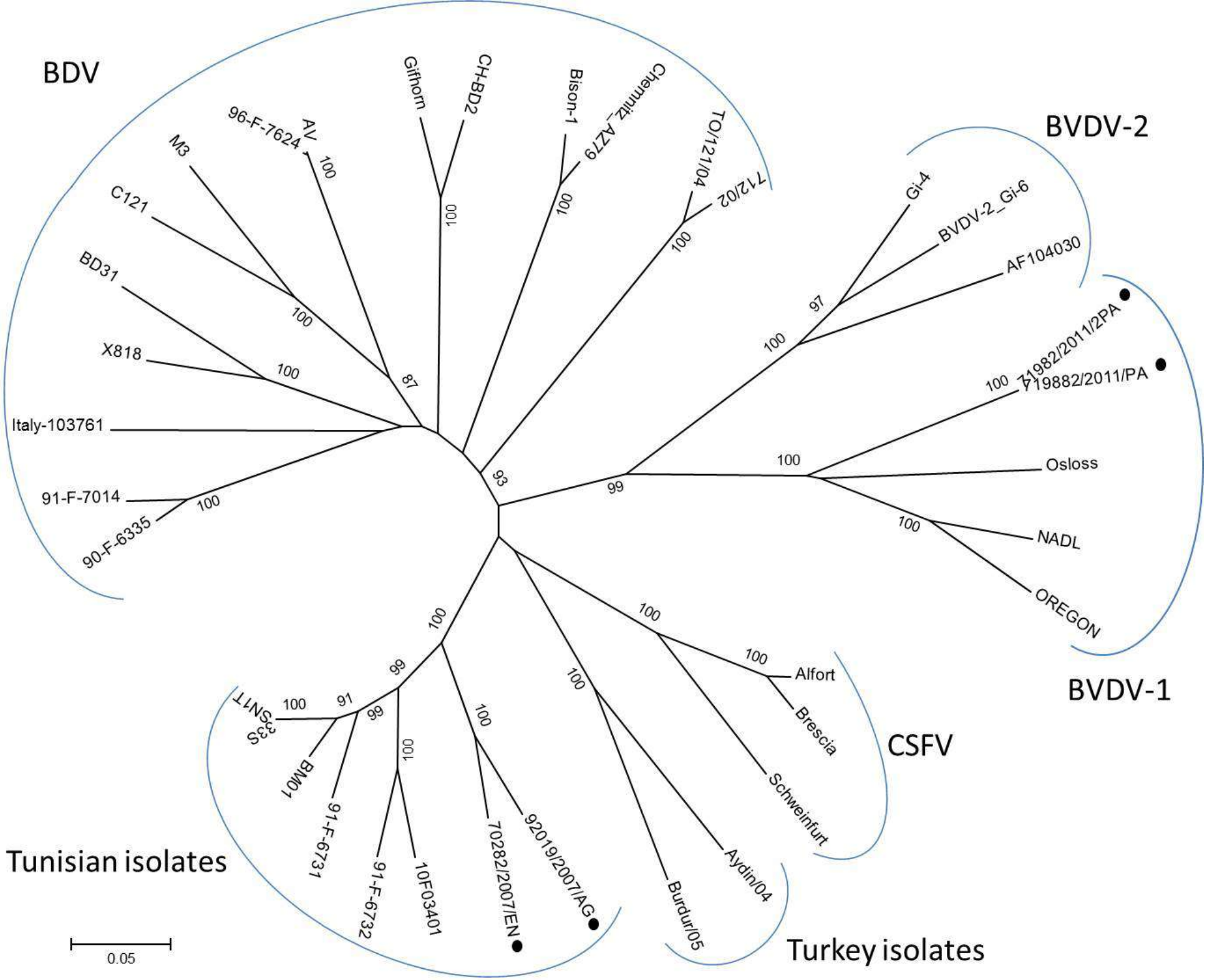
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