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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.

ABSTRACT

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

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

INTRODUCTION

Marek's disease (**MD**) is a worldwide, contagious, lymphoprolipherative 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+ 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+ 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 × *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 *Eco*R-Q for 5'-GGT GAT ATA AAG ACG ATA GTC ATG-3' and the reverse primer
130 *Eco*R-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 $MgCl_2$
133 solution, 0.5 µl of dNTPs, 13 µl of H_2O 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 80Vand 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 *Eco*R-Q for, *Eco*R-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

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

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

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

205 **DISCUSSION**

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,

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

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

Molecular characterization and clinical findings are not sufficient to ascertain the level of virulence of the detected viruses, therefore, in vivo pathotyping assays are needed. For this purpose, viral isolation should be attempted. Moreover, the isolation of weakly virulent strains could offer the 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[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.

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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] Attenuated
[2] Unknown
[3] Mild
[4] Virulent
[5] Very virulent
[6] 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	*Meq* gene length (bp)	GenBank accession number
"Very long *meq*" 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 *meq*" 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 *meq*" 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	- [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 GaHV-2/ Italy/Ck/599/16	**R**	R	**E**	R	**D**	**N**	A	**I**	I	H	L	T	V	W
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 GaHV-2/ Italy/Ck/848/17	**R**	R	**E**	R	**D**	**N**	A	**I**	F	H	L	T	V	W

[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.

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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 GaHV-2/Italy/Ck/852/17 GaHV-2/Italy/Ck/853/17 GaHV-2/Italy/Ck/854/17	R	A	Y	S	V	P	S	C	G	T

[1] Amino acid position with respect to Italian "standard *meq*" GaHV-2 stains.
[2] Amino acid position with respect to CVI988 strain.

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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.

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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.

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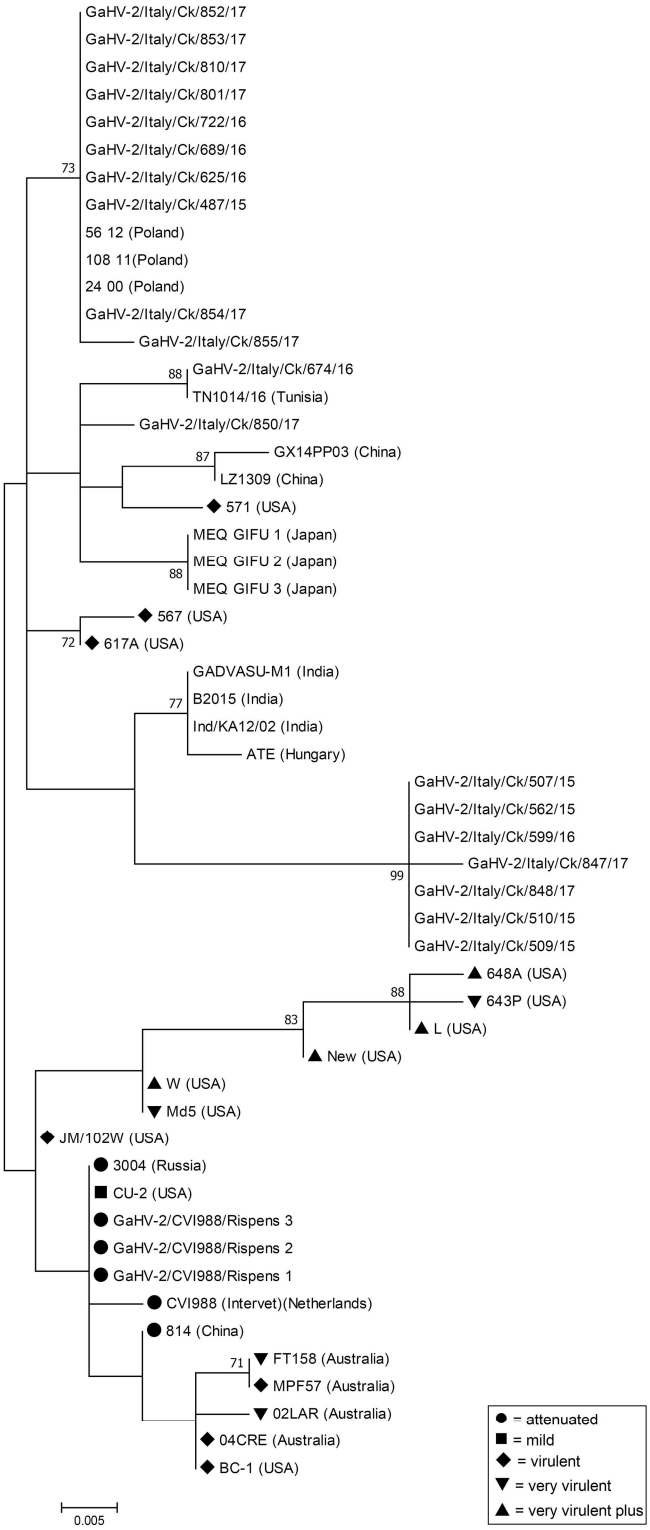
GaHV-2/Italy/Ck/852/17
GaHV-2/Italy/Ck/853/17
GaHV-2/Italy/Ck/810/17
GaHV-2/Italy/Ck/801/17
GaHV-2/Italy/Ck/722/16
GaHV-2/Italy/Ck/689/16
73
GaHV-2/Italy/Ck/625/16
GaHV-2/Italy/Ck/487/15
56 12 (Poland)
108 11(Poland)
24 00 (Poland)
GaHV-2/Italy/Ck/854/17
GaHV-2/Italy/Ck/855/17
88
GaHV-2/Italy/Ck/674/16
TN1014/16 (Tunisia)
GaHV-2/Italy/Ck/850/17
87
GX14PP03 (China)
LZ1309 (China)
571 (USA)
MEQ GIFU 1 (Japan)
MEQ GIFU 2 (Japan)
88
MEQ GIFU 3 (Japan)
567 (USA)
72
617A (USA)
GADVASU-M1 (India)
77
B2015 (India)
Ind/KA12/02 (India)
ATE (Hungary)
GaHV-2/Italy/Ck/507/15
GaHV-2/Italy/Ck/562/15
GaHV-2/Italy/Ck/599/16
GaHV-2/Italy/Ck/847/17
99
GaHV-2/Italy/Ck/848/17
GaHV-2/Italy/Ck/510/15
GaHV-2/Italy/Ck/509/15
648A (USA)
88
643P (USA)
83
L (USA)
New (USA)
W (USA)
Md5 (USA)
JM/102W (USA)
3004 (Russia)
CU-2 (USA)
GaHV-2/CVI988/Rispens 3
GaHV-2/CVI988/Rispens 2
GaHV-2/CVI988/Rispens 1
CVI988 (Intervet)(Netherlands)
814 (China)
71
FT158 (Australia)
MPF57 (Australia)
02LAR (Australia)
04CRE (Australia)
BC-1 (USA)
0.005
= attenuated
= mild
= virulent
= very virulent
= very virulent plus

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