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This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1654953> since 2022-01-27T09:48:13Z

Published version:

DOI:10.1177/1040638717746202

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

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13 Running title: Divergent BRSV strains in Italy

14

15 **Abstract**

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

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

30

31 Key words: Bovine respiratory syncytial virus, divergent strains

32

33 **Body of manuscript**

34 Bovine respiratory syncytial virus (BRSV) belongs to the *Pneumovirus* genus
35 within the family Pneumoviridae. Since the end of 1960s it has been recognized as
36 responsible for causing an acute respiratory disease syndrome in beef and dairy
37 calves¹³ and regular winter outbreaks of respiratory disease in cattle.¹⁸ Its distribution
38 is worldwide and its impact on the cattle industry is associated with economic losses
39 due to morbidity, mortality, costs of treatment and prevention, loss of production and
40 reduced carcass value.¹⁶ While BRSV is mainly transmitted by direct contact with
41 infected animals or by aerosol,¹¹ its transmission can also be influenced by biotic and
42 abiotic risk factors.¹²

43 The presence of maternally-derived antibodies is known to pose a major obstacle
44 to efficacious vaccination: recent findings indicate that this problem may now be
45 overcome,¹ but vaccine failure could be at least partially attributed to a possible
46 broader antigenic spectrum of the BRSV population. Like most RNA viruses, BRSV
47 has high genetic heterogeneity and a rapid evolutionary rate¹⁵ forming different viral
48 subpopulations within a single host. The complex mixture of viral variants, called
49 *quasispecies*, can lead to new divergent strains. This viral feature is particularly
50 important in relation to the efficacy of BRSV prophylaxis.

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

56 Several recent studies have revealed its high prevalence both within and among
57 herds in Europe.^{7,6,20} Moreover, genetic characterization studies have reported a strict
58 geographic correlation between viral variants and the emergence of new variants in
59 Northern European countries¹⁷ since the late 1990s.

60 The few studies published to date on BRSV distribution in Italy have focused on
61 wildlife,^{3,5} and little is known about the genetic features of BRSV strains circulating
62 among cattle herds. In this study samples testing positive on diagnostic tests for
63 BRSV were characterized to identify circulating viral strains and to determine the
64 presence of new variants. To do this, a sample set was selected from among the
65 samples tested by the Istituto Zooprofilattico Sperimentale dell'Umbria e Marche
66 (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 previously¹⁹ 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 Valarcher¹⁷ (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 jModelTest⁴ 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

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

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

128 Though a small region of the G protein was analyzed, the similarity between the
129 new Italian sequences and the reference ones suggests that vaccination could still be
130 useful for animal protection; nonetheless, the genetic and antigenic divergence found
131 in Italy and in several other countries as well constitutes evidence for BRSV
132 circulation and evolution. Moreover, estimation of the evolutionary rate of G protein
133 coding sequence is in line with the previously published data¹⁷ ($4.38 \cdot 10^{-3}$
134 substitutions/site/year, ESS>200), supporting the notion that BRSV evolves after its

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

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

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

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

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

170 **Acknowledgments**

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

172 **Declaration of conflicting interests**

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

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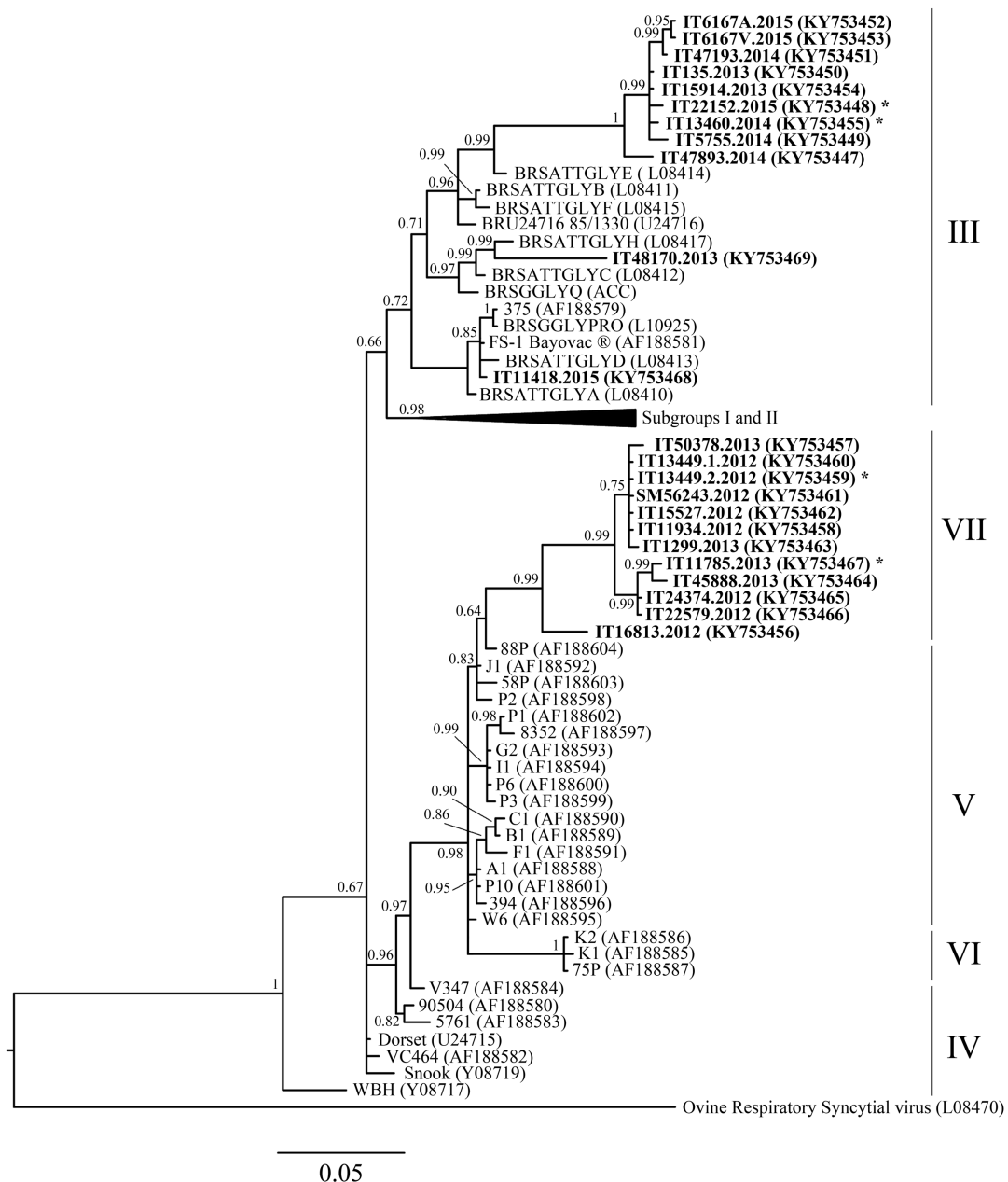
226 **Table 1.** Samples used for study of bovine respiratory syncytial virus in Italy.

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.

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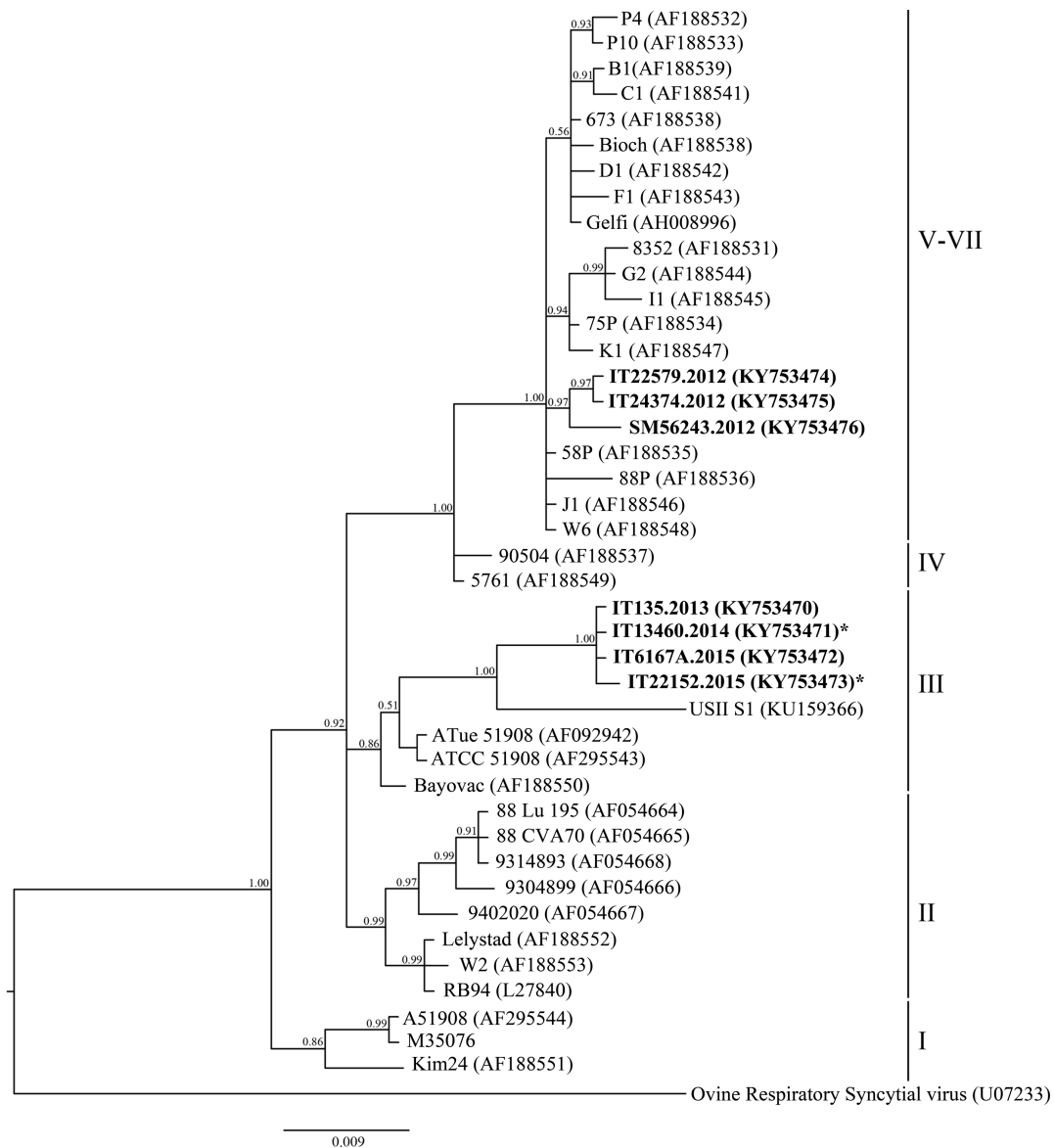
229 Figure 1. Bayesian tree of G gene partial sequence. Designations at the ends of the
 230 branches refer to the subgroup based on Valarcher et al., 2000. New Italian sequences
 231 are reported in bold. The year of collection is indicated as the last part of the sample
 232 name. Sequences obtained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



234

235

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



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