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Abstract

Fifty-seven *Gallid alphaherpesvirus* 2 (GaHV-2) isolates, collected during a 30-year period (1990–2019) from commercial poultry flocks affected by Marek's disease (MD), were molecularly characterised. The GaHV-2 *meq* gene was amplified and sequenced to evaluate the virus virulence, based on the number of PPPPs within the proline-rich repeats (PRRs) of its transactivation domain. The present illustration of virus virulence evaluation on a large scale of field virus isolates by molecular analysis exemplifies the practical benefit and usefulness of the molecular marker in commercial GaVH-2 isolates. The alternative assay of GaVH-2 virulence pathotyping is the classical Gold Standard ADOL method, which is difficult and impossible to employ on a large scale using the Specific Pathogen Free (SPF) chicks of the ADOL strains kept in isolators for two months. The phylogenetic analysis performed in the present study showed that the *meq* gene amino acid sequences of the 57 Israeli strains divide into 16 phylogenetic branches. The virulence evaluation was performed in comparison with 36 GaHV-2 prototype strains, previously characterised by the in vivo Gold Standard ADOL assay. The results obtained revealed that the GaHV-2 strains circulating in Israel have evolved into a higher virulence potential during the years, as the four-proline stretches number in the *meq* gene decreased over the investigated period, typically of very virulent virus prototypes. The present study supports the *meq* gene molecular markers for the assessment of field GaVH-2 strains virulence.

Keywords

Marek's disease · *Gallid alphaherpesvirus* 2 · *meq* gene · Molecular characterisation · Phylogenetic analysis · Virulence evaluation.

Introduction

Marek's disease (MD) is a highly contagious and potentially lethal viral disease that affects the poultry industry worldwide, while causing an economically important lymphoproliferative disease. The infection leads to clinical disease, expressed mainly with visceral lymphomas, increased mortality and reduced growth, as well as sub-clinical immunosuppression, causing the exacerbation of other diseases and decreasing vaccinal immunity (Gimeno, 2014; Gimeno et al., 2018; Schat & Nair, 2013). The virus belongs to the genus *Mardivirus*, subfamily *Alphaherpesvirinae*. The current nomenclature describes 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) (Gatherer et al., 2021). Vaccination protects from MD in commercial poultry, but failure due to increased virulence and greater fitness of GaHV-2 isolates is emerging worldwide (Nair, 2018; Trimpert et al., 2017). The virulence of GaHV-2 isolates has increased

over the years from mild (m) to virulent (v), very virulent (vv) and very virulent plus (vv+) (Witter, 1997; Witter et al., 2005). Moreover, in addition to the virulence evolution of the GaHV-2 isolates in commercial poultry, complex factors may also be involved in the occurrence of MD outbreaks in vaccinated chicken flocks, such as the inability of vaccines to protect against MD due to several potential reasons, including the emergence of novel isolates which are immunologically unsuitable to the existing vaccines, non-optimal vaccine application (Davidson et al., 2018a; 2018b) molecular recombination with other viruses in co-infected chickens (Davidson, 2020), co-infection with immunosuppressive viruses (Schat & van Santen, 2013) as well as non-optimal management conditions. The intrinsic virulence of GaHV-2 isolates is mediated by a number of viral genes. The most important is the *meq* gene which was the first discovered GaHV-2 oncogene. The *meq* gene contains a transactivation N-terminal basic-leucine zipper (bZIP) domain and a C-terminal proline-rich transrepression domain (Liu et al., 1999; Qian et al., 1995; Ross, 1999). The *meq* oncogene encodes a 339 amino acid unspliced open reading frame in vv and vv+ GaHV-2 pathotypes and a larger form of 398 amino acids in low virulence strains. The *meq* protein C-terminal comprises a pattern of genetic polymorphism (Shamblin et al., 2004). Unlike the relatively low evolutionary rates of the entire GaHV-2 genome, which is typical for dsDNA viruses (Duffy et al., 2008; Firth et al., 2010), the *meq* gene sequence evolves much faster, resembling the evolutionary rate of RNA viruses (Padhi et al., 2016). In particular, the GaHV-2 *meq* gene evolves at a rate of 1.02×10^{-4} substitutions per site per year, as compared to the range of 10^{-7} to 10^{-5} that characterise other DNA viruses. Trimpert et al. (2017) also calculated that GaHV-2 had a mean evolutionary rate of 1.58×10^{-5} substitutions per site per year. As the *meq* gene harbours the highest number of point mutations over time, Shamblin et al. (Shamblin et al., 2004) and Renz et al. (2012) evidenced that the number of the four-proline stretches (PPPP) in the *meq* gene transactivation domain indicates the pathogenicity of GaHV-2 strains; in the most virulent isolates, the lowest number of PPPP repeats was detected, whereas the attenuated and low pathogenicity isolates showed a highest number of repeats. The determination of GaHV-2 virulence by molecular sequencing was demonstrated as a helpful tool to indicate GaHV-2 isolates' pathogenicity. That molecular assay is much simple and attainable mean of virulence evaluation than the previously established Gold Standard in vivo pathotyping assays, established by Witter et al. (2005). The in vivo examination employed complex experimental infection trials using specific genetic lines of chickens which were grown as Specific Pathogen Free (SPF) over many generations. The chicks have to be kept in strict biosecurity management conditions in isolator units and the GaVH-2 virulence examinations require an experimental system of many and big groups of chicks, for which suitable infrastructures are not available in many laboratories. To enable the performance of GaVH-2 virulence evaluation examinations in more laboratories, Dudnikova et al. (2007) described a simplified method, but it still remains unattainable for most laboratories worldwide. Following the studies of Shamblin et al. [16] and

Renz et al. [20], the pathogenicity evaluation of GaHV-2 strains by molecular analysis of the *meq* gene and its number of PPPPs repeats was explored to verify whether it could serve as a molecular marker of GaHV-2 virulence. Dunn et al. (2019) also confirmed that the *meq* gene is associated with virulence, but suggested that this gene is not the only predictor of virulence. The *meq* gene polymorphism was further employed to create epidemiological molecular linkages between various GaHV-2 strains, according to the numerous studies that have been published from various countries as previously detailed (Mescolini et al., 2019; 2020a; 2020b). GaHV-2 isolates have been identified and described in Israel since 1988 (Davidson et al., 1988; 1999; 2007). Despite the request to determine their virulence, the practical possibilities that were available to evaluate their virulence were limited, therefore, until the present study, the virulence of the Israeli GaVH-2 isolates was not evaluated. The emergence of *meq* gene molecular configuration regarding the number of four-proline repeats as a marker for virulence offered now a possibility to challenge that mission. Therefore, in the present study, we aimed to molecularly classify GaHV-2 isolates from a 30-year collection of Israeli commercial poultry flocks clinically affected with MD and to evaluate the virulence of these GaHV-2 field isolates. To reflect the findings on a solid ground, about 36 previously characterised international GaHV-2 prototype strains were included in the study for reference and comparison.

Materials and methods

Commercial flocks and sampling

Fifty-seven commercial Israeli poultry flocks from 1990 to 2019, which were affected with clinical MD, were sampled and included in the study. Details of the examined flocks are reported in Table 1. Since the collection of samples was dated 20–30 years ago, there are missing details in Table 1, such as the age at sampling of flocks 1–18 and the type of flocks 1–13. However, the source of DNA was blood buffy coat enriched for peripheral blood lymphocytes. These flocks were diagnosed with visceral tumours; therefore, samples were submitted to the laboratory of Avian Diseases at KVI, Israel, for molecular diagnosis of GaHV-2. The flocks included various types of flocks of various ages, including layers, broilers, broiler breeders, turkeys and even zoo chickens. The flocks originated in different Israeli locations and various epidemiological linkages may have occurred between them. The sampling included the visceral tumour tissues of liver and spleen, but also feather tips from affected birds and buffy coat cells, as detailed in Table 1. The samples were kept frozen at −70 ℃ until analysis.

Genomic DNA extraction

The clinical samples were thawed and applied by smearing on FTA® Cards for shipment in dry and stable conditions. For extraction and DNA purification, the commercial kit 'NucleoSpin® Tissue' (MACHEREY-NAGEL

GmbH & Co. KG, Düren, Germany) was used following the manufac- turer's instructions outlined below. From each smear, 8–10 pieces of about 5 mm2 of area were excised and placed in a 1.5 ml Eppendorf tube containing 180 µl buffer T1. The tube was vortexed and heated at 94 ℃ for 10 min on a heating block. After cooling down, 25 µl of Proteinase k solution was added, vortexed and incubated for 1 h at 56 ℃, followed by the addition of 200 µl of buffer B3, vortexed and incubated at 56 ℃ for 10 min. The DNA was precipitated by the addition of 210 µl of ethanol 96–100% and vortexed. Each sample was applied to a one column NucleoSpin® Tissue Column, centrifuged for 1 min at 11,000 × g and the flow-through discarded. The membrane was washed twice with 500 µl and 600 µl of buffer BW and centrifuged for 1 min at 11,000 × g and dried again by centrifugation. DNA elution was performed using 100 µl of buffer BE and incubation for 1 min at room temperature before final centrifugation for 1 min at 11,000 × g. The DNA was kept at −20 ℃ until use.

Amplification and sequencing of the *meq* gene

The entire *meq* gene was amplified as previously described (Mescolini et al., 2019) using two primers external to the *meq* gene, *EcoR*-Q for and *EcoR*-Q rev. The negative samples were subsequently subjected to a second round of PCR amplification using two heminested PCR protocols including external primers from the first PCR protocol (hereinafter referred to as PCR1) and internal primers *meq*-R (5'-GGG TCT CCC GTC ACC TGG-3') and *meq*-F (5'-ATG TCT CAG GAG CCA GAG CCG-3') (Hassanin et al., 2013). In the first heminested PCR protocol (PCRhe1), the primer pair EcoF–MeqR was used generating a 1363 bp-long amplicon and in the second heminested PCR protocol (PCRhe2), the primer pair MeqF and EcoR was used generating a 1276 bp-long amplicon. In a total reaction volume of 25 µl, 8 µl of eluted template DNA for PCR1 (reduced to 3 µl for PCRhe1 and for PCRhe2) was mixed with 0.125 µl of GoTaq G2 Flexi DNA Polymerase (Promega, Madison, WI), 5 µl of 5X Colourless GoTaq Flexi Buffer, 1.75 µl of MgCl2 25 mM solution, 0.5 µl of dNTPs, 1 µl of each primer and 8 µl of H2O for molecular biology (13 µl for PCRhe1 and for PCRhe2). PCR1 cycling conditions were as previously described (Mescolini et al., 2019); PCRhe1 and 2 cycling conditions were as follows: 2 min of denaturation at 95 °C followed by 25 cycles, each consisting of denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min (PCRhe1) or at 65 °C for 1 min 10 s, and extension at 72 °C for 1 min 30 s. 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 visualised under ultraviolet light after an electrophoretic run at 80 V and 400 mA for 50 min. The *meq* amplicons products obtained were purified using ExoSAP-IT™ Express PCR Product Cleanup (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's instructions and sequenced in both directions using PCR1 primers EcoR-Q for and EcoR-Q rev coupled with PCRhe2 MeqF primer by a commercial sequencing service (Macrogen Europe, Amsterdam, The Netherlands). Some

sequences obtained using the EcoR-Q for primer were not satisfactory; therefore, a further sequencing was carried out for these samples using a new primer specially designed on the already obtained sequences of the Israeli strains, which was called MeqF1 (5'-TGA CAG GTG AAT TGT GAC CGT T-3'). The obtained sequences were named using the following nomenclature: GaHV-2/Israel/Host species (Turkey: Ty, Chicken: Ck)/ID number/year of detection.

Sequence and phylogenetic analysis

The obtained nucleotide (nt) sequences were edited using BioEdit Sequence Alignment Editor, Version 7.0.5.3 (Tom Hall, Ibis Therapeutics, Carlsbad, California, USA). Nucleotide similarities between the Israeli sequences and *meq* gene sequences, available in the NCBI database and retrieved from GenBank (Table 2), were investigated through the Basic Local Alignment Search Tool (BLAST) and the Clustal W software (Thompson et al., 1994). Deduced amino acid (aa) sequences were analysed focus- ing 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 the maximum likelihood method under the Jones–Taylor–Thornton model in MEGA X (Kumar et al., 2018). Nodal supports were estimated with 1000 bootstrap replicates and considered significant when equal to or greater than 70. Table 3 details the reference GaVH-2 strains that were used for molecular alignment and comparison with the field isolates analysed. The characterised molecular configuration of the *meq* gene, country, and year of isolation and publication reference are detailed.

Accession numbers

The nucleotide sequences of the *meq* genes obtained in this study were submitted to GenBank and are available under accession numbers OQ926484 through OQ926538.

Results

Phylogenetic analysis of the GaVH-2 *meq* gene of commercial isolates and reference strains

Fifty-seven out of 58 FTA samples tested GaHV-2 positive, either directly when subjected to PCR1 (35 samples out of 57) or after PCRhe1 and PCRhe2 were carried out (the remaining 22 samples). All the Israeli isolates collected (Table 1) showed a *meq* protein of 339 aa apart from one strain which showed a 338aa-long *meq* (Table 4). Some strains had genetically identical sequences (100% nucleo- tide identity between each other); therefore, to facilitate the description of the results, the strains were divided into 16 homogeneous groups where the members within each group had an identical nt sequence (Table 2). Within each of these groups, a sample representative of the entire group was

selected to perform the amino acid sequence analysis and phylogenetic analysis.

Figure 1 shows the phylogenetic tree of the *meq* gene of representative isolates aligned together with the *meq* gene of reference strains retrieved from the GenBank (Table 3). The phylogenetic tree illustrates the division of these isolates into 16 branches (from A to R). In each branch, the Israeli GaVH-2 isolates were intercalated with the reference strains. Table 2 summarises the phylogenetic distribution of the Israeli GaVH-2 commercial isolates by grouping the identical sequences and assigning a representative isolate for each phylogenetic tree branch. The phylogenetic groups A–M contain isolates from ascending years (1990–2017) except groups P, Q and R which intercalated into groups E and F. In general, the phylogenetic analysis indicated that the GaVH-2 Israeli isolates display a trend of genetic diversification which might reflect the virulence of these viruses and the severity of disease. As the GaVH-2 isolates were obtained from commercial poultry flocks over a long period of time, and from many sources, the epidemiological data, linkages between the various flocks, status of vaccination and severity of disease, were not available. However, almost all these isolates were collected from tumour-bearing flocks with considerable morbidity and mortality, otherwise they would not be submitted for diagnosis at the Division of Avian Diseases by veterinarians, poultry flock owners or sampled for research reasons by us.

Number of PPPPs in the *meq* gene protein of the Israeli isolates

Table 3 shows the number of PPPPs contained in the *meq* gene protein of each phylogenetic group of Israeli GaHV-2 isolates, ranging from two to five repeats. The molecular features and the virulence of these isolates were evaluated in the light of the reference strains for which the virulence was experimentally determined in previous studies and in the light of the most recent molecularly characterised but unpathotyped GaHV-2 strains (Table 3). In Table 3, several strains of each virulence degree were included: 4 attenuated and mildly virulent strains, 11 virulent strains, 9 very virulent strains and 12 very virulent plus strains. The number of PPPPs in each group varied; therefore, we calculated the mean PPPPs' repeat average. The reference strain group (n = 4) with attenuated and mild pathogenicity had 7 PPPPs repeats, the group of virulent reference strains (n = 11) had an average of 5 PPPPs repeats (standard deviation of 1.41), the group of very virulent reference strains (n = 9) had an average of 3.89 PPPPs repeats (standard deviation of 1.45) and the group of very virulent plus reference strains (n = 12) had an average of 2.66 PPPPs repeats (standard deviation of 1.23). The *meq* protein sequence analysis of the Israeli isolates (Tables 2 and 4) illustrates that the phylogenetic groups A–D (with the exception of group B) had 5 PPPPs repeats and the repeat number subsequently decreased. The decrease in the PPPPs number indicates the occurrence of a gradual increase in virulence of the Israeli field isolates of GaVH-2 until the years 2000, when the isolates had *meq* proteins with only 2–3 PPPPs repeats, typically found in very virulent plus reference strains. Accordingly, it can be assumed that the

virulence potential of the Israeli GaVH-2 isolates from commercial poultry flocks is continuously evolving towards greater virulence. This trend reflects the processes which are documented worldwide, probably as a result of continued selection pressure due to vaccination and other factors, as described previously in North America and Eurasia (Trimpert et al., 2017). Our findings are in accordance with previous studies that reported the geographically restricted evolution of field GaHV-2 strains in Italy (Mescolini et al., 2019; 2020a) China (Yu et al., 2013), India (Suresh et al., 2017), Egypt (Hassanin et al., 2013; Abdallah et al., 2018), Poland (Woźniakowski & Samorek-Salamonowicz, 2014), Japan (Abd-Ellatieff et al., 2018), Iran (Ghalyanchilangeroudi et al., 2022) and others. 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.

Discussion

The present study reports for the first time the attempt to evaluate the virulence of 57 GaVH-2 isolated in commercial chicken flocks in Israel according to the number of PPPPs repeats in their *meq* gene. The study also further solidifies the validity of employing the *meq* gene molecular marker for the assessment of virulence in field GaVH-2 isolates. This approach demonstrated previously its accuracy on reference prototype strains for which in vivo experimental evaluation of virulence were performed according to the Gold Standard ADOL method described by Witter et al. (2005). As in vivo pathotyping is difficult to perform and raises ethical issues, the *meq* gene molecular characterisation is a valuable tool for assessing strains' virulence. In particular, the number of PPPPs repeats in the transactivation domain of the Meq protein can be accepted for use as a valid molecular marker for the GaVH-2 virulence. However, the molecular findings should be supported by clinical observations, necropsy findings and vaccination status. The flocks sampled over 30 years from commercial farms represent the viruses that circulated in poultry and eventually infected also a zoo chicken group. Because the infection caused considerable and unusual morbidity and mortality in those flocks that were significantly higher than normal, those flocks were submitted by the field veterinarians for our laboratory diagnosis. The clinical conditions these flocks included visible tumours mainly in visceral organs, which were confirmed also histopathologically, enhanced mortality, immunodepression as well as poor efficacy of other vaccines. As those flocks were commercial, the information was kept confidential. However, the fact that the samples were collected from commercial flocks emphasises the diagnostic value of the findings, unlike prototype virus strains which served to reproduce the disease under biosecurity conditions in SPF chicks, as detailed in Table 3. In addition, it is notable that in commercial poultry industry, there are multifactorial components that do not exist in

experimental disease reproduction by prototype viruses in SPF chicks grown in isolators. Because of that complexity, it was important to confront the molecular assay, to show its efficacy for virulence evaluation in native conditions. In addition, it is important to mention that the sampling was performed quite a while ago, and then the original purpose for submitting those samples was only for diagnosis.

Declarations

Competing interests

The authors declare no competing interests.

Ethical statement

The study reported in this paper was non-interventional and only used existing samples that were submitted for routine diagnostic purposes.

Table 1. Details of the Israeli commercial flocks examined.

Identification number	Year of isolation	Type of flock	Age (weeks)	Source of DNA	Sequence number
1	1990	Not known	Not known	Buffy coat	1439/20
2	1990	Not known	Not known	Buffy coat	1440/20
3	1990	Not known	Not known	Buffy coat	1441/20
4	1990	Not known	Not known	Buffy coat	1442/20
5	1990	Not known	Not known	Buffy coat	1443/20
6	1991	Not known	Not known	Buffy coat	1444/20
7	1991	Not known	Not known	Buffy coat	1445/20
8	1991	Not known	Not known	Buffy coat	1446/20
9	1991	Not known	Not known	Buffy coat	1447/20
10	1992	Not known	Not known	Buffy coat	1448/20
11	1992	Not known	Not known	Buffy coat	1449/20
12	1992	Not known	Not known	Buffy coat	1450/20
13	1992	Not known	Not known	Buffy coat	1451/20
14	1995	Turkey	Not known	Buffy coat	1452/20
15	1996	Turkey	Not known	Buffy coat	1453/20
16	1996	Turkey	Not known	Buffy coat	1454/20
17	1999	Broilers	Not known	Buffy coat	1455/20
18	2009	Layers	28	Liver + spleen	1456/20
19	2009	Layers	25	Liver + spleen	1457/20
20	2009	Layers	28	Liver + spleen	1458/20
21	2009	Layers	24	Liver + spleen	1459/20
22	2009	Layers	24	Liver + spleen	1460/20
23	2009	Layers	25	Liver + spleen	1461/20
24	2009	Layers	21	Liver + spleen	1462/20
25	2009	Layers	35	Liver + spleen	1467/20
26	2009	Layers	24	Liver + spleen	1468/20
27	2011	Layers	41	Liver + spleen	1469/20
28	2011	Layers	29	Liver + spleen	1470/20
29	2016	Layers	26	Liver + spleen	1471/20
30	2016	Layers	31	Liver + spleen	1472/20
31	2016	Layers	26	Liver + spleen	1473/20
32	2016	Broiler breeder	14	Liver + spleen	1474/20
33	2016	Layers	31	Liver + spleen	1475/20
34 34	2017	Broiler breeder	2	Liver + spleen Feathers	1476/20 1488/20
35 35	2017	Layers	27	Liver + spleen Feathers	1477/20 1489/20
36 36	2017	Broiler breeder	19	Liver + spleen Feathers	1478/20 1490/20
37	2018	Zoo	Not known	Liver + spleen	1479/20
38	2018	Not known	Not known	Liver + spleen	1480/20
39	2018	Not known	Not known	Feathers	1481/20
40	2019	Layers	32	Liver + spleen	1482/20
41	2019	Layers	29	Liver + spleen	1483/20
42	2019	Layers	41	Liver + spleen	1484/20
43	2019	Layers	27	Liver + spleen	1485/20
44	2019	Layers	46	Liver + spleen	1486/20

45	2019	Layers	36	Liver + spleen	1487/20
46	1991	Not known	Not known	Liver + spleen	1491/20
47	1991	Not known	Not known	Liver + spleen	1492/20
48	1998	Not known	Not known	Liver + spleen	1493/20
49	2000	Not known	Not known	Liver + spleen	1494/20
50	2000	Not known	Not known	Liver + spleen	1495/20
51	1999	Not known	Not known	Liver + spleen	1496/20
52	1999	Not known	Not known	Liver + spleen	1497/20
53	1999	Not known	Not known	Liver + spleen	1498/20
54	2001	Not known	Not known	Liver + spleen	1499/20
55	2000	Not known	Not known	Liver + spleen	1500/20
56	2003	Not known	Not known	Liver + spleen	1501/20
57	2000	Not known	Not known	Liver + spleen	1502/20
58	2000	Not known	Not known	Liver + spleen	1503/20

Table 2. Grouping of the examined GaHV-2 isolates according to their nucleotide sequences.

Phylogenetic Group	No. of PPPPs	Selected Isolate	Isolates with identical sequences
A	5	GaHV-2/Israel/Unknown/1439-20/1990 (1)	GaHV-2/Israel/ Unknown/1439-20/1990 (1) GaHV-2/Israel/ Unknown/1445-20/1991 (7) GaHV-2/Israel/ Unknown/1446-20/1991 (8) GaHV-2/Israel/ Unknown/1447-20/1991 (9) GaHV-2/Israel/ Unknown/1448-20/1992 (10) GaHV-2/Israel/Turkey/1452-20/1995 (14) GaHV-2/Israel/ Unknown/1491-20/1991 (46) GaHV-2/Israel/ Unknown/1492-20/1991 (47) GaHV-2/Israel/ Unknown/1494-20/2000 (49) GaHV-2/Israel/ Unknown/1497-20/1999 (52) GaHV-2/Israel/ Unknown/1498-20/1999 (53) GaHV-2/Israel/ Unknown/1503-20/2000 (58)
B	4	GaHV-2/Israel/ Unknown/1441-20/1990 (3)	GaHV-2/Israel/ Unknown/1440-20/1990 (2) GaHV-2/Israel/ Unknown/1441-20/1990 (3)
C	5	GaHV-2/Israel/ Unknown/1442-20/1990 (4)	GaHV-2/Israel/ Unknown/1442-20/1990 (4)
D	5	GaHV-2/Israel/ Unknown/1443-20/1990 (5)	GaHV-2/Israel/ Unknown/1443-20/1990 (5)
E	3	GaHV-2/Israel/ Unknown/1444-20/1991 (6)	GaHV-2/Israel/ Unknown/1444-20/1991 (6) GaHV-2/Israel/Turkey/1454-20/1996 (16)
F	3	GaHV-2/Israel/ Unknown/1449-20/1992 (11)	GaHV-2/Israel/ Unknown/1449-20/1992 (11) GaHV-2/Israel/ Unknown/1450-20/1992 (12) GaHV-2/Israel/ Unknown/1451-20/1992 (13) GaHV-2/Israel/ Unknown/1495-20/2000 (50) GaHV-2/Israel/ Unknown/1500-20/2000 (55)
G	5	GaHV-2/Israel/Turkey/1453-20/1996 (15)	GaHV-2/Israel/Turkey/1453-20/1996 (15)
H	3	GaHV-2/Israel/Ck/1456-20/2009 (18)	GaHV-2/Israel/Ck/1456-20/2009 (18) GaHV-2/Israel/ Ck/1457-20/2009 (19) GaHV-2/Israel/ Ck/1458-20/2009 (20) GaHV-2/Israel/ Ck/1459-20/2009 (21) GaHV-2/Israel/ Ck/1460-20/2009 (22) GaHV-2/Israel/ Ck/1461-20/2009 (23) GaHV-2/Israel/ Ck/1462-20/2009 (24) GaHV-2/Israel/ Ck/1467-20/2009 (25) GaHV-2/Israel/ Ck/1468-20/2009 (26) GaHV-2/Israel/ Ck/1469-20/2011 (27) GaHV-2/Israel/ Ck/1470-20/2011 (28) GaHV-2/Israel/ Ck/1473-20/2016 (31) GaHV-2/Israel/ Ck/1474-20/2016 (32) GaHV-2/Israel/ Ck/1475-20/2016 (33)

			GaHV-2/Israel/ Ck/1478-20/2017 (36) GaHV-2/Israel/ Unknown/1479-20/2018 (37) GaHV-2/Israel/ Unknown/1480-20/2018 (38) GaHV-2/Israel/ Unknown/1481-20/2018 (39) GaHV-2/Israel/ Ck/1487-20/2019 (45) GaHV-2/Israel/ Unknown/1501-20/2003 (56)
I	4	GaHV-2/Israel/Ck/1471-20/2016 (29)	GaHV-2/Israel/Ck/1471-20/2016 (29) GaHV-2/Ck/Israel/1482-20/2019 (40) GaHV-2/Israel/ Ck/1483-20/2019 (41) GaHV-2/Israel/ Ck/1484-20/2019 (42) GaHV-2/Israel/ Ck/1485-20/2019 (43) GaHV-2/Israel/ Ck/1486-20/2019 (44)
L	3	GaHV-2/Israel/Ck/1472-20/2016 (30)	GaHV-2/Israel/Ck/1472-20/2016 (30)
M	4	GaHV-2/Israel/Ck/1488-20/2017 (34)	GaHV-2/Israel/Ck/1488-20/2017 (34)
N*	NA	GaHV-2/Israel/Ck/1477-20/2017 (35)*	GaHV-2/Israel/Ck/1477-20/2017 (35)*
O*	NA	GaHV-2/Israel/Unknown/1493-20/1998 (48)*	GaHV-2/Israel/Unknown/1493-20/1998 (48)*
P	2	GaHV-2/Israel/ Unknown/1496-20/1999 (51)	GaHV-2/Israel/ Unknown/1496-20/1999 (51)
Q	3	GaHV-2/Israel/ Unknown/1499-20/2001 (54)	GaHV-2/Israel/ Unknown/1499-20/2001 (54)
R	3	GaHV-2/Israel/ Unknown/1502-20/2000 (57)	GaHV-2/Israel/ Unknown/1502-20/2000 (57)

NA not available; * sequence not suitable for analysis.

Table 3. Details of the reference GaHV-2 strains included in the phylogenetic analysis.

GaHV-2 strain	Country	Year	Pathotype	Meq gene size (aa)	PPPPs (number)	Accession no.	Reference
CVI988	The Netherland	1969	att[a]	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	1970	m[b]	398	7	AY362708	Shamblin et al., 2004
MD70/13	Hungary	1970	v[c]	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	
MPF57	Australia	1994	v	398	5	EF523774	Renz et al., 2012
04CRE	Australia	2004	v	398	5	EF523773	
573	USA	NA	v	339	4	AY362711	Shamblin et al., 2004
567	USA	NA	v	339	4	AY362709	
637	USA	NA	v	339	4	AY362713	
BC-1	USA	NA	v	398	7	AY362707	
JM	USA	NA	v	398	7	AY243331	
JM/102W	USA	NA	v	399	7	DQ534539	Spatz & Silva, 2007
Md5	USA	1977	vv[d]	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	
C12/130	UK	1992	vv	339	5	FJ436096	Spatz et al., 2007
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	
RB1B	USA	NA	vv	339	5	AY243332	Shamblin et al., 2004
648A	USA	1994	vv+[e]	339	2	AY362725	
New	USA	1999	vv+	339	2	AY362719	
W	USA	1999	vv+	339	4	AY362723	
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	
L	USA	NA	vv+	339	2	AY362717	
N	USA	NA	vv+	339	2	AY362718	
RL	USA	NA	vv+	339	2	AY362720	
TK	USA	NA	vv+	339	2	AY362721	
U	USA	NA	vv+	339	2	AY362722	
X	USA	NA	vv+	339	2	AY362724	
Polen5	Poland	2010	NA	339	4	MF431496	Trimpert et al., 2017
B2015	India	2015	NA	339	5	LC195187	Puro et al., 2018
GaHV-2/Italy/Ck/625/16	Italy	2016	NA	339	4	MK139666	Mescolini et al., 2019
GaHV-2/Italy/Ck/689/16	Italy	2016	NA	339	4	MK139668	
GaHV-2/Italy/Ck/513/15	Italy	2015	NA	339	4	MK855056	
GaHV-2/Italy/Ck/757/17	Italy	2017	NA	339	4	MK855062	
GaHV-2/Italy/Ck/921/18	Italy	2018	NA	339	4	MK855065	
GaHV-2/Italy/Turkey/601/16	Italy	2016	NA	339	4	MN017102	

NA not available

att[a] = attenuated

m[b] = moderate

v[c] = virulent

vv[d] = very virulent

vv+[e] = very virulent plus

Table 4. Meq protein sequence analysis of Israeli isolates.

Phylogenetic group	No. of PPPPs	Selected isolate	Isolates with identical sequences
A	5	GaHV-2/Israel/Unknown/1439-20/1990 (1)	GaHV-2/Israel/ Unknown/1439-20/1990 (1)
			GaHV-2/Israel/Unknown/1445-20/1991 (7)
			GaHV-2/Israel/Unknown/1446-20/1991 (8)
			GaHV-2/Israel/Unknown/1447-20/1991 (9)
			GaHV-2/Israel/Unknown/1448-20/1992 (10)
			GaHV-2/Israel/Turkey/1452-20/1995 (14)
			GaHV-2/Israel/Unknown/1491-20/1991 (46)
			GaHV-2/Israel/Unknown/1492-20/1991 (47)
			GaHV-2/Israel/Unknown/1494-20/2000 (49)
			GaHV-2/Israel/Unknown/1497-20/1999 (52)
			GaHV-2/Israel/Unknown/1498-20/1999 (53)
			GaHV-2/Israel/Unknown/1503-20/2000 (58)
B	4	GaHV-2/Israel/Unknown/1441-20/1990 (3)	GaHV-2/Israel/Unknown/1440-20/1990 (2)
			GaHV-2/Israel/Unknown/1441-20/1990 (3)
C	5	GaHV-2/Israel/Unknown/1442-20/1990 (4)	GaHV-2/Israel/Unknown/1442-20/1990 (4)
D	5	GaHV-2/Israel/Unknown/1443-20/1990 (5)	GaHV-2/Israel/Unknown/1443-20/1990 (5)
E	3	GaHV-2/Israel/Unknown/1444-20/1991 (6)	GaHV-2/Israel/Unknown/1444-20/1991 (6)
			GaHV-2/Israel/Turkey/1454-20/1996 (16)
F	3	GaHV-2/Israel/Unknown/1449-20/1992 (11)	GaHV-2/Israel/Unknown/1449-20/1992 (11)
			GaHV-2/Israel/Unknown/1450-20/1992 (12)
			GaHV-2/Israel/Unknown/1451-20/1992 (13)
			GaHV-2/Israel/Unknown/1495-20/2000 (50)
			GaHV-2/Israel/Unknown/1500-20/2000 (55)
G	5	GaHV-2/Israel/Turkey/1453-20/1996 (15)	GaHV-2/Israel/Turkey/1453-20/1996 (15)
H	3	GaHV-2/Israel/Ck/1456-20/2009 (18)	GaHV-2/Israel/Ck/1456-20/2009 (18)
			GaHV-2/Israel/Ck/1457-20/2009 (19)
			GaHV-2/Israel/Ck/1458-20/2009 (20)
			GaHV-2/Israel/Ck/1459-20/2009 (21)
			GaHV-2/Israel/Ck/1460-20/2009 (22)
			GaHV-2/Israel/Ck/1461-20/2009 (23)

			GaHV-2/Israel/Ck/1462-20/2009 (24)
			GaHV-2/Israel/Ck/1467-20/2009 (25)
			GaHV-2/Israel/Ck/1468-20/2009 (26)
			GaHV-2/Israel/Ck/1469-20/2011 (27)
			GaHV-2/Israel/Ck/1470-20/2011 (28)
			GaHV-2/Israel/Ck/1473-20/2016 (31)
			GaHV-2/Israel/Ck/1474-20/2016 (32)
			GaHV-2/Israel/Ck/1475-20/2016 (33)
			GaHV-2/Israel/Ck/1478-20/2017 (36)
			GaHV-2/Israel/Unknown/1479-20/2018 (37)
			GaHV-2/Israel/Unknown/1480-20/2018 (38)
			GaHV-2/Israel/Unknown/1481-20/2018 (39)
			GaHV-2/Israel/Ck/1487-20/2019 (45)
			GaHV-2/Israel/Unknown/1501-20/2003 (56)
I	4	GaHV-2/Israel/Ck/1471-20/2016 (29)	GaHV-2/Israel/Ck/1471-20/2016 (29)
			GaHV-2/Ck/Israel/1482-20/2019 (40)
			GaHV-2/Israel/Ck/1483-20/2019 (41)
			GaHV-2/Israel/Ck/1484-20/2019 (42)
			GaHV-2/Israel/Ck/1485-20/2019 (43)
			GaHV-2/Israel/Ck/1486-20/2019 (44)
L	3	GaHV-2/Israel/Ck/1472-20/2016 (30)	GaHV-2/Israel/Ck/1472-20/2016 (30)
M	4	GaHV-2/Israel/Ck/1488-20/2017 (34)	GaHV-2/Israel/Ck/1488-20/2017 (34)
N[a]	NA	GaHV-2/Israel/Ck/1477-20/2017 (35)[a]	GaHV-2/Israel/Ck/1477-20/2017 (35)[a]
O[a]	NA	GaHV-2/Israel/Unknown/1493-20/1998 (48) [a]	GaHV-2/Israel/Unknown/1493-20/1998 (48) [a]
P	2	GaHV-2/Israel/Unknown/1496-20/1999 (51)	GaHV-2/Israel/Unknown/1496-20/1999 (51)
Q	3	GaHV-2/Israel/Unknown/1499-20/2001 (54)	GaHV-2/Israel/Unknown/1499-20/2001 (54)
R	3	GaHV-2/Israel/Unknown/1502-20/2000 (57)	GaHV-2/Israel/Unknown/1502-20/2000 (57)

[a] = amino acid not available.

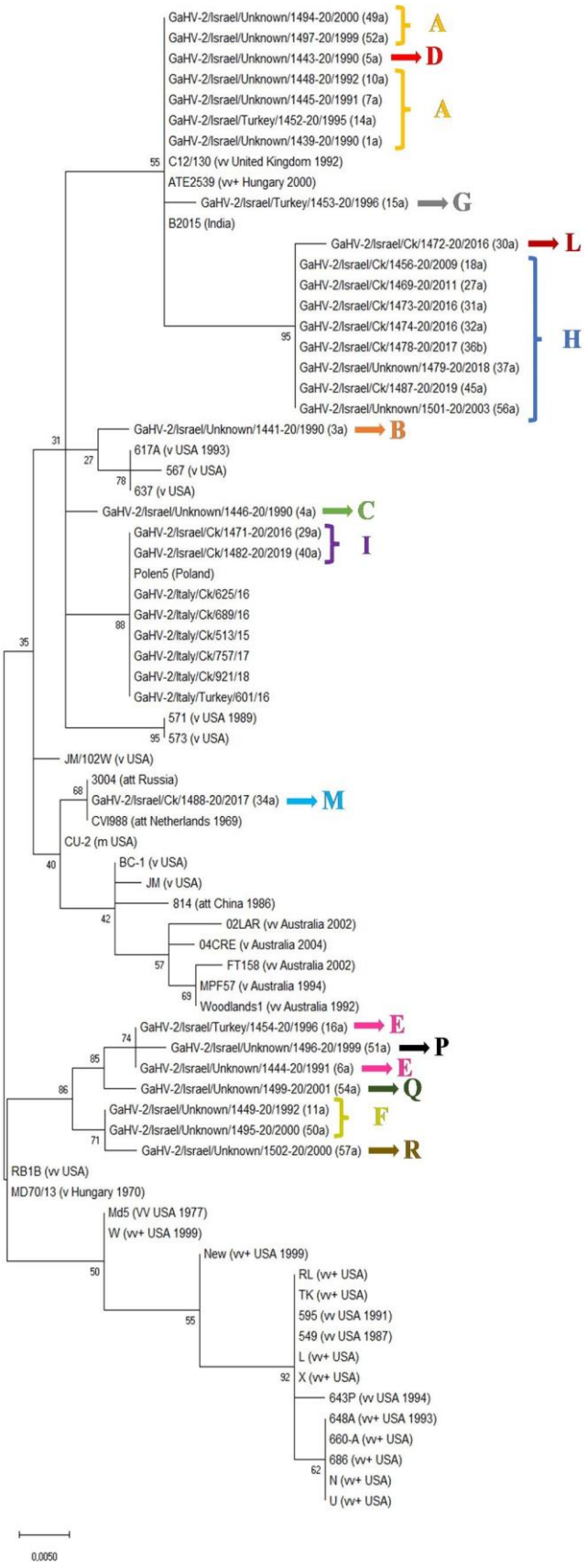

Fig. 1 The phylogenetic tree of the *meq* gene of representative isolates aligned together with the *meq* gene of reference strains retrieved from the GenBank.

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