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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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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. Whereas 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 (aa) 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 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 PPPP. 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

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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 nononcogenic 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 2 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 2 forms: classical and acute. Classical MD (also known as fowl paralysis) is characterized by spastic paralysis due to nerve lesions; it was mainly

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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, 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 (**aa**) 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). The gene *meq* is polymorphic, 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 aa, 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 are 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 9 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, 5 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, noninvasive, and nonlethal (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, 5 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, MO). 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.

PCR Amplification of the meq Gene

The full-length *meq* gene was amplified, according to Shamblin et al. (2004), using the forward primer *Eco*R-Q for 5′-GGT GAT ATA AAG ACG ATA GTC ATG-3′ and the reverse primer *Eco*R-Q rev 5′-CTC ATA CTT CGG AAC TCC TGG AG-3′. In a total reaction volume of 25 μL, 3 μL of eluted template DNA was mixed with 0.125 μL of GoTaq G2 Flexi DNA Polymerase (Promega, Madison, WI), 5 μL of 5X Colorless GoTaq Flexi Buffer, 1.75 μL of $MgCl_2$ solution, 0.5 μL of

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 to 24
509/15	Lazio	Classical	Araucana, Marans, Satsumadori	5 to 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 to 4.5
674/16	Emilia-Romagna	NS[2]	Padovana, Polish	6 to 12
689/16	Lazio	Acute	Cochin, Padovana	6 to 8
722/16	Tuscany	NS	Sussex	2 to 2.5
801/17	Sicily	NS	Wyandotte	3.5 to 4
810/17	Sicily	Transient paralysis	Padovana	3 to 4.5
847/17	Lombardy	Classical	Brahma	12
848/17	Emilia-Romagna	Classical—R	Silkie	2 to 4
850/17	Tuscany	NS	Brahma, Silkie	6
852/17	Campania	Acute	Australorp, Satsumadori, Sumatra	6 to 9
853/17	Lombardy	Acute	Ayam Cemani	4 to 7
854/17	Trentino-Alto Adige	NS	Serama	9 to 24
855/17	Tuscany	NS	Leghorn, Valdarno	8 to 12

[1]Birds experienced a complete recovery.
[2]Clinical signs and gross lesions were not specific for MD. High mortality was often reported.

dNTPs, 13 μL of H_2O for molecular biology, and 1 μL of each primer. Cycling conditions were as follows: 2 min of denaturation at 95°C followed by 35 cycles, each consisting of denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1.5 min. A final elongation step at 72°C for 5 min completed the reaction. The PCR products were separated on agarose gel (1%), stained with ethidium bromide, and visualized under ultraviolet light after an electrophoretic run at 80 V and 400 mA for 50 min.

DNA Sequencing and Sequence Analysis

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

The nucleotide sequences were assembled and edited using Bioedit Sequence Alignment Editor Version 7.2.5.0 (Tom Hall, Ibis Therapeutics, Carlsbad, CA), then aligned and compared, using Clustal W software (Thompson et al., 1994), with the *meq* gene sequences of 32 selected GaHV-2 field and vaccine strains retrieved from the GenBank database (Table 2) and with the sequences of three CVI988/Rispens vaccine strains currently used in Italy. The number of four consecutive prolines (**PPPP**) contained in the PRR of the transactivation domain, the proline content, and the aa substitutions in the deduced aa sequence of *meq* genes were evaluated.

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

RESULTS

All 19 backyard chicken flocks tested in the present study were positive for GaHV-2. The obtained complete *meq* gene sequences were submitted to the GenBank database under the accession numbers listed in Table 3. Sequence analysis revealed that GaHV-2 strains had *meq* gene sequences of variable sizes: 1,257 bp, 1,020 bp, or 897 bp, which were named "very long *meq*," "standard *meq*," and "short *meq*" strains, respectively, based on a slightly modified version of the *meq* open reading frames classification reported by Chang et al. (2002b) (Table 3).

Length, insertion size, number of PPPP motifs within the transactivation domain, and the proline content of *meq*-deduced aa sequences of the Italian GaHV-2 strains and one representative GaHV-2 strain for each pathotype were evaluated (Table 4). Seven GaHV-2 strains showed a long Meq isoform (418 aa, "very long *meq*" strains), with an insertion of 79 aa and a high number of PPPP motifs (9 to 10). Eleven strains had a short Meq isoform (339 aa, "standard *meq*" strains) without insertion in the transactivation domain and a lower number of PPPP (4 to 5). Only one strain showed a very short Meq isoform (298 aa, "short *meq*" strain) with 2 PPPP in its transactivation domain.

The aa substitutions found in the Meq proteins of the analysed strains compared to the vaccine strain CVI988 (Intervet), chosen as a reference strain, are

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.

reported in Tables 5 to 7. Sequences of "very long *meq*" strains, which differ among themselves with respect to very few aa changes, showed 10 to 14 aa 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. A total of 5 to 8 aa substitutions were found when "standard *meq*" (Table 6) and "short *meq*" (Table 7) strains were compared with the CVI988 vaccine strain. Almost all aa changes of "standard *meq*" and "short *meq*" strain-encoded Meq 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 positions. In particular, the GaHV-2/Italy/Ck/855/17 strain showed a substitution at position 177 (P177S), interrupting a stretch of 4 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 aa 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. A total of 9 out of 11 Italian "standard *meq*" strains and the "short *meq*" strain were 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.

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	1,257	MK139661
	GaHV-2/Italy/Ck/509/15	1,257	MK139662
	GaHV-2/Italy/Ck/510/15	1,257	MK139663
	GaHV-2/Italy/Ck/562/15	1,257	MK139664
	GaHV-2/Italy/Ck/599/16	1,257	MK139665
	GaHV-2/Italy/Ck/847/17	1,257	MK139672
	GaHV-2/Italy/Ck/848/17	1,257	MK139673
"Standard *meq*" strain	GaHV-2/Italy/Ck/487/15	1,020	MK139660
	GaHV-2/Italy/Ck/625/16	1,020	MK139666
	GaHV-2/Italy/Ck/674/16	1,020	MK139667
	GaHV-2/Italy/Ck/689/16	1,020	MK139668
	GaHV-2/Italy/Ck/722/16	1,020	MK139669
	GaHV-2/Italy/Ck/801/17	1,020	MK139670
	GaHV-2/Italy/Ck/810/17	1,020	MK139671
	GaHV-2/Italy/Ck/850/17	1,020	MK139674
	GaHV-2/Italy/Ck/852/17	1,020	MK139675
	GaHV-2/Italy/Ck/853/17	1,020	MK139676
	GaHV-2/Italy/Ck/854/17	1,020	MK139677
"Short *meq*" 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/102 W (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.

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 aa 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 3 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 3 out of 7 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 3 aa substitutions found in the bZIP domain might have altered the Meq binding capacity and contributed to the low oncogenicity of the Italian "very long *meq*" strains.

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.

	Amino acid substitution position													
Strain	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	**R**	R	**E**	R	**D**	**N**	A	**I**	I	H	L	T	V	W
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	**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	**R**	R	**E**	R	**D**	**N**	A	**I**	F	H	L	T	V	W
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.

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

	Amino acid substitution position									
Strain	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	R	A	Y	S	V	P	S	C	G	T
GaHV-2/Italy/Ck/689/16	R	A	Y	S	V	P	S	C	G	T
GaHV-2/Italy/Ck/722/16	R	A	Y	S	V	P	S	C	G	T
GaHV-2/Italy/Ck/801/17	R	A	Y	S	V	P	S	C	G	T
GaHV-2/Italy/Ck/810/17	R	A	Y	S	V	P	S	C	G	T
GaHV-2/Italy/Ck/852/17	R	A	Y	S	V	P	S	C	G	T
GaHV-2/Italy/Ck/853/17	R	A	Y	S	V	P	S	C	G	T
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.

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

	Amino acid substitution position					
Strain	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 the CVI988 strain.

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

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

The heterogeneity of the viral population, supported by the allocation of the analyzed strains into 3 major clusters, suggests that the introduction of GaHV-2 to Italy could have occurred over multiple occasions. Ornamental chicken owners regularly enter their birds into international "beauty contests," where chickens are

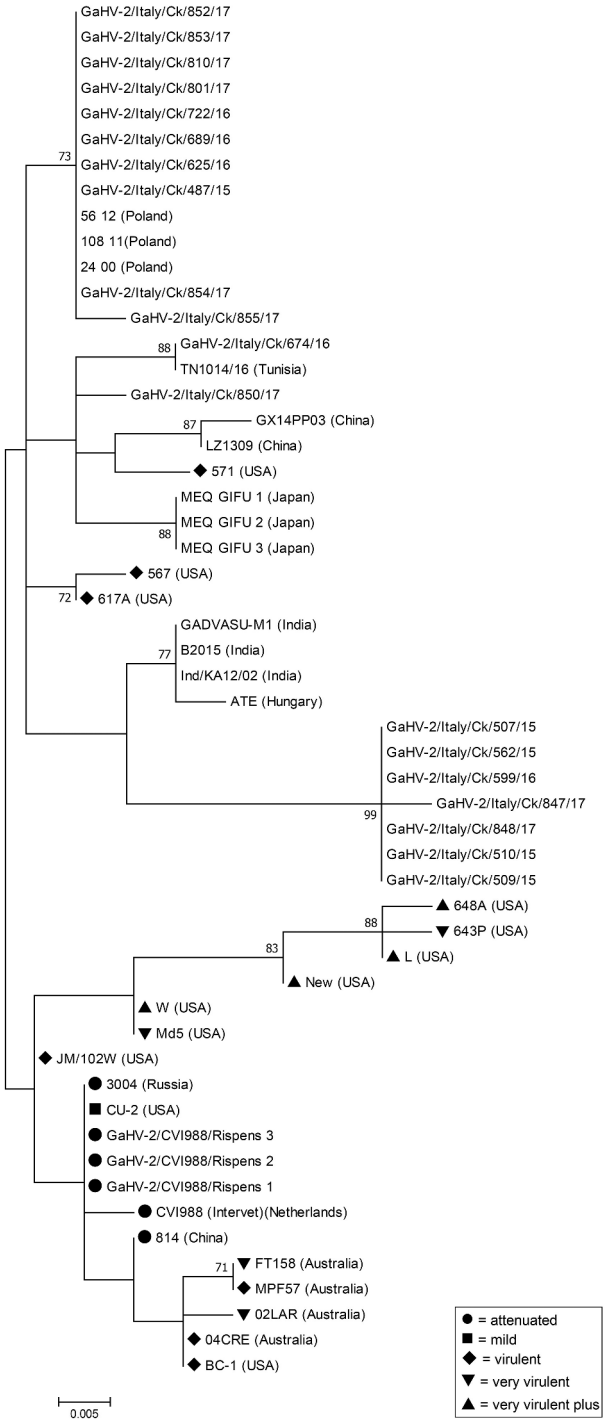

Figure 1. Phylogenetic tree based on Meq amino acid sequences of 19 Italian GaHV-2 strains, 32 international GaHV-2 strains, and 3 CVI988/Rispens vaccine strains currently used in Italy. Only bootstrap values ≥ 70 are reported.

generally kept in adjacent cages, facilitating the transmission of the virus from bird to bird. The national and international trade of live, valuable breeders is another possible route of entry.

Viruses could also have reached the rural context by overcoming the biosecurity measures applied in commercial poultry houses to find a highly variable poultry population with different species, breeds, ages, and immune statuses, with unknown susceptibility to MD. The reverse could be also true: backyard flocks could act as a reservoir for GaHV-2 strains of various and unknown pathotypes, representing a potential threat for commercial poultry flocks located in the same area. Biosecurity 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 detection of low-virulence viruses dates back to the 1970s (Smith and Calnek, 1973, 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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