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# FEATURES OF MAREK'S DISEASE VIRUS IN RURAL CHICKENS

## **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**

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The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide 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.

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

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## INTRODUCTION

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

### ***Backyard Flocks***

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

### ***Sampling***

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

### ***DNA Extraction***

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

## 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 MgCl<sub>2</sub>  
133 solution, 0.5 µl of dNTPs, 13 µl of H<sub>2</sub>O 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.

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## 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.

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## 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.

## DISCUSSION

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

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

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

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**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 <sup>1</sup>	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 <sup>2</sup>	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

<sup>1</sup> Birds experienced a complete recovery;<sup>2</sup> 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 <sup>1</sup>	- <sup>2</sup>	DQ534538
814	China	att	1980s	AF493551
3004	Russia	att	-	EU032468
CU-2	USA	m <sup>3</sup>	1970s	AY362708
04CRE	Australia	v <sup>4</sup>	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 <sup>5</sup>	2002	EF523771
02LAR	Australia	vv	2002	EF523772
Md5	USA	vv	1977	AF243438
643P	USA	vv	1994	AY362716
L	USA	vv+ <sup>6</sup>	-	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

<sup>1</sup> Attenuated<sup>2</sup> Unknown<sup>3</sup> Mild<sup>4</sup> Virulent<sup>5</sup> Very virulent<sup>6</sup> Very virulent plus

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**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

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**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	- <sup>1</sup>	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

<sup>1</sup> 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 <sup>1</sup>	352 <sup>3</sup> / 371 <sup>4</sup>	373/ 392	386/ 405	390/ 409	391/ 410	
CVI988 (Intervet)	H	G	D	Q	H	K	T	F	- <sup>2</sup>	H	L	I	V	W	
GaHV-2/ Italy/Ck/847/17	<b>R</b>	R	<b>E</b>	R	<b>D</b>	<b>N</b>	A	<b>I</b>	I	P	S	T	L	C	
GaHV-2/ Italy/Ck/507/15															
GaHV-2/ Italy/Ck/562/15	<b>R</b>	R	<b>E</b>	R	<b>D</b>	<b>N</b>	A	<b>I</b>	I	H	L	T	V	W	
GaHV-2/ Italy/Ck/599/16															
GaHV-2/ Italy/Ck/510/15	<b>R</b>	R	<b>E</b>	R	<b>D</b>	<b>N</b>	A	<b>I</b>	I	H	S	T	V	W	
GaHV-2/ Italy/Ck/509/15															
GaHV-2/ Italy/Ck/848/17	<b>R</b>	R	<b>E</b>	R	<b>D</b>	<b>N</b>	A	<b>I</b>	F	H	L	T	V	W	

<sup>1,4</sup> Amino acid position with respect to Italian “very long *meq*” strains.

<sup>2</sup> Deletion of CVI988 compared with Italian “very long *meq*” strains.

<sup>3</sup> 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 <sup>1</sup> / 277 <sup>2</sup>	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										

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

<sup>2</sup> 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 <sup>1</sup> / 386 <sup>2</sup>
CVI988 (Intervet)	G	S	D	C	P	I
GaHV-2/Italy/Ck/855/17	R	A	Y	S	S	T

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

<sup>2</sup> 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  $\geq 70$  are reported.

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