

A Highly Pathogenic Marek's Disease Virus Isolated From a Immunized to Bivalent Vaccines Chicken Farm in China

Huimin Li (✉ lihuimin3465@163.com)

Jinggangshan Medical School: Jinggangshan University <https://orcid.org/0000-0002-8177-8513>

Zengxu Ge

Jinggangshan Medical School: Jinggangshan University

Qiong Luo

South China Agricultural University College of Veterinary Medicine

Qiang Fu

Jinggangshan Medical School: Jinggangshan University

Ruai Chen

South China Agricultural University College of Veterinary Medicine

Research Article

Keywords: Marek's disease virus, virulence, pathogenicity, vaccine efficacy

Posted Date: September 28th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-933666/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Marek's disease virus (MDV) is an important oncogenic poultry pathogen that can generally be controlled by vaccination. However, MDV still occasionally occurs on vaccinated farms owing to possible genetic variation among MDV strains as well as management-related issues. In this study, a novel MDV strain (designated LZ1309) was isolated from a poultry flock that had been previously vaccinated using the HVT plus CVI988 vaccine strains. Animal experiments showed that LZ1309 infection led higher morbidity (100%) and mortality (90%). Moreover, existing vaccines only provided partial protection against LZ1309, which protection indexes of HVT, CVI988, and HVT plus CVI988 were 68.4%, 85%, and 90%, respectively. In conclusion, we have shown that the more virulent of Marek's disease virus existed in vaccinated with HVT plus CVI988 in poultry farms in China. And the emergence of LZ1309 poses a new potential threat to poultry farms. In future studies, the development of new treatment strategies should be of high priority.

1. Introduction

Marek's disease (MD) is a highly contagious lymphoproliferative and immunosuppressive disease of chickens and is responsible for significant economic losses to the poultry industry. The causative agent of MD is an oncogenic cell-associated alphaherpesvirus known as Marek's disease virus (MDV). The International Committee on Taxonomy of Viruses classifies MDV-related viruses into three groups: gallid alphaherpesvirus 2 (GaHV-2, also known as MDV-1), GaHV-3 (previously referred to as MDV-2), and meleagrid herpesvirus type 1 (MeHV-1; turkey herpesvirus 1, HVT, previously MDV-3)[1]. GaHV-2 is pathogenic and induces tumours in susceptible chickens, whereas the other two species are non-pathogenic. According to its pathogenicity, GaHV-2 is further classified into four pathotypes: mild (m), virulent (v), very virulent (vv), and very virulent plus (vv+)[2].

MD can be controlled by vaccination, with great efficacy against the development of the disease, resulting in reductions in economic losses to poultry farms since the 1970s[3, 4]. The live vaccines HVT (non-pathogenic MeHV-1), SB-1 (non-pathogenic GaHV-3), 814 (an effective attenuated GaHV-2 vaccine developed and used in China), and CVI988 (an effective attenuated GaHV-2 vaccine used commercially worldwide) as well as the bivalent vaccines HVT plus SB-1 and HVT plus CVI988 are some of the vaccines that have been used worldwide on poultry farms[5]. Although these vaccines protect against tumours and mortality and provide lifelong immunity, vaccination does not induce sterile immunity, allowing for the replication and shedding of virulent challenge MDVs in chickens[6, 7]. Due to complicated interactions among the pathogens, vaccines, and hosts, the virulence of field MDV strains has increased, with viruses acquiring the ability to overcome the immune responses induced by the vaccines[8]. The emergence of more virulent MDV variants may have devastating consequences for the poultry industry in the future. Thus, it is necessary to conduct surveillance studies to assess the prevalence of MDV on commercial poultry farms and develop more sustainable strategies against MDV in a timely manner.

In China, the vaccines HVT, CVI988, and 814 and the bivalent vaccine HVT plus SB-1 have been widely used on laying and breeding chicken farms for a few decades. However, MD outbreaks frequently occur in several provinces in China, even among chickens that have been inoculated with the commercial HVT plus CVI988 vaccines. Recently, a MDV strain isolated from a commercial layer farm vaccinated with HVT plus CVI988 in Colombia clusters with other vv + MDV strains by phylogenetic analysis and has similar characteristics as found in the proline rich region of Meq gene[9]. Nevertheless, no field MDV strains have yet been reported from chicken farms vaccinated with HVT plus CVI988 in China.

Here, we isolated a highly virulent MDV strain (LZ1309) from a layer flock in the Gansu province of China that had received the HVT plus CVI988 vaccine. This represents the first instance of the isolation of a highly virulent MDV from chickens vaccinated with HVT plus CVI988 in China. Thus, the primary purpose of this study was to explore the pathogenic characteristics of the novel isolated MDV strain, the causes of MD vaccination failure, and the evolution of MDVs. Moreover, to gain a comprehensive understanding of the pathogenicity of the LZ1309 strain, our results indicate that a highly virulent MDV with the ability to override the immunoprotection of HVT plus CVI988, which is presently the most effective vaccine combination, is emerging in China. This study provides a basis for future investigations into more effective MDV prevention and treatment measures.

2. Materials And Methods

2.1 Ethics statement

The animal experiments and study protocol were approved by South China Agriculture University's Institutional Animal Care and Use Committee and performed in accordance with approved animal care guidelines and protocols. The animal ethics committee approval number is SYXK (Yue) 2014 – 0136.

2.2 Clinical samples and virus isolation

Suspected MD chickens were identified in a commercial Hyline-brown chicken flock that had been previously vaccinated at 1 day of age with HVT plus CVI988 vaccines. The infected chickens showed depressed activity, progressive emaciation, and decreased egg production rates of 60%. Death and culling rates in this flock increased by 40%, and at necropsy, the livers and spleens showed visible tumours. Heparin anticoagulated peripheral blood samples were collected from the diseased chickens for virus isolation. Tumour samples were collected from livers and spleens and stored at 4°C for transport.

Primary CEF cells were prepared from 10-day-old SPF chicken eggs purchased from Guangdong Wens Dahuanong Biotechnology (Guangzhou, China). Lymphocytes were purified using a commercial lymphocyte-separating medium and were inoculated into CEF monolayers. Then, the inoculated CEF cells were cultured at 37°C in a 5% CO₂ humidified atmosphere for 120 h. Clear CPEs were observed followed by three blind passages. After plaque purification and five passages in CEF cells, the purified virus LZ1309 was stored in 20% foetal bovine serum and 10% dimethyl sulfoxide (DMSO) in liquid nitrogen.

2.3 Virus identification

PCR amplification of an MDV-specific 132-bp repeat sequence was used to distinguish LZ1309 from vaccine strains[10]. The serotype of LZ1309 was identified by indirect immunofluorescence using the GB2 monoclonal antibody (prepared and stored in our laboratory) comprised of the gB protein-specific antibody of MDV serotype 1. PCR was used to detect exogenous viruses including ALV, CIAV, and REV (Table 1)[11, 12].

Table 1
Primers for Marek's disease virus (MDV) genes and for MDV, ALV, REV and CIAV detection

| Virus/Gene | Primers | Sequence | Product length |
|------------|---------|-------------------------------|----------------|
| MDV | F1 | 5'- ATGCGATGAAAGTGCTATGGAG-3' | 264bp |
| | R1 | 5'-ATCCCTATGAGAAAGCGCTTGA-3' | |
| ALV | F2 | 5'-GGATGAGGTGACTAAGAAAG-3' | 545bp |
| | R2 | 5'-CGAACCAAAGGTAACACACG-3' | |
| REV | F3 | 5'-GCCTTAGCCGCCATTGTA-3' | 300bp |
| | R3 | 5'-CCAGCCAACACCACGAACA-3' | |
| CIAV | F4 | 5'-GCATTCCGAGTGGTTACTATT-3' | 843bp |
| | R4 | 5'-CGTCTTGCCATCTTACAGTCTT-3' | |
| Meq | F5 | 5'-ATAGACGATGTGCTGCTGAGAG-3' | 1020bp |
| | R5 | 5'-CTTCAATACTTTCCGGTCTGTG-3' | |
| pp38 | F6 | 5'-TCATCTTCAACCCACAGCCATCC-3' | 873bp |
| | R6 | 5'-TCGCTTAATCTCCGCCTCCAAC-3' | |
| vIL-8 | F7 | 5'-GAGACCCAATAACAGGGAAATC-3' | 738bp |
| | R7 | 5'-TAGACCGTATCCCTGCTCCATC-3' | |

2.4 Virulence study of LZ1209 strain

One-day-old White Leghorn SPF chicks (n = 207) were obtained from Guangdong Wens Dahuanong Biotechnology and randomly divided into nine groups. The birds were fed and watered ad libitum and housed separately in negative-pressure isolators. The chickens of group 3 and group 7 were vaccinated with HVT (FC126 strain) while those of group 4 and group 8 were inoculated with CVI988 by intraperitoneal injection of 2000 plaque forming units (PFU) on day 1. The birds of group 5 and group 9 were vaccinated with HVT (FC126 strain) plus CVI988 at 2000 PFU simultaneously on day 1. Groups 2 and group 6 were unvaccinated. At 10 days old, the chickens of groups 2–5 were challenged with 2000 PFU of LZ1309 in 0.2 ml while those of group 6–9 received the vv MDV strain Md5 at the same dose.

Inoculation and infection were both via intraperitoneal injection. Chickens in the control group 1 were inoculated with 0.2 ml of DMEM as the control group.

Clinical symptoms, gross post-mortem lesions, and mortality rates were observed and recorded every day until day 90, when all chickens were humanely sacrificed. Three chickens were randomly selected from each group, and immune organ indexes [organ index = organ weight (g) / body weight (kg)] were measured after euthanasia at 35 days post-infection (dpi). Organs from diseased or dead chickens with pathological changes were diagnosed by PCR, and those without macroscopic lesions were assessed via histopathological observation.

2.5 Statistical analyses

Survival rate curves were drawn using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, USA). Protective indices were calculated using the following formula: Protective index (PI) = (% MD in unvaccinated chickens – % MD in vaccinated chickens) / % MD in unvaccinated chickens × 100 [13]. All data are presented as the mean ± standard deviation (SD). One-way ANOVA was employed to evaluate statistical differences among groups using GraphPad Prism 6 software. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Isolation and identification of MDV strain LZ1309

Local outbreaks of MDV were reported from 2010 to 2016, and thus the active surveillance of MDV on chicken farms was undertaken nationwide in China. Suspected MD with visceral tumours was observed on a large-scale poultry farm in Lanzhou in the Gansu province of China. Subsequently, an MDV strain named LZ1309 was isolated from diseased chickens. PCR detection of the MDV 132-bp repeat (Fig. 1A) showed that the LZ1309 strain carries two copies of the repeat, consistent with the unique molecular characteristics of pathogenic MDV strains. Chicken embryo fibroblast (CEF) cultures inoculated with LZ1309 exhibited typical cytopathic effects (CPEs) under a light inverted microscope, in agreement with those induced by MDV infection (Fig. 2A). CPEs were also confirmed via immunofluorescence assay using monoclonal antibodies specific for the gB protein of GaHV-2 (produced by our laboratory) (Fig. 2C), and no CPEs were observed in uninfected CEF cultures under a fluorescence microscope (Fig. 2B and 2D). In addition, the virus preparation tested negative for the adventitious agents avian leukosis virus (ALV), reticuloendotheliosis virus (REV), and chicken infectious anaemia virus (CIAV) based on PCR analysis (Fig. 1B–D).

3.2 Pathogenicity of LZ1309 isolate in chickens

3.2.1. Survival and mortality analysis

We performed virulence studies by infecting nine groups of healthy, specific pathogen-free (SPF) chickens with cell culture-purified virus. The chickens of group 3 and group 7 were vaccinated with HVT (FC126 strain), while those of group 4 and group 8 were inoculated with CVI988, and group 5 and group 9 were vaccinated with HVT (FC126 strain) plus CVI988. Groups 2 and group 6 were not vaccinated. Then, groups 2–5 were challenged with LZ1309, while group 6–9 received the vv MDV strain Md5. Group 1 was inoculated with Dulbecco's modified Eagle's medium (DMEM) as a control.

There were no clinical signs of infection observed in any of the nine experimental groups during the first 30 days, except for one bird that died before day 6 due to a quality problem acknowledged by the supplier. The incidence of infection in group 2 using the LZ1309 strain was 100%, and the mortality reached 90% (Table 2). In comparison, both the morbidity and mortality of the chickens in group 6, infected with the vv MDV Md5, were 100% at 64 dpi. The earliest deaths appeared at 34 and 14 days post-challenge in chickens infected with LZ1309 and Md5, respectively. The mortality peaks occurred on days 71–88 and 54–64 post-challenge in groups infected with the LZ1309 and Md5 strains, respectively (Fig. 3). The results showed LZ1309 had almost as high pathogenicity as Md5 strain, but the mortality peak was much later than Md5.

Table 2
 Statistics of chickens challenged with MDV strains (n = 20) at 90 days post-challenge

| Groups | Vaccine strain | Challenge strain | N | MD cases(n) | Dead cases(n) | PI ¹ | Tumor cases (n) |
|--------|----------------|------------------|----|--------------|---------------|-----------------|-----------------|
| | | | | Morbidity | Mortality | | Tumor Incidence |
| 1 | None | None | 20 | 0 | 0 | 0 | 0 |
| 2 | None | LZ1309 | 20 | 20/20 (90%) | 18/20 (90%) | - | 5/20 (25%) |
| 3 | HVT | LZ1309 | 19 | 6/19 (31.6%) | 2/19 (10.5%) | 68.4% | 2/19 (10.5%) |
| 4 | CVI988 | LZ1309 | 20 | 3/20 (15%) | 1/20 (5%) | 85% | 0/20 (0%) |
| 5 | HVT + CVI988 | LZ1309 | 20 | 2/20 (10%) | 0/20 (0%) | 90% | 0/20 (0%) |
| 6 | None | Md5 | 20 | 20/20 (0%) | 20/20 (100%) | - | 9/20 (45%) |
| 7 | HVT | Md5 | 20 | 8/20 (40%) | 2/20 (10%) | 60% | 2/20 (10%) |
| 8 | CVI988 | Md5 | 20 | 5/20 (25%) | 0/20 (0%) | 75% | 0/20 (0%) |
| 9 | HVT + CVI988 | Md5 | 20 | 1/20 (5%) | 0/20 (0%) | 95% | 0/20 (0%) |

Note: ¹ PI, protective index.

3.2.2 Vaccine protection index and tumour incidence

The vaccine protection indices for the groups challenged with strain LZ1309 after vaccination with HVT, CVI988, and HVT plus CVI988 were 68.4%, 85%, and 90%, while as for Md5 were 60%, 75%, 95%, respectively. Post-mortem examination of the chickens in the positive-control LZ1309 group (group 2) identified tumours in the liver, spleen, and heart. The incidences of tumours in chickens infected with LZ1309 and Md5 were 25% and 45%, respectively (Table 2). The CVI988 vaccine prevented the LZ1309 and Md5 strains from causing tumours, but the HVT vaccine did not. Two infected-LZ1309 birds vaccinated with HVT died from organ tumours.

3.2.3 Immune Organ Damages of LZ1309-infected chickens

The body weights and immune organ indexes of three chickens randomly selected from each group were measured and analyzed at 35 dpi. Compared with control group, both LZ1309 and Md5 strain could significantly reduce body weight infected-chickens ($p < 0.001$). There was no difference of the body

weight between LZ1309 and Md5 group ($p > 0.05$). The results of indexes of immune organs showed that both LZ1309 and Md5 could induce bursal and thymic atrophy more severely than control group ($p < 0.001$). Moreover, LZ1309 and Md5 caused severe spleen enlargement compared to the control group ($p < 0.01$, $p < 0.001$, respectively) (Fig. 4). The results indicated that LZ1309 strain could affect the growth and development of chickens, as well as the damages of immune organ induced severe immunosuppression.

3.2.4 Histopathology

The pathological results of the unvaccinated LZ1309 group indicated the presence of focally distributed lymphocytic proliferation in the liver tumours. The red and white pulp of the spleen edges was not sharply demarcated, and there was atrophy in the white pulp, which in some cases was completely absent and replaced by a diffuse distribution of lymphocytes. In the heart tissue, lymphocyte proliferation was observed between myocardial fibres. The cortex of the bursa of Fabricius was reduced or disappeared. Necrosis of the medullary cells formed a cavity, with shrunken follicles and decreased numbers of lymphocytes (Fig. 5).

4. Discussion

Highly virulent MDVs causing tumours and death have been frequently reported in chicken flocks immunized with HVT, HVT plus SB1, or CVI988 in Japan, Australia, Turkey, the USA[14–17]. Recently, UDEACO-2013 strain clustered with other vv + MDVs according to phylogenetic analysis, was reported from a poultry farm in Colombia that had vaccinated chickens with the MeHV-1 strain FC126 and GaHV-2 Rispens strain[9]. However, in China, MDVs isolated from chicken flocks immunized with HVT plus CVI988 have not previously been reported. In this study, a highly virulent MDV designated LZ1309 was first isolated from a chicken flock vaccinated with HVT plus CVI988 from Gansu Province, China.

The gold standard of Avian Disease and Oncology Laboratory (ADOL) method for determination of pathotype is based on the induction of lymphoproliferative lesions in vaccinated chickens[18]. Its limitations include requirements for a specific type of chickens, large numbers of animals, and a statistical method to compare which is not conducive to be used in other laboratories. A modification of the ADOL method that utilizes local SPF chickens and comparisons with prototype strains is proposed to evaluate the pathogenicity and vaccine efficacy of field MDV isolates[2]. By this method, we found the morbidity and mortality of LZ1309 were 100% and 90% respectively in unvaccinated SPF chickens at 90 dpi. These results were similar to those found with the vv MDV strain Md5, in which both the morbidity and mortality were 100% under the same experimental conditions at 64 dpi. Compared with Md5, LZ1309 had a longer latent infection period, with the first death occurring at 35 dpi. The mortality peak at 71–88 dpi was much later than that of Md5 at 54–64 dpi, but was similar to those of strains SD2012-1 at 60–85 dpi and BS/15 at 63–84 dpi[19, 20]. The tumour incidence of LZ1309 was 25% which was lower than Md5 strain. Tumours in LZ1309-challenged chickens were most commonly found in the liver, heart, and spleen and it was confirmed by histopathological sections. Moreover, LZ1309 caused more severe

atrophy of the immune organs, especially in the thymus and bursa which was more likely to induce immunodepression seriously.

The protection indices of HVT, CVI988, and HVT plus CVI988 against LZ1309 challenge were 68.4%, 85%, and 90%, respectively. This indicates that the HVT vaccine does not provide the ideal protective effect. CVI988 vaccines provide fairly well protection against LZ1309 with an index of 85% and the HVT plus CVI988 vaccine provides even better protection with an index of 90% under experimental condition. However, this is not a negligible percent of vaccine protection rates and it is expected to be decreased under field condition.

Previous studies have suggested that virulences among field MDV strains are diverse, and several newly isolated MDV strains from China and other countries have obtained the ability to counter vaccines[21–23]. Existing commercial vaccines do not provide complete protection against LZ1309 and other MDV strains, indicating that these viruses are most likely still spreading on farms and may cause greater economic losses to the poultry industry. According to the trade-off hypothesis, which focuses on whether there is a transmission–virulence relationship in a particular disease interaction, vaccination can select for higher virulence[24]. Therefore, epidemiological investigations and constant MDV monitoring is important. Moreover, it is necessary to develop better preventive measures and establish a healthier feeding environment in order to reduce chicken morbidity and mortality on these farms.

5. Conclusions

In conclusion, we isolated a highly virulent MDV strain, LZ1309, from an HVT plus CVI988 vaccine-immunized chicken flock in China. Animal experiments showed that the virulence of LZ1309 was similar to that of the reference vv MDV strain Md5, with morbidity and mortality rates of 100% and 90%, respectively. The commercial vaccines CVI988 and HVT plus CVI988 provided effective protection against LZ1309 at 85% and 90%, respectively. The most important MD preventive measures are the monitoring of MD occurrence in real time and the development of strict immunization procedures.

Declarations

Acknowledgments: This study was funded by the Educational Department of Jiangxi Province (Grant No. GJJ190568).

Author Contributions: Huimin Li and Zengxu Ge contributed equally to this work. All authors reviewed and improved the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Osterrieder N, Kamil JP, Schumacher D, Tischer BK, Trapp S (2006) Marek's disease virus: from miasma to model. *Nat Rev Microbiol* 4:283–294
2. Witter RL, Calnek BW, Buscaglia C, Gimeno IM, Schat KA (2005) Classification of Marek's disease viruses according to pathotype: philosophy and methodology. *Avian Pathol* 34:75–90
3. Nair V (2004) Successful control of Marek's disease by vaccination. *Dev Biol (Basel)* 119:147–154
4. Witter RL (2001) Protective efficacy of Marek's disease vaccines. *Curr Top Microbiol Immunol* 255:57–90
5. Schat KA (2016) History of the First-Generation Marek's Disease Vaccines: The Science and Little-Known Facts. *Avian Dis* 60:715–724
6. Atkins KE, Read AF, Savill NJ, Renz KG, Islam AF, Walkden-Brown SW, Woolhouse ME (2013) Vaccination and reduced cohort duration can drive virulence evolution: Marek's disease virus and industrialized agriculture. *Evolution* 67:851–860
7. Read AF, Baigent SJ, Powers C, Kgosana LB, Blackwell L, Smith LP, Kennedy DA, Walkden-Brown SW, Nair VK (2015) Imperfect Vaccination Can Enhance the Transmission of Highly Virulent Pathogens. *Plos Biol* 13:1–18
8. Boodhoo N, Gurung A, Sharif S, Behboudi S (2016) Marek's disease in chickens: a review with focus on immunology. *Vet Res* 47:119–138
9. Lopez-Osorio S, Piedrahita D, Espinal-Restrepo MA, Ramirez-Nieto GC, Nair V, Williams SM, Baigent S, Ventura-Polite C, Aranzazu-Taborda DA, Chaparro-Gutierrez JJ (2017) Molecular characterization of Marek's disease virus in a poultry layer farm from Colombia. *Poult Sci* 96:1598–1608
10. Silva RF (1992) Differentiation of pathogenic and non-pathogenic serotype 1 Marek's disease viruses (MDVs) by the polymerase chain reaction amplification of the tandem direct repeats within the MDV genome. *Avian Dis* 36:521–528
11. Chacon RD, Astolfi-Ferreira CS, Guimaraes MB, Torres LN, De la Torre DI, Sa LRM, Piantino Ferreira AJ (2019) Detection and Molecular Characterization of a Natural Coinfection of Marek's Disease Virus and Reticuloendotheliosis Virus in Brazilian Backyard Chicken Flock. *Vet Sci* 6:1–14
12. Li Y, Wang Y, Fang L, Fu J, Cui S, Zhao Y, Cui Z, Chang S, Zhao P (2016) Genomic Analysis of the Chicken Infectious Anemia Virus in a Specific Pathogen-Free Chicken Population in China. *Biomed Res Int* 2016:1–5
13. Shamblin CE, Greene N, Arumugaswami V, Dienglewicz RL, Parcels MS (2004) Comparative analysis of Marek's disease virus (MDV) glycoprotein-, lytic antigen pp38- and transformation antigen Meq-encoding genes: association of meq mutations with MDVs of high virulence. *Vet Microbiol* 102:147–167
14. Dunn JR, Black Pyrkosz A, Steep A, Cheng HH (2019) Identification of Marek's disease virus genes associated with virulence of US strains. *J Gen Virol* 100:1132–1139
15. Murata S, Machida Y, Isezaki M, Maekawa N, Okagawa T, Konnai S, Ohashi K (2020) Genetic characterization of a Marek's disease virus strain isolated in Japan. *Virol J* 17:186–199

16. Ozan E, Muftuoglu B, Sahindokuyucu I, Kurucay HN, Inal S, Kuruca N, Elhag AE, Karaca E, Tamer C, Gumusova S, Albayrak H, Barry G, Gulbahar MY, Yazici Z (2021) Marek's disease virus in vaccinated poultry flocks in Turkey: its first isolation with molecular characterization. *Arch Virol* 166:559–569
17. Wajid SJ, Walkden-Brown SW, Vanselow BA, Islam AF, Renz KG (2015) In vivo characterisation of two Australian isolates of Marek's disease virus including pathology, viral load and neuropathotyping based on clinical signs. *Aust Vet J* 93:240–247
18. Witter RL (1997) Increased virulence of Marek's disease virus field isolates. *Avian Dis* 41:149–163
19. Sun GR, Zhang YP, Lv HC, Zhou LY, Cui HY, Gao YL, Qi XL, Wang YQ, Li K, Gao L, Pan Q, Wang XM, Liu CJ (2017) A Chinese Variant Marek's Disease Virus Strain with Divergence between Virulence and Vaccine Resistance. *Viruses* 9:1–11
20. Yu ZH, Teng M, Luo J, Wang XW, Ding K, Yu LL, Su JW, Chi JQ, Zhao P, Hu B, Zhang GP, Liu JX (2013) Molecular characteristics and evolutionary analysis of field Marek's disease virus prevalent in vaccinated chicken flocks in recent years in China. *Virus Genes* 47:282–291
21. Abd-Elattief HA, Abou Rawash AA, Ellakany HF, Goda WM, Suzuki T, Yanai T (2018) Molecular characterization and phylogenetic analysis of a virulent Marek's disease virus field strain in broiler chickens in Japan. *Avian Pathol* 47:47–57
22. Kozdrun W, Stys-Fijol N, Czekaj H, Piekarska K, Niczyporuk JS, Stolarek A (2020) Occurrence of Marek's Disease in Poland on the Basis of Diagnostic Examination in 2015–2018. *J Vet Res* 64:503–507
23. Shi M, Li M, Wang P, Wang W, Li H, Gao Y, Lin L, Huang T, Wei P (2021) An outbreak in three-yellow chickens with clinical tumors of high mortality caused by the coinfection of reticuloendotheliosis virus and Marek's disease virus: a speculated reticuloendotheliosis virus contamination plays an important role in the case. *Poult Sci* 100:19–25
24. Boots M (2015) The Need for Evolutionarily Rational Disease Interventions: Vaccination Can Select for Higher Virulence. *Plos Biol* 13:1–6

Figures

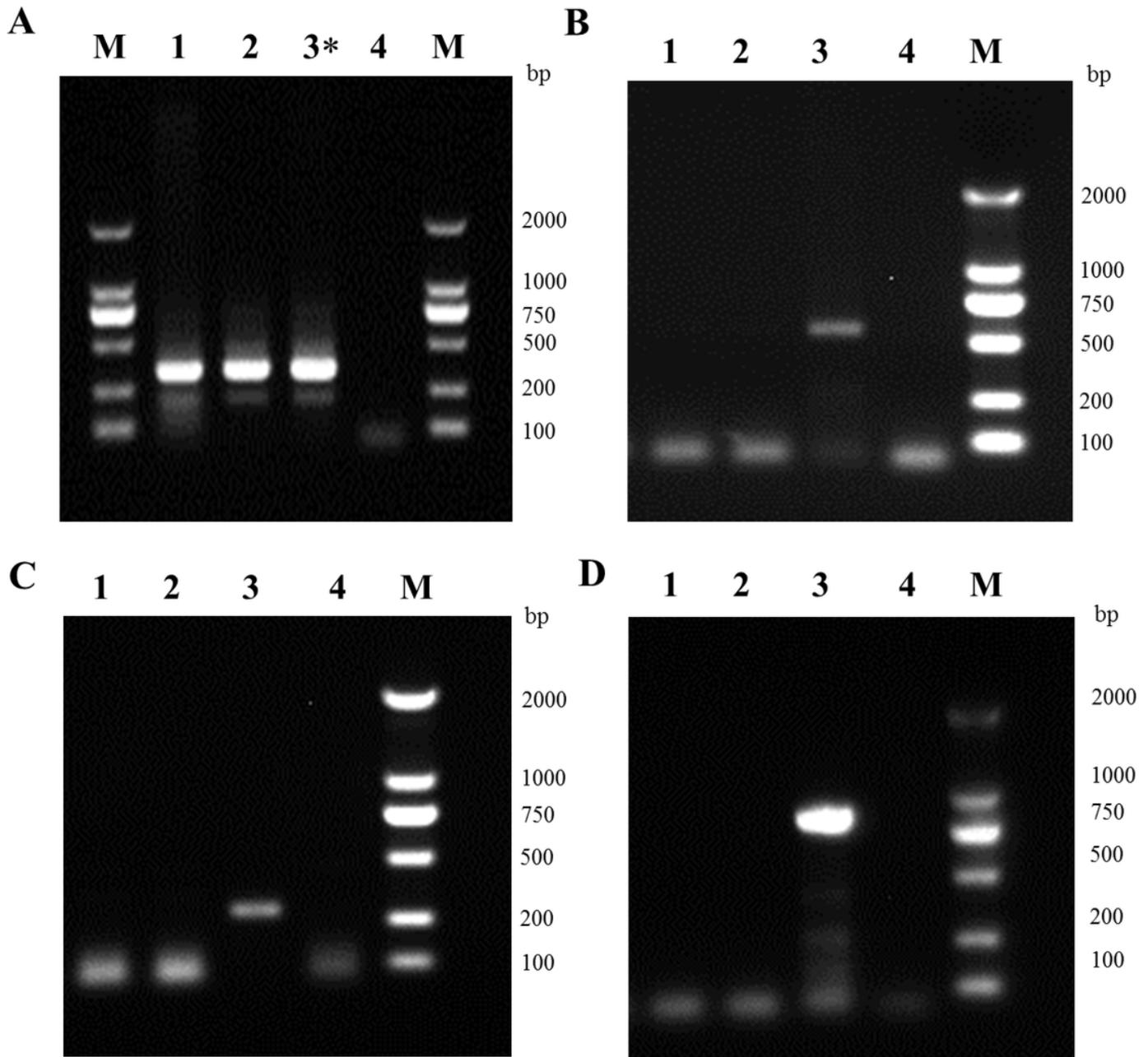


Figure 1

Detection of MDV, ALV and REV CIAV by PCR using primers specific for (A) MDV (B) ALV (C) REV (D) CIAV. Lane M, DL 2000 DNA Marker; Lane 1, Spleen of infected chicken from farm with an MD outbreak; Lane 2, DEF culture infected with MDV LZ1309; Figure 1-A Lane 3*: Md5 a very virulent Positive MDV control with two copies of 132 bp; Figure 1-B-D Lane 3: Positive control for ALV, REV and CIAV, respectively; Lane 4, Negative control, CEF culture without infection.

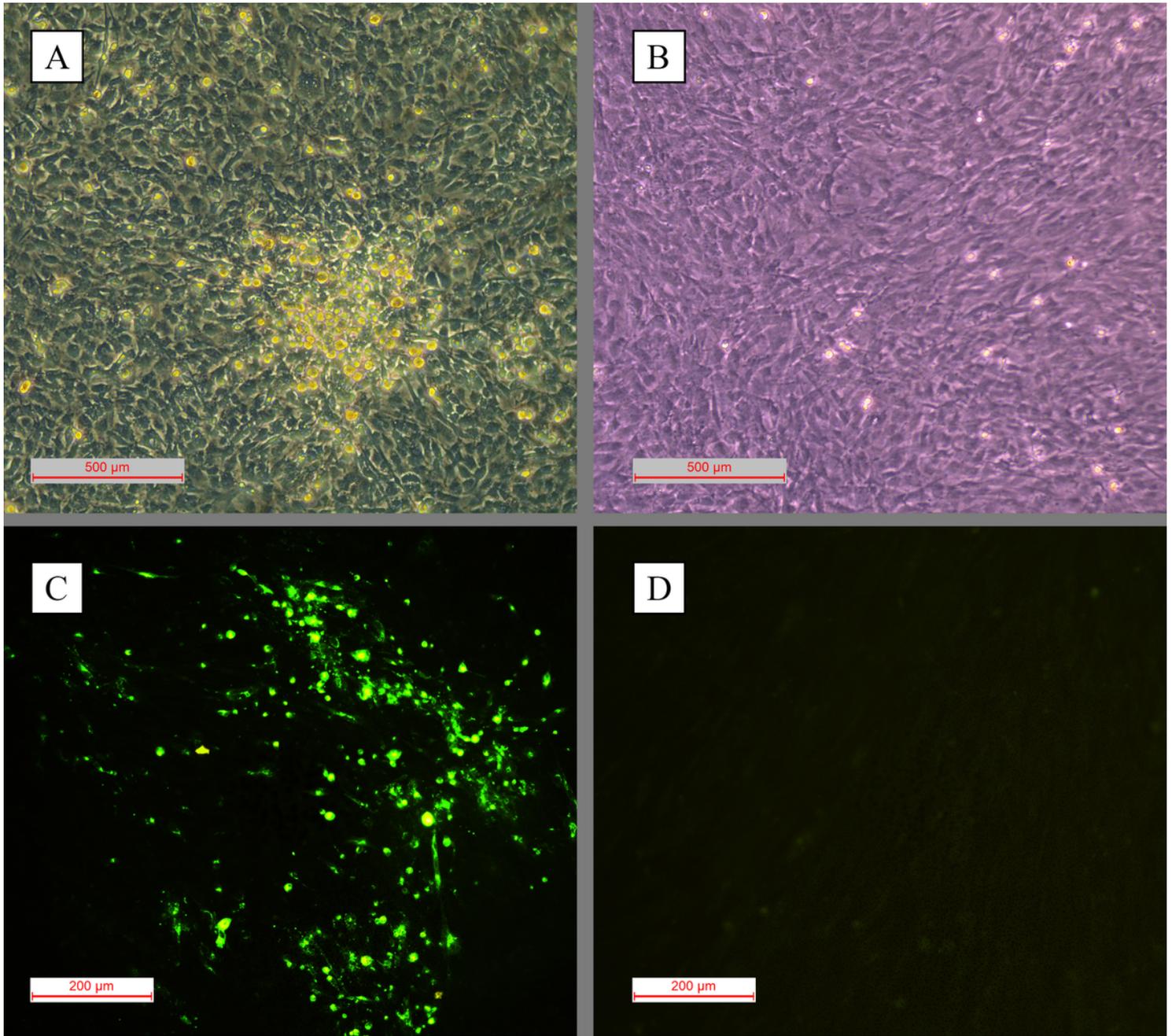


Figure 2

Cytopathogenic effects in CEF cell monolayers infected with LZ1309. (A). Infected cells 0. (B).Uninfected cells 0. (C). Indirect immunofluorescence of infected cells stained with the GB2 monoclonal antibody 0 (D). Indirect immunofluorescence of uninfected cells .

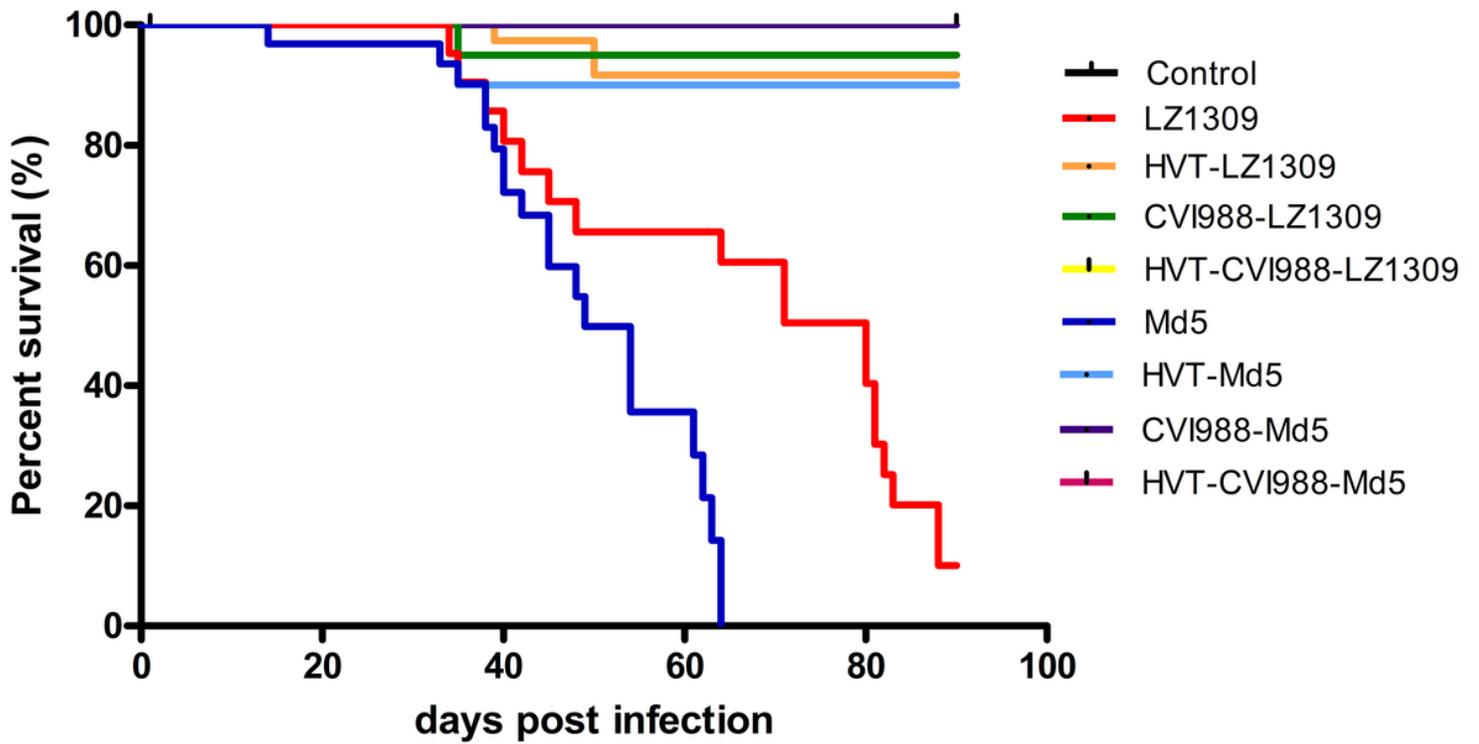


Figure 3

Survival of SPF chickens challenged with virus strains LZ1309 strain, HVT, CVI988 and HVT plus CVI988 vaccines against strains LZ1309, vvMDV Md5 and non-infected control.

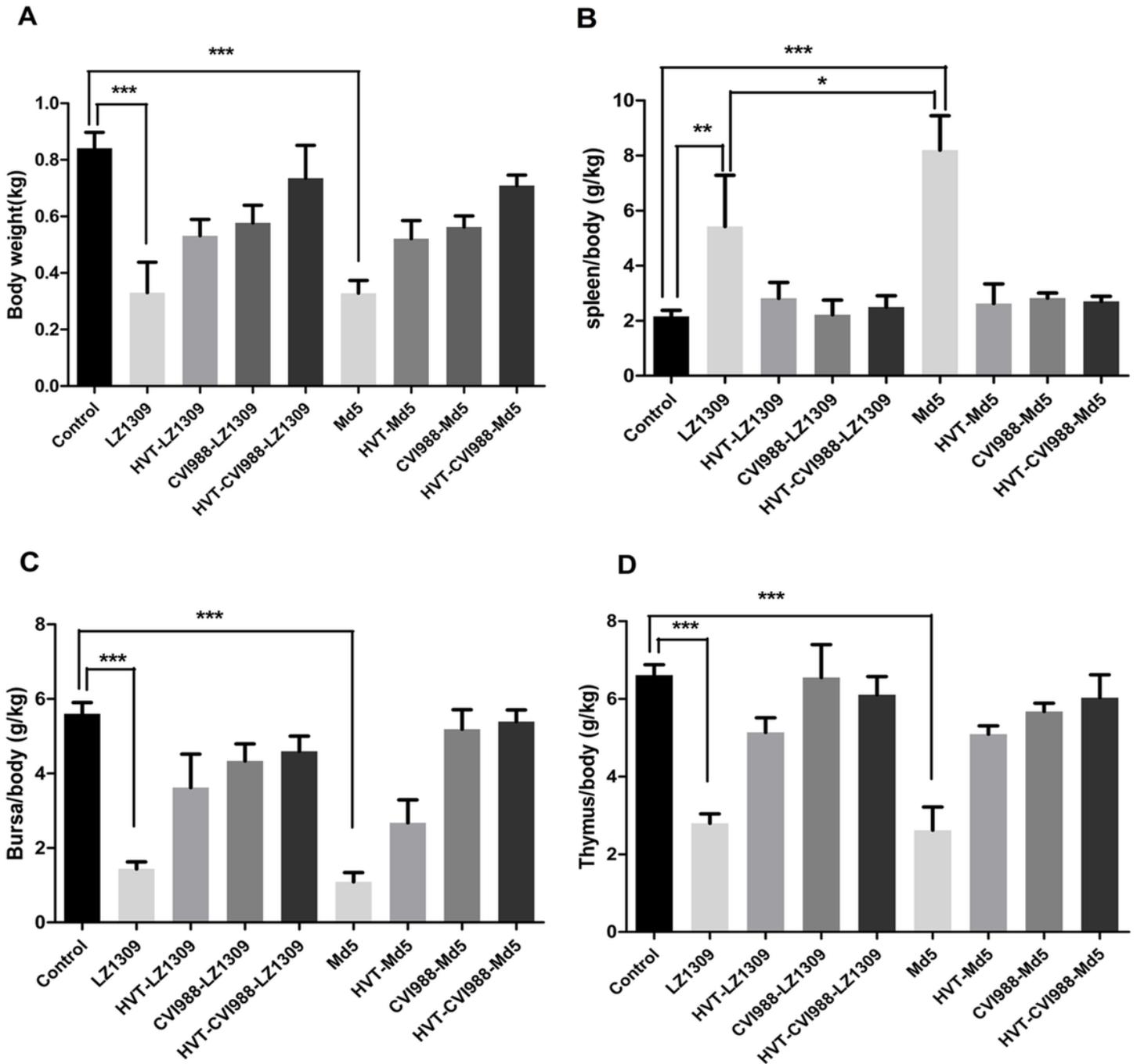


Figure 4

Body weights and immune organ index of three chickens randomly selected from each group at 35 dpi. The data are shown as Mean \pm SD and differences were considered to be statistically significant at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). (A) Body weights (kg) of three chickens in each group; (B) Spleen/body weight (g/kg) of three chickens in each group; (C) Bursa/body weight (g/kg) of three chickens in each group; (D) Thymus/ body weight (g/kg) of three chickens in each group.

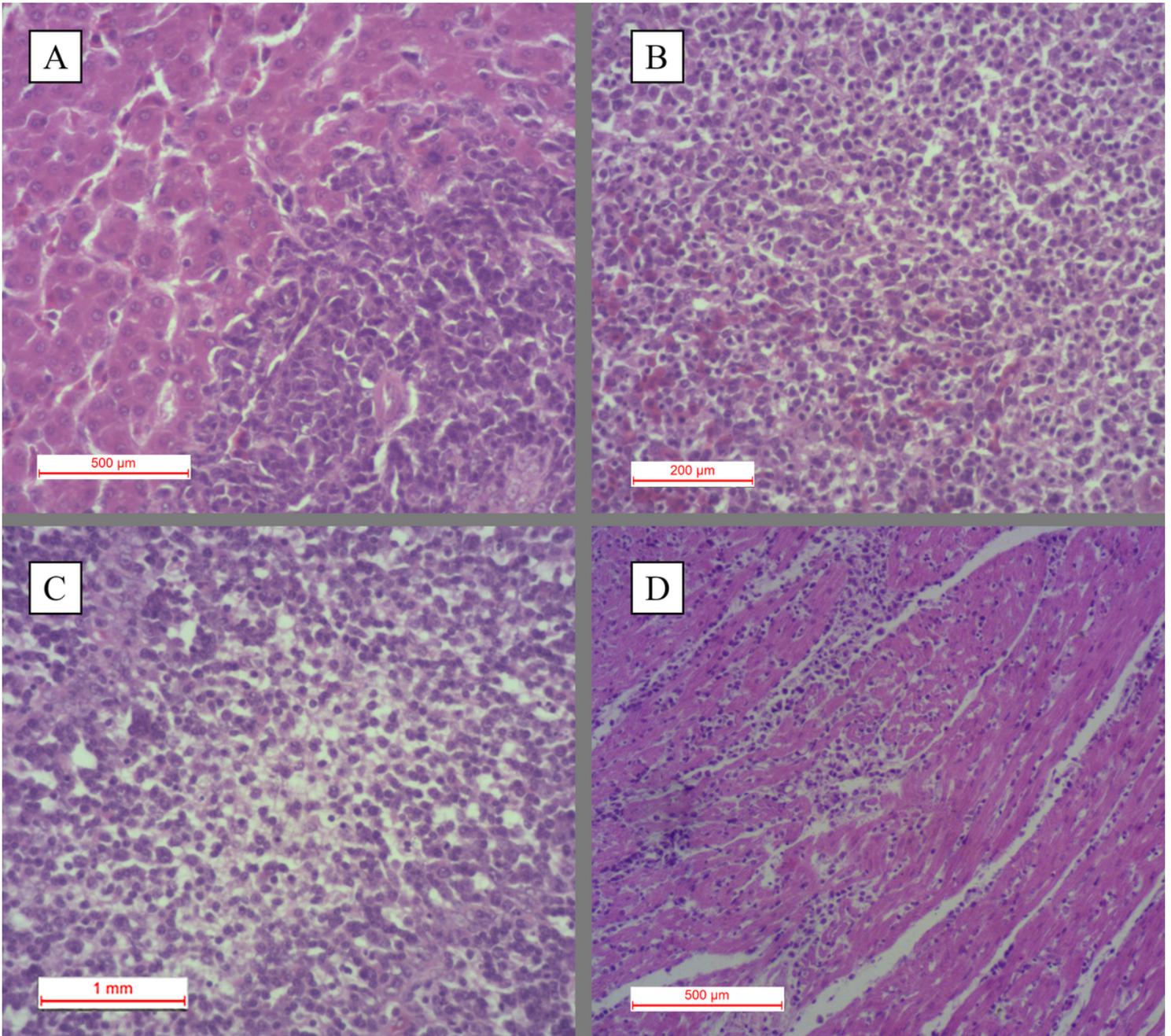


Figure 5

Histopathologic observation of diseased chickens in groups infected with the LZ1309 strain. (A) Liver (100×); (B) Spleen (200×); (C) bursa (40×). H and E staining