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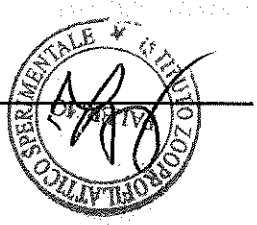
ORIGINAL ARTICLE



Genotyping of bovine viral diarrhea viruses (BVDV) isolated from cattle in Sicily

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Abstract Bovine Viral Diarrhea–Mucosal Disease (BVD–MD) is a widely spread infectious disease that causes important economic losses in farms. Several epidemiological studies indicate a high genetic heterogeneity among Bovine Viral Diarrhea Virus (BVDV) strains circulating in Italy. The aim of this study was to investigate the genotypes of BVDV in Sicily, a region in the South of Italy. For this purpose, 17 BVDV strains collected from cattle breed in Sicily between 2005 and 2008 were genetically typed by sequencing of the 5′-untraslated region (5′-UTR) of the viral genome. In this study, phylogenetic analysis showed that all 17 examined strains were clustered within the BVDV genotype 1. Particularly, 14 of them were clustered with the BVDV-1b subgroup, while the remaining three strains were clustered with the BVDV-1e. Moreover, the restriction analysis indicated a bovine origin for all of the 17 strains typed in this study. These results could be useful to carry out an epidemiological survey and to create vaccines that protect cattle against BVDV different subgroups.

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Introduction

Bovine Viral Diarrhea–Mucosal Disease (BVD–MD) is an infectious disease occurring in cattle; it is a widespread problem to beef and dairy herds that reduces productivity and increases death loss. The BVDV infection has been observed in various forms that cause reduced milk production, reduced conception rate, respiratory disorders, other diseases, and even death among animals acquiring acute infection, and abortions, congenital defecs, and growth retardation after fetal infection. In addition, fetal infection leads to persistent infection (PI) calves that often are small and unthrifty, with increased susceptibility to other diseases, and an increased risk to die due to mucosal disease (Hamers et al., 1998; Houe, 1999). Whereas animals acutely infected shed virus and can potentially expose susceptible contact cattle, PI animals have an important role in the perpetuation of BVDV infections since they are the main source of virus transmission as they continuously shed large amounts of virus in the environment and are seronegative (Baker, 1995).

The various disease syndromes noted in cattle infected with BVDV are mainly attributed to the age of the animal at the onset of the infectious disease and the virus biotype. The BVD virus (BVDV) is a positive sense single stranded RNA virus (ssRNA+), it is a member of the *Pestivirus* genus of the Flaviviridae family, and like all flaviviruses, it has a lipid envelope (Fauquet et al., 2005). Two antigenically distinct genotypes are recognized: BVDV-1 and BVDV-2 (Ridpath et al., 1994; Tajima et al., 2001; Vilcek

et al., 2001; Couvreur et al., 2002); each of the genotypes has been divided into two biotypes, cytopathic (CP) and noncytophatic (NCP), according to their ability to produce visible changes in cell cultures. As with most of the RNA viruses, the BVDV shows a high mutation rate causing the presence of new variants with different antigenicity and pathogenicity and making diagnosis and control plans difficult (Fulton et al., 2003; Bolin and Grooms, 2004).

The BVDV genome includes a single long Open Reading Frames (ORF) sequence, flanked by two non-coding regions: 3′UTR and 5′UTR (Collet et al., 1988; Deng and Brock, 1992). The ORF sequence encodes for a single polyprotein cleaved by cellular and viral proteases giving origin to structural and nonstructural viral proteins (Meyers and Thiel, 1996). On the basis of 5′UTR sequence comparison, the BVDV-I can be separated into 15 sub-genotypes, among which BVDV-Ia and BVDV-Ib are most relevant (Pratelli et al., 2001; Vilcek et al., 2001; 2004; Jackova et al., 2008; Yesilbag et al., 2008; Hornberg et al., 2009). The BVDV-2 has been divided only into 2a and 2b subtypes (Ridpath and Neill 2000; Vilcek et al., 2001; Mishra et al., 2008). It was identified in North America in late 1980s (Perdrizet et al., 1987; Corapi et al., 1989; Ridpath et al., 1994). Now, BVDV-2 infection is widely distributed in Japan (Nagai et al., 2001, 2008), Korea (Kim and Dubovi, 2003), India (Mishra et al., 2008) and China (Zhu et al., 2009). Rarely BVDV-2 has been described in Europe (Falcone et al., 2003, Letellier et al., 1999; Vilcek et al., 2002). In Italy, it was isolated from animals' death following the use of a live IBR vaccine contaminated with genotype BVDV-2 (Falcone et al., 1999, 2001; Luzzago et al., 2001). The Molecular Epidemiology study and knowledge about the diversity of BVDV are important to control the appearance of new variants in a country and to achieve new diagnostic tools. They are also essential to achieve effective vaccination strategies against the genotypes present in a country. So far, phylogenetic analysis has been used to characterize the BVDV genotypes in various regions of Italy (Di Marco et al., 2002; Purpari et al., 2005). The aim of this study was to improve knowledge about genotypes circulating in Sicily, a region on the south of Italy, through a phylogenetic analysis performed on isolated strains.

Materials and methods

Data included in this study were collected from a pool of bovine samples received by the Department of Virology of the Experimental Zooprophylactic Institute of Sicily "A. Mirri" and collected in some farms of Eastern Sicily during the routine tests conducted between 2005 and 2008. All vaginal smears were collected from cows with ipofertility and organs were collected from death animals for unknown causes.

Serological tests

A total of 15,326 serum samples were analyzed for the detection of BVDV specific antibodies, using an indirect Enzyme Linked Immunosorbent Assay (ELISA), following the manufacturer's instructions (Svanova, Biotech AB). Each serum sample was tested in duplicate and the final results were read by a spectrophotometer, measuring the optical density (OD) at 450 nm.

Capture ELISA

Virus detection was carried out by means of a capture ELISA (IDEXX) on 862 buffy coats, 5 vaginal smears and 38 organs, according to the manufacturer's instruction. Results were read through the use of a spectrophotometer at 450 nm.

Real-time polymerase chain reaction

A specific real-time polymerase chain reaction (RT-PCR) to investigate the viral genome was performed on 1,284 buffy coats, 56 vaginal smears 157 organs. For this purpose, total RNA was extracted from samples using a commercial kit, (High pure isolation RNA kit—Roche), according to the manufacturer's instructions. RT-PCR was realized using a Master Mix ready to use (Access Quick Master Mix—Promega), with 0.4 μM of each primer DL1 and DL4 (Table 1), specific for the highly conserved 5′UTR region of the *Pestivirus's* genome, in a total volume of 25 μl (Kim and Dubovi 2003).

Virus isolation

Virus isolation on Madin Derby Bovine Kidney (MDBK) cell monolayers was performed on 17 buffy coats, 110 vaginal smears and 215 organs. The infected cells were incubated for 6 days at 37°C and checked daily. After three passages, criolysates were analyzed through the RT-PCR to identify strains of BVDV (Fig. 1).

Phylogenetic analysis

For genetic typing of the viral strains isolated, the phylogenetic analysis was realized on a nucleotide

Table 1 Nucleotide sequence of primers

Primer	Sequence (5′→3′)	Position in NADL	Product size in base pairs (bp)
DL1	GCCATGCCCTTAGTAGGACTAGC	105–127	290
DL4	CAACTCCATGTGCCATGTACAGC	394–371	
324	ATGCCC$_A^T$TAGTAGGACTAGCA	108–128	288
326	TCAACTCCATGTGCCATGTAC	395–375	

sequence of 210 bp long within the 5′-UTR region of the *Pestivirus's* genome. For this purpose, RNA from each strain was retrotranscribed and then amplified as previously described (Vilcek et al., 1994). The reverse transcription was performed using the forward primer 324 (Table 1) and a commercial kit (First strand cDNA syntesis—Roche). The cDNA amplification by PCR was performed in a total volume of 50 µl containing a 1× PCR Buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl), 0.8 mM of dNTP, 1 µM of each primer 324 and 326 (Table 1) and 2.5 U of Taq Polymerase (Go Taq DNA Polymerase—Promega). Sequence analysis was carried out in both directions. Obtained sequences were aligned and compared to other reference sequences representative of phylogenetic groups of BVDV, previously identified and available in GenBank using Clustal W program (Thompson et al., 1994). The alignment was manually edited using the BioEdit program ver. 5.0.9 (Hall, 1999), and phylogenetic analysis was carried out using the Phylip program ver.3.6. The pairwise genetic distance was calculated using the 84 method (F84) and the phylogenetic tree was constructed using the Neighbor-Joining method. The bootstrap analysis was carried out on 1,000 replicates (Felsenstein 1985; 2001).

In order to verify the bovine origin of the strains isolated, restriction analysis was also performed. For this purpose, the *AvaI* restriction enzyme was used to digest the amplified products, obtained with the 324/326 primers. The restriction pattern was visualized in a 2% agarose gel (Vilcek et al., 1994).

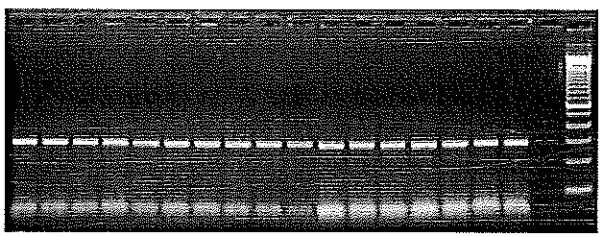
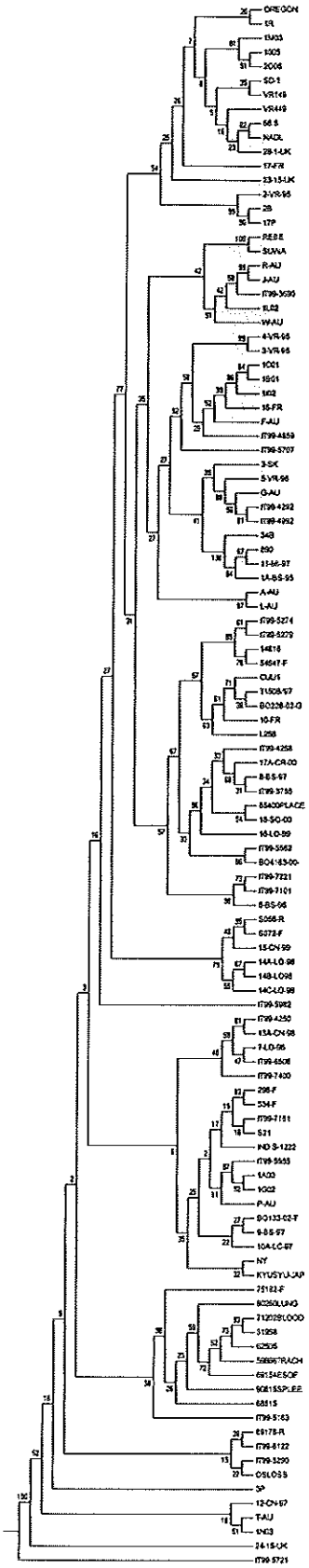


Fig. 1 Two hundred eighty-eight base pair amplification products. Wells 1–17, BVDV strains analyzed in this study; well 18, negative control; well 19, 100-bp ladder

Results and discussion

The results of serological tests show that 7,081 (46.21%) out of a total of 15,326 serum samples analyzed through ELISA were positive, while 8,245 (53.79%) were negative. The results of virological assays showed that 95 (4%) out of a total of 2,402 samples of blood, organs and smears tested by virological methods were positive. A total of 35 virus strains were isolated on monolayers cell. All isolated strains were the NCP biotype. Their identity was confirmed by RT-PCR on cryolisates. Phylogenetic analysis was conducted on 17 strains derived from one sample for each cow. In particular, the viral strains derived from 11 blood samples (collected from 11 PI animals), from four organ samples (spleen, trachea, oesophagus and lung collected from four found dead animals), from one placensta sample (collected from an aborted fetus) and from one vaginal smear (collected from a bovine with reproductive problems). The phylogenetic analysis performed on the 5′UTR region characterized the strains within the genotype BVDV-1. Comparison with other Italian, European and global sequences retrieved from GenBank (Luzzago et al., 2001; Ciulli et al., 2008) showed their affiliation with the subgenotypes BVDV-Ie and BVDV-Ib, showing a low genetic heterogeneity. Particularly, three strains isolated from samples collected during 2005–2006 were classified into the subgenotype BVDV-Ie. Fourteen strain isolates from samples collected during 2007–2008 were clustered into subgenotype BVDV-Ib like the strains NY-1, Osloss and most of Italian reference strains. The BVDV-2 was not detected in this study. These results are supported by a bootstrap analysis, respectively, of 57% for Ie group and 77% for Ib group (Fig. 2). In addition, digestion with *AvaI* restriction enzyme confirmed the bovine origin for all BVDV isolates tested, providing the expected fragments of 117 bp and 171 bp (Fig. 3) (Vilcek et al., 1994). This is the first work that describes the molecular characterization of BVDV strains in Sicily and extends epidemiological studies performed in Italy from many authors. Previous studies on the BVD viruses collected in Northern Italy have shown a high level of heterogeneity among strains indicating the presence of different groups: BVDV-1b, BVDV-Ia, BVDV-Ic,



◀ **Fig. 2** Phylogenetic tree based on the analysis of 210 nucleotides derived from the sequence 5′-UTR. The tree shows the relationship between the 17 BVDV-I sequenced strains in this study and other Italian and reference strains. Samples indicated as 14816, 54647-F and 65400 placenta, cluster into BVDV-Ie group, while samples indicated as S056R, S072F, 296-F, 534-F, 75182-F, 80260 lung, 71202 blood, 31958, 62505, 59866 trachea, 69154 esoph., 90815 splee., 68515, and 69178-R cluster into BVDV-Ib group

BVDV-Id, BVDV-Ie, BVDV-If, BVDV-Ih, BVDV-Ig, and BVDV-Ij (Luzzago et al., 1999, 2001; Pratelli et al., 2001; Falcone et al., 2003; Ciulli et al., 2008; Giammarioli et al., 2008; Martucciello et al., 2009). As is known, certain factors relating to the management livestock, such as trade and movement, contribute to exposing the animals to a high risk of infection and to the introduction of new genetic variants. In the last years, some eradication programs have been used for BVDV infection in some European countries (Obritzhauser et al., 2005). In Italy, many infectious diseases are subjected to National Control Plans in order to provide a periodic monitoring of susceptible animals and to identify possible outbreaks. However, to date, there is no National Control Plan for the BVDV but only in some provinces of northern and central areas (Ferrari et al., 1999; Luzzago et al., 2006; 2008). In Sicily, BVDV infection is widespread in many farms. In this regard, it should be noted that in 1999, a severe hemorrhagic outbreak of BVDV was described in some wild herds. In that case, sequence analysis showed the presence of BVDV-Ib and BVDV-Ia (Di Marco et al., 2002) subgenotype. Therefore, the genetic characterization of viral strains isolated from infected animals represents a valid tool to better define the BVDV strains circulating in a specific area and to study their evolution. The epidemioloc study on biomolecular basis could allow implementing an aimed control plan to monitor an infectious disease as well as to realize vaccines capable of protecting susceptible animals.

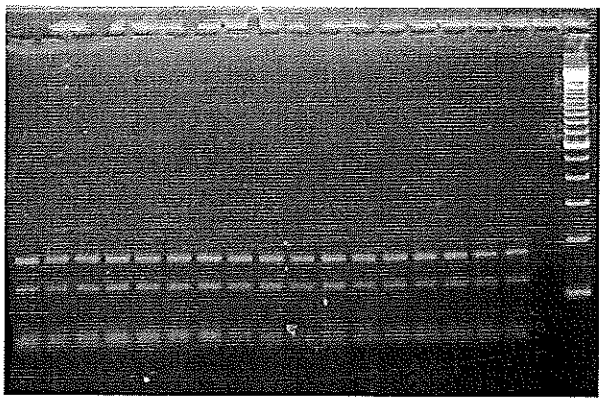


Fig. 3 *AvaI* restriction pattern: all 17 strains of BVDV show a bovine origin, presenting fragments of 171 bp and 117 bp deriving from a fragment of 288 bp

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