Genetic characterization of bovine respiratory syncytial virus strains isolated in Italy: evidence for the circulation of new divergent clades

Genetic characterization of bovine respiratory syncytial virus strains isolated in Italy: evidence for the circulation of new divergent clades

Luigi Bertolotti[1], Monica Giammarioli, Sergio Rosati

Department of Veterinary Science, University of Torino, Grugliasco, Torino, Italy (Bertolotti, Rosati), Istituto Zooprofilattico Sperimentale dell'Umbria e Marche (Giammarioli)

[1]Corresponding Author: Luigi Bertolotti, Department of Veterinary Science, University of Torino, Largo Paolo Braccini 2, 10095, Grugliasco, Torino, Italy, luigi.bertolotti@unito.it

Running title: Divergent BRSV strains in Italy

Abstract

Bovine respiratory syncytial virus (BRSV) is circulating across Europe. Though vaccination helps control the disease, its prevalence within and among herds remains high. Recent genetic characterization studies revealed a strict geographic correlation between viral variants; on the other hand they showed the emergence of new variants in Northern Europe.

Few studies to date have described BRSV distribution and little is known about the genetic features of BRSV strains circulating in Italy. Samples testing positive on diagnostic tests for BRSV were characterized, and the coding regions of the G and N proteins were sequenced to determine the presence of divergent variants. Two different sets of sequences were found, also in samples obtained from animals from vaccinated herds. The two groups of sequences correspond to two time periods and suggest an active role of herd immunity in preventing the spread of infection. Our findings that different strains of BRSV are circulating in Italy and that the virus is evolving rapidly highlight the importance of updating vaccination strategies.

Key words: Bovine respiratory syncytial virus, divergent strains

Body of manuscript

Bovine respiratory syncytial virus (BRSV) belongs to the *Pneumovirus* genus within the family Pneumoviridae. Since the end of 1960s it has been recognized as responsible for causing an acute respiratory disease syndrome in beef and dairy calves[13] and regular winter outbreaks of respiratory disease in cattle.[18] Its distribution is worldwide and its impact on the cattle industry is associated with economic losses due to morbidity, mortality, costs of treatment and prevention, loss of production and reduced carcass value.[16] While BRSV is mainly transmitted by direct contact with infected animals or by aerosol, [11] its transmission can also be influenced by biotic and abiotic risk factors.[12]

The presence of maternally-derived antibodies is known to pose a major obstacle to efficacious vaccination: recent findings indicate that this problem may now be overcome,[1] but vaccine failure could be at least partially attributed to a possible broader antigenic spectrum of the BRSV population. Like most RNA viruses, BRSV has high genetic heterogeneity and a rapid evolutionary rate[15] forming different viral subpopulations within a single host. The complex mixture of viral variants, called *quasispecies*, can lead to new divergent strains. This viral feature is particularly important in relation to the efficacy of BRSV prophylaxis.

Among viral proteins, the G protein was identified as the major attachment protein because antibodies specific to the G protein were shown to block binding of the virus to cells.[10] Owing to its genetic and antigenic heterogeneity, the G protein, together with the nucleoprotein (N protein) and the fusion protein (F protein), was used as a target to better classify the viral strains of BRSV.[17]

Several recent studies have revealed its high prevalence both within and among herds in Europe.[7,6,20] Moreover, genetic characterization studies have reported a strict geographic correlation between viral variants and the emergence of new variants in Northern European countries[17] since the late 1990s.

The few studies published to date on BRSV distribution in Italy have focused on wildlife, [3,5] and little is known about the genetic features of BRSV strains circulating among cattle herds. In this study samples testing positive on diagnostic tests for BRSV were characterized to identify circulating viral strains and to determine the presence of new variants. To do this, a sample set was selected from among the samples tested by the Istituto Zooprofilattico Sperimentale dell'Umbria e Marche (IZSUm) diagnostic lab, including specimens from BRSV outbreaks throughout Italy

67 that had occurred between 2012 and 2015. Positivity to BRSV was determined using a
68 diagnostic real-time PCR assay described previously [19] and by targeting the gene
69 encoding glycoprotein F. Table 1 presents the sample collection time period, animal
70 tissue, and geographic area where the outbreaks occurred.
71 RNA was extracted using a Qiagen EZ1 Virus Mini kit (Qiagen, Hilden,
72 Germany). Eluted RNA was used as a template for amplification of the G coding
73 sequence. Amplification was performed applying the nested protocol previously
74 published by Valarcher [17] (Table S1) and using a Qiagen One-step RT-PCR kit
75 (Qiagen) following the manufacturer's instructions.
76 After the first amplification step (primer pairs G2.5-F2.7 and N2.1-N2.2, Table
77 S1), the PCR results were checked by agarose electrophoresis: samples showing the
78 expected band (about 1kb) were directly sequenced. The nested protocol (primers
79 pairs VG1-VG4 and N2.3-N2.4) was applied only to the samples that did not test
80 positive after the first amplification cycle. A set of G sequence positive samples was
81 used for amplifying a partial region of the N protein to confirm the subgroup
82 association.
83 All PCR positive samples were sequenced in both directions (BMR Genomics,
84 Padua, Italy), and the electropherograms were manually checked. A set of reference
85 sequences was selected from GenBank, including the six subgroups previously
86 proposed.[17] The sequences were aligned with respect to the coding frame, and the
87 genetic heterogeneity was evaluated. The uncorrected p distance was calculated for
88 the samples, and a phylogenetic tree was drawn by applying the best evolutionary
89 model selected by the jModelTest [4] and the Bayesian approaches included in the
90 MrBayes v. 3.2.5 software.[14] Evolutionary rate was evaluated using BEAST v. 2.4.3
91 software.[2]
92 Sequence analysis revealed the presence of different BRSV strains circulating in
93 Italy. For the G protein gene sequence (Fig. 1), two samples (IT111418-2015 and
94 IT48170-2013) were from non-vaccinated farms and were strictly related to old
95 subgroup III, similar to vaccine strains like the FS-1 Bayovac strain. The other
96 sequences formed two separate monophyletic clades derived from two separate
97 subgroups. In more detail, nine sequences formed a divergent clade within subgroup
98 III. The Italian samples forming this group came from outbreaks dating from between
99 2013 and 2015 that had occurred throughout the country. The remaining 12 new
100 sequences were related to subgroups V and VI, creating a new clade tentatively called

subgroup VII. Also in this case they came from outbreaks that had occurred around the country, but during an earlier period (between 2012 and 2013). The average nucleotide similarity along the G protein gene sequence within each clade was equivalent (98.65% and 98.84% within subgroups III and VII, respectively), suggesting comparable evolutionary behavior. Even if the tree topology based on the less variable N protein gene sequences (Fig. S1) does not allow a clear separation of the subgroups V and VI as already reported,[17] the new Italian sequences formed a supported subclade. However, given the small number of sequences, the sequences are included in a more general V-VII clade, following the previous topology interpretation.[17] The high similarity among the Italian sequences was maintained: nucleotide identity was 99.74% within subgroups III and 99.58% within the subclade of V-VII groups, respectively.

Some sequences included in the divergent clade within subgroup III and new subgroup VII came from farms where vaccination measures were in place (Table 1, marked by an asterisk in Fig.1 and Fig. S1), probably due to poor implantation of a vaccine protocol or due to selective pressure from a non-sterile immune response. However, if we consider only the linear immunodominant epitope region along G protein ,[8,9,17] the new Italian sequences are quite similar to the previously described ones (Table S2). The epitopes were characterized as a crucial region along the G protein for its folding. All the new Italian sequences showed a serine at position 184, the amino acid change that typically differentiates subgroup I from the others. The presence of threonine at position 205 associates the new Italian sequences to subgroups III, IV, and V, as well as the leucine-serine at positions 183-184. The Italian sequences belonging to subgroup III showed a mutation from proline to serine at position 194: serine was present at that position only in the samples from subgroups IV and V. The Italian clade forming subgroup VII showed the pattern SxSxS at position 190: this pattern was typical of BRSV subgroup V.

Though a small region of the G protein was analyzed, the similarity between the new Italian sequences and the reference ones suggests that vaccination could still be useful for animal protection; nonetheless, the genetic and antigenic divergence found in Italy and in several other countries as well constitutes evidence for BRSV circulation and evolution. Moreover, estimation of the evolutionary rate of G protein coding sequence is in line with the previously published data [17] ($4.38 \cdot 10^{-3}$ substitutions/site/year, ESS>200), supporting the notion that BRSV evolves after its

introduction into a susceptible area and before its extinction due to natural immune response or vaccination.

Genetic characterization of the circulating viral strains revealed the presence of at least three different variants, demonstrating that BRSV is still evolving. This is particularly important in areas where vaccination protocols are in place. As reported by Valarcher and colleagues, viral strains belonging to subgroups V and VI were identified in vaccinated calves, whereas all the vaccine strains belonged to subgroups II (i.e., Rispoval) and III (i.e., Bayovac).[17] Given the genetic and antigenic divergence of those strains, the authors suggested that vaccination sometimes does not prevent infection of calves with BRSV of subgroups V and VI, indicating that vaccinated calves may be poorly protected against infection by recent BRSV isolates. Italian strains are closely related to both old and recent subgroups. No geographical clustering was evident within the country, probably because of animal trade movements, but the spatial aggregation was maintained when different countries were compared.

However, the Italian sequences belong to two different collection periods; interestingly, the sequences belonging to new subgroup VII were obtained from samples collected before 2013 and they shared the same evolutionary path with the French and Belgian sequences collected at the end of the 1990s (subgroups V and VI). In contrast, none of the more recently collected samples (2013-2015) belonged to this clade yet all of them descended from the older strains included in subgroup III. Epitope analysis supports the field data, showing similar amino acid sequences of the two Italian subgroups, though the tree topology shows a clear viral evolution along its branches. This temporal separation supports the active role of herd immunity (natural or by vaccination) in preventing the spread and the maintenance of viral infection. Nevertheless the sequences were all monophyletic and formed a separate clade within subgroup 3. The clade topology suggests that, within each subgroup, viral isolates could show a continuum evolution and that their spread could be limited to very short time periods.

Our results highlight that vaccination is fundamental for BRSV control but knowledge about the genetic and antigenic features of circulating strains is extremely helpful for preventing vaccination plans from failing. Continuous investigation and genetic characterization of positive diagnostic samples are useful tools for updating

our knowledge about BRSV evolution and can inform our understanding of the emergence of new viral strains that may escape vaccination protection.

Acknowledgments

The authors wish to thank Luigi Trosso for his excellent technical work.

Declaration of conflicting interests

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

175 **References**

176 1 Blodörn K, et al. Vaccine safety and efficacy evaluation of a recombinant
177 bovine respiratory syncytial virus (BRSV) with deletion of the SH gene and
178 subunit vaccines based on recombinant human RSV proteins: N-nanorings, P
179 and M2-1, in calves with maternal antibodies. PLoS One 2014;9:e100392.
180 2 Bouckaert R, et al. BEAST 2: A software platform for bayesian evolutionary
181 analysis. PLoS Comput Biol 2014;10:e1003537.
182 3 Citterio C V, et al. Serological study of a population of alpine chamois
183 (Rupicapra r rupicapra) affected by an outbreak of respiratory disease. Vet Rec
184 2003;153:592–596.
185 4 Darriba D, et al. jModelTest 2: more models, new heuristics and parallel
186 computing. Nat Methods 2012;9:772.
187 5 Gaffuri A, et al. Serosurvey of roe deer, chamois and domestic sheep in the
188 central Italian Alps. J Wildl Dis 2006;42:685–690.
189 6 Klem TB, et al. Bovine respiratory syncytial virus: infection dynamics within
190 and between herds. Vet Rec 2013;173:476.
191 7 Klem TB, et al. Occurrence and phylogenetic analysis of bovine respiratory
192 syncytial virus in outbreaks of respiratory disease in Norway. BMC Vet Res
193 2014;10:15.
194 8 Langedijk JP, et al. Type-specific serologic diagnosis of respiratory syncytial
195 virus infection, based on a synthetic peptide of the attachment protein G. J
196 Immunol Methods 1996;193:157–166.
197 9 Langedijk JP, et al. Antigenic structure of the central conserved region of
198 protein G of bovine respiratory syncytial virus. J Virol 1997;71:4055–4061.
199 10 Levine S, et al. Demonstration that glycoprotein G is the attachment protein of

respiratory syncytial virus. J Gen Virol 1987;68 (Pt 9):2521–2524.

11 Mars MH, et al. Airborne transmission of BHV1, BRSV, and BVDV among cattle is possible under experimental conditions. Vet Microbiol 1999;66:197–207.

12 Ohlson A, et al. Risk factors for seropositivity to bovine coronavirus and bovine respiratory syncytial virus in dairy herds. Vet Rec 2010;167:201–206.

13 Paccaud MF, Jacquier C. A respiratory syncytial virus of bovine origin. Arch Gesamte Virusforsch 1970;30:327–342.

14 Ronquist F, et al. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol 2012;61:539–542.

15 Sarmiento-Silva RE, et al. Epidemiology, molecular epidemiology and evolution of bovine respiratory syncytial virus. Viruses 2012;4:3452–3467.

16 Schneider MJ, et al. An evaluation of bovine respiratory disease complex in feedlot cattle: Impact on performance and carcass traits using treatment records and lung lesion scores. J Anim Sci 2009;87:1821–1827.

17 Valarcher JF, et al. Evolution of bovine respiratory syncytial virus. J Virol 2000;74:10714–10728.

18 Valarcher J-F, Taylor G. Bovine respiratory syncytial virus infection. Vet Res 2007;38:153–180.

19 Vilcek S, et al. Development of nested PCR assays for detection of bovine respiratory syncytial virus in clinical samples. J Clin Microbiol 1994;32:2225–2231.

20 Wolff C, et al. Bovine respiratory syncytial virus and bovine coronavirus in Swedish organic and conventional dairy herds. Acta Vet Scand 2015;57:2.

226 **Table 1.** Samples used for study of bovine respiratory syncytial virus in Italy.

Sample	Origin	Year	Tissue	Vaccination
IT16813.2012	Southern Italy	2012	Lung	No
IT11934.2012	Central Italy	2012	Lung	No
IT13449.2012	Central Italy	2012	Lung	No
IT15527.2012	Central Italy	2012	Lung	No
IT22579.2012	Central Italy	2012	Lung	No
IT24374.2012	Central Italy	2012	Lung	No
SM56243.2012	Central Italy	2012	Lung	na
IT13449.2012	Central Italy	**2012**	**Lung**	**Yes**
IT48170.2013	Northern Italy	2013	Swab	No
IT135.2013	Southern Italy	2013	Lung	No
IT15914.2013	Northern Italy	2013	Lung	No
IT11785.2013	Central Italy	**2013**	**Lung**	**Yes**
IT45888.2013	Northern Italy	2013	Swab	No
IT50378.2013	Central Italy	2013	Organs	No
IT1299.2013	Central Italy	2013	Lung	No
IT13460.2014	Northern Italy	**2014**	**Organs**	**Yes**
IT47193.2014	Northern Italy	2014	Organs	No
IT47893.2014	Northern Italy	2014	Organs	No
IT5755.2014	Northern Italy	2014	Organs	No
IT11418.2015	Central Italy	2015	Organs	No
IT22152.2015	Central Italy	**2015**	**Organs**	**Yes**
IT6167A.2015	Southern Italy	2015	Organs	No
IT6167v.2015	Southern Italy	2015	Organs	No

227 NA = unknown.

228

Figure 1. Bayesian tree of G gene partial sequence. Designations at the ends of the branches refer to the subgroup based on Valarcher et al., 2000. New Italian sequences are reported in bold. The year of collection is indicated as the last part of the sample name. Sequences obtained from animals from vaccinated herds are marked with an asterisk.

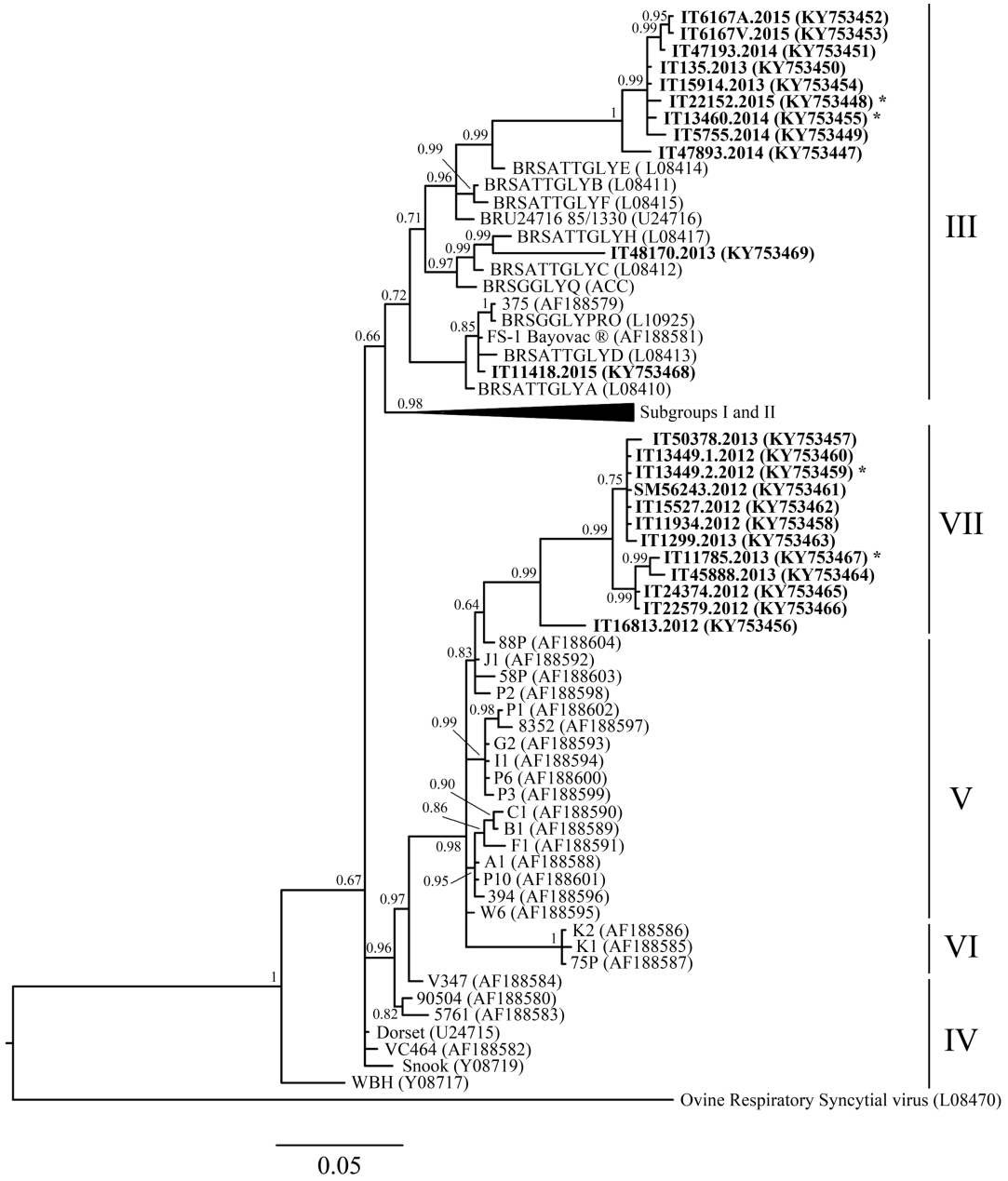

Figure S1. Bayesian tree of N gene partial sequence. Designations at the ends of the branches refer to the subgroup based on Valarcher et al., 2000. New Italian sequences are reported in bold. The year of collection is indicated as the last part of the sample name. Sequences obtained from animals from vaccinated herds are marked with an asterisk.