Marek's disease viruses circulating in commercial poultry in Italy in the years 2015–2018 are closely related by their meq gene phylogeny

Title

Marek's disease viruses circulating in commercial poultry in Italy in the years 2015-2018 are closely related by their *meq* gene phylogeny.

Running Title

Virulence of GaHV-2 strains circulating in Italian commercial chickens

Giulia Mescolini[1, *], Caterina Lupini[1], Irit Davidson[2], Paola Massi[3], Giovanni Tosi[3], Elena Catelli[1]

[1] Department of Veterinary Medical Sciences, University of Bologna, Via Tolara di Sopra 50, 40064, Ozzano dell'Emilia (BO), Italy

[2] Division of Avian Diseases, Kimron Veterinary Institute, Bet Dagan P.O. Box 12 5025001, Israel

[3] Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Sezione Diagnostica di Forlì, Via don Eugenio Servadei 3/E-3/F, 47122, Forlì (FC), Italy

[*]Corresponding author: Giulia Mescolini, Department of Veterinary Medical Sciences -University of Bologna, Via Tolara di Sopra, 50; 40064 – Ozzano dell'Emilia (BO) Italy; Phone +39 051 2097560; e-mail: giulia.mescolini3@unibo.it

Summary

Marek's disease (MD) is a lymphoproliferative disease important to the poultry industry worldwide; it is caused by Gallid alphaherpesvirus 2 (GaHV-2). The virulence of the GaHV-2 isolate has shifted over the years from mild to virulent, very virulent and very virulent +. Nowadays the disease is controlled by vaccination, but field strains of increased virulence are emerging worldwide. Economic losses due to MD are mostly associated with its acute form, characterised by visceral lymphomas. The present study aimed to molecularly classify a group of 13 GaHV-2 strains detected in vaccinated Italian commercial chicken flocks during acute MD outbreaks, and to scrutinise the ability of predicting GaHV-2 virulence, according to the *meq* gene sequence. The full-length *meq* genes were amplified and the obtained amino acid (aa) sequences were analysed, focusing mainly on the number of stretches of four proline molecules (PPPP) within the transactivation domain. Phylogenetic analysis was carried out with the Maximum Likelihood method using the obtained aa sequences and the sequences of Italian strains detected in backyard flocks and of selected strains retrieved from GenBank. All the analysed strains showed 100% sequence identity in the *meq* gene, which encodes a Meq protein of 339 aa. The Meq protein includes four PPPP motifs in the transactivation domain and an interruption of a PPPP motif due to a proline-to-serine substitution at position 218. These features are typically encountered in highly virulent isolates. Phylogenetic analysis revealed that the analysed strains belonged to a cluster that includes high-virulence GaHV-2 strains detected in Italian backyard flocks and a hypervirulent Polish strain. Our results support the hypothesis that the virulence of field isolates can be suggested by *meq* aa sequence analysis.

Keywords: commercial chickens; Italy; GaHV-2; *meq* gene; strain virulence

Introduction

Marek's disease (MD) is an economically-important lymphoproliferative disease to the poultry industry worldwide due to its capacity to cause clinical disease, increased mortality and reduced growth, as well as sub-clinical immunosuppression, causing the exacerbation of other diseases and decreased vaccinal immunity (Schat & Nair, 2013). The virus belongs to the genus *Mardivirus*, subfamily *Alphaherpesvirinae*. According to the most recent nomenclature, it consists of three viral species: *Gallid alphaherpesvirus* 2 (GaHV-2) (aetiological agent of MD), *Gallid alphaherpesvirus* 3 (GaHV-3), and *Meleagrid alphaherpesvirus* 1 (MeHV-1) or Herpesvirus of turkeys (HVT) (International Committee on Taxonomy of Viruses, 2017). GaHV-3 and HVT are antigenically related to GaHV-2 and are widely used as vaccines, usually in association with live attenuated GaHV-2 strains (e.g. CVI988/Rispens). In the field, economic losses due to MD are mostly associated with the acute form of the disease, characterised by visceral lymphomas, however virus-induced immunosuppression might be relevant too, even if its impact is very difficult to assess (Gimeno, 2014; Gimeno & Schat, 2018).

The virulence of GaHV-2 isolates has shifted over the years from mild (m) to virulent (v), very virulent (vv) and very virulent + (vv+) (Witter, 1997; Witter et al., 2005). The disease is controlled by vaccination, but field GaHV-2 strains with increased virulence and greater fitness are emerging worldwide (Trimpert et al., 2017; Nair, 2018). Complex factors might be involved in the occurrence of MD outbreaks in vaccinated chicken flocks, such as: the increased virulence of GaHV-2 over time (Witter, 1997); the inability of the vaccine to offer protection; non-optimal vaccine application, due to its labile cell-associated form (Davidson et al., 2018; Davidson, Natour-Altory, & Shimshon, 2018); co-infection with immunosuppressive viruses (Schat & van Santen, 2013); and other factors.

The GaHV-2 genome encodes more than 200 genes, including the *meq* gene, which was the first discovered GaHV-2 oncogene (Jones et al., 1992). The *meq* gene encodes a protein with homology to the leucine-zipper class nuclear oncogenes, which is composed of a trans-activation N-terminal

74 basic-leucine zipper (bZIP) domain and a C-terminal proline-rich trans-repression domain (Qian et
75 al., 1995; Liu et al., 1999; Ross, 1999). The oncogenic activities of the Meq protein are mediated by
76 its dimerisation, through the bZIP domain, with itself, as well as with c-Jun-like proteins, such as
77 JunB, c-Jun and c-Fos. Meq also binds to cellular transcription factors such as ATF, CREB and
78 C/EBP (Deng et al., 2010) and interacts with cellular proteins without a bZIP domain, such as the
79 cellular tumour suppressors p53, the retinoblastoma protein (pRb) and the cyclin-dependent kinase
80 2 (CDK-2) or the heat shock protein Hsp70 (Deng et al., 2010; Gennart et al., 2015). The *meq*
81 oncogene encodes a 339 amino acid unspliced open reading frame in vv and in vv+ GaHV-2
82 pathotypes and a larger form of 398 amino acids in low virulence strains, having amplifications in
83 the C-terminal proline-rich repeat region (Shamblin et al., 2004).

84 Concurrently with the stepwise evolution of the virulence of GaHV-2 (Schat & Baranowski, 2007),
85 an increased pattern of genetic polymorphism at the C-terminus domain of the *meq*-encoded
86 oncoprotein has been described (Shamblin et al., 2004). High genetic diversity has been reported for
87 the *meq* gene in spite of the relatively low evolutionary rates of change thought to commonly
88 characterise dsDNA viruses (Duffy, Shackelton, & Holmes, 2008; Firth et al., 2010). Findings by
89 Padhi & Parcells (2016) and Trimpert et al. (2017) reveal that the *meq* gene sequence evolves at a
90 much faster rate than most dsDNA viruses, and is comparable with the evolutionary rate of RNA
91 viruses. By analysing the complete *meq* gene sequence of 84 GaHV-2 strains, Padhi & Parcells
92 (2016) estimated the mean evolutionary rate, beginning from the year 1935, to be greater than other
93 dsDNA viruses, namely 1.02×10^{-4} substitutions per site per year, as compared to the range of 10^{-7}
94 to 10^{-5} for other DNA viruses. Trimpert et al. (2017) analysed 18 complete GaHV-2 genomes and
95 calculated that the GaHV-2 had a mean evolutionary rate of 1.58×10^{-5} substitutions per site per
96 year, in which the *meq* open reading frame (MDV076) was identified as one of the loci harbouring
97 the highest number of point mutations over time. The *meq* gene is evolving under positive selection,
98 most likely imposed by vaccination, reflecting viral adaptation against the host immune responses.

Shamblin et al. (2004) and Renz et al. (2012) demonstrated that the number of the four-proline stretches (PPPP) in the *meq* gene transactivation domain are an indicative marker for the pathogenicity of GaHV-2 strains isolated from chickens; the most virulent isolates showed the lowest number of PPPP repeats, unlike the attenuated and the low pathogenicity isolates, which showed a highest number of repeats. The determination of GaHV-2 virulence by molecular sequencing could be valuable compared to the *in vivo* pathotyping assays, which require complex experimental infection trials of specific genetic lines of chickens (Witter et al., 2005; Dudnikova et al., 2007). At the moment *in vivo* studies are mandatory for an accurate inclusion of GaHV-2 strains into one of the known pathotypes. The *meq* gene polymorphism is also useful to create epidemiological molecular linkages between various GaHV-2 strains, according to the numerous studies that have been recently published from various countries: China (Tian et al., 2011; Zhang et al., 2011; Yu et al., 2013), Poland (Woźniakowski & Samorek-Salamonowicz, 2014), U.S.A. (Padhi & Parcell, 2016), Colombia (Lòpez-Osorio et al., 2017), Egypt (Hassanin, Abdallah, & El-Araby, 2013; Abdallah et al., 2018), India (Gupta, Deka, & Ramneek, 2016; Suresh et al., 2017; Prathibha, Sreedevi, Vinod Kumar, & Srilatha, 2018) and Japan (Abd-Ellatieff et al., 2018).

In the present study, we aimed to molecularly classify a group of 13 GaHV-2 strains, detected during acute MD outbreaks in vaccinated Italian commercial flocks, according to the *meq* gene sequence. We also aimed to scrutinise the ability of suggesting GaHV-2 virulence according to the *meq* gene sequence.

Materials and Methods

Commercial flocks and sampling

MD outbreaks occurred between 2015 and 2018 in 13 commercial chicken flocks located in 6 different Italian regions; flock specifics are reported in Table 1. The chicken flocks were different in

terms of production type, genetic line and age. MD vaccination status was known for 10 out of 13 flocks. Flocks were vaccinated with the association of CVI988/Rispens and HVT vaccines. The vaccination of broiler breeders was performed *in ovo* and repeated at 1 or 7 days of age. Cockerels were vaccinated with the association of CVI988/Rispens +HVT at 1 day old. All the examined flocks experienced acute MD with an increased mortality rate due to visceral lymphomas. At necropsy, portions of the spleen, liver or ovary, showing gross lymphomatous lesions, were sampled and stored at -20 °C until analysis.

Genomic DNA extraction

Genomic DNA was extracted separately from lymphomatous liver, spleen or ovary using the commercial kit "NucleoSpin® Tissue" (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany), following the manufacturer's instructions.

Amplification and sequencing of the *meq* gene

The entire *meq* gene was amplified and sequenced as previously described (Mescolini et al., 2019).

Sequence and phylogenetic analysis of the *meq* gene

The obtained nucleotide (nt) sequences were edited using BioEdit Sequence Alignment Editor Version 7.0.5.3 (Tom Hall, Ibis Therapeutics, Carlsbad, California, USA). Similarities between Italian sequences and *meq* gene sequences available in the NCBI database were investigated through the Basic Local Alignment Search Tool (BLAST).

The obtained sequences were aligned against and compared with previously published complete *meq* gene sequences of Italian strains detected in backyard flocks and with 57 selected complete *meq* gene sequences retrieved from GenBank (Table 2), using Clustal W software (Thompson, Higgins, & Gibson, 1994). Deduced amino acid (aa) sequences were analysed focusing on the number of PPPPs within the proline-rich repeats (PRRs) of the transactivation domain and on the presence of aa substitutions. A phylogenetic tree, based on *meq* gene aa sequences, was built using

147 the Maximum Likelihood method under the Jones–Taylor–Thornton model in MEGA version X
148 (Kumar et al., 2018). Nodal supports were estimated with 1000 bootstrap replicates and considered
149 significant when equal to or greater than 70.

150

151 **Results**

152 Thirteen GaHV-2 strains were detected by the specific PCR protocol in as many investigated
153 chicken flocks. The *meq* genes of the detected strains were genetically identical, being 1020 bp in
154 length; the obtained sequences were deposited into GenBank under the following names and
155 accession numbers: GaHV-2/Italy/Ck/456/15 - MK855054; GaHV-2/Italy/Ck/498/15 - MK855055;
156 GaHV-2/Italy/Ck/513/15 - MK855056; GaHV-2/Italy/Ck/515/15 - MK855057; GaHV-
157 2/Italy/Ck/559/15 - MK855058; GaHV-2/Italy/Ck/561/15 - MK855059; GaHV-2/Italy/Ck/565/15 -
158 MK855060; GaHV-2/Italy/Ck/567/15 - MK855061; GaHV-2/Italy/Ck/757/17 - MK855062; GaHV-
159 2/Italy/Ck/875/18 - MK855063; GaHV-2/Italy/Ck/876/18 - MK855064; GaHV-2/Italy/Ck/921/18 -
160 MK855065; GaHV-2/Italy/Ck/1083/18 - MK855066.

161 The molecular characteristics of the deduced Meq protein, compared to prototype strains CVI988,
162 CU-2, JM/102W, Md5 and 648A, are presented in Table 3. All the currently-detected-strains
163 showed a Meq protein of 339 aa that contained, in the transactivation domain, four PPPP motifs
164 and, at position 218, a proline-to-serine substitution, interrupting a hypothetical PPPP sequence at
165 the third position (PPPP → PPSP). Distinctive aa substitutions were found in the Italian GaHV-2
166 strains at positions 110 (C110S) and 218 (P218S).

167 The BLAST search showed 100% homology of the currently detected *meq* gene sequences with
168 those of the 8 Italian GaHV-2 strains (GaHV-2/Italy/Ck/625/16; GaHV-2/Italy/Ck/689/16; GaHV-
169 2/Italy/Ck/722/16; GaHV-2/Italy/Ck/801/17; GaHV-2/Italy/Ck/810/17; GaHV-2/Italy/Ck/852/17;
170 GaHV-2/Italy/Ck/853/17; GaHV-2/Italy/Ck/854/17) detected between 2016 and 2017 in Italian

backyard flocks that experienced acute MD, transient paralysis or sudden death, and with one of a Polish strain named Polen5 (Table 2). The phylogenetic tree (Figure 1) confirmed the previously reported findings, revealing that the strains detected in the present study clustered together with the Italian strains detected in backyard chickens, and with the Polish strain.

Discussion

The present study reports the sequence analysis of the *meq* gene of 13 GaHV-2 strains detected in several cases of MD-related visceral tumours that occurred in Italian vaccinated commercial chicken flocks collected during the years 2015–2018. Surprisingly, all the *meq* gene sequences of the analysed strains were identical, despite the fact that viruses were detected in flocks differing in terms of production type (broiler breeders, layers or cockerels), geographical location and ownership. The viruses coded for Meq proteins of 339 aa possessing features typically encountered in highly virulent isolates (Shamblin et al., 2004; Renz et al., 2012) and were phylogenetically related to the GaHV-2 strains currently circulating in Italian backyard flocks (Mescolini et al., 2019) and with a hypervirulent strain isolated in Poland in 2010 (Trimpert et al., 2017). Common trade routes may have hypothetically served as a source of dissemination for GaHV-2 between European countries and between industrial and rural compartments, where biosecurity breaches may have also occurred.

Our findings are in accordance with previous studies that reported the geographically-restricted evolution of field GaHV-2 strains in China (Yu et al., 2013), India (Suresh et al., 2017), Egypt (Abdallah et al., 2018) and Poland (Woźniakowski & Samorek-Salamonowicz, 2014). Recently, a comprehensive time-scaled phylogeny study, performed on complete genomes, revealed evidence of the geographical structuring of GaHV-2 strains, supporting the emergence of virulent viruses independently in North America and Eurasia (Trimpert et al., 2017).

Although GaVH-2, as a dsDNA virus, was foreseen to possess high genetic stability to mutations, unexpectedly, its *meq* gene sequence is mutating at a high evolutionary rate, namely 10^{-4} substitutions per site per year (Padhi & Parcells, 2016). The GaHV-2 *meq* gene evolutionary rate is typical for highly mutating RNA viruses, ranging between 10^{-2} to 10^{-5} substitutions per site per year; the avian influenza virus, subgroup H9N2 mutate as an example, with a value of $6.1x10^{-3}$ substitutions per site per year (Davidson et al., 2014). High evolutionary rates reflect the strong positive selection that exists for GaHV-2 in commercial flocks, probably resulting from the vaccination with highly effective, but imperfect vaccines that are increasing viral diversity (Padhi & Parcells, 2016). The fitness and replication of highly virulent strains seems to be favoured in vaccinated flocks (Read et al., 2015), in which strains able to avoid vaccine-induced protection could be selected.

There is a growing need for new MD vaccines that are efficacious against currently circulating viruses, due to the occurrence of breaks in vaccine immunity. Since vaccine failures are occurring worldwide (López-Osorio et al., 2017; Sun et al., 2017; Abdallah et al., 2018; Abd-Ellatieff et al., 2018), more studies must be conducted to evaluate the protection conferred by Rispens-type vaccines against more recent, and not yet pathotyped, field strains with a history of high virulence. Naturally circulating low virulence strains could represent a solution if they offer improved protection over CVI988/Rispens vaccine.

The rapid increase in the sequencing activities of the *meq* gene of field GaHV-2 strains all over the world has made possible to epidemiologically correlate a significant number of molecular data. The *meq* gene aa sequence has been correlated to GaHV-2 strains virulence (Shamblin et al., 2004; Renz et al., 2012). Our data support this last finding, having our strains molecular features of high virulence and having been detected during severe MD outbreaks in vaccinated chickens.

Meq gene sequencing alone is known to be an insufficient method to include field strains into defined pathotypes, therefore, recently, the research is focusing on finding other genetic predictors

of GaHV-2 virulence using complete or targeted DNA sequencing (Dunn et al., 2019). This will provide a highly advantageous alternative to the classical "gold standard" method of *in vivo* pathotyping (Witter et al., 2005), which requires the experimental infection of a large number of a specific type of chickens with standard GaHV-2 prototype strains. As that pathotype classification assay is difficult and not feasible worldwide, Dudnikova et al. (2007) developed an alternative "best fit" pathotyping assay. Although the "best fit" pathotyping assay is simplified, it also employs long-term trials using specific pathogen free chicks. *In vivo* pathotyping assays are not generally accessible, as those experimental infections require the use of chicks vaccinated at 1 day of age, challenged with virulent isolates and housed in poultry isolation units for 56 days post challenge.

As *in vivo* pathotyping is not easily achievable, the *meq* gene molecular characterisation would be the most rapid and accessible way to suggest virulence of field strains. However, the molecular findings should be supported by clinical observations, necropsy findings and vaccination status.

Conflict of Interest Statement

The authors declare no conflict of interest.

Ethical Statement

Ethical statement is not applicable.

References

Abd-Ellatieff, H. A., Abou Rawash, A. A., Ellakany, H. F., Goda, W. M., Suzuki, T., & Yanai, T. (2018). Molecular characterization and phylogenetic analysis of a virulent Marek's disease virus field strain in broiler chickens in Japan. Avian Pathology, 47(1), 47–57.

https://doi.org/10.1080/03079457.2017.1362497

Abdallah, F., Hassanin, O., Attar, E., Ali, H., Megahed, M., & Nair, V. (2018). Marek's Disease Virus in Egypt: Historical Overview and Current Research Based on the Major MDV-Encoded Oncogene Meq. Hosts and Viruses, 5, 35–43. http://dx.doi.org/10.17582/journal.hv/2018/5.3.35.43

Davidson, I., Fusaro, A., Heidari, A., Monne, I., & Cattoli, G. (2014). Molecular evolution of H9N2 avian influenza viruses in Israel. Virus Genes, 48(3), 457–463. https://doi.org/10.1007/s11262-014-1037-0

Davidson, I., Natour-Altory, A., Raibstein, I., Kin, E., Dahan, Y., Krispin, H., & Elkin, N. (2018). Monitoring the uptake of live avian vaccines by their detection in feathers. Vaccine, 36, 637–643. https://doi.org/10.1016/j.vaccine.2017.12.052

Davidson, I., Natour-Altory, N., & Shimshon Y. (2018). Evaluation of Live Vaccine Application in Commercial Poultry Flocks Using Feathers – In Practice. Israel Journal of Veterinary Medicine, 73, 8–13.

Deng, X., Li, X., Shen, Y., Qiu, Y., Shi, Z., Shao, D., Jin, Y., Chen, H., Ding, C., LiLi, Chen, P., & Ma, Z. (2010). The Meq oncoprotein of Marek's disease virus interacts with p53 and inhibits its transcriptional and apoptotic activities. Virology Journal, 7: 348. https://doi.org/10.1186/1743-422X-7-348

Dudnikova, E., Norkina, S., Vlasov, A., Slobodchuk, A., Lee, L. F., & Witter, R. L. (2007). Evaluation of Marek's disease field isolates by the "best fit" pathotyping assay. Avian Pathology, 36(2), 135–143. https://doi.org/10.1080/03079450701209857

Duffy, S., Shackelton, L. A., & Holmes, E. C. (2008). Rates of evolutionary change in viruses: Patterns and determinants. Nature Reviews Genetics, 9, 267–276. https://doi.org/10.1038/nrg2323

267 Dunn, J.R., Black Pyrkosz, A., Steep, A. & Cheng, H.H. (2019). Identification of Marek's disease
268 virus genes associated with virulence of US strains. Journal of General Virology, 100, 1132-
269 1139. https://doi.org/10.1099/jgv.0.001288

270 Firth, C., Kitchen, A., Shapiro, B., Suchard, M. A., Holmes, E. C., & Rambaut, A. (2010). Using
271 time-structured data to estimate evolutionary rates of double-stranded DNA viruses. Molecular
272 Biology and Evolution, 27(9), 2038–2051. https://doi.org/10.1093/molbev/msq088

273 Gennart, I., Coupeau, D., Pejaković, S., Laurent, S., Rasschaert, D., & Muylkens, B. (2015).
274 Marek's disease: Genetic regulation of gallid herpesvirus 2 infection and latency. Veterinary
275 Journal, 205, 339–348. https://doi.org/10.1016/j.tvjl.2015.04.038

276 Gimeno, I. M. (2014). Marek's Disease and Differential Diagnosis with Other Tumor Viral
277 Diseases of Poultry. In *Encyclopedia of Agriculture and Food Systems* (pp. 156–171).
278 https://doi.org/https://doi.org/10.1016/B978-0-444-52512-3.00193-5

279 Gimeno, I.M., & Schat K.A. (2018). Virus-Induced Immunosuppression in Chickens. Avian
280 Diseases, 62, 272–285. https://doi.org/10.1637/11841-041318-Review.1

281 Gupta, M., Deka, D., & Ramneek. (2016). Sequence analysis of Meq oncogene among Indian
282 isolates of Marek's disease herpesvirus. Meta Gene, 9, 230–236.
283 https://doi.org/10.1016/j.mgene.2016.07.009

284 Hassanin, O., Abdallah, F., & El-Araby, I. E. (2013). Molecular characterization and phylogenetic
285 analysis of Marek's disease virus from clinical cases of Marek's disease in Egypt. Avian
286 Diseases, 57(Suppl 2), 555–561. https://doi.org/10.1637/10337-082912-Reg.1

287 International Committee on Taxonomy of Viruses, 2017. Master Species List 2017 [WWW
288 Document]. ICTV. Retrieved from https://talk.ictvonline.org/files/master-species-
289 lists/m/msl/7185

290 Jones, D., Lee, L., Liu, J.L., Kung H. J., & Tillotson, J. K. (1992). Marek's disease virus encodes a

basic-leucine zipper gene resembling the fos/jun oncogenes that is highly expressed in lymphoblastoid tumors. Proceedings of the National Academy of Sciences, 89, 4042–4046. https://doi.org/10.1073/pnas.89.9.4042

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution, 35(6), 1547–1549. https://doi.org/10.1093/molbev/msy096

Liu, J. L., Lin, S. F., Xia, L., Brunovskis, P., Li, D., Davidson, I., Lee, L.F., & Kung, H. J. (1999). Meq and V-IL8: Cellular genes in disguise? Acta Virologica, 43, 94–101.

López-Osorio, S., Piedrahita, D., Espinal-Restrepo, M. A., Ramírez-Nieto, G. C., Nair, V., Williams, S. M., Baigent, S., Ventura-Polite, C., Aranzazu-Taborda, D.A., & Chaparro-Gutiérrez, J. J. (2017). Molecular characterization of Marek's disease virus in a poultry layer farm from Colombia. Poultry Science, 96(6), 1598–1608. https://doi.org/https://doi.org/10.3382/ps/pew464

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, pii: pez095. doi: 10.3382/ps/pez095. [Epub ahead of print]

Nair, V. (2018). Spotlight on avian pathology: Marek's disease. Avian Pathology, 47, 440–442. https://doi.org/10.1080/03079457.2018.1484073

Padhi, A., & Parcells, M. S. (2016). Positive selection drives rapid evolution of the meq oncogene of Marek's disease virus. PLoS ONE, 11(9). https://doi.org/10.1371/journal.pone.0162180

Prathibha, Y., Sreedevi, B., Vinod Kumar, N., & Srilatha, C. (2018). Molecular characterization and phylogenetic analysis of oncogenes from virulent serotype-1 Marek's disease virus in India. Acta Virologica, 62(3), 277–286. https://doi.org/10.4149/av_2018_221

315 Qian, Z., Brunovskis, P., Rauscher 3rd, F., Lee, L., & Kung, H. J. (1995). Transactivation activity
316 of Meq, a Marek's disease herpesvirus bZIP protein persistently expressed in latently infected
317 transformed T cells. Journal of Virology, 69(7), 4037–4044.
318 http://www.ncbi.nlm.nih.gov/pubmed/7769661

319 Read, A. F., Baigent, S. J., Powers, C., Kgosana, L. B., Blackwell, L., Smith, L. P., Kennedy, D.A.,
320 Walkden-Brown, S.W., & Nair, V. K. (2015). Imperfect Vaccination Can Enhance the
321 Transmission of Highly Virulent Pathogens. PLOS Biology, 13(7), e1002198.
322 https://doi.org/10.1371/journal.pbio.1002198

323 Renz, K. G., Cooke, J., Clarke, N., Cheetham, B. F., Hussain, Z., Fakhrul Islam, A. F. M., Tannock,
324 G.A., & Walkden-Brown, S. W. (2012). Pathotyping of Australian isolates of Marek's disease
325 virus and association of pathogenicity with meq gene polymorphism. Avian Pathology, 41(2),
326 161–176. https://doi.org/10.1080/03079457.2012.656077

327 Ross, N. L. J. (1999). T-cell transformation by Marek's disease virus. Trends in Microbiology, 7,
328 22–29. https://doi.org/10.1016/S0966-842X(98)01427-9

329 Schat, K. A., & Baranowski, E. (2007). Animal vaccination and the evolution of viral pathogens.
330 Revue Scientifique Et Technique-Office International Des Epizooties, 26(2), 327–338.

331 Schat, K.A., & Nair V. (2013). Neoplastic diseases: Marek's disease. In D. E. Swayne, J. R.
332 Glisson, L. R. McDougald, L. K. Nolan, D. L. Suarez, V. L. Nair, (Eds.) *Diseases of Poultry*
333 (13th ed., pp. 515–552). Ames, Iowa, USA: Wiley-Blackwell Hoboken.

334 Schat, K.A., & van Santen, V.L. (2013). Chicken infectious anemia. In D. E. Swayne, J. R. Glisson,
335 L. R. McDougald, L. K. Nolan, D. L. Suarez, V. L. Nair (Eds.) *Diseases of Poultry* (13th ed.,
336 pp. 248–275). Ames, Iowa, USA: Wiley-Blackwell Hoboken.

337 Shamblin, C. E., Greene, N., Arumugaswami, V., Dienglewicz, R. L., & Parcells, M. S. (2004).
338 Comparative analysis of Marek's disease virus (MDV) glycoprotein-, lytic antigen pp38- and

transformation antigen Meq-encoding genes: Association of meq mutations with MDVs of high virulence. Veterinary Microbiology, 102(3–4), 147–167. https://doi.org/10.1016/j.vetmic.2004.06.007

Spatz, S. J., Petherbridge, L., Zhao, Y., & Nair, V. (2007). Comparative full-length sequence analysis of oncogenic and vaccine (Rispens) strains of Marek's disease virus. Journal of General Virology, 88(4), 1080–1096. https://doi.org/10.1099/vir.0.82600-0

Spatz, S.J., & Silva, R.F. (2007). Polymorphisms in the repeat long regions of oncogenic and attenuated pathotypes of Marek's disease virus 1. Virus Genes, 35, 41–53. doi:10.1007/s11262-006-0024-5

Spatz, S.J., Smith, L.P., Baigent, S.J., Petherbridge, L., & Nair, V. (2011). Genotypic characterization of two bacterial artificial chromosome clones derived from a single DNA source of the very virulent gallid herpesvirus-2 strain C12/130. Journal of General Virology, 92, 1500–1507. doi:10.1099/vir.0.027706-0

Sun, G. R., Zhang, Y. P., Lv, H. C., Zhou, L. Y., Cui, H. Y., Gao, Y. L., Qi, X. Le, Wang, Y. Q., Li, K., Gao, L., Pan, Q., Wang, X. M., & Liu, C. J. (2017). A Chinese variant Marek's disease virus strain with divergence between virulence and vaccine resistance. Viruses 9, 71. https://doi.org/10.3390/v9040071

Suresh, P., Johnson Rajeswar, J., Sukumar, K., Harikrishnan, T. J., & Srinivasan, P. (2017). Complete nucleotide sequence analysis of the oncogene "Meq" from serotype 1 Marek's disease virus isolates from India. British Poultry Science, 58, 111–115. https://doi.org/10.1080/00071668.2016.1257780

Teng, L. Q., Wei, P., Song, Z. B., He, J. J., & Cui, Z. Z. (2011). Molecular epidemiological investigation of Marek's disease virus from Guangxi, China. Archives of Virology, 156, 203–206. doi:10.1007/s00705-010-0840-8

363 Thompson, J.D., Higgins, D.G., & Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of
364 progressive multiple sequence alignment through sequence weighting, position-specific gap
365 penalties and weight matrix choice. Nucleic Acids Research, 22, 4673–4680. doi:
366 10.1093/nar/22.22.4673

367 Tian, M., Zhao, Y., Lin, Y., Zou, N., Liu, C., Liu, P., Cao, S., Wen, X., & Huang, Y. (2011).
368 Comparative analysis of oncogenic genes revealed unique evolutionary features of field
369 Marek's disease virus prevalent in recent years in China. Virology Journal, 8, 121.
370 https://doi.org/10.1186/1743-422X-8-121

371 Trimpert, J., Groenke, N., Jenckel, M., He, S., Kunec, D., Szpara, M. L., Spatz, S. J., Osterrieder,
372 N., & McMahon, D. P. (2017). A phylogenomic analysis of Marek's disease virus reveals
373 independent paths to virulence in Eurasia and North America. Evolutionary Applications,
374 10(10), 1091–1101. https://doi.org/10.1111/eva.12515

375 Tulman, E. R., Afonso, C. L., Lu, Z., Zsak, L., Rock, D. L., & Kutish, G. F. (2000). The Genome of
376 a Very Virulent Marek's Disease Virus. Journal of Virology, 74, 7980–7988.
377 doi:10.1128/jvi.74.17.7980-7988.2000

378 Witter, R. L. (1997). Increased Virulence of Marek's Disease Virus Field Isolates. Avian Diseases,
379 41(1), 149. https://doi.org/10.2307/1592455

380 Witter, R. L., Calnek, B. W., Buscaglia, C., Gimeno, I. M., & Schat, K. A. (2005). Classification of
381 Marek's disease viruses according to pathotype: Philosophy and methodology. Avian
382 Pathology, 34, 75–90. https://doi.org/10.1080/03079450500059255

383 Woźniakowski, G., & Samorek-Salamonowicz, E. (2014). Molecular Evolution of Marek's Disease
384 Virus (MDV) Field Strains in a 40-Year Time Period. Avian Diseases, 58(4), 550–557.
385 https://doi.org/10.1637/10812-030614-Reg.1

386 Yu, Z.-H., Man, T., Lou, J., Wang, X.-W., Ding, K., Yu, L-L, Su, J-W, Chi, J.-Q., Zhao, P., Hu, B.,

Zhang, G.-P., & Liu, J.-X. (2013). Molecular characteristics and evolutionary analysis of field Marek's disease virus prevalent in vaccinated chicken flocks in recent years in China. Virus Genes, 47, 282–291. doi: 10.1007/s11262-013-0942-y

Zhang, F., Liu, C. J., Zhang, Y. P., Li, Z. J., Liu, A. L., Yan, F. H., Cong, F., & Cheng, Y. (2012). Comparative full-length sequence analysis of Marek's disease virus vaccine strain 814. Archives of Virology, 157, 177–183. doi:10.1007/s00705-011-1131-8

Zhang, Y. P., Liu, C. J., Zhang, F., Shi, W., & Li, J. (2011). Sequence analysis of the meq gene in the predominant Marek's disease virus strains isolated in China during 2006-2008. Virus Genes, 43(3), 353–357. https://doi.org/10.1007/s11262-011-0645-1

Table 1. Description of Italian commercial flocks affected by acute Marek's Disease and their vaccination status.

Flock ID	Region of origin	Year	Production type	Genetic line	Age (weeks)	Vaccine strain	1st vaccination	2nd vaccination	MD form
456/15	Emilia-Romagna	2015	Broiler breeders	Ross 708	NA†	CVI988+HVT	*In ovo*	1 day	Acute
498/15	Emilia-Romagna	2015	Broiler breeders	NA	NA	NA	NA	NA	Acute
513/15	Emilia-Romagna	2015	Layers	Hy-Line	31	NA	NA	NA	Acute
515/15	Emilia-Romagna	2015	Layers	NA	NA	NA	NA	NA	Acute
559/15	Veneto	2015	Broiler breeders	Ross 308	41	CVI988+HVT	*In ovo*	1 day	Acute
561/15	Friuli-Venezia Giulia	2015	Broiler breeders	Ross 708	56	CVI988+HVT	*In ovo*	1 day	Acute
565/15	Veneto	2015	Broiler breeders	Ross 708	36	CVI988+HVT	*In ovo*	1 day	Acute
567/15	Marche	2015	Cockerels	Hy-Line	11	CVI988+HVT	1 day	- ‡	Acute
757/17	Emilia-Romagna	2017	Broiler breeders	Ross 308	51	CVI988+HVT	*In ovo*	1 day	Acute
875/18	Emilia-Romagna	2018	Broiler breeders	Ross 308	40	CVI988+HVT	*In ovo*	7 days	Acute
876/18	Emilia-Romagna	2018	Broiler breeders	Ross 308	31	CVI988+HVT	*In ovo*	7 days	Acute
921/18	Abruzzo	2018	Broiler breeders	Ross 308	27	CVI988+HVT	*In ovo*	1 day	Acute
1083/18	Tuscany	2018	Cockerels	Hy-Line	10	CVI988+HVT	1 day	-	Acute

† Not available
‡ Not performed

Table 2. Details of the GaHV-2 strains, retrieved from GenBank, which were used for the phylogenetic analysis.

GAHV-2 strain	Country	Year	Pathotype	Size of Meq (aa)	PPPPs (N°)	GenBank Accession N°	Reference
CVI988	Netherlands	1969	att	398	7	DQ530348	Spatz et al., 2007
814	China	1986	att	398	7	JF742597	Zhang et al., 2012
3004	Russia	NA[†]	att	398	7	EU032468	NA
CU-2	USA	1970s	m	398	7	AY362708	Shamblin et al., 2004
MD70/13	Hungary	1970	v	339	5	MF431495	Trimpert et al., 2017
571	USA	1989	v	339	3	AY362710	Shamblin et al., 2004
617A	USA	1993	v	339	4	AY362712	Shamblin et al., 2004
MPF57	Australia	1994	v	398	5	EF523774	Renz et al., 2012
04CRE	Australia	2004	v	398	5	EF523773	Renz et al., 2012
573	USA	NA	v	339	4	AY362711	Shamblin et al., 2004
567	USA	NA	v	339	4	AY362709	Shamblin et al., 2004
637	USA	NA	v	339	4	AY362713	Shamblin et al., 2004
BC-1	USA	NA	v	398	7	AY362707	Shamblin et al., 2004
JM	USA	NA	v	398	7	AY243331	Shamblin et al., 2004
JM/102W	USA	NA	v	399	7	DQ534539	Spatz & Silva, 2007
Md5	USA	1977	vv	339	4	AF243438	Tulman et al., 2000
549	USA	1987	vv	339	2	AY362714	Shamblin et al., 2004
595	USA	1991	vv	339	2	AY362715	Shamblin et al., 2004
C12/130	UK	1992	vv	339	5	FJ436096	Spatz et al., 2011
Woodlands1	Australia	1992	vv	399	5	EF523775	Renz et al., 2012
643P	USA	1994	vv	339	2	AY362716	Shamblin et al., 2004
02LAR	Australia	2002	vv	398	5	EF523772	Renz et al., 2012
FT158	Australia	2002	vv	398	5	EF523771	Renz et al., 2012
RB1B	USA	NA	vv	339	5	AY243332	Shamblin et al., 2004
648A	USA	1994	vv+	339	2	AY362725	Shamblin et al., 2004
New	USA	1999	vv+	339	2	AY362719	Shamblin et al., 2004

W	USA	1999	vv+	339	4	AY362723	Shamblin et al., 2004
ATE2539	Hungary	2000	vv+	339	5	MF431493	Trimpert et al., 2017
660-A	USA	NA	vv+	339	2	AY362726	Shamblin et al., 2004
686	USA	NA	vv+	339	2	AY362727	Shamblin et al., 2004
L	USA	NA	vv+	339	2	AY362717	Shamblin et al., 2004
N	USA	NA	vv+	339	2	AY362718	Shamblin et al., 2004
RL	USA	NA	vv+	339	2	AY362720	Shamblin et al., 2004
TK	USA	NA	vv+	339	2	AY362721	Shamblin et al., 2004
U	USA	NA	vv+	339	2	AY362722	Shamblin et al., 2004
X	USA	NA	vv+	339	2	AY362724	Shamblin et al., 2004
EU-1	Italy	1992	NA	339	5	MF431494	Trimpert et al., 2017
0093	China	2002	NA	339	3	AF493550	NA
0095	China	2002	NA	339	3	AF493552	NA
0297	China	2002	NA	339	3	AF493553	NA
0304	China	2002	NA	339	2	AF493554	NA
G2	China	2002	NA	339	4	AF493556	NA
YLO40920	China	2005	NA	339	3	DQ174459	Teng et al., 2011
GXY2	China	2007	NA	339	3	EF546430	Teng et al., 2011
GX070060	China	2008	NA	339	3	EU427303	Teng et al., 2011
GX070079	China	2008	NA	339	3	EU427304	Teng et al., 2011
Polen5	Poland	2010	NA	339	4	MF431496	Trimpert et al., 2017
tn-n1	India	2010	NA	339	5	HM749324	NA
tn-n2	India	2010	NA	339	4	HM749325	NA
UDEACO-04/13	Colombia	2013	NA	339	2	KU058701	López-Osorio et al., 2017
UDEACO-06/13	Colombia	2013	NA	339	2	KU058696	López-Osorio et al., 2017
UDEACO-07/13	Colombia	2013	NA	339	3	KU058697	López-Osorio et al., 2017
bd2	USA	2015	NA	339	2	KU173119	Trimpert et al., 2017
bf1	USA	2015	NA	339	2	KU173117	Trimpert et al., 2017
bf2	USA	2015	NA	339	2	KU173118	Trimpert et al., 2017
sd1	USA	2015	NA	339	2	KU173116	Trimpert et al., 2017
sd2	USA	2015	NA	339	2	KU173115	Trimpert et al., 2017

GaHV-2/Italy/Ck/487/15	Italy	2015	NA	339	5	MK139660	Mescolini et al., 2019
GaHV-2/Italy/Ck/507/15	Italy	2015	NA	418	9	MK139661	Mescolini et al., 2019
GaHV-2/Italy/Ck/509/15	Italy	2015	NA	418	9	MK139662	Mescolini et al., 2019
GaHV-2/Italy/Ck/510/15	Italy	2015	NA	418	9	MK139663	Mescolini et al., 2019
GaHV-2/Italy/Ck/562/15	Italy	2015	NA	418	9	MK139664	Mescolini et al., 2019
GaHV-2/Italy/Ck/599/16	Italy	2016	NA	418	9	MK139665	Mescolini et al., 2019
GaHV-2/Italy/Ck/625/16	Italy	2016	NA	339	4	MK139666	Mescolini et al., 2019
GaHV-2/Italy/Ck/674/16	Italy	2016	NA	339	4	MK139667	Mescolini et al., 2019
GaHV-2/Italy/Ck/689/16	Italy	2016	NA	339	4	MK139668	Mescolini et al., 2019
GaHV-2/Italy/Ck/722/16	Italy	2016	NA	339	4	MK139669	Mescolini et al., 2019
GaHV-2/Italy/Ck/801/17	Italy	2017	NA	339	4	MK139670	Mescolini et al., 2019
GaHV-2/Italy/Ck/810/17	Italy	2017	NA	339	4	MK139671	Mescolini et al., 2019
GaHV-2/Italy/Ck/847/17	Italy	2017	NA	418	10	MK139672	Mescolini et al., 2019
GaHV-2/Italy/Ck/848/17	Italy	2017	NA	418	9	MK139673	Mescolini et al., 2019
GaHV-2/Italy/Ck/850/17	Italy	2017	NA	339	5	MK139674	Mescolini et al., 2019
GaHV-2/Italy/Ck/852/17	Italy	2017	NA	339	4	MK139675	Mescolini et al., 2019
GaHV-2/Italy/Ck/853/17	Italy	2017	NA	339	4	MK139676	Mescolini et al., 2019
GaHV-2/Italy/Ck/854/17	Italy	2017	NA	339	4	MK139677	Mescolini et al., 2019
GaHV-2/Italy/Ck/855/17	Italy	2017	NA	298	2	MK139678	Mescolini et al., 2019

† Not available

Table 3. Molecular characteristics of the deduced Meq protein, compared to prototype strains. Amino acid substitutions interrupting PPPPs are underlined. Distinctive aa substitutions of Italian GaHV-2 strains are framed.

Strain	Meq length (aa)	PPPPs (n°)	Amino acid substitutions														
			71	77	80	110	119	153	176	180	216†	217	218	277	283	320	326
CVI988	398	7	S	E	D	C	C	P	P	T	P	P	P	L	A	I	I
CU-2	398	7	S	E	D	C	C	P	P	T	P	P	P	L	A	I	T
JM/102W	399	7	A	E	D	C	C	P	P	T	S	P	P	L	A	I	T
Md5	339	4	A	K	D	C	C	P	P	T	P	A	P	L	V	T	T
648A	339	2	A	K	D	C	R	Q	A	A	P	A	P	P	A	I	T
GaHV-2/Italy/Ck/456/15	339	4	A	E	Y	S	C	P	P	T	P	P	S	L	A	I	T
GaHV-2/Italy/Ck/498/15	339	4	A	E	Y	S	C	P	P	T	P	P	S	L	A	I	T
GaHV-2/Italy/Ck/513/15	339	4	A	E	Y	S	C	P	P	T	P	P	S	L	A	I	T
GaHV-2/Italy/Ck/515/15	339	4	A	E	Y	S	C	P	P	T	P	P	S	L	A	I	T
GaHV-2/Italy/Ck/559/15	339	4	A	E	Y	S	C	P	P	T	P	P	S	L	A	I	T
GaHV-2/Italy/Ck/561/15	339	4	A	E	Y	S	C	P	P	T	P	P	S	L	A	I	T
GaHV-2/Italy/Ck/565/15	339	4	A	E	Y	S	C	P	P	T	P	P	S	L	A	I	T
GaHV-2/Italy/Ck/567/15	339	4	A	E	Y	S	C	P	P	T	P	P	S	L	A	I	T
GaHV-2/Italy/Ck/757/17	339	4	A	E	Y	S	C	P	P	T	P	P	S	L	A	I	T
GaHV-2/Italy/Ck/875/18	339	4	A	E	Y	S	C	P	P	T	P	P	S	L	A	I	T
GaHV-2/Italy/Ck/876/18	339	4	A	E	Y	S	C	P	P	T	P	P	S	L	A	I	T
GaHV-2/Italy/Ck/921/18	339	4	A	E	Y	S	C	P	P	T	P	P	S	L	A	I	T
GaHV-2/Italy/Ck/1083/18	339	4	A	E	Y	S	C	P	P	T	P	P	S	L	A	I	T

† Amino acid position according to the 339 aa-long Meq isoform

405 **Figure legend**

406 **Figure 1.** Phylogenetic tree based on *meq* gene complete amino acid sequences of the 13 GaHV-2
407 strains detected in Italian commercial flocks (marked with a black dot, ●) and of the 76 strains
408 retrieved from GenBank.