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Increased genetic diversity of BVDV-1: recent findings and implications thereof

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Abstract Sequence-based genotyping was recently used to distinguish between the BVDV-1 and BVDV-2 species of the *bovine viral diarrhoea virus* (*BVDV*). Quite recently, a new putative species, BVDV-3, was also detected. The phylogenetic analysis of the 5′-untranslated region (UTR) and Npro region has revealed at least 17 distinct subtypes for BVDV-1 to date. The aim of this study was to further investigate the genetic heterogeneity of BVDV-1 in Italy, by analysing 173 virus sequences from isolates collected over an 18-year period (1997–2014). Viral RNA was extracted from the original biological samples identified as BVDV-1-positive. Reverse transcription (RT) and polymerase chain reaction (PCR) assays targeting a 288-base pair (bp) region of the 5′-UTR and a 428-bp region encoding the autoprotease Npro were performed, and the RT-PCR products were sequenced. The phylogenetic analysis of the 5′-UTR and Npro sequences re-confirmed the circulation of ten out of eleven subtypes previously discovered in Italy. Interestingly, four isolates differed significantly from all of the bovine pestiviruses identified so far, thereby providing evidence for the circulation of three novel subtypes that have not been documented so far. The growing number of reports on BVDV-1 heterogeneity, including the recent findings reported herein, raises concern related to the emergence and spread of new BVDV variants, with possible implications for animal health and disease control. This global issue needs to be addressed with the highest priority.

Keywords BVDV-1 · Molecular typing · Phylogenetic analysis · Genetic diversity

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Introduction

Pestivirus is a genus within the family *Flaviviridae*, comprising four recognized species, *bovine viral diarrhoea virus 1* and *2* (BVDV-1 and BVDV-2), *classical swine fever virus* (CSFV), and *border disease virus* (BDV). Pestivirus infections in cattle have raised worldwide concern owing to the associated economic losses. The pestivirus genome comprises a positive single-stranded RNA molecule approximately 12.3 kb in length, containing a single open reading frame (ORF) flanked by two untranslated regions (5′- and 3′-UTR) [1]. Owing to the nature of the single-stranded genome, these viruses display high mutation rates, which, in some cases, may lead to the emergence of new virus lineages. Although the correlation of high mutation rates with the emergence of new lineages has not been proven, the rise of new pestivirus species has been observed. Besides the previously identified species, four new *pestivirus* species have now been discovered. However, the newly discovered species have not received official recognition so far. These putative species include the Giraffe virus, associated with the outbreak of a mucosal-like disease in Kenyan giraffes; the Pronghorn virus, isolated from a pronghorn antelope in the United States; the Bungowannah virus, detected in pigs following an outbreak of stillbirths and neonatal deaths in Australia; and a group of viruses referred to as the HoBi-like, BVDV-3, or atypical pestiviruses [2–6]. Based on the phylogenetic

Table 1 BVDV-1 isolates analysed in this study

Region	Year	Subtype
Basilicata (1 virus)	2010	1g
Calabria (1 virus)	2010	1r*
Campania (18 viruses)	2003–2013	1a, 1b, 1c
Emilia Romagna (7 viruses)	2000–2010	1a, 1b, 1d, 1e,
Lazio (19 viruses)	2004–2014	1a, 1b, 1f, 1h
Lombardia (21 viruses)	2001–2014	1b, 1g, 1h, 1f, 1e, 1d, 1k
Marche (20 viruses)	2004–2014	1a, 1b, 1d, 1e, 1h
Piemonte (17 viruses)	2003–2011	1b, 1e
Puglia (6 viruses)	2000–2012	1b, 1e
Sardegna (4 viruses)	2005	1a, 1k
Sicilia (4 viruses)	1997–2013	1a, 1b, 1e, 1t*
Toscana (3 viruses)	2003–2011	1a, 1b, 1k
Umbria (22 viruses)	2005–2012	1a, 1b, 1c, 1e, 1g, 1h, 1l, 1s*
Veneto (30 viruses)	2002–2012	1a, 1b, 1d, 1g, 1f, 1r*

* Novel nucleotide sequences

analysis of the 5′-UTR and Npro regions, at least 17 distinct subtypes of BVDV-1 (BVDV-1a to BVDV-1q) have been recognized so far [7–12]. In Italy, previous studies on the genetic diversity of BVDV provided evidence for the presence of at least eleven subtypes within BVDV-1 [13, 14]. Thus, Italy is one of the countries with the highest genetic diversity of BVDV. As an extension of these studies, a comparative analysis of the newly discovered BVDV-1 isolates was performed to further investigate the genetic heterogeneity of BVDV-1.

Materials and methods

In order to accurately define the genetic pattern of the Italian BVDV-1 isolates and to assess the potential risk of the emergence of novel subtypes, a total of 173 sequences were analysed, by comparing isolates collected from 14 regions in Italy during an 18-year period (1997–2014) (Table 1). The sequences were obtained from a previous study [13] and from the viruses isolated recently from the sick or persistently infected cattle from farms located all over Italy. Our genetic study was based on the 5′-UTR, supported by selected comparison within the Npro-coding region. Viral RNA was extracted from the original biological samples identified as BVDV-1-positive. Reverse transcription and polymerase chain (RT-PCR) assays targeting a 288-bp region of the 5′-UTR and a 428-bp region encoding the autoprotease Npro were performed as described previously [15, 16].

The purified amplicons were sequenced on both strands. Nucleotide sequences were aligned using Clustal X2 algorithm. Manual editing was performed using BioEdit software, version 7.0. The phylogeny was estimated using the neighbour-joining algorithm and the maximum likelihood method, respectively.

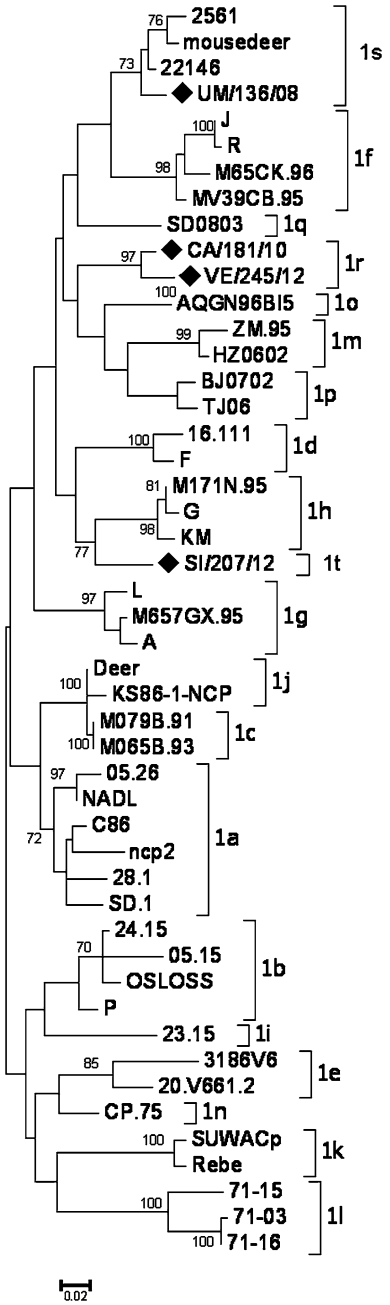

Fig. 1 Phylogenetic tree based on the 5′-UTR sequences. Molecular evolutionary genetics analyses were performed with MEGA6 using the NJ method. Distances were computed using the Kimura two-parameter model. Bootstrap values of over 70 % are shown for 1,000 replicate data sets. The tree describes the relationship between selected 5′-UTR sequences retrieved from the GenBank database and the Italian novel nucleotide sequences labelled by the symbol *filled diamond*. Their GenBank accession numbers are as follows: CA/181/10 (LM994672); VE/245/12 (LM994671); UM/136/08 (LM994673); and SI/207/12 (LM994674)

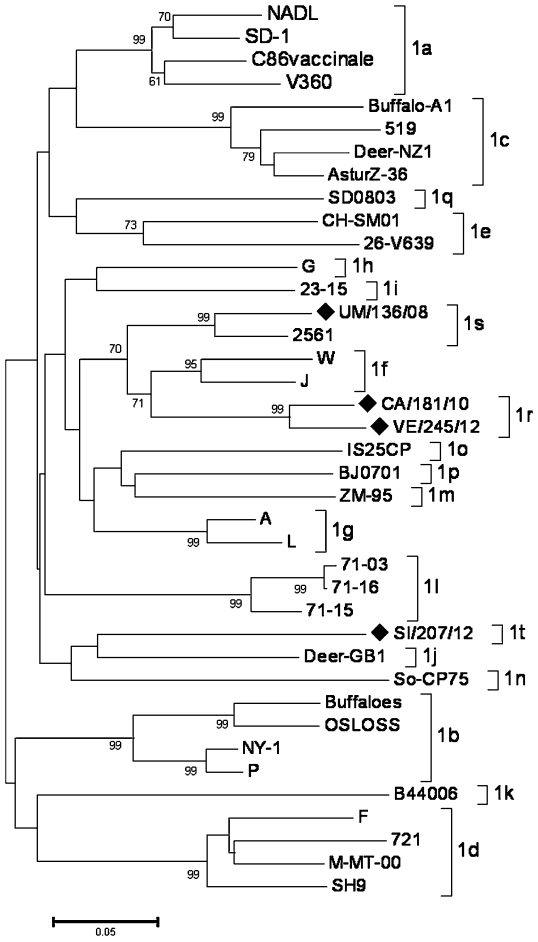

Fig. 2 Phylogenetic tree based on the Npro sequences. Molecular evolutionary genetics analyses were performed with MEGA6 using the NJ method. Distances were computed using the Kimura two-parameter model. Bootstrap values of over 70 % are shown for 1,000 replicate datasets. The tree describes the relationship between selected Npro sequences retrieved from the GenBank database and the Italian novel nucleotide sequences labelled by the symbol *filled diamond*. Their GenBank accession numbers are as follows: CA/181/10 (LN515609); VE/245/12 (LN515610); UM/136/08 (LN515612); and SI/207/12 (LN515611)

Results

The topology of the tree indicated that the isolates belong to several distinct subtypes, namely BVDV-1a ($n = 27$), BVDV-1b ($n = 81$), BVDV-1c ($n = 2$), BVDV-1d ($n = 4$), BVDV-1e ($n = 27$), BVDV-1f ($n = 7$), BVDV-1g ($n = 5$), BVDV-1h ($n = 10$), BVDV-1k ($n = 5$), and BVDV-1l ($n = 1$) (data not shown), thus re-confirming the circulation of ten out of eleven subtypes already identified in Italy. Interestingly, four isolates significantly differed from all the ruminant pestiviruses described so far (Figs. 1, 2). Two BVDV-1 isolates, originating (i) from Southern Italy in 2010, from an acute transient form of BVD; and (ii) from Northern Italy in 2012, from an immunotolerant and persistently infected animal, clustered together into a new phylogenetic group, the putative subtype BVDV-1r. These isolates displayed an identity of 72.9–83.4 % to the corresponding BVDV-1 reference strains. These sporadic subtypes were isolated from distant geographical regions, epidemiologically unrelated to each other, and they did not belong to the same chain of infection.

One particular virus isolate, collected in Central Italy in 2008, shared a 96.6 % identity to strain 22146/81 isolated in Lower Saxony, Germany [17], and 95.0 % identity to the BVDV-1 strain 2561 isolated from England [18], identified as a 1f-like in a previous study; it was closely related to the pestivirus isolated from a persistently infected mousedeer (*Tragulus javanicus*) in the Copenhagen Zoo [19], sharing an identity of 95.4 %. The sequence similarity ranged from 82.9 to 86.3 % between the various subtypes. All together, these viruses form a novel phylogenetic group, distinct from the 1-f subtype, tentatively named as BVDV-1s.

The fourth isolate was collected in Sicily in 2012 and shared an 81.0 % identity to the Deer-GB1 isolate [7]. This isolate displayed an identity of 72.9–81.5 % between the various subtypes. It remains to be confirmed whether the Sicilian isolate falls into a novel phylogenetic group, representing the first member of a separate cluster tentatively named as subtype BVDV-1t.

To confirm the grouping found in the 5′-UTR, we analysed the Npro region 27 viruses representative of each subtype, selected on the basis of their bootstrap values. The resulting phylogenetic tree showed that these viruses clustered in the same phylogenetic branches as those of the 5′-UTR-based tree and displayed similar bootstrap values (data not shown).

Discussion

The aim of this work was to study the genetic diversity of a broad range of BVD viruses circulating in Italy. For this purpose, an extensive collection of BVDV isolates collected from all over Italy was subjected to sequence-based genotyping. At the subgroup level, pairwise similarity and cluster analysis provided a direct assignation to 13 distinct subtypes. Most farm cattle were infected with the predominant BVDV-1a, BVDV-1b, and BVDV-1e isolates, thereby showing a widespread geographical distribution. The remaining subtypes only occurred sporadically. According to the criterion adopted for the segregation of pestiviruses into pre-defined genetic groups, as proposed by Becher et al. [20], our results provided clear evidence for the presence of three additional novel subtypes, which had never been described previously. We propose to name them as BVDV-1r, BVDV-1s, and BVDV-1t, respectively. However, it needs to be mentioned that the grouping of strains into branches depends not only on the nucleotide

sequences, but also on the number of sequences analysed. Genotyping indeed becomes complicated for branches represented by a limited number of strains or only by a single sequence, as is the case with the Sicilian isolate.

Genetic characterization provides insights into the process of viral evolution, especially that of highly variable RNA viruses such as BVDV. We anticipate that in the near future, additional genetic groups of BVDV-1 will be discovered, thereby adding to the genetic diversity of this virus. However, this could possibly lead to taxonomic discrepancies between the identified genetic groups. As additional data become available, we would be able to precisely identify the various genetic groups, which would then facilitate the development of accurate diagnostic procedures and the development of effective vaccines.

In summary, the results presented in this work revealed a high level of BVDV genetic heterogeneity, which is mainly attributable to the absence of any BVDV systematic control measure. In Italy, the introduction and spatial distribution of BVDV is mainly caused by animals moving within the country or by the introduction of viral strains from other countries. In addition, livestock management practices, including cattle trade and movement, expose cattle herds to a high risk of introduction of BVDV infection as well as of new genetic variants as a consequence of a high diversity of BVDV. This raises the question as to whether the BVD vaccines that have historically harboured only the BVDV-1a and BVDV-1b subtypes can effectively protect cattle from the other highly diverse BVDV-1 isolates. On the other hand, although the different subtypes reflect remarkable heterogeneity, there is by far no indication that these strains can render the currently used vaccines ineffective. Previous studies have shown pronounced antigenic differences among the various pestivirus species as well as between the individual subtypes of BVDV-1 [21, 22]. This is of importance to the BVD control programs where vaccination protocols are applied, as the antigenic differences limit the cross-protection among the highly divergent species and types of bovine pestiviruses. However, to date, no attempt has been made to address this situation. Moreover, the current paucity of biological information hinders the process of vaccine development. Therefore, more definitive cross-protection studies should be carried out to address the importance of the epitopic diversity and to determine whether future vaccines should harbour a mixture of several BVDV subtypes. The genetic diversity of the BVDV isolates is equally important for laboratory diagnosis, as it may impact the ability of current diagnostic methods that are targeted at the established pestiviruses to enable detection and monitoring of emerging strains, highlighting the need for new molecular tools that can unambiguously identify animals infected with any of the bovine pestiviruses. The genetic changes in viruses can lead to dramatic alterations in their biological properties, including changes in their virulence or adaptation to new hosts, and may even result in the emergence of new infectious diseases. Moreover, the rapid evolution of viruses poses a major threat to human and animal health, as well as to the economic welfare.

In conclusion, the growing number of reports on BVDV-1 heterogeneity, including the recent findings reported herein, raises significant concerns about the emergence and spread of new BVDV variants, with possible impacts on animal health and disease control. This issue needs to be addressed urgently at the global level.

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