

Alma Mater Studiorum Università di Bologna
Archivio istituzionale della ricerca

Molecular characterization of the meq gene of Marek's disease viruses detected in unvaccinated backyard chickens reveals the circulation of low- and high-virulence strains

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:

Molecular characterization of the meq gene of Marek's disease viruses detected in unvaccinated backyard chickens reveals the circulation of low- and high-virulence strains / Giulia Mescolini; Caterina Lupini; Viviana Felice; Alessandro Guerrini; Flavio Silveira; Mattia Cecchinato; Elena Catelli. - In: POULTRY SCIENCE. - ISSN 0032-5791. - STAMPA. - 98:8(2019), pp. 3130-3137. [10.3382/ps/pez095]

Availability:

This version is available at: <https://hdl.handle.net/11585/722542> since: 2020-02-06

Published:

DOI: <http://doi.org/10.3382/ps/pez095>

Terms of use:

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>).
When citing, please refer to the published version.

(Article begins on next page)

This is the final peer-reviewed accepted manuscript of:

Mescolini, G., Lupini, C., Felice, V., Guerrini, A., Silveira, F., Cecchinato, M., Catelli, E. (2019). Molecular characterization of the meq gene of Marek's disease viruses detected in unvaccinated backyard chickens reveals the circulation of low- and high-virulence strains. Poultry Science, 98: 3130-3137.

The final published version is available online at: <https://doi.org/10.3382/ps/pez095>

Rights / License:

The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

1 **FEATURES OF MAREK'S DISEASE VIRUS IN RURAL CHICKENS**

2

3 **Molecular characterization of the *meq* gene of Marek's disease viruses detected in**
4 **unvaccinated backyard chickens reveals the circulation of low and high virulence strains**

5

6 Giulia Mescolini, ^{*,1} Caterina Lupini, ^{*} Viviana Felice, ^{*} Alessandro Guerrini, ^{*} Flavio Silveira, ^{*}
7 Mattia Cecchinato, [†] and Elena Catelli^{*}

8

9 ^{*} Department of Veterinary Medical Sciences, University of Bologna, Via Tolara di Sopra 50,
10 40064, Ozzano dell'Emilia (BO), Italy

11 [†] Department of Animal Medicine, Production and Health, University of Padua, Viale
12 dell'Università 16, 35020, Legnaro (PD), Italy

13

14 ¹ Corresponding author: giulia.mescolini3@unibo.it

15 Giulia Mescolini

16 Department of Veterinary Medical Sciences, University of Bologna, via Tolara di Sopra 50, 40064,
17 Ozzano dell'Emilia (BO), Italy.

18 Full telephone: 0039 0512097560

19 E-mail address: giulia.mescolini3@unibo.it

20 Scientific Session: Immunology, Health and Disease

21 The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide
22 sequence database, and accession numbers from MK139660 to MK139678 have been assigned.

23 **ABSTRACT**

24 Marek's disease (MD) is an important lymphoproliferative disease of chickens, caused by *Gallid*
25 *alphaherpesvirus 2* (GaHV-2). Outbreaks are commonly reported in commercial flocks, but also in
26 backyard chickens. While the molecular characteristics of GaHV-2 strains from the commercial
27 poultry sector have been reported, no recent data are available for the rural sector. To fill this gap,
28 19 GaHV-2 strains detected in 19 Italian backyard chicken flocks during suspected MD outbreaks
29 were molecularly characterized through an analysis of the *meq* gene, the major GaHV-2 oncogene.
30 The number of four consecutive prolines (PPPP) within the proline-rich repeats of the Meq
31 transactivation domain, the proline content and the presence of amino acid substitutions were
32 determined. Phylogenetic analysis was performed using the Maximum Likelihood method.
33 Sequence analysis revealed a heterogeneous population of GaHV-2 strains circulating in Italian
34 backyard flocks. Seven strains, detected from birds affected by classical MD, showed a unique *meq*
35 isoform of 418 amino acids (aa) with a very high number of PPPP motifs. Molecular and clinical
36 features are suggestive of a low oncogenic potential of these strains. The remaining 12 strains,
37 detected from flocks experiencing acute MD, transient paralysis or sudden death, had shorter Meq
38 protein isoforms (298 or 339 aa) with a lower number of PPPP motifs and point mutations
39 interrupting PPPPs. These features allow us to assert the high virulence of these strains. These
40 findings reveal the circulation of low and high virulence GaHV-2 strains in the Italian rural sector.

41

42 **Key words:** backyard chicken; Marek's disease virus; *meq* gene; molecular characterization

43

44

45

46

47

48

INTRODUCTION

49

50 Marek's disease (**MD**) is a worldwide, contagious, lymphoproliferative disease of chickens caused
51 by a lymphotropic and oncogenic virus, *Gallid alphaherpesvirus 2* (**GaHV-2**); it is also known as
52 Marek's disease virus, belonging to the genus *Mardivirus* of the *Alphaherpesvirinae* subfamily.
53 Genus *Mardivirus* includes two other viral species: *Gallid alphaherpesvirus 3* (**GaHV-3**) and
54 *Meleagrid alphaherpesvirus 1* or Turkey herpesvirus (**HVT**). GaHV-3 and HVT are both non-
55 oncogenic and used as vaccines, being antigenically related to GaHV-2. Four GaHV-2 pathotypes
56 are currently recognized: mild, virulent, very virulent and very virulent plus (Witter, 1997; Witter et
57 al., 2005). Birds become infected by inhalation of infectious viral particles that are present in the
58 environment. GaHV-2 is capable of replicating and establishing latency in T lymphocytes and may
59 induce neoplastic transformation of latently-infected CD4⁺ T cells, leading to the development of
60 multiple lymphomas in the visceral organs (Nair, 2013). GaHV-2 causes several pathologic
61 syndromes, which can be divided into two types: neoplastic and nonneoplastic (Gimeno, 2014).
62 Neoplastic syndromes, characterized by GaHV-2-induced lymphoproliferative lesions, are the most
63 frequently reported syndromes in the field, having prominent economic significance. Within this
64 category, MD can be subdivided into two forms: classical and acute. Classical MD (also known as
65 fowl paralysis) is characterized by spastic paralysis due to nerve lesions; it was mainly observed
66 prior to the 1950s, concomitantly with infection with low virulence strains (Witter, 1997). The more
67 severe form of the disease, termed acute MD (Biggs et al., 1965), was observed from the late 1950s
68 and is characterized by visceral lymphomas, with or without nerve lesions, and associated with
69 infection with more virulent GaHV-2 strains (Witter, 1997). Nonneoplastic syndromes, such as
70 transient paralysis, panophthalmitis, atherosclerosis and lymphodegenerative syndromes, are rare in
71 the field as they normally occur in unvaccinated, susceptible chickens without specific maternally-
72 derived antibodies (Gimeno, 2014).
73 Among the more than 200 genes of the GaHV-2 genome, the Marek's *Eco* RI-Q (*meq*) gene, unique
74 to GaHV-2 and highly expressed in latently-infected and transformed T CD4⁺ cells (Tai et al.,

2017), is proposed to play a key role in the GaHV-2-induced transformation process of latently-
infected T lymphocytes. The *meq* gene encodes the Meq protein, a basic leucine zipper transcription
factor composed of an N-terminal basic leucine zipper (**bZIP**) domain and a proline-rich C-terminal
transactivation domain (Qian et al., 1995). The last 33 carboxy-terminal amino acids are essential
for transcriptional transactivation (Qian et al., 1995), whereas the number of proline-rich repeats
(PRR) in the transactivation domain seems to be related with repression of transcription (Chang et
al., 2002a). *Meq* is a polymorphic gene, with various recognized sizes: long-*meq* (*L-meq*), *meq*,
short-*meq* (*S-meq*) and very short-*meq* (*VS-meq*); these encode Meq protein isoforms with 399,
339, 298 and 247 amino acids, respectively (Chang et al., 2002b). The existence of these different
length Meq isoforms is due to the presence of insertions or deletions in the transactivation domain,
resulting in a variable number of PRR. This number, along with specific point mutations in the
PRR, appears to correlate with GaHV-2 virulence (Shamblin et al., 2004; Renz et al., 2012).
Moreover, the *meq* gene has been recently included in a list of candidate genes associated with an
increase of GaHV-2 virulence due to a greater-than-average number of point mutations found in the
virulent Eurasian and North American GaHV-2 strains (Trimpert et al., 2017). This gene is evolving
at a fast rate for a dsDNA virus, and most of its polymorphisms have evolved under positive
selection (Padhi and Parcells, 2016).

MD is a major cause of mortality in backyard chickens (Pohjola et al., 2015; Mete et al., 2016) and
GaHV-2 strains can circulate freely because flocks composed of birds with different immune
statuses, ages and breeds, are more susceptible to infection. Backyard farm owners do not generally
vaccinate their birds and backyard production methods imply a low biosecurity level (Cecchinato et
al., 2011); this facilitates the circulation of infectious agents, including GaHV-2, and constitutes a
threat to any commercial poultry holdings nearby. To our knowledge, recent data about molecular
characteristics of Marek's disease virus circulating in backyard flocks worldwide is not available. In
the present study, we analyzed the complete *meq* gene sequences of 19 GaHV-2 strains detected
from suspected MD outbreaks in 19 Italian backyard chicken flocks.

MATERIALS AND METHODS

101

Backyard Flocks

102 From 2015 to 2017, 19 Italian backyard chicken flocks were sampled for routine molecular
103 diagnostic activity for MD. All flocks were unvaccinated for MD and showed clinical signs or
104 lesions suggestive of MD. Several chicken breeds were involved in the outbreaks (Table 1). The
105 farms were located in nine different Italian regions (Table 1) and consisted of a variable number of
106 chickens (from 40 to 150), kept mainly for exhibition or hobby and marginally for eggs and meat.
107 Other poultry species, such as turkey, quail, peacock, pigeon, goose, duck, guinea fowl and Roul
108 Roul partridge, were reared alongside the affected chickens on most farms.
109

110

Sampling

111 For GaHV-2 PCR detection, five feathers/bird were collected from the axillary feather tracts, as
112 suggested by Baigent et al. (2013). Feather sampling was chosen because it is easy, fast, non-
113 invasive and non-lethal (Davidson et al., 2018), and is suitable for sampling ornamental chicken
114 breeds that have economic and emotional value.
115

116

DNA Extraction

117 Total DNA was extracted from feather tips using a commercial kit (High Pure PCR Template
118 Preparation Kit, Roche Diagnostics GmbH, Mannheim, Germany), with a subtle adjustment to the
119 manufacturer's instructions. Briefly, five feather tips from each bird were pooled together, cut,
120 ground and digested overnight at 55°C in a digestion buffer containing tissue lysis buffer,
121 proteinase K and DL-Dithiothreitol solution (Sigma-Aldrich, Saint Louis, Missouri, USA). After
122 digestion, binding buffer followed by isopropanol was added and samples were placed in spin
123 columns and centrifuged at 8000 × g for 1 min. After two washings, DNA was eluted with 200 µl of
124 elution buffer.
125

126

127 **PCR Amplification of the *meq* Gene**

128 The full-length *meq* gene was amplified, according to Shamblin et al. (2004), using the forward
129 primer *EcoR-Q* for 5'-GGT GAT ATA AAG ACG ATA GTC ATG-3' and the reverse primer
130 *EcoR-Q rev* 5'-CTC ATA CTT CGG AAC TCC TGG AG-3'. In a total reaction volume of 25 µl, 3
131 µl of eluted template DNA was mixed with 0.125 µl of GoTaq G2 Flexi DNA Polymerase
132 (Promega, Madison, Wisconsin, USA), 5 µl of 5X Colorless GoTaq Flexi Buffer, 1.75 µl of MgCl₂
133 solution, 0.5 µl of dNTPs, 13 µl of H₂O for molecular biology, and 1 µl of each primer. Cycling
134 conditions were as follows: 2 min of denaturation at 95°C followed by 35 cycles, each consisting of
135 denaturation at 95°C for 1 min, annealing at 58°C for 1 min and extension at 72 °C for 1.5 min. A
136 final elongation step at 72 °C for 5 min completed the reaction. The PCR products were separated
137 on agarose gel (1%), stained with ethidium bromide and visualized under ultraviolet light after an
138 electrophoretic run at 80V and 400mA for 50 min.

139

140 **DNA Sequencing and Sequence Analysis**

141 The amplification products were sequenced using a commercial sequencing service (Macrogen
142 Europe, Amsterdam, The Netherlands). In order to obtain a complete and reliable *meq* gene
143 sequence, primers *EcoR-Q* for, *EcoR-Q rev* (Shamblin et al., 2004) and an internal primer (*meq-F*,
144 5'-ATG TCT CAG GAG CCA GAG CCG-3') (Hassanin et al., 2013) were used. The obtained
145 sequences were named using the following nomenclature: GaHV-2 / Italy / Chicken (Ck) / ID
146 number / year of detection.

147 The nucleotide sequences were assembled and edited using Bioedit Sequence Alignment Editor
148 Version 7.2.5.0 (Tom Hall, Ibis Therapeutics, Carlsbad, California, USA), then, aligned and
149 compared, using Clustal W software (Thompson et al., 1994), with the *meq* gene sequences of 32
150 selected GaHV-2 field and vaccine strains retrieved from the GenBank database (Table 2) and with
151 the sequences of three CVI988/Rispens vaccine strains currently used in Italy. The number of four
152 consecutive prolines (**PPPP**) contained in the proline-rich repeats of the transactivation domain, the

153 proline content and the amino acid (**aa**) substitutions in the deduced aa sequence of *meq* genes were
154 evaluated.

155 A phylogenetic tree based on the *meq* gene sequences of Italian and selected GaHV-2 strains from
156 GenBank was generated with the Maximum Likelihood method, using MEGA7 (Kumar et al.,
157 2016). Only the nodes of the tree with bootstrap values equal or greater than 70, calculated based on
158 1000 replicates, were considered reliable.

159

160

RESULTS

161 All 19 backyard chicken flocks tested in the present study were positive for GaHV-2. The obtained
162 complete *meq* gene sequences were submitted to the GenBank database under the accession
163 numbers listed in Table 3. Sequence analysis revealed that GaHV-2 strains had *meq* gene sequences
164 of variable sizes: 1257 bp, 1020 bp or 897 bp, which were named “very long *meq*”, “standard *meq*”
165 and “short *meq*” strains, respectively, based on a slightly modified version of the *meq* open reading
166 frames classification reported by Chang et al. (2002b) (Table 3).

167 Length, insertion size, number of PPPP motifs within the transactivation domain and the proline
168 content of *meq* deduced amino acid sequences of the Italian GaHV-2 strains and one representative
169 GaHV-2 strain for each pathotype were evaluated (Table 4). Seven GaHV-2 strains showed a long
170 Meq isoform (418 aa, “very long *meq*” strains), with an insertion of 79 amino acids and a high
171 number of PPPP motifs (9–10). Eleven strains had a short Meq isoform (339 aa, “standard *meq*”
172 strains) without insertion in the transactivation domain and a lower number of PPPPs (4–5). Only
173 one strain showed a very short Meq isoform (298 aa, “short *meq*” strain) with two PPPPs in its
174 transactivation domain.

175 The amino acid substitutions found in the Meq proteins of the analysed strains compared to the
176 vaccine strain CVI988 (Intervet), chosen as reference strain, are reported in Tables 5, 6 and 7.

177 Sequences of “very long *meq*” strains, which differ among themselves with respect to very few
178 amino acid changes, showed 10 to 14 amino acid substitutions when compared with the CVI988

179 vaccine strain. Five of these mutations, at positions 37 (H37R), 80 (D80E), 98 (H98D), 101
180 (K101N), and 242 (F242I) of the Meq protein (Table 5), were only found in the Italian strains. The
181 uniqueness of this mutation pattern was further confirmed by a BLAST search. Five to eight amino
182 acid substitutions were found when “standard *meq*” (Table 6) and “short *meq*” (Table 7) strains
183 were compared with the CVI988 vaccine strain. Almost all amino acid changes of “standard *meq*”
184 and “short *meq*” strain-encoded Meqs have already been reported in previously published
185 International sequences.

186 “Standard *meq*” and “short *meq*” strains contained interruptions of PPPP motifs in the PRR of the
187 transactivation domain, both at the second and third position. In particular, the GaHV-
188 2/Italy/855/17 strain showed a substitution at position 177 (P177S), interrupting a stretch of four
189 prolines at position 3 (PPPP → PPSP). The GaHV-2/Italy/Ck/674/16 strain showed a substitution at
190 position 217 (P217A), interrupting a PPPP sequence at position 2 (PPPP → PAPP). Finally, the
191 strains GaHV-2/Italy/Ck/625/16, GaHV-2/Italy/Ck/689/16, GaHV-2/Italy/Ck/722/16, GaHV-
192 2/Italy/Ck/801/16, GaHV-2/Italy/Ck/810/16, GaHV-2/Italy/Ck/852/16, GaHV-2/Italy/Ck/853/16
193 and GaHV-2/Italy/Ck/854/16 showed substitutions at position 218 (P218S), interrupting the PPPP
194 sequence at position 3 (PPPP → PPSP).

195 The phylogenetic tree, based on the Meq amino acid sequences of the Italian strains, the vaccine
196 strains and 32 selected GaHV-2 strains, is shown in Figure 1. The “very long *meq*” Italian strains
197 form an independent cluster, phylogenetically related to a cluster formed by Hungarian and Indian
198 strains. Nine out of eleven Italian “standard *meq*” strains and the “short *meq*” strain clustered
199 together with selected Polish isolates. Two Italian “standard *meq*” strains (GaHV-2/Italy/Ck/674/16
200 and GaHV-2/Italy/Ck/850/17) did not belong to the above-mentioned group and the GaHV-
201 2/Italy/Ck/674/16 strain appeared to be connected with a recent Tunisian strain.

202

203

204

DISCUSSION

205

206 For the first time, the present study provides molecular insights into the GaHV-2 strains currently
207 circulating in backyard chickens, expanding the knowledge on MD in the rural sector. Nineteen
208 strains, detected from 2015 to 2017 in Italian backyard chickens exhibiting typical MD clinical
209 signs or gross lesions, were molecularly characterized on the basis of their *meq* gene sequences,
210 revealing the circulation of a heterogeneous viral population.

211 Previous studies highlighted a correlation between the *meq* gene sequence and GaHV-2 virulence
212 (Shamblin et al., 2004; Renz et al., 2012). In particular, strains showing a low number of PRR
213 within the transactivation domain, and amino acid substitutions interrupting PPPP motifs within the
214 PRR, exhibit higher virulence. In the sequence analysis, the Italian strains were subdivided,
215 according to *meq* gene length, into three categories: “very long *meq*”, “standard *meq*” and “short
216 *meq*”.

217 The “very long *meq*” strains detected in the present study showed a Meq isoform of 418 aa with a
218 high number (from 9 to 10) of PPPP motifs in their transactivation domains. These molecular
219 features could be suggestive of low oncogenic potential. Moreover, all “very long *meq*” strains were
220 detected from birds affected by classical MD, macroscopically not showing visceral tumours and
221 experiencing a complete recovery in three out of seven outbreaks. These strains share diverse and
222 sometimes unique aa substitutions that, in part (H98D, K101N and Q93R), fall within the bZIP
223 domain. This domain is responsible for Meq dimerization with itself or with other dimerization
224 partners, forming homodimers or heterodimers, respectively. The ability to form one interaction or
225 the other is influenced by the bZIP sequence and the presence of mutations in this domain could
226 disrupt the formation of one or both types of dimers (Brown et al., 2009; Suchodolski et al., 2009;
227 Suchodolski et al., 2010). This interaction allows the adjacent basic region of Meq to anchor to
228 specific DNA binding sites with different affinities, depending on the dimer type, consequently
229 transactivating or transrepressing viral and host genes exerting different biological effects, mostly
230 linked to oncogenesis (Qian et al., 1996; Liu et al., 1998; Levy et al., 2005). The three amino acid

231 substitutions found in the bZIP domain might have altered the Meq binding capacity and
232 contributed to the low oncogenicity of the Italian “very long *meq*” strains.

233 On the other hand, “standard *meq*” and “short *meq*” strains were detected from flocks experiencing
234 acute MD, transient paralysis or sudden death, occasionally preceded by neurologic signs. These
235 also featured a low number of PPPP motifs in the transactivation domain, and the presence of point
236 mutations in the PRR that interrupted stretches of four prolines in most of the “short *meq*” or
237 “standard *meq*” strains; this allows us to assert, according to Shamblin et al. (2004), the high
238 virulence of these strains. These findings reveal the circulation of both low and high virulence
239 GaHV-2 strains in the Italian rural sector.

240 The variability of observed MD clinical forms could be also due to different disease susceptibilities
241 amongst the different breeds involved. Genetic resistance to MD is well known and while breeding
242 programs for commercial poultry generally include genetic selection for resistance to MD (Schat
243 and Nair, 2013), selection programs for ornamental chickens are mainly focused on the selection of
244 phenotypic traits compliant with the breed standard.

245 The heterogeneity of the viral population, supported by the allocation of the analyzed strains into
246 three major clusters, suggests that the introduction of GaHV-2 to Italy could have occurred over
247 multiple occasions. Ornamental chicken owners regularly enter their birds into international ‘beauty
248 contests’, where chickens are generally kept in adjacent cages, facilitating the transmission of the
249 virus from bird to bird. The national and international trade of live, valuable breeders is another
250 possible route of entry.

251 Viruses could also have reached the rural context by overcoming the biosecurity measures applied
252 in commercial poultry houses to find a highly variable poultry population with different species,
253 breeds, ages and immune statuses, with unknown susceptibility to MD. The reverse could be also
254 true: backyard flocks could act as reservoir for GaHV-2 strains of various and unknown pathotypes,
255 representing a potential threat for commercial poultry flocks located in the same area. Biosecurity
256 measures are not generally applied to backyard farms (Cecchinato et al., 2011) and, in most cases,

257 birds have continuous daytime access to open-air pens, and contact with wild birds; these birds have
258 been identified as carriers of presumably pathogenic GaHV-2 strains (Murata et al., 2012), so this
259 may facilitate the introduction of foreign viruses.

260 Finally, the last detections of low virulence viruses dates back to the 1970s (Smith and Calnek,
261 1973; Smith and Calnek, 1974), presumably because of the poultry industry's major interest in
262 investigating highly virulent strains responsible for MD outbreaks in vaccinated commercial poultry
263 flocks (López-Osorio et al., 2017; Suresh et al., 2017; Abd-Elattieff et al., 2018). Weakly virulent
264 viruses are more likely to circulate naturally in backyard flocks, probably due to the absence of
265 vaccine-induced selective pressure and weak biosecurity measures.

266 Molecular characterization and clinical findings are not sufficient to ascertain the level of virulence
267 of the detected viruses, therefore, in vivo pathotyping assays are needed. For this purpose, viral
268 isolation should be attempted. Moreover, the isolation of weakly virulent strains could offer the
269 opportunity to evaluate their potential as candidate vaccines.

270

271

272

273

274

275

276

277

278

279

280

281

282

- 284 Abd-Ellatieff, H.A., A.A. Abou Rawash, H.F. Ellakany, W.M. Goda, T. Suzuki, and T. Yanai.
285 2018. Molecular characterization and phylogenetic analysis of a virulent Marek's disease virus field
286 strain in broiler chickens in Japan. *Avian Pathol.* 47: 47–57.
- 287 Baigent, S.J., L.B. Kgosana, A.A. Gamawa, L.P. Smith, A.F. Read, and V.K. Nair. 2013.
288 Relationship Between Levels of Very Virulent MDV in Poultry Dust and in Feather Tips from
289 Vaccinated Chickens. *Avian Dis.* 57: 440–447.
- 290 Biggs, P.M., H.G. Purchase, B.R. Bee, and P.J. Dalton. 1965. Preliminary report on acute Marek's
291 disease (fowl paralysis) in Great Britain. *Vet. Rec.* 77: 1339–1340.
- 292 Brown, A.C., L.P. Smith, L. Kgosana, S.J. Baigent, V. Nair, and M.J. Allday. 2009.
293 Homodimerization of the Meq Viral Oncoprotein Is Necessary for Induction of T-Cell Lymphoma
294 by Marek's Disease Virus. *J. Virol.* 83: 11142–11151.
- 295 Cecchinato, M., A. Comin, L. Bonfanti, C. Terregino, I. Monne, M.Lorenzetto, and S. Marangon.
296 2011. Epidemiology and Control of Low Pathogenicity Avian Influenza Infections in Rural Poultry
297 in Italy. *Avian Dis.* 55: 13–20.
- 298 Chang, K.-S., K. Ohashi, and M. Onuma. 2002a. Suppression of transcription activity of the MEQ
299 protein of oncogenic Marek's disease virus serotype 1 (MDV1) by L-MEQ of non-oncogenic
300 MDV1. *J. Vet. Med. Sci.* 64: 1091–1095.
- 301 Chang, K.-S., K. Ohashi, and M. Onuma. 2002b. Diversity (polymorphism) of the meq gene in the
302 attenuated Marek's disease virus (MDV) serotype 1 and MDV-transformed cell lines. *J. Vet. Med.*
303 *Sci.* 64: 1097–1101.
- 304 Davidson, I., A. Natour-Altory, I. Raibstein, E. Kin, Y. Dahan, H. Krispin, and N. Elkin. 2018.
305 Monitoring the uptake of live avian vaccines by their detection in feathers. *Vaccine.* 36: 637–643.

306 Gimeno, I.M. 2014. Marek's Disease and Differential Diagnosis with Other Tumor Viral Diseases
307 of Poultry. Pages 156–171 in Encyclopedia of Agriculture and Food Systems, 2nd Edition. N. Van
308 Alfen, ed. Academic Press, Elsevier Science & Technology, San Diego, California, USA.

309 Hassanin, O., F. Abdallah, and I.E. El-Araby. 2013. Molecular characterization and phylogenetic
310 analysis of Marek's disease virus from clinical cases of Marek's disease in Egypt. *Avian Dis.* 57:
311 555–561.

312 Kumar, S., G. Stecher, and K. Tamura. 2016. MEGA7: Molecular Evolutionary Genetics Analysis
313 Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.* 33: 1870–1874.

314 Levy, A.M., O. Gilad, L. Xia, Y. Izumiya, J. Choi, A. Tsalenko, Z. Yakhini, R. Witter, L. Lee, C.J.
315 Cardona, and H.-J. Kung. 2005. Marek's disease virus Meq transforms chicken cells via the v-Jun
316 transcriptional cascade: a converging transforming pathway for avian oncoviruses. *Proc. Natl.*
317 *Acad. Sci. U. S. A.* 102: 14831–14836.

318 Liu, J.-L., Y. Ye, L.F. Lee, and H.-J. Kung. 1998. Transforming potential of the herpesvirus
319 oncoprotein MEQ: morphological transformation, serum-independent growth, and inhibition of
320 apoptosis. *J. Virol.* 72: 388–395.

321 López-Osorio, S., D. Piedrahita, M.A. Espinal-Restrepo, G.C. Ramírez-Nieto, V. Nair, S.M.
322 Williams, S.J. Baigent, C. Ventura-Polite, D.A. Aranzazu-Taborda, and J.J. Chaparro-Gutiérrez.
323 2017. Molecular characterization of Marek's disease virus in a poultry layer farm from Colombia.
324 *Poult. Sci.* 96: 1598–1608.

325 Mete, A., R. Gharpure, M.E. Pitesky, D. Famini, K. Sverlow, and J. Dunn. 2016. Marek's Disease
326 in Backyard Chickens, A Study of Pathologic Findings and Viral Loads in Tumorous and
327 Nontumorous Birds. *Avian Dis.* 60: 826–836.

328 Murata, S., Y. Hayashi, A. Kato, M. Isezaki, S. Takasaki, M. Onuma, Y. Osa, M. Asakawa, S.
329 Konnai, and K. Ohashi. 2012. Surveillance of Marek's disease virus in migratory and sedentary
330 birds in Hokkaido, Japan. *Vet. J.* 192: 538–540.

331 Nair, V. 2013. Latency and Tumorigenesis in Marek's Disease. *Avian Dis.* 57: 360–365.

332 Padhi, A., and M.S. Parcells. 2016. Positive selection drives rapid evolution of the meq oncogene
333 of Marek's disease virus. *PLoS One* 11, e0162180.

334 Pohjola, L., L. Rossow, A. Huovilainen, T. Soveri, M.L. Hänninen, and M. Fredriksson-Ahomaa.
335 2015. Questionnaire study and postmortem findings in backyard chicken flocks in Finland. *Acta*
336 *Vet. Scand.* 57.

337 Qian, Z., P. Brunovskis, L. Lee, P.K. Vogt, and H.J. Kung. 1996. Novel DNA binding specificities
338 of a putative herpesvirus bZIP oncoprotein. *J. Virol.* 70: 7161–7170.

339 Qian, Z., P. Brunovskis, F. Rauscher 3rd, L. Lee, and H.J. Kung. 1995. Transactivation activity of
340 Meq, a Marek's disease herpesvirus bZIP protein persistently expressed in latently infected
341 transformed T cells. *J. Virol.* 69: 4037–4044.

342 Renz, K.G., J. Cooke, N. Clarke, B.F. Cheetham, Z. Hussain, A.F.M. Fakhru'l Islam, G.A. Tannock,
343 and S.W. Walkden-Brown. 2012. Pathotyping of Australian isolates of Marek's disease virus and
344 association of pathogenicity with meq gene polymorphism. *Avian Pathol.* 41: 161–176.

345 Schat, K.A., and V. Nair. 2013. Neoplastic diseases: Marek's disease. Pages 515–552 in *Diseases of*
346 *Poultry* 13th edn. D.E. Swayne, J.R. Glisson, L.R. McDougald, L.K. Nolan, D.L. Suarez, V.L. Nair,
347 ed. Wiley-Blackwell Hoboken, Ames, Iowa, USA.

348 Shamblin, C.E., N. Greene, V. Arumugaswami, R.L. Dienglewicz, and M.S. Parcells. 2004.
349 Comparative analysis of Marek's disease virus (MDV) glycoprotein-, lytic antigen pp38- and
350 transformation antigen Meq-encoding genes: Association of meq mutations with MDVs of high
351 virulence. *Vet. Microbiol.* 102: 147–167.

352 Smith, M.W., and B.W. Calnek. 1973. Effect of virus pathogenicity on antibody production in
353 Marek's disease. *Avian Dis.* 17: 727–736.

354 Smith, M.W., and B.W. Calnek. 1974. Comparative features of low-virulence and high-virulence
355 marek's disease virus infections. *Avian Pathol.* 3: 229–246.

356 Suchodolski, P.F., Y. Izumiya, B. Lupiani, D.K. Ajithdoss, O. Gilad, L.F. Lee, H.-J. Kung, and
357 S.M. Reddy. 2009. Homodimerization of Marek's Disease Virus-Encoded Meq Protein Is Not
358 Sufficient for Transformation of Lymphocytes in Chickens. *J. Virol.* 83: 859–869.

359 Suchodolski, P.F., Y. Izumiya, B. Lupiani, D.K. Ajithdoss, L.F. Lee, H.J. Kung, and S.M. Reddy.
360 2010. Both homo and heterodimers of Marek's disease virus encoded Meq protein contribute to
361 transformation of lymphocytes in chickens. *Virology* 399: 312–321.

362 Suresh, P., J. Johnson Rajeswar, K. Sukumar, T.J. Harikrishnan, and P. Srinivasan. 2017. Complete
363 nucleotide sequence analysis of the oncogene “Meq” from serotype 1 Marek's disease virus isolates
364 from India. *Br. Poult. Sci.* 48: 111–115.

365 Tai, S.H.S., C. Hearn, S. Umthong, O. Agafitei, H.H. Cheng, J.R. Dunn, and M. Niikura. 2017.
366 Expression of Marek's Disease Virus Oncoprotein Meq During Infection in the Natural Host.
367 *Virology* 503: 103–113.

368 Thompson J.D., D.G. Higgins, and T.J. Gibson. 1994. CLUSTAL W: improving the sensitivity of
369 progressive multiple sequence alignment through sequence weighting, position-specific gap
370 penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673–4680.

371 Trimpert, J., N. Groenke, M. Jenckel, S. He, D. Kunec, M.L. Szpara, S.J. Spatz, N. Osterrieder, and
372 D.P. McMahon. 2017. A phylogenomic analysis of Marek's disease virus reveals independent paths
373 to virulence in Eurasia and North America. *Evol. Appl.* 10: 1091–1101.

374 Witter, R.L. 1997. Increased Virulence of Marek's Disease Virus Field Isolates. *Avian Dis.* 41:
375 149–163.

376 Witter, R.L., B.W. Calnek, C. Buscaglia, I.M. Gimeno, and K.A. Schat. 2005. Classification of
377 Marek's disease viruses according to pathotype: Philosophy and methodology. *Avian Pathol.* 34:
378 75-90.

379

380

381

382

383

384

385

386

387

388

389

390

391

392

393

394

395

396

Table 1. Geographical location of the studied backyard flocks, with the observed clinical forms of Marek's disease (MD) and the age and breed of affected chickens.

Flock ID	Italian region	MD form	Chicken breeds	Age range (months)
487/15	Piedmont	Acute	Silkie	4
507/15	Sardinia	Classical - R ¹	Amrock, Millefiori di Lonigo	7 - 24
509/15	Lazio	Classical	Araucana, Marans, Satsumadori	5 - 36
510/15	Lazio	Classical	Campine	36
562/15	Lazio	Classical - R	Sebright	6
599/16	Lazio	Classical	Sebright	24
625/16	Tuscany	Acute	Robusta Lionata	2 - 4.5
674/16	Emilia-Romagna	NS ²	Padovana, Polish	6 - 12
689/16	Lazio	Acute	Cochin, Padovana	6 - 8
722/16	Tuscany	NS	Sussex	2 - 2.5
801/17	Sicily	NS	Wyandotte	3.5 - 4
810/17	Sicily	Transient paralysis	Padovana	3 - 4.5
847/17	Lombardy	Classical	Brahma	12
848/17	Emilia-Romagna	Classical - R	Silkie	2 - 4
850/17	Tuscany	NS	Brahma, Silkie	6
852/17	Campania	Acute	Australorp, Satsumadori, Sumatra	6 - 9
853/17	Lombardy	Acute	Ayam Cemani	4 - 7
854/17	Trentino-Alto Adige	NS	Serama	9 - 24
855/17	Tuscany	NS	Leghorn, Valdarno	8 - 12

¹ Birds experienced a complete recovery;

² Clinical signs and gross lesions were not specific for MD. High mortality is often reported.

Table 2. GaHV-2 strains, retrieved from GenBank, which were included in the molecular analysis.

Strain	Country of origin	Pathotype	Year	GenBank accession number
CVI988 (Intervet)	Netherlands	att ¹	- ²	DQ534538
814	China	att	1980s	AF493551
3004	Russia	att	-	EU032468
CU-2	USA	m ³	1970s	AY362708
04CRE	Australia	v ⁴	2004	EF523773
MPF57	Australia	v	1994	EF523774
BC-1	USA	v	1970s	AY362707
JM/102W	USA	v	1962	DQ534539
567	USA	v	-	AY362709
571	USA	v	1989	AY362710
617A	USA	v	1993	AY362712
FT158	Australia	vv ⁵	2002	EF523771
02LAR	Australia	vv	2002	EF523772
Md5	USA	vv	1977	AF243438
643P	USA	vv	1994	AY362716
L	USA	vv+ ⁶	-	AY362717
New	USA	vv+	-	AY362719
W	USA	vv+	-	AY362723
648A	USA	vv+	1994	AY362725
ATE	Hungary	-	-	AY571784
24_00	Poland	-	2000	KJ464764
108_11	Poland	-	2011	KJ464831
56_12	Poland	-	2012	KJ464839
Ind/KA12/02	India	-	2012	KP342383
GX14PP03	China	-	2014	KX506775
LZ1309	China	-	2015	KX966280
B2015	India	-	2015	LC195187
GADVASU-M1	India	-	2016	KY651231
MEQ_GIFU_1	Japan	-	2016	LC208801
MEQ_GIFU_2	Japan	-	2016	LC208802
MEQ_GIFU_3	Japan	-	2016	LC208803
TN1014/16	Tunisia	-	2016	KY113150

¹ Attenuated² Unknown³ Mild⁴ Virulent⁵ Very virulent⁶ Very virulent plus

Table 3. Lengths of the *meq* genes of Italian GaHV-2 strains, with GenBank accession numbers.

Strain classification	Strain	<i>Meq</i> gene length (bp)	GenBank accession number
“Very long <i>meq</i> ” strain	GaHV-2/Italy/Ck/507/15	1257	MK139661
	GaHV-2/Italy/Ck/509/15	1257	MK139662
	GaHV-2/Italy/Ck/510/15	1257	MK139663
	GaHV-2/Italy/Ck/562/15	1257	MK139664
	GaHV-2/Italy/Ck/599/16	1257	MK139665
	GaHV-2/Italy/Ck/847/17	1257	MK139672
	GaHV-2/Italy/Ck/848/17	1257	MK139673
“Standard <i>meq</i> ” strain	GaHV-2/Italy/Ck/487/15	1020	MK139660
	GaHV-2/Italy/Ck/625/16	1020	MK139666
	GaHV-2/Italy/Ck/674/16	1020	MK139667
	GaHV-2/Italy/Ck/689/16	1020	MK139668
	GaHV-2/Italy/Ck/722/16	1020	MK139669
	GaHV-2/Italy/Ck/801/17	1020	MK139670
	GaHV-2/Italy/Ck/810/17	1020	MK139671
	GaHV-2/Italy/Ck/850/17	1020	MK139674
	GaHV-2/Italy/Ck/852/17	1020	MK139675
	GaHV-2/Italy/Ck/853/17	1020	MK139676
GaHV-2/Italy/Ck/854/17	1020	MK139677	
“Short <i>meq</i> ” strain	GaHV-2/Italy/Ck/855/17	897	MK139678

Table 4. Meq protein features of Italian GaHV-2 strains, compared to selected reference strains, with one of each pathotype.

Strain	Meq protein length (aa)	Insertion size (aa)	PPPPs (n°)	Proline content (%)
CVI988 (Intervet) (att)	399	60	8	23.25
CU-2 (m)	398	59	7	23.06
JM/102W (v)	399	60	7	23.06
Md5 (vv)	339	- ¹	4	21.24
648A (vv+)	339	-	2	20.88
GaHV-2/Italy/Ck/847/17	418	79	10	23.87
GaHV-2/Italy/Ck/507/15	418	79	9	23.63
GaHV-2/Italy/Ck/509/15	418	79	9	23.63
GaHV-2/Italy/Ck/510/15	418	79	9	23.63
GaHV-2/Italy/Ck/562/15	418	79	9	23.63
GaHV-2/Italy/Ck/599/16	418	79	9	23.63
GaHV-2/Italy/Ck/848/17	418	79	9	23.63
GaHV-2/Italy/Ck/487/15	339	-	5	21.47
GaHV-2/Italy/Ck/850/17	339	-	5	21.47
GaHV-2/Italy/Ck/625/16	339	-	4	21.18
GaHV-2/Italy/Ck/674/16	339	-	4	21.18
GaHV-2/Italy/Ck/689/16	339	-	4	21.18
GaHV-2/Italy/Ck/722/16	339	-	4	21.18
GaHV-2/Italy/Ck/801/17	339	-	4	21.18
GaHV-2/Italy/Ck/810/17	339	-	4	21.18
GaHV-2/Italy/Ck/852/17	339	-	4	21.18
GaHV-2/Italy/Ck/853/17	339	-	4	21.18
GaHV-2/Italy/Ck/854/17	339	-	4	21.18
GaHV-2/Italy/Ck/855/17	298	-	2	19.40

¹ Absence of insertion.

Table 5. Amino acid substitutions in the Meq proteins of “very long *meq*” Italian GaHV-2 strains, using the CVI988 vaccine strain as consensus sequence. Italian unique mutations, after comparison with all available sequences, are reported in bold.

Strain	Amino acid substitution position														
	37	66	80	93	98	101	139	242	261 ¹	352 ³ / 371 ⁴	373/ 392	386/ 405	390/ 409	391/ 410	
CVI988 (Intervet)	H	G	D	Q	H	K	T	F	- ²	H	L	I	V	W	
GaHV-2/ Italy/Ck/847/17	R	R	E	R	D	N	A	I	I	P	S	T	L	C	
GaHV-2/ Italy/Ck/507/15															
GaHV-2/ Italy/Ck/562/15	R	R	E	R	D	N	A	I	I	H	L	T	V	W	
GaHV-2/ Italy/Ck/599/16															
GaHV-2/ Italy/Ck/510/15	R	R	E	R	D	N	A	I	I	H	S	T	V	W	
GaHV-2/ Italy/Ck/509/15	R	R	E	R	D	N	A	I	F	H	L	T	V	W	
GaHV-2/ Italy/Ck/848/17															

^{1,4} Amino acid position with respect to Italian “very long *meq*” strains.

² Deletion of CVI988 compared with Italian “very long *meq*” strains.

³ Amino acid position with respect to CVI988 strain.

Table 6. Amino acid substitutions in the Meq proteins of “standard *meq*” Italian GaHV-2 strains, using the CVI988 vaccine strain as consensus sequence.

Strain	Amino acid substitution position									
	66	71	80	110	115	217 ¹ / 277 ²	218/ 278	244/ 304	271/ 331	326/ 386
CVI988 (Intervet)	G	S	D	C	V	P	P	C	G	I
GaHV-2/Italy/Ck/850/17	R	A	Y	C	A	P	P	G	G	T
GaHV-2/Italy/Ck/487/15	R	A	Y	S	V	P	P	C	G	T
GaHV-2/Italy/Ck/674/16	R	A	Y	R	A	A	P	C	R	T
GaHV-2/Italy/Ck/625/16										
GaHV-2/Italy/Ck/689/16										
GaHV-2/Italy/Ck/722/16										
GaHV-2/Italy/Ck/801/17										
GaHV-2/Italy/Ck/810/17	R	A	Y	S	V	P	S	C	G	T
GaHV-2/Italy/Ck/852/17										
GaHV-2/Italy/Ck/853/17										
GaHV-2/Italy/Ck/854/17										

¹ Amino acid position with respect to Italian “standard *meq*” GaHV-2 stains.

² Amino acid position with respect to CVI988 strain.

Table 7. Amino acid substitutions in the Meq protein of “short *meq*” Italian GaHV-2 strain, using the CVI988 vaccine strain as consensus sequence.

Strain	Amino acid substitution position					
	66	71	80	110	177	285 ¹ / 386 ²
CVI988 (Intervet)	G	S	D	C	P	I
GaHV-2/Italy/Ck/855/17	R	A	Y	S	S	T

¹ Amino acid position with respect to the Italian “short *meq*” strain.

² Amino acid position with respect to CVI988 strain.

409 **Figure caption.**

410 **Figure 1.** Phylogenetic tree based on Meq amino acid sequences of 19 Italian GaHV-2 strains, 32
411 international GaHV-2 strains and 3 CVI988/Rispens vaccine strains currently used in Italy. Only
412 bootstrap values ≥ 70 are reported.

413

