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A high frequency of *Gallid herpesvirus-2 co-infection with Reticuloendotheliosis virusis* associated with high tumor rates in Chinese chicken farms

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ABSTRACT

The prevalence of Marek's disease (MD) caused by *Gallid herpesvirus-2* (GaHV-2) has been increasing in chickens in China despite universal vaccination. Among the possible reasons for this trend, of *Reticuloendotheliosis virus* (REV) contamination in vaccines could lead to co-infection and reduce the vaccine efficacy. Here, we report the epidemiological findings of our continuous surveillance of MD, and an examination of the effects of REV and/or GaHV-2 co-infection. A total of 1230 samples were collected between 2011 and 2015 from 305 flocks covering many of the chicken-raising regions of China. Among these, 606 samples were determined to be GaHV-2-positive, 13.0% of which were found to be co-infected with REV from 18.8% of the flocks. One GaHV-2 strain (HS/1412), a REV strain (HS/1412R), and a GaHV-2 and REV-co-infected strain (HS/1412 GR) were isolated from different chickens of a GaHV-2 and REV co-infected flock. Pathogenicity tests showed that HS/1412 and HS/1412 GR caused disease in all chickens and that HS/1412R induced morbidity in 84.6% of the infected chickens. HS/1412 GR induced 100% mortality and 76.9% tumor formation, which were significantly higher frequencies than those observed with strain HS/1412 (38.5% and 15.4%, respectively) and HS/1412R (0% and 0%). These results indicate that co-infection with GaHV-2 and REV might explain the persistent, sporadic outbreaks of neoplastic disease in some commercial flocks, resulting in a significant economic burden to the poultry industry of China.

1. Introduction

Marek's disease (MD) is a lymphoproliferative disease of chickens caused by the highly contagious alpha-herpesvirus *Gallid herpesvirus 2* (GaHV-2) of the genus *Mardivirus*. GaHV-2 transforms T lymphocytes and is thus characterized by marked immunosuppression, lymphoid infiltration of neuronal and other tissues, and subsequent development of neurological symptoms and lymphomas in a wide range of tissues (Osterrieder et al., 2006). Thus, the control of MD through vaccination is a critical step to maintain viability in the modern poultry industry. However, sporadic outbreaks still occur in vaccinated flocks worldwide due to various factors.

Most studies using the Rispens vaccine have shown levels of protection similar to or better than those of the herpesvirus of turkeys (HVT) and bivalent vaccines for MD; however, some studies have shown variable protection against the virulent-plus GaHV-2 strains with only partial protection conferred against particular strains (Witter, 2005; Zhang et al., 2015). Therefore, uniformly high protection levels against MD cannot be assumed. Multiple oncogenic viral infections were found in 25% of commercial chicken and turkey flocks surveyed in Israel between 1993 and 2004 (Davidson, 2009). Moreover, infection with another retrovirus, *Reticuloendotheliosis virus* (REV) of the genus *Gammaretrovirus,* has been reported to decrease the effectiveness of the MD vaccine (Bulow, 1977), resulting in significant losses to vaccinated flocks. REV causes reticuloendotheliosis (RE), which is another tumorigenic and immunosuppressive disease. Infection of chicken flocks with REV is moderately prevalent, but clinical disease is rarely recognized in chickens and turkeys. Furthermore, REV is a potential contaminant of avian vaccines, with reported cases of such contaminated vaccines emerging in China and other countries in recent years (Awad et al., 2010; Fadly and Garcia, 2006; Fadly and Witter, 1997; Koyama et al., 1976; Li et al., 2013; Liu et al., 2009; Zavala and Cheng, 2006). In addition, co-infections with GaHV-2 and REV have been reported in China, in which the flocks became affected by both diseases at approximately 6 weeks of age, and the morbidity was approximately 30% or higher until 10 weeks of age (Bao et al., 2015).

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However, the specific role of REV in the pathogenicity of MD remains unclear (Buscaglia, 2013).

The aim of the present study was to investigate the occurrence of GaHV-2 and REV co-infection in chicken flocks of China, and to determine the effect of experimental co-infection with isolates of GaHV-2 and REV using specific-pathogen-free (SPF) chickens.

2. Materials and methods

2.1. Ethics statement

The animal experiments were approved by the Animal Ethics Committee of the Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences (CAAS) and were performed in accordance with animal ethics guidelines and approved protocols (SYXK (hei) 2011-022). All SPF chickens were cared for in accordance with humane procedures.

2.2. Clinical samples and viral identification

We collected 1230 clinical samples, consisting of 1144 feather pulps and 86 peripheral blood lymphocytes (PBLs), from 305 flocks of diseased chickens. The samples were diagnosed with MD by polymerase chain reaction (PCR) of a 132-bp repeat (132bpr) fragment of GaHV-2 and REV infection was assessed by amplification of the long terminal repeat (LTR) of the REV pro-viral genome. Isolates were analyzed by PCR and enzyme-linked immunosorbent assays using a p27 mAb for avian leukosis virus (ALV) (Gopal et al., 2012; Yun et al., 2013), and by PCR for chicken infectious anemia virus (CIAV) (Qin et al., 2010). The positive control strains and the primers used for PCR amplification of GaHV-2, REV, ALV, and CIAV were previously described (Zhang et al., 2015) and are shown in Table S1.

In addition, the samples were identified by hotspot PCR to amplify the integrated REV LTR with the GaHV-2 sequence at the junction using a series of primers shown in Table S2 (Zhang and Cui, 2005). In brief, the LTR inserts were examined by PCR with one of four GaHV-2 primers and one of four REV primers (Zhang and Cui, 2005). The direction and position of the LTR inserts can be determined through PCR using four REV primers as well as GaHV-2 primers, which are located in the unique short (Us) region of the GaHV-2 genome near the internal repeat short (IRs)/Us junction or the Us/terminal repeat short (TRs) junction, respectively (Zhang et al., 2017). The positive samples were stored at −80 °C in our laboratory until viral isolation.

2.3. Viral isolation and identification

Viral isolation was performed as described previously for GaHV-2 (Zhang et al., 2011). Primary chicken embryonic fibroblasts (CEFs) were prepared from 10-day-old SPF chicken embryos using a standard procedure (Schat and Purchase, 1998). The infectious titers of isolates were determined by secondary CEF monolayer cultures grown in 6-well plates for GaHV-2 and in 96-well plates for REV. In brief, the selected samples were aseptically inoculated into CEF cultures and 2 − 5 blind passages were performed until cytopathogenic effects (CPEs) were observed. Some of the infected cells were propagated in CEF cultures, harvested in M199 medium supplemented with 20% fetal bovine serum and 10% dimethyl sulfoxide, and stored in liquid nitrogen. The other infected cells were frozen and thawed, followed by centrifugation and incubation with fresh CEF cells for three passages, which were used for REV isolation to eliminate the possibility of detecting GaHV-2. The isolates were identified by PCR amplification of the 132bpr of the GaHV-2 genome, and the LTR of the REV pro-viral genome (Zhang et al., 2011). They were also detected by immunofluorescence assays using an anti-gI monoclonal antibody (mAb) for GaHV-2, which was produced by our laboratory and is specific for the gI protein of GaHV-2, as well as an anti-gp90 mAb for REV (Xue et al., 2012). Fluorescence was observed from the isolates and the GaHV-2 reference strain GA or the REV reference strain 07I.

2.4. Experimental infection

The animal experiment was performed in a blinded manner. A total of 88 1-day-old SPF White Leghorn chicks (Experimental Animal Center of HVRI, CAAS) were divided into four groups, with 22 birds per group. The birds in the different groups were maintained in separate isolators with negative pressure, and food and water were provided ad libitum. The chicks in group 1 were inoculated intra-abdominally with the GaHV-2 isolate at a dose of 1000 plaque-forming units (PFUs) in 200 μl. The chicks in group 2 were inoculated with the co-infection isolate mixture containing 1000 PFUs of GaHV-2 and $3 \times 10^{3.6}$ $TCID_{50}$ REV. The chicks in group 3 were inoculated with only the REV isolate at $3 \times 10^{3.6}$ $TCID_{50}$ with the same volume of diluent via the same route. The chicks in group 4 received an equal amount of the diluent only delivered via the same route, and served as the control. Chicks were examined daily for clinical signs of MD. Three chickens selected randomly in each group were euthanized humanely at 4, 14, and 28 days post challenge (dpc), and spleen samples were collected to determine the GaHV-2 or REV genome load. In addition, the body weights and weights of immune organs (bursa, thymus, and spleen) were recorded for further analysis. Postmortem examination was performed on all birds that died or were euthanized humanely at the end of the experiment (80 dpc).

2.5. Quantitative PCR

The collected spleen tissues were homogenized in phosphate-buffered saline, and DNA was extracted using the AxyPrep Body Fluid Viral DNA/RNA Miniprep Kit (Corning Life Sciences Co., Ltd., Suzhou, China) according to the manufacturer's instructions. The GaHV-2 *meq* gene was used as the GaHV-2 genome target gene, the REV *gp90* gene was used as the REV genome target gene, and the chicken *ovotransferrin* gene was used as a reference gene for host cells. Quantitative PCR detection was performed as previously described (Li et al., 2010; Zhang et al., 2007). The absolute numbers of GaHV-2 or REV gene copies per million cells from the collected spleens were calculated based on the standard curves generated. The normalized viral load was calculated using the following formula: $\log_{10}$ [(viral genome copy number/chicken genome copy number) $\times 10^6$].

2.6. Statistical analyses

Survival analysis, body weight, immune organ index, and viral load data were analyzed using GraphPad Prism (Version 7.02; GraphPad Software, Inc., San Diego, CA, USA). Comparisons of body weights, immune organ indexes, and viral load between groups at each time point were performed using multiple t-tests (Holm–Sidak method, with alpha = 0.05, by GraphPad Prism). Differences were considered statistically significant at $p \leq 0.05$.

3. Results

3.1. Epidemiological investigation

A total 1230 samples, consisting of 1144 feather pulps and 86 PBLs, were obtained from 305 farms located in Heilongjiang, Jilin, Liaoning, Shandong, Hebei, Jiangsu, Hunan, Guangdong, Tianjin, Ningxia, Anhui, and Gansu province or city of China from 2011 to 2015. Among these, 606 samples obtained from 165 farms were found to harbor two or three copies of the GaHV-2 genomic 132bpr, and were determined to be GaHV-2-positive based on PCR amplification, accounting for a 49.3% positivity rate (Table 1). All GaHV-2-positive samples were tested for the presence of the LTR sequence of the REV pro-viral genome,

Table 1
Gallid herpesvirus-2 (GaHV-2) infection in samples collected from different flocks across several provinces of China.

Year	Provinces	flocks			samples		
		n	positive	Rate (%)	*n*	positive	Rate (%)
2015	10	138	52	37.7	526	209	39.7
2014	9	31	18	58.1	182	63	34.6
2013	7	55	37	67.3	159	87	54.7
2012	10	51	39	76.5	214	152	71.0
2011	6	30	19	63.3	149	95	63.8
Average	12	305	165	54.1	1230	606	49.3

Table 2
Co-infection with *Reticuloendotheliosis virus* (REV) in *Gallid herpesvirus-2* (GaHV-2)-positive samples.

Year	MD positive flocks	MDV + REV flocks		MD positive samples	MDV + REV samples	
		positive	Rate (%)		positive	Rate (%)
2015	52	15	28.8	209	32	15.3
2014	18	2	11.1	63	8	12.7
2013	37	6	16.2	87	12	13.8
2012	39	4	10.3	152	16	10.5
2011	19	4	21.1	95	11	10.5
Average	165	31	18.8	606	79	13.0

revealing co-infection in 79/606 (13.0%) samples from 31/165 (18.8%) flocks. This survey demonstrated that some flocks were severely co-infected by the two viruses, especially in 2015 (Table 2).

The diseased flocks showing typically MD signs and infected with GaHV-2 were observed at approximately 8 or 9 weeks of age, and the morbidity rate was 1–30% and usually below 5% until 20 weeks. Disease resulting from co-infection with GaHV-2 and REV was observed as early as 6 weeks of age, and the morbidity rate was 20–60% and usually approximately 30% until 20 weeks of age. Furthermore, in the co-infected flocks, approximately 13.0% of GaHV-2-positive chickens were also co-infected with REV. In addition, since the REV LTR can integrate into the GaHV-2 genome in co-infected CEFs or chickens (Jones et al., 1996; Kost et al., 1993), recombinant GaHV-2 with the REV LTR was detected by hotspot PCR for the GaHV-2 and REV-co-infected samples; however, no chimeric molecules of fragments were amplified in any of the samples.

3.2. Viral isolation and identification

One GaHV-2-positive sample and one GaHV-2 and REV-co-infected sample collected from a severely-affected layer flock in Liaoning Province were used for viral isolation, and the two isolates were denoted HS/1412 and HS/1412 GR, respectively. These isolates were found to be well-adapted to CEF and induced CPEs indicative of MD after two blind passages at 5 days post inoculation. PCR detection showed that HS/1412 and HS/1412 GR contained two copies of the 132bpr genomic GaHV-2 region. Furthermore, HS/1412 GR was positive for the LTR of the REV pro-viral genome and was negative for chimeric molecules of GaHV-2 and REV fragments.

Some HS/1412 GR-infected cells were frozen and thawed, followed by centrifugation and incubation with fresh CEF cells for three passages. PCR detection confirmed that these cells were positive for the LTR of REV and negative for the 132bpr of GaHV-2, and the strain was denoted HS/1412R.

Immunofluorescence analysis and PCR showed that HS/1412 and HS/1412 GR isolates were GaHV-2 positive, whereas HS/1412R and HS/1412 GR were REV-positive (Fig. 1). The cultures of the 44 isolates were negative. Cultures of the isolates HS/1412, HS/1412R, and HS/1412 GR were confirmed to be free of ALV and CIAV by PCR and/or ELISA, and the CEF controls were free of GaHV-2, REV, ALV, and CIAV (PCR results shown in Fig. 1 and ELISA results not shown).

3.3. Virulence assays

To verify the pathogenicity of the isolates, a pathogenicity test in 52 chicks was performed using the GaHV-2 isolate HS/1412, REV isolate HS/1412R, and GaHV-2 and REV-co-infected isolate HS/1412 GR, which were all isolated from the same flock. HS/1412 and HS/1412 GR caused disease in all infected chickens, and HS/1412R induced morbidity in 84.6% of the chickens. HS/1412 GR induced mortality and tumor formation in 100% and 76.9% of the chickens, representing significantly higher frequencies than those induced by strains HS/1412 (38.5% and 15.4%, respectively) and HS/1412R (0% and 0%) by the end of the experiment (80 dpc). The mortality and morbidity analysis included 52 chicks (Table 3).

Survival analysis revealed significant effects of the challenge treatments with the isolate HS/1412 GR ($P \leq 0.05$) on the pattern of mortality (Fig. 2). Challenge treatments with isolate HS/1412 GR showed a distinct pattern of sudden mortality between 20 and 40 dpc. The first MD tumor was detected in the third dead chicken, and all of the chicks that died subsequently had clear MD tumors formed by 52 dpc, with most gross tumors found in the proventriculus, followed by the liver, spleen, and kidney. However, HS/1412 and HS/1412R induced clinical signs from approximately 6 weeks of age. Only two chicks in the strain HS/1412-infected group and no chicks in the HS/1412R-infected group died by 80 dpc. Infection with HS/1412 was predominantly characterized by neurological signs, chronic wasting, and later visceral lymphomas, whereas infection with HS/1412R could be predominantly characterized by runting–stunting syndrome.

3.4. Immune organ damage

The body weights and immune organ index of three chickens in each group were calculated and analyzed at 4, 14, and 28 dpc (Fig. 3). The body weights of the HS/1412 GR infection group were much lower than those of the control group ($P \leq 0.05$ for each comparison, Fig. 3A) at 28 dpc, although there was no such difference ($P > 0.05$ for each comparison) among the other groups. HS/1412 GR infection caused severe thymus atrophy (Fig. 3C) and splenic enlargement (Fig. 3D) in some cases. Similar to that with HS/1412 GR, both HS/1412 and HS/1412R infection could also cause severe thymus atrophy compared to that in control chicks at all time points, except for HS/1412R infection at 4 dpc (Fig. 3C); however, splenic enlargement was only observed at 14 dpc (Fig. 3D). These results suggested that HS/1412 GR more seriously affects the growth of chickens and causes more severe immune organ damage than HS/1412 and HS/1412 G.

3.5. GaHV-2 and REV genome load in the chicken spleen

Finally, to evaluate the effects of co-infection on the replication of GaHV-2 and REV in vivo, the GaHV-2 and/or REV genome load in the spleens of HS/1412-, HS/1412R-, and HS/1412 GR-challenged chickens was detected over time by quantitative PCR (Fig. 4). Interestingly, the GaHV-2 loads showed similar trends with no significant differences observed between the HS/1412- and HS/1412 GR-challenged groups at any time point (Fig. 4A). However, the REV load in the spleens of chickens in the HS/1412R-challenged group was higher than that in the HS/1412 GR-challenged group at 4 dpc ($P \leq 0.05$), although no differences were detected at 14 and 28 dpc (Fig. 4B). These results suggested that GaHV-2 co-infection reduces the replication rate of REV only at the early stage of infection (4 dpc), but has no discernible effect on the replication and proliferation of GaHV-2 and REV in the chicken spleen.

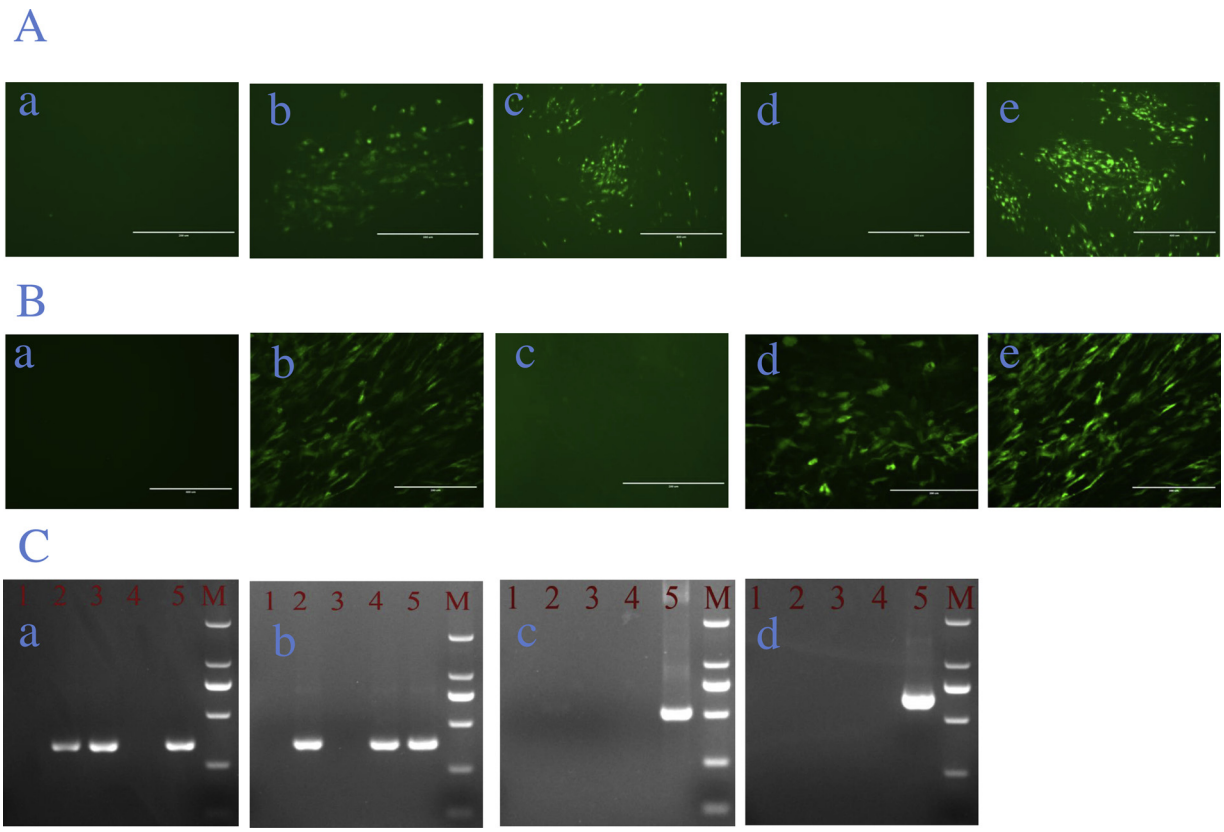

Fig. 1. Identification of the isolates using immunofluorescence assays and PCR. **(A)** Specific staining of the viral plaques from isolates with *Gallid herpesvirus-2* (GaHV-2) gI-specific monoclonal antibody (mAb) observed by fluorescence microscopy. Scale bar: 200 μm. (a) Uninfected chicken embryonic fibroblasts (CEFs), (b) HS14/1412 GR, (c) HS14/1412, (d) HS14/1412R, and (e) GaHV-2 reference strain GA. **(B)** Specific staining of the viral-infected CEF with an *Reticuloendotheliosis virus* (REV) gp90-specific mAb observed by fluorescence microscopy. Scale bar: 200 μm. (a) Uninfected CEFs, (b) HS14/1412 GR, (c) HS14/1412, (d) HS14/1412R, and (e) REV reference strain 07I. **(C)** PCR for (a) GaHV-2, (b) REV, (c) Avian leukosis virus (ALV), (d) Chicken infectious anaemia virus (CIAV); Lane 1: uninfected CEFs, Lane 2: HS14/1412 GR, Lane 3:HS14/1412, Lane 4: HS14/1412R, Lane 5: reference strain (a: GaHV-2 GA, b: REV 07I, c: ALV HLJ09SH01, d:CIAV M9905), M: DL2000 marker.

Table 3
Morbidity and mortality at 80 days post-challenge in the various treatment groups.

Groups	N	Diseased cases (n)	Morbility (%)	Dead cases (*n*)	Mortality (%)	Tumor cases (n)	Tumor incidence (%)
HS/1412 GR	13	13	100	13	100	10	76.9
HS/1412	13	13	100	5	38.5	2	15.4
HS/1412R	13	11	84.6	0	0	0	0
Control	13	0	0	0	0	0	0

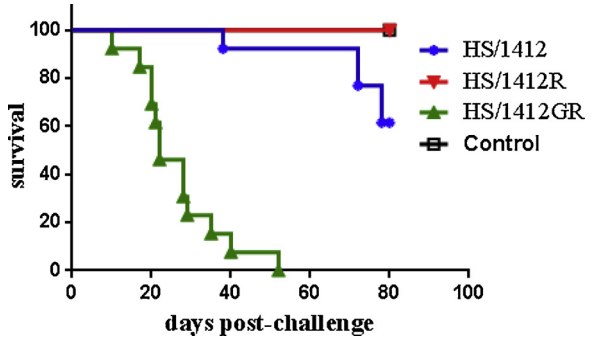

Fig. 2. Survival patterns showing the overall effects of infection with various strains.

4. Discussion

Previous studies have indicated the high prevalence of simultaneous infections by several tumorigenic viruses in chickens (Cui, 2003; Davidson and Borenstein, 1999; Jin et al., 2001; Lupiani et al., 2006; Zhang et al., 2004). For example, the co-occurrence of GaHV-2 and REV genetic material was found in 24 of 25 (96%) isolates examined from Polish chicken flocks, which was attributed to contaminated CVI988/Rispens + HVT vaccine stocks (Wozniakowski et al., 2015). A serological survey showed that 42.6% of broiler breeders from different provinces of China were infected with REV from September 2008 to November 2009 (Cheng et al., 2011). Quantitative PCR detection showed that the positive rate of REV was 59.0% among chickens of Sichuan Province in 2010 (Yue et al., 2010). The LTR region of REV was also shown to be capable of integrating into the GaHV-2 genome when REV-contaminated cell cultures were used to grow GaHV-2 (Isfort et al., 1992; Jones et al., 1996; Kost et al., 1993; Witter et al., 1997). Indeed, chimeric molecules of GaHV-2 and REV fragments can be amplified by

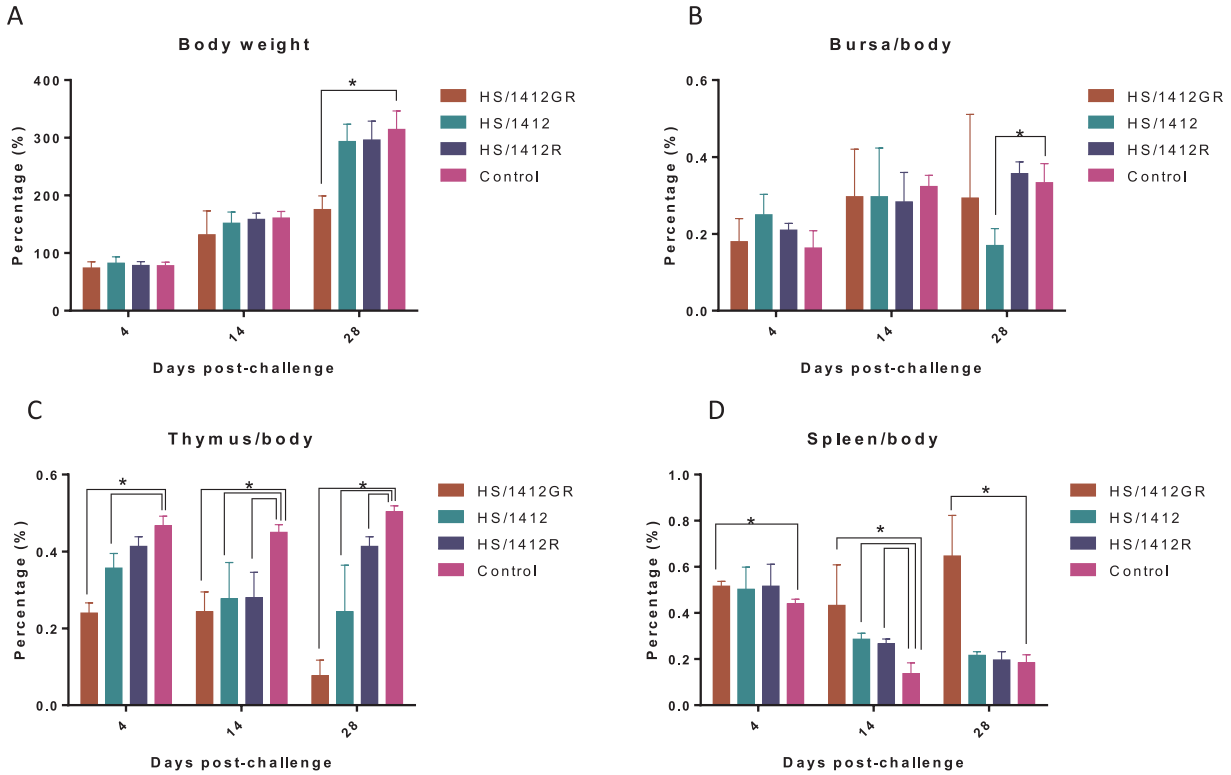

Fig. 3. Body weights and ratios of immune organ weight/body weight in the chickens of each group. The data are shown as mean ± SD (n = 3 per group), and differences were considered statistically significant at $p \leq 0.05$ (*). (**A**) Body weights of the chickens in each group. (**B**) Ratios of bursa weight/body weight in the chickens of each group. (**C**) Ratios of thymus weight/body weight in the chickens of each group. (**D**) Ratios of spleen weight/body weight in the chickens of each group.

PCR from GaHV-2 tumor samples, indicating that genetic recombination between the two viruses can occur in vivo (Davidson and Borenshtain, 2001). In addition, two field strains of GaHV-2 (GX0101 and GD0202) containing the REV LTR insertion fragment were reported (Zhang and Cui, 2005). In the current study, 606 GaHV-2-positive samples were obtained from flocks assessed between 2011 and 2015, covering many of the chicken-raising regions of China, 18.8% of which were co-infected with REV, and 13.0% of the chickens were co-infected in these dual-infected flocks. However, no chimeric molecules representing GaHV-2 and REV fragments could be amplified by PCR from any of the GaHV-2 and REV-co-infected samples. However, we have detected chimeric molecules of fragments of the LCY strain in CEFs, which was isolated from GaHV-2 and REV-co-infected samples (Zhang et al., 2017), indicating that the rate of recombination between the two viruses is very low, or that the recombinant GaHV-2 strains do not have a growth advantage in vivo. Moreover, in our assessed flocks, those co-infected with GaHV-2 and REV had a higher incidence of tumor development, and the time to first death of diseased chickens was earlier than that of flocks solely infected with GaHV-2. This suggests that GaHV-2 might enhance the infection process of REV, or vice versa, and that such co-infections could partially account for outbreaks of neoplastic disease in some farms in China.

In our previous study, the GaHV-2 isolates LCC, LLY, LTS, ZY/1203, WC/1203, WC/1110, ZW/15, and BS/15 exhibited different pathogenic characteristics, and induced mortality rates ranging from 10% to 85.7% in unvaccinated chickens (Sun et al., 2017a, b; Zhang et al., 2015, 2016). In the present study, infection with the GaHV-2 isolate HS/1412 resulted in neurological signs, chronic wasting, and the subsequent

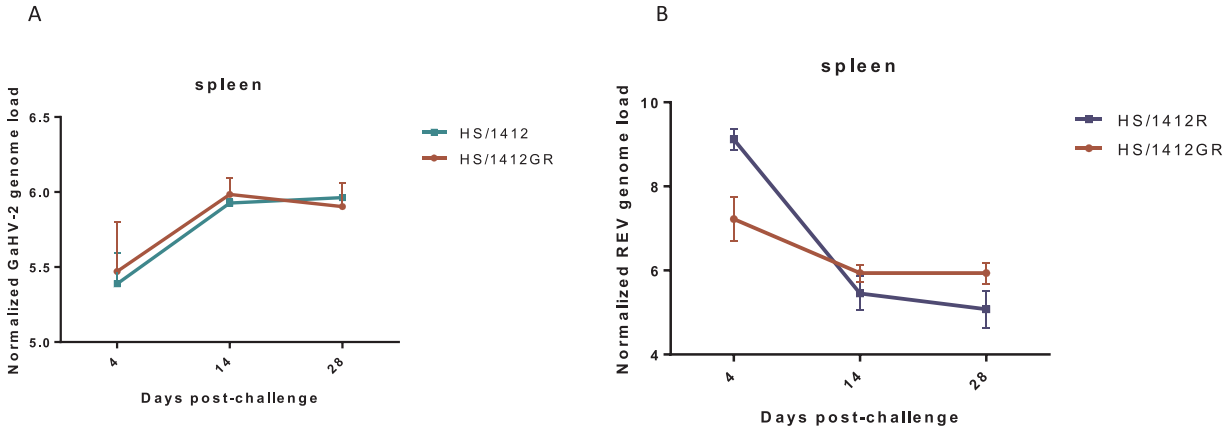

Fig. 4. *Gallid herpesvirus-2* (GaHV-)2 and genome loads of the spleens of challenged chicks. The viral genome loads were calculated as the logarithm of the viral copy numbers per million cells and are shown as mean ± SD. (**A**) Normalized GaHV-2 genome loads; (**B**) normalized *Reticuloendotheliosis virus* (REV) genome loads.

formation of visceral lymphomas, demonstrating similar pathogenic characteristics to the previously reported isolate LCC. However, the GaHV-2 and REV-co-infected isolate HS/1412 GR induced earlier death and a higher tumor rate than the isolate HS/1412 and any of our previous isolates.

HS/1412 GR was isolated from a field diseased chicken co-infected with GaHV-2 and REV, and was only propagated for three passages in CEF cultures; however, this co-infected isolate might lead to similar clinical signs compared to those observed in field diseased chickens. Since the same cells were infected with both viruses, it was difficult to purify GaHV-2 from the co-infected isolate; however, REV purification from the co-infected isolate was possible. In short, after freeze-thawing, the isolate HS/1412 GR was centrifuged and incubated with fresh CEFs for three passages, which could eliminate the presence of GaHV-2. We isolated the GaHV-2 strain HS/1412 from the same flock, and used the REV strain HS/1412R purified from the co-infected isolate HS/1412 GR as the reference virus. The GaHV-2 isolate HS/1412 or REV isolate HS/1412R alone did not or only slightly caused tumor formation and mortality by the end of the experiment, whereas the co-infected isolate HS/1412 GR resulted in significantly higher mortality and tumor rates. Such synergistic pathogenicity between GaHV-2 and REV has been reported previously, and the protective efficacy of existing MD vaccines was shown to be attenuated by co-infection with Chinese field GaHV-2 and REV strains (Sun et al., 2017b).

Overall, these data suggest that co-infection with GaHV-2 and REV occurs in many MD-vaccinated chicken flocks in China, leading to an earlier disease onset and higher morbidity, and indicate that such co-infection were likely to be a main contributor to the sporadic cases of neoplastic diseases in layer and breed flocks in China reported in recent years. Moreover, the co-infected isolate HS/1412 GR was highly pathogenic and induced significantly higher mortality and tumor rates than the GaHV-2 isolate HS/1412 and the REV isolate HS/1412R (both $P \leq 0.05$). The synergetic effects of GaHV-2 and REV co-infection lead to higher mortality and tumor rates in SPF chickens, which could contribute to even more severe losses to the poultry industry in the future.

Declaration of Competing Interest

The authors declare no competing financial interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.108418.

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