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Partial genome analysis of murine gammaherpesvirus 4556

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Summary. – Murine gammaherpesvirus 68 (MHV-68) -infected mouse is an animal model of gammaherpesvirus infection in man and domestic animals. Murine gammaherpesvirus 4556 (MHV-4556), isolated from *Apodemus flavicollis* ticks has been considered a close relative of MHV-68 but different in some features of infection *in vitro* and *in vivo*. Previous comparison of MHV-4556 with MHV-68 has revealed their diversity in immune evasion protein MK3. In this study, *Hind*III and *Eco*RI restriction profiles of the MHV-4556 genome disclosed absence of the deletion that has been identified previously at the left end of genomes of murine gammaherpesvirus 76 (MHV-76) and murine gammaherpesvirus Šumava (MHV-Šumava). A 22, 565 bp portion of MHV-4556 genome sequence was sequenced, analyzed and compared with that of MHV-68. Nucleotide sequences of 21 genes of MHV-4556 and deduced amino acid sequences revealed their identity to those of MHV-68 except for differences in 15 nucleotides and 8 amino acids in 5 genes and their proteins, respectively. Due to these differences, immune evasion protein M4 and structural proteins encoded by ORF8 (gB), ORF11 (p43), ORF26 and ORF52, respectively, are predicted to have a reduced hydrophilicity and surface exposure compard with their MHV-68 counterparts. These differences obviously contribute to some different pathogenetical features of these viruses and could explain the weaker immunogenicity of MHV-4556 in comparison with MHV-68.

Keywords: murine gammaherpesvirus 4556; restriction analysis; partial genome sequence

Introduction

In 1980, the isolation of first five murine gammaherpesviruses was reported (Blaškovič *et al.,* 1980). MHV-76 and MHV-78 were isolated from *Apodemus flavicollis*, while MHV-60, MHV-68, and MHV-72 originated from *Myodes glareolus.* About 13 years later, three other murine gammaherpesviruses, namely MHV-4556, MHV-5682 and MHV-Šumava were found in *Apodemus flavicollis* in Slovakia and Bohemia (Kožuch *et al.*, 1993).

Studies of antigenic relationships between some murine gammaherpesviruses isolated from different hosts and areas using a panel of monoclonal antibodies revealed antigenic specificity of MHV-Šumava (Matúšková *et al.,* 2003). Till now, infections of mice with MHV-68, MHV-72, and MHV-76, respectively, have been intensively studied (Sunil-Chandra *et al.,* 1992, 1993, 1994a,b; Mistríková *et al.,* 1994, 1996; Macrae *et al.*, 2001; Rašlová *et al.,* 2000a, 2001). The sequencing of MHV-68 genome confirmed assumptions of a close genetic relationship of MHV-68 to herpesvirus saimiri (SaHV-2), Epstein-Barr virus (EBV) and Kaposi´s sarcoma-associated herpesvirus (KSHV) (Virgin *et al.,* 1997; Efstatiou *et al.*, 1990; Kúdelová and Rajčáni, 2009). In 2000, MHV-68 was classified as Murid herpesvirus 4 (MuHV-4) (synonyms mouse herpesvirus strain 68 and murine gammaherpesvirus 68) into a new species, *Murid herpesvirus 4*, the genus *Rhadinovirus*, the subfamily *Gammaherpesvirinae* (van Regenmortel *et al.,* 2000). Its genome contains unique sequence of 118,237 bp that is flanked by multiple copies of a 1,213 bp terminal repeat (Virgin *et al.,* 1997).

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Abbreviations: BRHV = Brest herpesvirus; EBV = Epstein-Barr virus; GAG = glycosaminoglycan; KSHV = Kaposi´s sarcoma-associated herpesvirus; LPDs = lymphoproliferative diseases; MHV-60, MHV-68, MHV-72, MHV-76, MHV-78, MHV-4556, MHV-5682, and MHV-Šumava = murine gammaherpesviruses 60, 68, 72, 76, 78, 4556, 5682, and Šumava, respectively; RFLP = restriction fragment length analysis; WMHV = wood mouse herpesvirus

MHV-68 belongs to the so far best characterized murine gammaherpesviruses. Following i.n. inoculation, viremia appears due to virus replication in the alveolar epithelium and endothelial cells of alveolar septa. The productive virus growth within lung epithelium ceases at 7 to 10 days p.i. During the viremic phase, mature B cells as well as macrophages become infected. At the same time, the virus spreads from the lungs via haematogenous route to the most of host organs (Sunil-Chandra *et al.,* 1992a; Mistríková *et al.,* 2000). In acute infection, an infectious mononucleosis (IM) -like syndrome develops showing splenomegaly, an increased number of proliferating B cells and atypical mononuclear cells (Blackman *et al.,* 2000; Doherty *et al.,* 2001). Similarly to other gammaherpesviruses, it establishes a long-term latency in B-lymphocytes (spleen and lymph nodes) and macrophages but in lung endothelial cells as well (Sunil-Chandra *et al.*, 1992b; Rajčáni *et al.,* 1985; Stewart *et al.,* 1998). The IM-like syndrome analogous to that induced by EBV is associated with establishment of the virus latency, lymphoproliferative diseases (LPDs) and solid tumors (lymphomas) (Sunil-Chandra *et al.,* 1994a; Mistríková *et al.,* 1996). A huge work was done on MHV-68 that is recently probably the most amenable animal model virus for studying the pathogenesis of LPDs caused by human gammaherpesviruses (Rajčáni and Kúdelová, 2007; Kúdelová and Rajčáni, 2009; Barton *et al.,* 2011). Vertical transmission of MHV-68 and its presence in *Ixodes ricinus* ticks feeding on free-living lizards that implicates the tick as a vector in the spread of MHV-68 within vertebrate hosts in nature was recently confirmed (Štiglicová *et al.,* 2011; Ficová *et al.,* 2011).

The second murine gammaherpesvirus with known complete genome, MHV-76 shows in comparison with MHV-68 a more rapid clearing from the lungs, reduced splenomegaly and absence of tumorigenicity in experimentally infected mice. This difference is attributed to the absence of a 9,538 bp portion at the at the left end of U_L region of genome that encompasses eight viral tRNA-like genes and the M1, M2, M3, and M4 genes unique to MHV-68. Since the rest of the MHV-76 genome is essentially identical to that of MHV-68, it was suggested as a deletion mutant of MHV-68 (Macrae *et al.,* 2001).

More recently, a complete genome sequence of wood mouse herpesvirus (WMHV), isolated from *A. sylvaticus* in UK, was disclosed. The pathogenesis of WMHV in wood mice was found extremely similar to that of MHV-68, except for the absence of inducible bronchus-associated lymphoid tissue at day 14 and a higher load of latently infected cells at day 21 p.i. Sequence analysis confirmed identical genome structure and gene content of WMHV to those of MHV-68, showing an overall 85% sequence identity. These are probably sufficient data to warrant classification of WMHV into a new species, *Murid herpesvirus 7* (Hughes *et al.,* 2010).

To date, partial genome sequence of three gammaherpesviruses is known. These viruses include a novel gammaherpesvirus designated Brest herpesvirus (BRHV), isolated from white-toothed shrew *Crocidura russula* in France, MHV-Šumava and MHV-72 (Blasdell *et al.,* 2003; Hughes *et al.,* 2010; Blaškovičová *et al.,* 2007; Halásová *et al.,* 2011). They all show similarity with MHV-68 in the growth in cell culture and pathogenesis in their natural hosts.

The partial genome sequence (71,913 bp) of BRHV was found 99.2 % identical to the corresponding portion of the WMHV genome (Hughes *et al.,* 2010). With regard to all so far described properties of BRHV this gammaherpesvirus was suggested to be classified into the same species as WMHV, *Murid herpesvirus 7*.

Sequencing of the ends of MHV-Šumava genome revealed at the left end a ~9.3 kbp deletion similar to that in MHV-76. However, an ~1.5 kbp deletion present at the right end of the MHV-Šumava genome was not found in other murine gammaherpesviruses (Blaškovičová *et al.,* 2007). Mistríková *et al*. (2002) identified some pathogenetical features distinguishing MHV-Šumava from MHV-68, MHV-72, and MHV-76. Namely, Balb/c mice infected with MHV-Šumava showed (i) a strong leukocytosis, (ii) as many as 60% atypical lymphocytes in peripheral blood during acute infection and (iii) the highest frequency of tumours in chronic infection among murine gammaherpesviruses (14.6% *vs* 11% for MHV-68 or MHV-72 and 0% for MHV-76) (Mistríková and Rajčáni, 2008).

Sequencing of the MHV-72 genome in the length of 22,899 bp ecompassing 22 genes revealed its diversity from MHV-68 in five structural proteins encoded by ORF20, ORF26, ORF48, M7, and ORF52, two immune evasion proteins (M3, MK3) and one non-structural protein encoded by ORF4 (Rašlová *et al.* 2000b; Mačáková *et al.,* 2003; Valovičová *et al.,* 2006; Belvončíková *et al.,* 2008; Halásová *et al.,* 2011). The observed diversity was suggested to contribute to some different pathogenetical features of MHV-72 in relation to MHV-68. Its pathogenesis in athymic and immuno-competent mice was found to be similar to that of MHV-68. An efficient MHV-72 replication in mammary glands and transmission via breast milk to newborn mice as a natural route of infection was reported by Rašlová *et al.* (2001). However, similar properties, namely shedding by milk and vertical virus transmission were more recently observed also with MHV-68 (Štiglicová *et al.,* 2011). Relatively high incidence of abnormal lymphocytes in the blood of virus-infected Balb/c or nude mice were described for MHV-72 but not for MHV-68, thus supporting the similarity between MHV-72 and EBV infections (Blackman *et al.,* 2000; Rašlová *et al.,* 2000a). A long-term-infection of immuno-suppressed as well as immuno-competent mice induced LPDs and neoplasmas in similar or higher rate compared to MHV-68 (Mistríková *et al.,* 2000; Mistríková and Rajčáni, 2008).

MHV-4556 was isolated from *Apodemus flavicolis*, the same host as in the case of MHV-Šumava, MHV-76, MHV-78, and MHV-5682. The isolation included three intracranial passages of the brain material from mouse to mouse, propagation in porcine kidney cell cultures and plaque-purification in murine mammary gland cell cultures. The findings of diversity in specific pathogenetical and molecular properties of various gammaherpesviruses support us in attempts to find some genetic diversity also between MHV-4556 and MHV-68. To date, the only known data for the MHV-4556 genome is a difference within the MK3 RING-CH finger domain known to be necessary for ubiquitination of MHC class I proteins (Valovičová *et al.,* 2006). In this study, we present the sequence of a portion of the MHV-4556 genome comprising 21 genes, its analysis and comparison with that of MHV-68. The obtained results show the same gene structure and almost identical gene sequences as with MHV-68 but also diversity of some genes, most likely involved in specific pathogenicity of MHV-4556.

Materials and Methods

Viruses. MHV-4556 clone 2.8 and MHV-68 clone f.2.6 were propagated in NMuMG cells at MOI of 0.001–0.1 PFU/cell, harvested at 80% CPE and stored at -70°C until use (Rašlová *et al.*, 2000b). The viruses were titrated by plaque assay on BHK-21 cells.

Cells. NMuMG (ATCC CRL-1636) or BHK 21 (ATCC CCL 10) cells were grown in DMEM (Gibco) supplemented with 10% FCS, 2 mmol/l glutamine (Invitrogen) and antibiotics at 37°C as previously described (Rašlová *et al.*, 2000b).

Viral DNA. The viruses were purified by linear sucrose gradient centrifugation (Rašlová *et al.*, 2000a), resuspended in 2xNPE buffer (0.2 mol/l NaCl, 20 mmol/l $Na_2HPO_4.2\,H_2O$, 1 mmol/l NaH_2PO_4, 2 mmol/l EDTA, pH 7.5), incubated with 100 µg/m1 RNase A (Sigma) for 30 min at 37°C and with 100 µg/ml proteinase K (Promega) and 1% SDS (Merck) for 4 hr at 56°C. DNA was extracted twice by phenol-chloroform, ethanol-precipitated and redissolved in TE buffer (0.01 mol/l Tris.HCl, 1 mmol/l EDTA, pH 8).

Restriction fragment length analysis (RFLP). Purified viral DNAwas digested with *Eco*RI or *Hind*III for 16 hrs at 37°C and electrophoresed in 0.7% agarose gels using standard procedures.

PCR. To amplify various fragments of the MHV-4556 genome by PCR the primers designed by Halásová *et al.,* (2011) for the MHV-68 genome (Virgin *et al.*, 1997) (Acc. No. U97553) were used. The PCR mixture contained ~20 ng of viral DNA, 0.3 mmol/l dNTPs, 2 mmol/l $MgCl_2$, 0.3 mmol/l primers, and 1 U of proof-reading Pfu DNA polymerase (Promega). The reaction, performed in the Mastercycler Personal (Eppendorf), consisted of one cycle of 95°C/5 min (initial denaturation), 35 cycles of 95°C/1 min, 54–60°C/45 sec and 72°C/1–2 min, and one cycle of 72°C/6–10 min. The obtained PCR products were electrophoresed in 1.5% agarose gels and purified using the Wizard DNA Clean-up System (Promega).

Cloning, sequencing, and sequence analysis. The products of at least two independent PCR reactions were cloned into the pGEM-T-Easy vector (Promega). Specificity and orientation of the clones were checked by restriction analysis. At least two clones were subjected to sequencing in the ABI PRISM 377 DNA Sequencer or ABI PRISM 3700 DNA Analyzer using the BigDye Terminator 3.1 Cycle Sequencing Kit (Perkin Elmer) or in the BITCET sequencing service. In addition to the MHV-68-specific primers, two universal pUC/M13 forward (24-mer) and reverse (22-mer) primers were used. The sequencing was done in both directions. The sequences of at least two clones of each DNA fragment had to be identical. Sequencing results were evaluated using Sequence Analysis 3.3 or Chromas Version 1.45 softver. The MHV-4556 nucleotide and deduced amino acid sequences were compared with their MHV-68 counterparts, using the sequence of MHV-68 WUMS strain as reference (Virgin *et al.*, 1997). Secondary structure of deduced proteins with identified amino acid differences was predicted according to Garnier *et al.* (1978) using the PROSIS program with probability of $1.25x10^{-4}$ for the occurrence of β-turns.

Results

RFLP of MHV-4556 genome

The MHV-4556 genome was compared with that of MHV-68 by RFLP. This approach revealed no differences between the *Eco*RI and *Hind*III restriction profiles of these two viruses and indicated their rough identity (Fig. 1). Further molecular analyses confirmed this conclusion by showing that the MHV-4556 genome, similarly to that of MHV-68, does not contain the ~9.5 kbp deletion at the left-hand end of the unique portion of the genome that had been found in MHV-76 or MHV-Šumava (data not shown) (Macrae *et al.,* 2001; Blaškovičová *et al.,* 2007).

Sequences of selected MHV-4556 genes and their analysis

The nucleotide sequence of a portion of the genome of MHV-4556 encompassing 14 structural and 7 non-structural genes was determined (Fig. 2). The obtained sequences were deposited at the EMBL/GenBank database under Acc. Nos. DQ124248, DQ378055, GQ421291-GQ421307, and GQ429004-GQ429006.

The sequence analysis of structural genes involved (*i*) ORF8 and ORF47, encoding important neutralization targets, envelope glycoproteins gB and gL, respectively, which both are epithelial cell-adapted accessories of the core gB/gH entry complex (Gillet and Stevenson, 2007), (*ii*) ORF11, ORF27, ORF20, ORF28, and ORF48, al encoding virion-associated proteins (Bortz *et al.,* 2003) (*iii*) ORF26 and ORF65, encoding the triplex component 2 protein (TRI-2) and the small capsid protein M9, respectively, and (*iv*) ORF38,

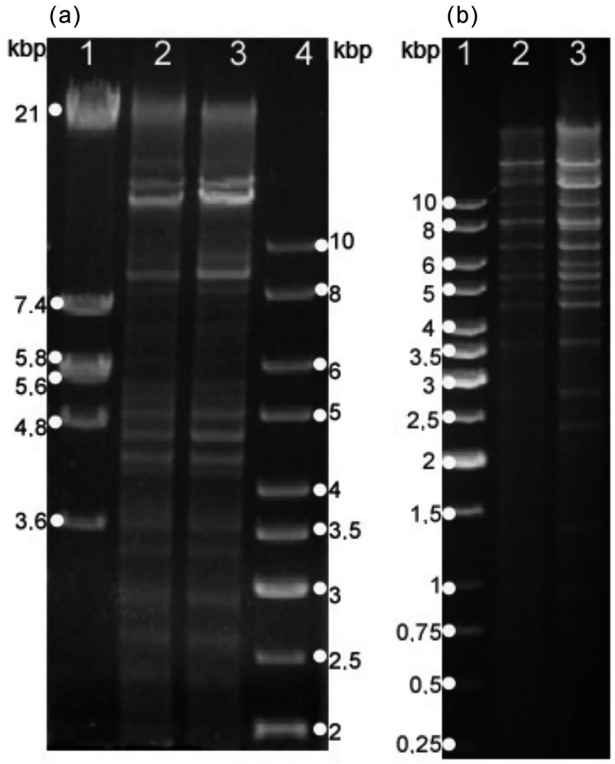

Fig. 1

***Hind*III (a) and *Eco*RI (b) restriction profiles of MHV-4556 and MHV-68**

(a) DNA size markers (lanes 1 and 4), MHV-68 (lane 2), MHV-4556 (lane 3).
(b) DNA size markers (lane 1), MHV-68 (lane 2), and MHV-4556 (lane 3).

ORF39, ORF45, and ORF52, all encoding tegument proteins engaged in virus egress, virion morphogenesis and modulation of innate immunity and virion infectivity (Bortz *et al.,* 2003, 2007; Jia *et al.,* 2005) (Table 1).

As for non-structural genes, (*i*) the M4 gene supposed to encode the immuno-evasion protein, (*ii*) ORF4 encoding the complement regulatory glycoprotein 70 that participates in herpesvirus entry, (*iii*) the M11 gene for the bcl-2 homolog, (*iv*) ORF72 encoding the cyclin D homolog, (*v*) ORF74 encoding the G-protein-coupled receptor, involved in tumour induction, and (*vi*) ORF73 encoding the homolog of KSHV LANA1, were analyzed (Table 2).

Comparison of identified MHV-4556 genes/proteins with their MHV-68 counterparts

The sequence analysis showed a near identity of genome structure and gene content of MHV-4556 and MHV-68. The identified MHV-4556 genes and proteins differred from their MHV-68 counterparts only in 15 nucleotides and 9 amino acids, respectively (Tables 1–4).

As for structural proteins (Tables 1 and 3), to quote only differences, the ORF8-encoded gB showed Cys instead of Tyr at position 66 (Table 3), implicating probably a major structural difference in relation to MHV-68 gB. The new Cys is predicted to create in the aa^{58}–aa^{68} region four new β-sheets and thus result in lower hydrophilicity and surface exposure of the aa^{62}–aa^{68} region of gB in comparison to the MHV-68 counterpart. Moreover, this difference implicated a loss of *Rsa*I restriction site.

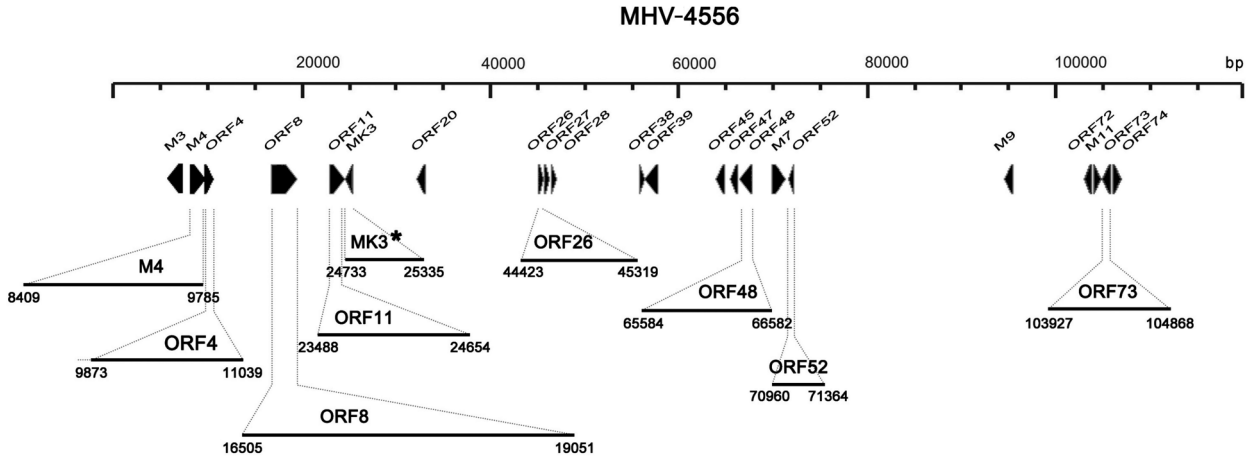

Fig. 2

Summary of sequenced genes/ORFs of MHV-4556

The sequenced genes/ORFs are shown as thick lines. The length of MHV-4556 genome (119–450 bp) and localization/orientation (arrows) of its individual genes/ORFs was predicted from MHV-68 (Acc. No. U97553). The MK3 gene (asterisk) was sequenced by Valovičová *et al.* (2006).

Table 1. Characteristics of structural genes/ORFs and proteins of MHV-4556

Gene/ORF				Protein	
Name	Acc. No.	Position (nt)	No. of nt differences in relation to MHV-68	Name/characteristics	Size (No. of aa)
ORF8	DQ378055	16 505-19 051	**2**	Glycoprotein B	849
ORF11	GQ421293	23 488-24 654	**1**	Virion-asociated protein 43	388
ORF20«	GQ421302	32 119-32 880	0	Virion-associated fusion protein	254
ORF26	GQ421294	44 423-45 319	**3**	Triplex component 2 protein	299
ORF27	GQ421303	23 488-24 654	0	Virion-associated protein 48	254
ORF28«	GQ421304	46 133-46 360	0	Virion-associated protein	76
ORF38	GQ421305	55 544-55 771	0	Tegument myristylated protein	75
ORF39«	GQ421306	55 802-56 950	0	Tegument glycoprotein M	383
ORF45«	GQ421307	63 655-64 272	0	IRL-7-binding protein homolog	206
ORF47«	GQ429004	65 027-65 545	0	Tegument glycoprotein L	173
ORF48«	GQ421295	65 584-66 582	**1**	Virion-associated protein	333
M7	GQ421299	69 466-70 914	0	Glycoprotein 150	483
ORF52«	GQ421296	70 960-71 364	**1**	Tegument protein	131
ORF65«	GQ421300	93 962-94 519	0	Small capsid protein M9	186

«Protein is encoded by complementary strand.

Table 2. Characteristics of non-structural genes/ORFs and proteins of MHV-4556

Gene/ORF				Protein	
Name	Acc. No.	Position (nt)	No. of nt differences in relation to MHV-68	Name/characteristics	Size (No. of aa)
M3*	GQ421298	6 060-7 277	0	Chemokine binding protein	406
M4	GQ421291	8 409-9 785	**2**	Unknown	459
ORF4*	GQ421292	9 873-11 039	**2**	Complement-regulatory glycoprotein 70	388
K3*[a]	DQ124248	24 733-25 335	**2**[a]	Immune evasion protein	201
M11	GQ421301	103 418-103 933	0	bcl-2 homolog	171
ORF72	GQ429005	102 426-103 181	0	Cyclin D homolog	252
ORF73*	GQ421297	103 927-104 868	**1**	KSHV LANAI homolog	314
ORF74	GQ429006	105 057-106 067	0	G-protein-coupled receptor	337

*Protein is encoded by complementary strand. [a]Valovičová *et al.* (2006).

The ORF11-encoded virion-associated protein 43 showed a difference at aa^{27}, implicating change of one α-helix to β-sheet and resulting in reduction of hydrophilicity and surface exposure of the aa^{16}–aa^{36} region of the MHV-4556 protein. Moreover, this difference implicated a new *Bpm*I restriction site.

The ORF26 gene and its TRI-2 protein showed differences in 3 nucleotides and 2 amino acids (aa^{43} and aa^{130}), respectively. These differences implicated a change of secondary structure of the aa^{120}–aa^{140} region, namely a loss of one β-sheet and a gain of another, and consequently resulted in lower hydrophilicity and surface exposure of the aa^{35}–aa^{55} region of the MHV-4556 protein.

The ORF48 gene showed a difference at the first nucleotide of the start codon, implicating its loss and truncation of the N-end of the encoded protein by 4 amino acids. This truncation was predicted to cause shortening of the respective α-helix without any change in hydrophilicity.

The ORF52 protein showed a difference at aa^{45}, implicating a strongly reduced hydrophilicity and surface exposure of the aa^{35}–aa^{55} region. This difference was predicted to influence the α-helix 2 region (aa^{46}–aa^{76}), which is important for self-association of protein monomers into dimers or tetramers and formation of final functional complex following interaction with other tegument and nucleocapsid components (Benach *et al.,* 2007). Moreover, this difference implicated a loss of *Pvu*II restriction site. This difference is the only common with those found in MHV-72 till now (Halásová *et al.,* 2011).

Table 3. Amino acid differences in structural proteins of MHV-4556 in relation to MHV-68

Gene/ORF	Nucleotide position	Difference in nucleotides	Amino acid position	Difference in amino acids	Difference in restriction sites
ORF8	16 801	A → G	66	Tyr → Cys	RsaI lost
	17 086	G → A	194	None	None
ORF11	23 567	A → G	27	Lys → Arg	BpmI new
ORF26	44 549	A → G	43	Thr → Ala	None
	44 788	A → G	122	None	None
	44 813	G → A	130	Asp → Asn	None
ORF48	66 581	T → C	1	Met → Thr	None
ORF52	71 231	C → T	45	Thr→Ile	PvuII lost

Table 4. Amino acid differences in non-structural proteins of MHV-4556 in relation to MHV-68

Gene/ORF	Nucleotide position	Difference in nucleotides	Amino acid position	Difference in amino acids	Difference in restriction sites
M4	8 765	A → G	119	None	None
	9 073	A → G	222	Glu → Gly	None
ORF4	9 862	T → A	11 nts upstream of start codon	None	None
	10 988	T → C	372	None	None
K3[a]	25 156	A → G	60	None	TauI new
	25 301	A → G	12	His → Arg[a]	None
ORF73	104 345	T → C	175	None	None

[a]Valovičová *et al.* (2006).

As for non-structural proteins (Tables 2 and 4), to quote only differences, the M4 protein showed difference at aa^{222}, implicating a reassortment of secondary structure of the aa^{215}–aa^{234} region, namley a change of eight β-sheets and five α-helices to seven β sheets, one turn and eight α-helices. This change implicated a decrease in both hydrophilicity and surface exposure.

The ORF4 gene showed differences not changing codons. However, the difference at nt^{9862} is situated within a potential promotor region, localized 11 nucleotides upstream of the start codon for the ORF4 protein.

The rest of genes/proteins did not exhibit any sequence differences between MHV-4556 and MHV-68.

Discussion

Although the MHV-4556 is considered a close relative to MHV-68 (Mistríková *et al.,* 2000), the kinetics of its replication in cell culture as well as infection in Balb/c mice are similar but not identical. Current studies on pathogenetical properties of MHV-4556 *in vitro* indicate that the main difference from MHV-68 in replication in BHK-21 cells is about a two log lower titer of infectious virus after 24 hrs p.i. Moreover, MHV-4556 exhibits features characterizing acute infection, establishment of latency, latency and reactivation from latency that are unique and thus different from those of MHV-68. For example, an acute infection in the lungs of Balb/c mice starts about 3 days earlier than that with MHV-68 in mice, reaching maximal levels of infectious virus at day 3 p.i. Furthermore, MHV-4556 causes a reduced and by about 10 days delayed splenomegaly in comparison to MHV-68 (Z. Halásová, personal communication). Long-term infection associated with neoplasm development that has been observed with murine gammaherpesviruses was so far not recorded for MHV-4556 (Mistríková and Rajčáni, 2008). Despite of these findings, the only genetic difference between MHV-4556 and MHV-68 is that within the RING-CH finger domain of MK3 that is known to play a major role in the establishment of latency and to be necessary for the ubiquitination of MHC class I proteins (Stevenson *et al.,* 2002; Lybarger *et al.,* 2003; Boname *et al.,* 2005b). The difference detected in MHV-4556 MK3 protein at aa 12 in relation to MHV-68 was suggested to implicate the aa^{9}–aa^{14} region as antigenic determinant (Valovičová *et al.,* 2006).

In this study, restriction analysis of MHV-4556 genome and sequencing and sequence analysis of a portion of the MHV-4556 genome encompassing 14 structural and 7 non-structural genes were done. The obtained results revealed no differences between the *Eco*RI and *Hind*III restriction profiles of MHV4556 and MHV68 genomes. For MHV-4556, we proved the presence of two Hind*III* fragments contain-

ing genes from the left end of genome, namely the 6.3 kbp fragment E with M1, M2, and M3 (a part) genes and the 4.8 kbp fragment H with M3 (a part), M4, and ORF4 genes. The absence of these two fragments in MHV-76 has allowed Macrae *et al.* (2001) to identify the ~9.5 kbp deletion at the left end of unique portion of the genome. A similar deletion was described for ΔγHV-68, a spontaneous mutant of MHV-68 (Clambey *et al.,* 2002), and MHV-Šumava as well (Blaškovičová *et al.,* 2007). The abovementioned differences in the *Hind*III profile of MHV-76 compared with MHV-68 corresponded to the absence of the 12.5 kbp *Eco*RI fragment B with M1, M2, M3, M4, ORF4, and ORF5 (a part) genes (Macrae *et al.,* 2001). Since we found out that the *Hind*III and *Eco*RI profiles of MHV-4556 and MHV-68 are comparable, we conclude that the MHV-4556 genome is most probably a full-length one without the ~9.5 kbp deletion mentioned above. Moreover, the presence of genes M3, M4, and ORF4 at the left end of the MHV-4556 genome was confirmed by our subsequent experiments.

We sequenced structural genes playing more or less important roles in membrane fusion, virus entry, direct spread of virus from lytically infected to uninfected cells, virion morphogenesis, modulation of innate immunity of infected cells, and virion infectivity. Amino acid differences between MHV-4556 and MHV-68 were identified in an important neutralizing target, a disulfide-linked virion envelope gB, and in two of five virion-associated proteins involved in the early phase of infection, virion assembly and egress (Bortz *et al.,* 2003).

In the ORF8-encoded gB, we identified Cys instead of Tyr at position 66, located only three amino acids upstream of Cys^{70}. This difference implicating four new β-sheets and a reduced hydrophilicity and surface exposure of the N-end of the protein may represent a major structural difference betwen MHV-4556 and MHV-68.

The difference found in the ORF48 protein, which is known to be essential for virus replication (Fuchs *et al.,* 2002), implicated truncation of its N-end without any effect on its secondary structure, while that in the ORF11 protein was predicted to change its secondary structure and consequently to reduce its hydrophilicity and surface exposure. The change in the MHV-4556 ORF11 protein in relation to its MHV-68 counterpart might have some effect upon the suggested functions of this protein in virus replication *in vivo*, immune evasion and apoptosis inhibition (Boname *et al.,* 2005a).

Of the four tegument proteins analyzed only the ORF52 protein differed from its MHV-68 counterpart in aa^{45}. This difference was predicted to reduce the hydrophilicity and surface exposure of the aa^{35}–aa^{55} region. Sequence alignment of ORF52 proteins of 15 herpesviruses revealed only five proteins with an amino acid differing from Thr at position 45 (Benach *et al.,* 2007; Halásová *et al.,* 2011). In this study, we found in MHV-4556 at this position Ile, a difference common with MHV-72. A recent crystal structure study revealed dimerization of the MHV-68 ORF52 protein, supposed to interact with other components of the tegument or nucleocapsid through its N terminal α-helix 1 as well as to create an asymmetrical tetramer, probably representing a latent form of the ORF52 protein not involved in virion assembly (Benach *et al.,* 2007). The difference at aa^{45} of ORF52 protein lies near the aa^{46}–aa^{76} region of α-helix 2, created by hydrophilic amino acids localized at the tetramer interface. Thus, the predicted reduction in hydrophilicity of this region of MHV-4556 and MHV-72 in relation to MHV-68 might influence at least the self-association of the ORF52 protein of these viruses.

Of the two capsid proteins encoded by ORF 26 and ORF65 only the former showed differences at aa^{43} and aa^{130} in relation to MHV-68, implicating slight changes at the N-end of protein but wide reassortment of secondary structure in the middle of protein, resulting in reduced hydrophilicity and surface exposure.

Of the six non-structural proteins only the M4 protein exhibited an amino acid difference at aa^{222} that implicated a reduced hydrophilicity and surface exposure as well. This protein was suggested to down-regulate the immune response of host to virus infection (Townsley *et al,* 2004). Data describing its binding domain/s and its activities against immune modulators, such as chemokines or cytokines are either unclear or unknown (Geere *et al,* 2006). Anyway, this different feature of the M4 protein might alter its binding to cells involved in the immune response or to extracellular immune modulators and thus alter their function in the response to MHV-4556 infection.

As for the ORF4 protein, it did not exhibit any differences chaging codons. However, the two nucleotide differences, situated within a potential promoter region of this gene, might alter expression of this major viral glycosaminoglycan-binding protein participating in virus entry (Kapadia *et al.,* 2002).

Summing up, we have determined the nucleotide sequence of a 22,565 bp portion of the MHV-4556 genome containing 21 genes and demonstrated that 5 genes (M4, ORF8, ORF11, ORF26, and ORF52) exhibit in relation to MHV-68 differences, which implicate an altered secondary structure and reduced hydrophilicity and surface exposure of the encoded proteins. Thus, the different pathogenic properties of MHV-4556 in relation to MHV-68 could be determined by the proteins with altered secondary structure, namely the immune evasion protein M4, envelope gB (ORF8), virion-associated p43 (ORF11), triplex component 2 protein (ORF26) and tegument protein (ORF52), which all play roles in immune evasion and virion entry, assembly and egress. However, other proteins not investigated in this study may also play a role in the pathogenicity of murine

gammaherpesviruses. Ongoing studies in this laboratory are expected to reveal a sequence of the MHV-4556 region localized at of left end of genome. This region playing an important role in the infection of mice with MHV-68 is known to be deleted in the length of ~9.5 kbp in MHV-76 and MHV-Šumava but not in MHV-72 and WMHV. Comparison of MHV-4556 sequences with those of WMHV revealed that all the MHV-4556 genes differing from their MHV-68 counterparts also differed from their WMHV counterparts. Analysis of 21 genes of MHV-4556 has shown that this virus is more divergent from MHV-68 than MHV-76. However, the variability in these MHV-4556 genes is far from that found in their WMHV counterparts (Hughes *et al.,* 2010). In addition to the previously reported MK3 protein (Valovičová *et al*., 2006), the presently described M4, ORF8, ORF11, ORF26, and ORF52 proteins of MHV-4556, all differing from their MHV-68 counterparts, may also contribute to some specific biological properties of MHV-4556. However, the relevance of sequence differences between MHV-4556 and MHV-68 for their pathogenicity and oncogenicity needs further investigation. Studies on specific pathogenetical features of MHV-4556 *in vitro* and *in vivo* are in progress. The results of this study might help to identify some mechanisms of virus-host interactions and some functions of viral genes in viral pathogenesis, thereby enhancing the potential of murine gammaherpesviruses as a model for study of human gammaherpesviruses.

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