Detection of p53 mutation and serum monitoring alert caused by Marek's disease virus

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Abstract

Background Marek's disease (MD), as a chicken neoplastic disease, brings huge economic losses to the global poultry industry. The tumor suppressor gene, wild type P53 plays a key role in blocking cell cycle, promoting apoptosis and maintaining stability of genome. The p53 function could change to that of an oncogene from a tumor inhibitory role, if a mutation happened. It will increase risk of cancer incidence.

Results It was found that the mutation rate of p53 was 60 percent in experimentally infected and naturally infected chickens. The mutations included point-mutation and deletions, and mostly located in the DNA-binding domain. The most common point mutation happened in five sites, which were 651, 786, 828, 864 and 879 respectively. The mutated P53 can be expressed in tumors of various tissues in an infected chicken because of the lengthening of the half-life of mutated P53. Due to the loss of nuclear localization function, most of mutated P53 were expressed in cytoplasm. The concentration of P53 was decrease in serum of MD infected chicken.

Conclusions Results of the current study suggested that p53 mutations with different types were common in MD, and most of mutated P53 were expressed in cytoplasm. Detecting the concertation of P53 and P53 antibody in serum could be helpful for diagnosis and monitor of MD.

Background

Marek's disease (MD) is a lymphoproliferative neoplastic disease caused by chicken Marek's disease virus (MDV or named Gallid alphaherpesvirus 2). It mainly leads to lymphocyte proliferation, tumor formation, immunosuppression, paralysis and mononuclear cell infiltration in peripheral nerves, gonads and immune organs [1, 2]. As one of highly contagious tumor diseases in chickens, it has been reported by about half of the world according to the data of OIE [3] and causes hug losses of up to 1–2 billion US dollars annually to the global poultry industry [4]. Described often as the "guardian of the genome" [5, 6] and the "cellular gatekeeper" [7], p53 is the most relevant and the important tumor suppressor gene with the highest mutation frequency in human and animal tumor diseases, playing a key role in maintaining the stability of genome and preventing tumor [5]. The chicken p53 gene has a full-length open reading frame, 5' and 3' untranslated regions and a polyadenylation signal, which encoded 367 amino acids and had 47% homology to the amino acids of human P53 [8]. It is divided into the wild type and the mutant type. The wild type is a normal tumor suppressor gene, while the mutant p53 cannot regulate cell growth, apoptosis and DNA repair due to spatial conformational change, and eventually transforms from a tumor suppressor gene into an oncogene [9]. Loss of p53 function is often a prerequisite for tumorigenesis and progression, and it has been confirmed that the proportion of p53 mutations in tumor tissues is between 10% and 100% [10].

It was found that p53 has a similar distribution to Meq gene in MD tumor cells [11]. Meq can directly interact with p53, and exogenous expression of Meq inhibits p53-mediated transcriptional activity and apoptosis [12]. Since the half-life of the wild type P53 is very short while the mutant P53 protein is

prolonged, abnormalities of P53 can lead to the accumulation of P53 antibodies in serum and P53 protein in tissues of tumor patients [13]. Several studies have shown that the emergence of P53 antibody in serum is an early event in the development of malignant tumors, which can play a predictive role in tumorigenesis [14, 15, 16]. P53 antibody has a significant correlation with clinical common neoplastic disease, including breast cancer, colon cancer, ovarian cancer and gastric cancer, but it was barely detectable in the serum of healthy people [17, 18, 19]. This correlation might also exist in chicken. However, there is knowledge gap about the role of mutant p53 in chicken Marek's disease.

In this study, chickens with MDV infection were collected and performed to histopathological diagnosis. In order to further analyze the mutation and expression of p53 in MD, immunohistochemistry was used to analyze the expression of P53, and mutations in the p53 were detected. In addition, to determine whether P53 can be used as a tumor marker to assist the clinical diagnosis and prognosis analysis of poultry tumor diseases, we further analyzed the dynamic changes of P53 antigens and antibodies in chicken serum after MDV infection.

Methods

Ethics Approval and Consent to Participate

All animal experiments in this study were approved by the Ethic Committee on the animals of Shandong Agricultural University (SDAUA-2015-012) and performed in accordance with approved animal care guidelines and protocols.

Infection of chickens with MDV

Twenty-one-day old SPF chickens were obtained from Shandong Academy of Agricultural Sciences Poultry Institute SPF Chicken Research Center (Jinan, China). They were randomly divided into two equal groups (10 in each group) and reared separately in isolators with filtered air under positive pressure. At one day of age, 2,000 plaque forming units (PFU) of MDV was inoculated intra-abdominally (i.a.) into each chicken in the infection group. The control group was inoculated with PBS. They were carbon dioxide euthanized at tenth week for next examine.

Sample collection and processing

Ten tissues of liver and spleen were collected from MDV-infected group to extract total RNA for p53 mutation rate analysis. And another ten samples, including liver, spleen, pancreas and bursa of fabricius, were collected all from 160-days laying hens with MD positive. They were fixed with 4% formaldehyde for 48 h for histopathological and immunohistochemical studies.

Histopathology and Immunohistochemical

The fixed liver, spleen, pancreas and bursa of fabricius were dehydrated, waxed, and cut into 3 μm slices, followed by Haematoxylin and eosin (H-E) staining for histopathology observation. In immunohistochemical (IHC) processing, tissues were cut into sections of 3 μm thickness mounted on

microslides treated with 0.1% poly-L-lysine. The sections were deparaffinized in xylene and rehydrated in a graded series of ethanol solutions into PBS, then were pre-treated in citrate buffer (0.01 mol/L; pH 6.0; 100 °C) for antigen retrieval by microwaving and cooled at room temperature for 20 min [20]. Endogenous alkaline peroxidase was quenched with 3% hydrogen peroxide solution in methanol for 60 min. Endogenous alkaline peroxidase was quenched with 3% hydrogen peroxide solution in methanol for 30 min [21]. Nonspecific antibody binding sites were prevented by incubating the sections with 5% fetal bovine serum for 60 min at room temperature. Following this, the anti-P53 polyclonal antibody (BOSTER, China) was diluted into 1:200 as primary antibody and immersed overnight at 4 °C in a black humid chamber. The secondary antibodies were HRP (horse radish peroxidase)-conjugated goat anti-mouse IgG (CWBIO, China). Immunoreactivity was then visualized with diaminobenzidine (DAB) staining. The sections were counterstained with hematoxylin and mounted. Negative controls were incubated with PBS instead of the primary antibody in the immunohistochemical analysis.

Total RNA isolation and transcription

Total RNA of liver and spleen tissue samples (50 mg/tissue) were isolated by using TRIzol reagent (Takara, Japan) following the manufacturer's instructions. Extracted total RNA (1ug) was reverse transcribed to cDNA with PrimeScript™ RT Reagent Kit (Roche, Switzerland). According to the published complementary DNA (cDNA) sequence of the chicken p53 (GeneBank accession number: nm205264), specific primers were designed to amplify the DNA-binding domain (DBD) of p53, which were shown in Table 1. Using PCR (Polymerase Chain Reaction) techniques, p53 cDNA genes sequences about DBD which were mutated mostly were amplification. The PCR reaction was performed by using thermal cycler (Takara, Japan) under the following conditions: 95℃ for 5 min; followed by 35 cycles of 95℃ for 30 s, 56℃ for 30 s, 72℃ for 45 s, and then a final 72℃ for 10 min extension cycle. DNA templates that were acquired from livers and spleens of the MDV positive chickens were amplified using primer pair p53-1/-2. The SPF chicken regarded as negative control. DNA fragments were successfully amplified with sizes of 687 bp. The target fragment was gel extracted and connected to pEASY-T1 vector. Then the recombinant plasmid was transformed in bacteria and made sequencing analysis. Finally, the sequencing results were compared with the wild type p53 cDNA sequences reported previously.

Table 1
Primers used to amplify chicken p53 cDNA

Primer	Nucleotide sequence	Annealing temperature (℃)	Size of fragments amplified(location)
p53-1	5'-GTGGCCGTCTATAAGAAATCAGA-3'	56	687 bp (496–1182)
p53-2	5'-AAAAAGGGGGCGTGGTCAGT-3'		

Enzyme-linked immunosorbent assay (ELISA) for P53 and P53 antibody

Seven serum samples were collected from clinical infected chicken. The other fourteen serum samples were gotten from two groups of experimental animals at 10 weeks of old. The antigen and antibody levels of chicken P53 were monitored by ELISA (mlbio, China). All processes were implemented in accordance with the instructions, and the absorbance (OD) of each sample was finally measured at a wavelength of 450 nm.

Statistical analysis

Statistical analyses were conducted with GraphPad. The Non-paramatic T test was used for statistical analysis of differences about the level of P53 and P53 antibody. Statistical significance was designated as *$p < 0.05$ or ** $p < 0.01$.

Result

Histopathological and Immunohistochemical analysis of P53 expression

Histopathologic examination revealed that the liver cells of infected SPF chicken undergo necrosis; the liver tissues were destroyed and showed focal infiltration and hyperplasia of lymphoid tumor cells with different sizes and shapes (Fig. 1A). The lymphoid follicle in the bursa of fabricius was atrophy to acinar, and the diffuse infiltration and proliferation of lymphoid tumor cells between the follicles were observed (Fig. 1B). In the livers of the clinical infected chickens, the cytoplasm of the lymphoid tumor cells that underwent multifocal proliferation were stained brown by IHC (Fig. 2A). Positive staining was also detected in the tumor cells in the spleen and the bursa of fabricius tissues (Fig. 2B; Fig. 2C). In contrast, no positive stained cells were observed in the control group (Fig. 2D).

Mutations in the p53

In the infected poultry, the mutation rate of p53 was 60 percent (12/20). Two types of mutations, deletions and point mutations (single-point mutations and multiple-point mutations), were detected in the p53, which was consistent with the results reported in previous studies [22]. In the 12 mutated p53 genes, the base sites with high mutation frequency were 651, 786, 828, 864 and 879. No mutation was found in the control group. Most of the mutations located in the core domain and the C-terminal domain. The mutation analysis was shown in Table 2.

Table 2

Mutations of p53 genes in MD

Mutation analysis			
Sample Info	Base mutation sites	amino acid mutations Site from to	Mutation area
NC	ND	ND	
MD (C)	611 T-C; 786 A-C; 828 C-G; 1026 A-G	204 Y A 329 E G	Core domain C-terminal domain
MD (C)	786 A-C; 803 G-A; 828 C-G; 864 C-A	268 R H	Core domain
MD (C)	786 A-C; 828 C-G; 864 C-A	ND	
MD (C)	828 C-G; 864 C-A	ND	
MD (C)	487 deletion; 539 T-C; 653 T-C; 786 A-C; 828 C-G	163 Stop	Core domain
MD (C)	828 C-G; 864 C-A		
MD (E)	631–677 delete; 864 C-A; 879 C-A; 1026 T-G	210 Stop	Core domain
MD (E)	786 A-C; 828 C-G; 864 C-A	ND	
MD (E)	651 G-A; 864 C-A; 879 C-A	ND	
MD (E)	674 A-G; 786 A-C; 828 C-G; 864 C-A; 878 C-T; 1094 G-A	225 N S 293 T I 365 G D	Core domain C-terminal domain
MD (E)	786 A-C; 828 C-G; 864 C-A	ND	
MD (E)	651 G-A; 701 G-T; 804 C-T; 828 C-G; 864 C-A; 907 G-A	234 R L 303 V M 152 E Y 153 H Y	Core domain C- terminal domain

NC was SPF chicken without diseases; (E) was experimental chicken; (C) was clinical chicken.

p53 antigen and p53 antibody levels for MD

The OD value of p53 antigen standard substance was obtained through ELISA detection. The P53 antigen standard curve was formed with the OD value as the Y-axis and the different groups of samples as the X-axis. Then the P53 levels in serum of each group were detected dynamically, statistically analyzed. The result showed that the concentration of P53 of the clinical and experimental MDV-infected group was significantly lower than control group (Fig. 3). The P53 antibody in experimental infection group was significantly higher than control group.

Discussion

It is widely acknowledged that P53 is an intra-nuclear phosphorylated protein, which half-life of wild-type P53 is only 20–30 minutes, and it exists in the nucleus for an extremely short time for hardly detected [23]. Meanwhile mutated P53 is not digested quickly inside tumor cells that has prolonged half-life of 20–40 hours, and therefore accumulates inside tumor cells [24]. In addition, as IHC has become an important indicator of various biomarker detection, P53 IHC is an effective substitute marker for TP53 mutation status [13]. At present, P53 IHC is mainly applied for human tumor diseases, such as ovarian cancer, hepatocellular carcinoma, esophageal cancer, etc., and as an important measure to detect the stage and grade of cancers. But there are few studies in poultry tumor diseases.

In this study, immunohistochemical analysis revealed that P53 was detected clearly in the tumor tissues of liver, spleen and the bursa of fabricius infected with MDV. It is worth noticed that P53 are almost expressed in the cytoplasm, which may be due to the mutation of p53 leading to the loss of nuclear localization function, which eventually caused the mutant P53 overexpressed in the cytoplasm.

It has been known that p53 as the “hotspots”, in which mutations were frequently occurred, is shown in a variety of tumors [25]. Of the 393 codons in the human p53, most mutations in the DBD occur at “mutation hotspots”, which had been found in R175, G245, R248, R249, R273, and R282. Previous studies have revealed that several point mutations were also found in neoplastic diseases of poultry such as MD, avian leukosis, but these mutations were not localized at the “hotspots” of the p53 [26]. The locations and types of p53 gene mutation vary largely in different tumors. The DBD of the p53 is a “hotspot” for mutation, as the majority of tumor-associated mutations in p53 occur within this region. These mutations are mainly point mutations, which are frequently inactivated by base deletions and insertions; majority of p53 mutations are missense mutations [27]. In the current study, p53 was detected to have multiple types of mutations dominated by point mutations in natural and experimental infections of MDV. Through mutational analyses, five base sites with the most frequent mutations were found, which were 651, 786, 828, 864, 879 respectively. And the altered codons compared with wild p53 were 217 (ACG-ACA), 262 (GCA-GCC), 276 (CGC-CGG), 288 (GCC-GCA), 293 (ACC-ACA), which encoded amino acid were synonymous mutations. It is generally believed that proteins are changed followed by gene mutation to participate in the regulation of tumorigenesis and development. However, in this study, p53 mutated at the frequent spots and amino acid occurred synonymy mutation. The amino acid of P53 was not changed.

At the same time, a short form of p53 transcript was detected in clinical cases infected with MDV. The deleted sequence was located at 631–677 bp in the open reading frame of the reference sequence, resulting in a frameshift with formation of missense mutations that would give rise to the ultimately termination of translation in amino acid at position 210. This was another short form of p53 transcript found after Qu’s research [22]. In the present research, it is found that the p53 has high mutation frequency of 60% in poultry oncology, and the mutation region is mainly concentrated in the DBD. The DBD allows the specific recognition of target sequences, at the same time, the central part and the C-

terminal base region comprise the nuclear localization signals, p53 oligomeric domain, and a domain that mediates non-sequence specific interaction of p53 with DNA and RNA [28]. Once the p53 is deleted and point mutations in this region, it may affect the formation of tetramers, which in turn leads to the conversion of wild type p53 into a mutant type and loss of normal function.

The conformation of mutate P53 extends its half-life to several hours, thereby accumulating in the cells. The accumulated mutant P53 acts as a target antigen to elicit an autoimmune response of the body producing serum P53 antibodies [29]. Several studies in human have shown that the production of serum P53 was closely related to the accumulation of P53 in the corresponding tumor. It had been found that the serum P53 concentration in cancer patients was significantly higher than that in healthy testers [30]. But in this study, the serum P53 antibody concentrations of experimental infected group was significantly higher than the control group. Meanwhile, the concentration of P53 in clinical and experimental MDV-infected group was significantly lower than control group, which was different from cancer research in human. This might be that p53, as a tumor suppressor, was largely consumed in response to the occurrence of MD. And then, the tumor promoted mutation of p53 and further reduced P53 concentration.

This research indicates that the p53 in MDV infected chicken trend to mutate, which is consistent with cancer patients. However, P53 antibody concentration in MD-infected chicken's serum did not show significant different from the control group. The concentration of P53 show a decrease in the MD-infected group, which may provide a new idea for the diagnosis and monitoring of MD.

Conclusion

Our results indicated that the mutation and expression of p53 were detected in MDV infection, and suggested that these mutations play a role in the development of MD. The mutant P53 was expressed in the tumor cells of various tissues of the diseased chicken and lost its nuclear localization function which transferred from the nucleus to cytoplasm. The concentration of P53 showed a significant decrease in the group of naturally and experimental infected, providing new ideas for the diagnosis and monitoring of MD.

Abbreviations

cDNA
complementary DNA;
DAB
diaminobenzidine;
DBD
DNA-binding domain;
ELISA
Enzyme-linked immunosorbent assay;
H-E

Haematoxylin and eosin;
IHC
immunohistochemical;
HRP
horse radish peroxidase;
PCR
Polymerase chain reaction;
PFU
plaque forming units.

Declarations

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Availability of data and materials
The data supporting the findings are included in the manuscript.

Authors' contributions

HZ carried out laboratory work, wrote the manuscript and performed the data analyses. ML designed the experiment, wrote the manuscript and performed the data analyses. ZH, SC, YL, SJ and YS carried out laboratory work. SL conceived and supervised this work, revised manuscript. All authors read and approved the final manuscript.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

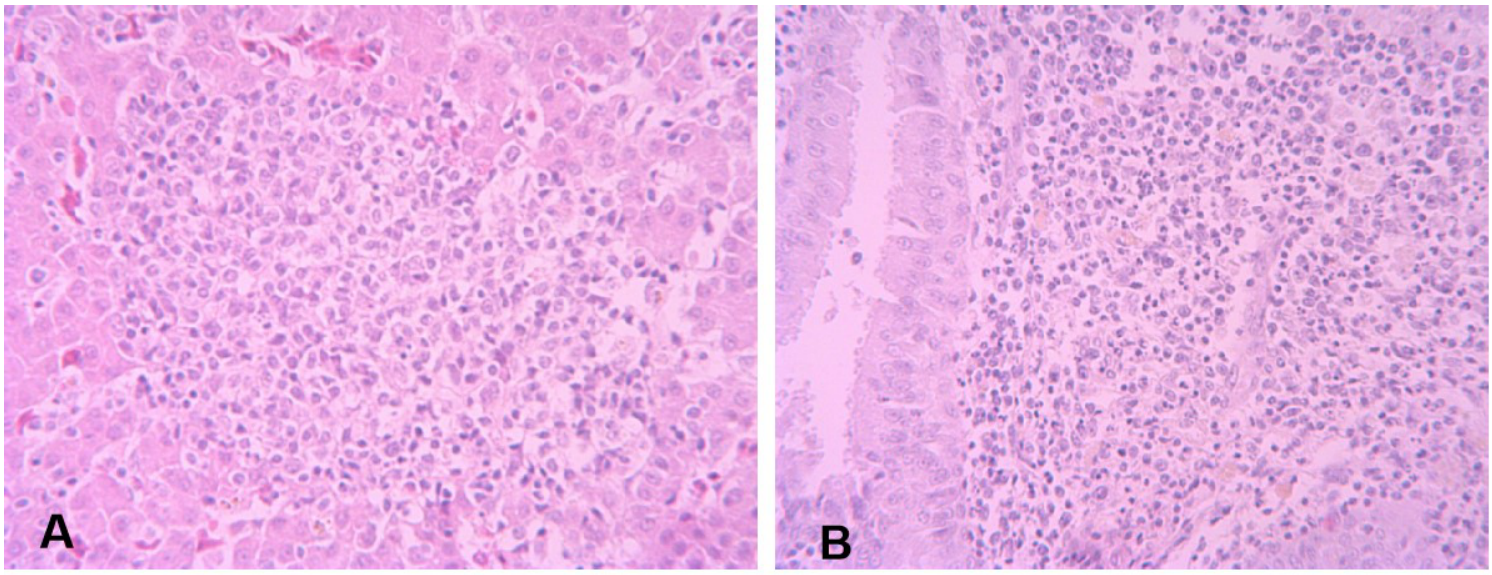

Figure 1

Histopathologic observation of diseased chickens infected with MDV. (A) Focal infiltration and hyperplasia of lymphoid tumor cells with different sizes and shapes were observed in the liver tissues (HE, 400×). (B) The diffuse infiltration and proliferation of lymphoid tumor cells between the follicles were observed (HE, 200×).

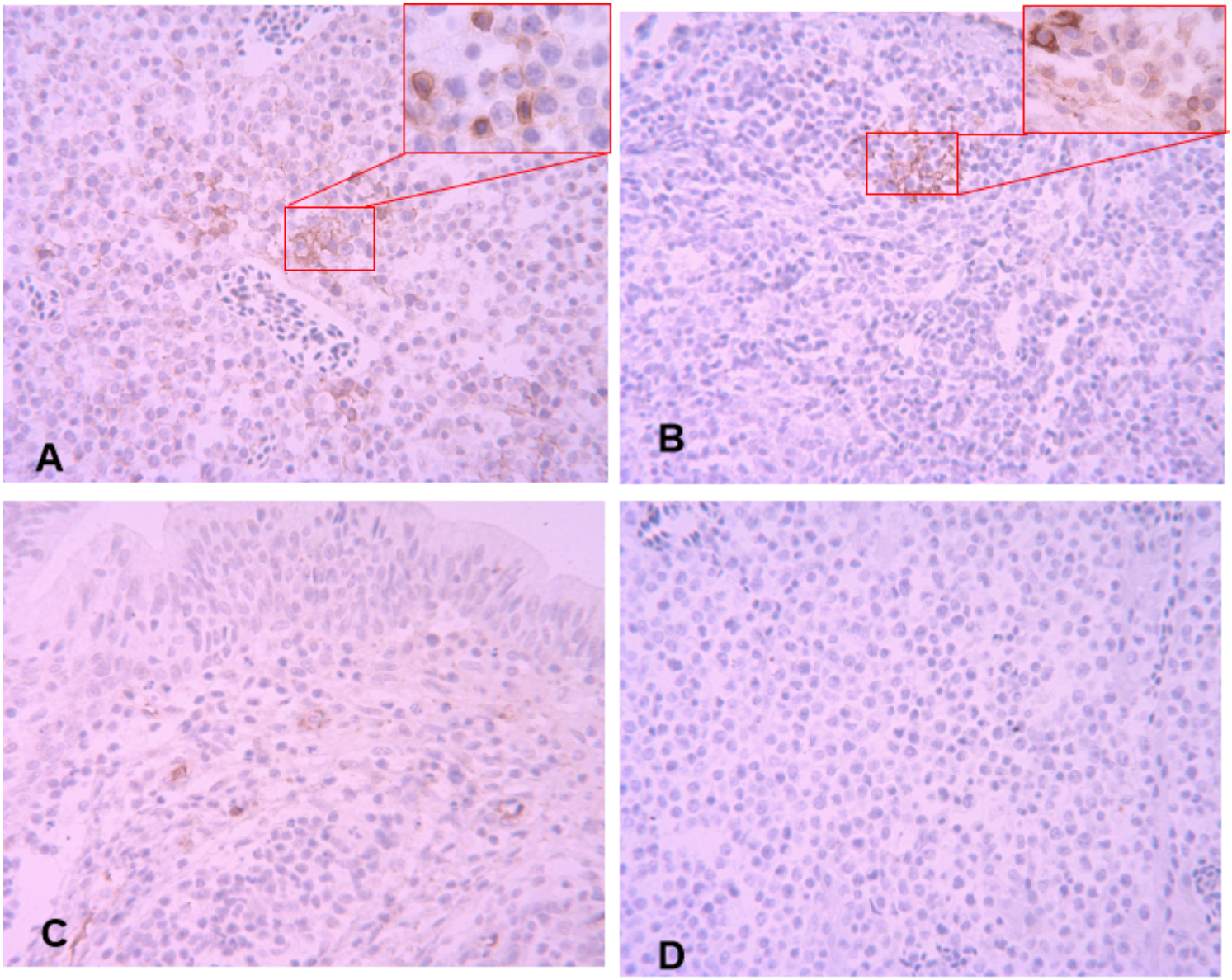

Figure 2

Immunohistochemical staining of p53 in infected chickens. (A) Liver. lymphoid tumor cells with cytoplasm expression. (B) Spleen. lymphoid tumor cells with cytoplasm expression. (C) Bursa of fabricius. lymphoid tumor cells with cytoplasm expression. (D) Liver. Negative controls were incubated with PBS.

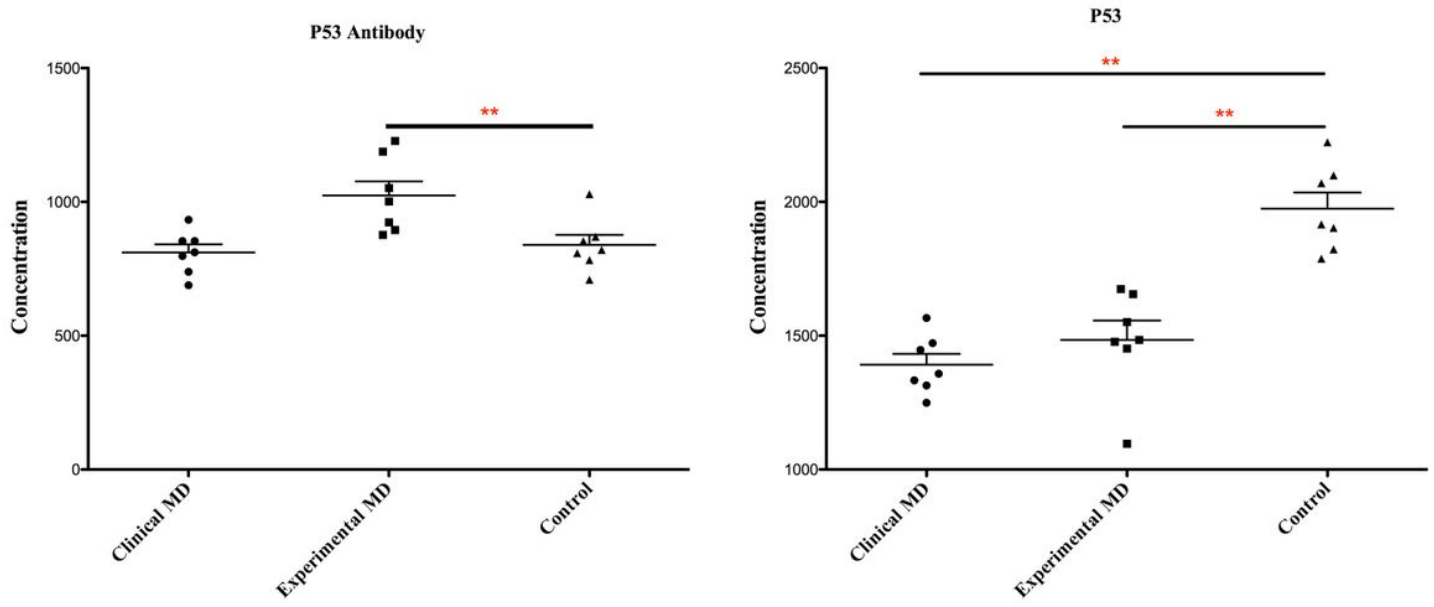

Figure 3

Level of P53 antigen and antibody in serum post infection with MDV.

Supplementary Files

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- p53inMDARRIVEChecklist.pdf