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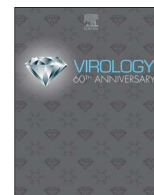

Phylogenetic and molecular epidemiological studies reveal evidence of recombination among Marek's disease viruses

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ABSTRACT

Marek's disease has brought enormous loss in chicken production worldwide and the increasing virulence of Marek's disease virus (MDV) became a severe problem. To better understand the genetic basis underlying, a Chinese MDV strain HNGS101 isolated from immunized chickens was sequenced. Phylogenetic analysis implied that HNGS101 showed more relatedness to Eurasian strains than GaHV-2 circulating in North America. Recombination networks analysis showed the evidence of recombination among MDV strains, and several recombination events in the UL and US region were found. Further analysis indicated that the HNGS101 strain seemed to be generated by the recombination of the earliest Eurasian strains and North American strains in the US region, which may be responsible for the MD outbreaks in China. In summary,this is the first report to demonstrate recombination events among MDV strains, which may shed light on the mechanism of virulence enhancement.

1. Introduction

Marek's disease virus (MDV), also known as gallid herpesvirus 2 (GaHV-2), is a highly oncogenic alpha-herpesvirus, causing immune suppression, malignant T-cell lymphomas and neurological disorders in chickens (Calnek, 2001). GaHV-2 belongs to the genus *Mardivirus*, which contains other two species: GaHV-3, a non-oncogenic herpesvirus also known as MDV-2, and turkey herpesvirus 1 (HVT) or Meleagrid herpesvirus type 1(MeHV-1), previously known as MDV-3. Based on mortality and morbidity rates in animals inoculated with MDV, a classification nomenclature has proposed five MDV pathotypes: attenuated (a), mildly virulent (m), virulent (v), very virulent (vv), and very virulent plus (vv+) (Witter, 1997).

MDV encodes more than 100 genes, the majority of which are present in the UL and US regions and share substantial homology with collinear genes of other alphaherpesviruses. Within the genome, many genes have been identified to be associated with the virulence of the virus, including *meq*, *icp4* and *vil-8*. The *meq* gene in the long-repeat regions of the MDV genome is associated with viral oncogenicity and pathogenicity (Lee et al., 2008). Previous reports showed that distinct polymorphisms and point mutations in *meq* were correlated with MDV virulence, and the number of four-proline repeats (PPPP repeats) in the *meq* had a strong negative association with virulence (Renz et al., 2012; Shamblin et al., 2004). The *icp4* gene in the short–repeat regions of the MDV genome is a transactivator of the replicative phase (Kato et al., 2002; Pratt et al., 1994) and may be involved in attenuation by multiple nonsynonymous mutations via in vitro serial passage (Hildebrandt et al., 2014). The *vil-8* gene encodes an interleukin-8 (IL-8) homolog that can attract chicken PBMC (Parcells et al., 2001).

In the 1970s, MD was controlled by vaccine derived from herpesvirus of turkeys (HVT) (Witter et al., 1970). After that, a bivalent vaccine consisting of SB-1 (serotype 2 MDV) (Schat and Calnek, 1978) and HVT was used. Since the 1990s, a vaccine using attenuated serotype 1 strain CVI988 was used and the disease was largely controlled until now (Rispens et al., 1972). However, MD vaccines do not prevent vaccinated birds from infection with virulent field strains, and the strain could replicate and evolve within the same bird (Atkins et al., 2013). Furthermore, the applied vaccines may contribute to the enhancement of field MDV virulence, as shown by the isolation of strains with higher pathogenicity even in birds vaccinated with the CVI988 strain (Nair, 2005). The mechanisms responsible for GaHV-2's increase in virulence and committal changes in disease have attracted great

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interest over the last few decades.

In China, MD was first reported in the 1970s. Several molecular epidemiologic studies have indicated that Chinese MDV isolates constitute a separate clade from MDV isolates obtained from other geographic regions (Tian et al., 2011; Zhang et al., 2016). Since the first complete genomic sequence of MDV (strain GA) was determined in the late 1990s (Lee et al., 2000), the complete genome sequences of more than twenty MDV strains were available in GenBank. Comparative genomics analysis with nucleotide sequences of virulent strains (Md5, Md11, RB-1B and GA) has provided some information on the genes and single nucleotide polymorphisms involving in virulence (Lee et al., 2000; Niikura et al., 2006; Spatz et al., 2007b). However, the available genetic data are still insufficient, especially for emerging strains of MDV with apparent different pathogenic characteristics and increased virulence. Unfortunately, little progress has been made correlating pathotype (or phenotype) with genotype. This is a formidable task since the genome of MDV is huge with 180 kb. In the past decade, next-generation sequencing (NGS) has led to a massive expansion in sequencing, allowing characterization at molecular genetic level of the complete genomes for many viruses and strains, which include serially passed MDV strains such as 648A (Spatz et al., 2012) and several classical MD vaccines as Rispens and SB-1 (Spatz et al., 2007a; Spatz and Schat, 2011). Here, using NGS, we sequenced and carried out genome-wide analysis of HNGS101. The main aim of this study is to characterize the MDV strain isolated from immunized chickens and investigate the recombination event among MDVs.

2. Materials and methods

2.1. Cells and viruses

Chicken embryo fibroblasts (CEFs) were isolated from 10-day-old specific pathogen-free (SPF) chicken embryos and maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) with antibiotics. Secondary CEFs (10^7/dish) were seeded overnight in 100 mm dishes, then infected with HNGS101 (Yu et al., 2013) in complete medium containing 1% FBS.

2.2. Purification of viral DNA from virus-infected cells

Viral DNA was isolated using the micrococcal nuclease procedure as described by Volkening and Spatz (Volkening and Spatz, 2009). Briefly, HNGS101 was propagated on CEFs in DMEM containing 1% fetal bovine serum and the cells were harvested when cytopathic effects (CPEs) reached to 80%. After permeabilization, cellular DNA was degraded using micrococcal nuclease. Viral capsid was then digested by proteinase K to release viral DNA, and high MW viral DNA was precipitated by PEG-8000 at concentrations of 6.5%. The purity of the viral DNA was confirmed after PEG-8000 precipitation by separating *Bam*HI-digested aliquots on an agarose gel.

2.3. DNA sequencing and analysis

Sequencing of 1 μg of the viral DNA was carried out using the Illumina HiSeq. 2000 sequencing platform (San Diego, CA, USA). The assembly results were optimized according to paired-end and overlap relationships by mapping the reads to contigs after removing the host sequence. Repetitive regions and ambiguities in the genome sequencing data were resolved by re-sequencing from PCR products using traditional Sanger sequencing.

2.4. Alignment and analysis

The sequences of 29 MDV strains were obtained from GeneBank (Table 1). Alignment of complete genome sequences and that of the sequences in four different sub-regions (UL, IRL, US, and IRS) of MDV strains were performed using MAFFT (Katoh and Toh, 2008). Multiple alignments of proteins and nucleotide sequences were generated using MUSCLE (Edgar, 2004). Phylogenetic analysis (Neighbor joining method) on alignments of the sequences was conducted by the MEGA6 (Kumar et al., 2008). All phylogenetic reconstructions were assessed statistically by analyzing one thousand bootstrap replications. Geneious was used to compare different sub-regions of MDV genome (Kearse et al., 2012).

Recombination networks on alignments of the whole genome, and the sequences in different sub-regions (UL, IRL, IRS, and US) of the 29 MDV strains were performed by using SplitsTree 4 (Huson, 1998). Statistical analysis of the recombination networks was generated by using the Phi test. To further analyze the possibility of recombination, bootscan analysis was generated to detect the crossover points for recombination events of selected sequences as representatives of different cluster using Simplot (Lole et al., 1999).

Table 1
Information of strains used for comparison.

Strain	Year isolated	Accession number	Country
HNGS101	2011	MG432697	China
GX0101	2001	JX844666.1	China
814	1986	JF742597.1	China
J-1	1974	KU744555	China
LCC	2011	KU744556	China
LTS	2011	KU744557	China
WC/1203	2012	KU744558	China
JL/1404	2014	KU744559	China
CC/1409	2014	KU744560	China
HS/1412	2014	KU744561	China
LCY	2011	KX290013.1	China
LMS	2007	JQ314003.1	China
pC12/130-15	1992	FJ436097.1	United Kingdom
pC12/130-10	1992	FJ436096.1	United Kingdom
CVI988	1969	DQ530348.1	Netherlands
CU-2	1968	EU499381.1	USA
Md5	1977	AF243438.1	USA
RB-1B	1981	EF523390.1	USA
684a p11	1994	JQ808361.1	USA
584A p80	1990	EU627065.1	USA
sd1	2015	KU173116	USA
sd2	2015	KU173115	USA
bf1	2015	KU173117	USA
bf2	2015	KU173118	USA
bd2	2015	KU173119	USA
EU-1	1992	MF431494	Italy
Polen5	2010	MF431496	Poland
MD70/13	1970	MF431495	Hungary
ATE2539	2000	MF431493	Hungary

3. Results

3.1. Genome organization

The genome of HNGS101 was assembled as 175,888 bp in length. The genome was organized into 6 regions as a characteristic of class E herpesvirus (Philip and Bernard, 2013). The UL, US, IRL and IRS (TRL is equal to IRL, TRS is equal to IRS) of HNGS101 were 113,541, 11,159, 12,736 and 12,141 bp in length, respectively. The complete genome sequence of HNGS101 has been deposited in GenBank with accession number MG432697.

3.2. Intragenic variations

To investigate the intragenic variations between the different MDV isolates, oncogenic genes including *meq*, *vil-8* together with *icp4* and *ul36* were analyzed based on a comparison of the complete genome sequences of the newly sequenced strain and those obtained from GenBank. The sequence of *meq* of HNGS101 was 1020 bp in length,

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