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

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Research article

Keywords: Marek's disease, p53, P53 antibody

Posted Date: May 12th, 2020

DOI: https://doi.org/10.21203/rs.2.23898/v2

Version of Record: A version of this preprint was published on August 24th, 2020. See the published version at https://doi.org/10.1186/s12917-020-02520-2.

Abstract

Background: Marek's disease 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. However, the p53 could become an oncogene from a tumor inhibitory role, if a mutation happened.

Results: The mutation rate of p53 was 60 percent in experimentally and naturally infected chickens. The mutations included point-mutations and deletions, and mostly located in the DNA-binding domain. The mutated P53 can be expressed in tumors of various tissues in an infected chicken. They accumulated in cytoplasm due to the loss of nuclear localization function. Unlike the researches on human cancer, the concentration of P53 serum of MD infected chicken was significantly lower than control group.

Conclusions: The p53 mutations were relevant with the development of MD. 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] and the "cellular gatekeeper" [6], p53 is the most relevant and important tumor suppressor gene with the highest mutation frequency in human and animal tumor diseases [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 [7]. 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 [8]. Loss of p53 function is often a prerequisite for tumorigenesis and progression [9]. The proportion of p53 mutations in tumor tissues is between 10% and 100% [9].

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 [10]. Several studies have shown that the P53 antibody plays a predictive role in tumorigenesis and its appearance in serum is an early event in the development of malignant tumors in human [11, 12, 13]. P53 antibody has a significant correlation with clinical common neoplastic disease, but it was barely detectable in the serum of healthy people [14]. This correlation might also exist in chicken. However, there is knowledge gap about the role of mutant p53 in chicken Marek's disease. This

study aimed to determine whether P53 can be used as a tumor marker to assist clinical diagnosis and prognosis analysis of MD.

Results

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 (Figure 1A). The lymphoid follicle in the bursa of Fabricius was atrophied; the diffuse infiltration and proliferation of lymphoid tumor cells between the follicles were observed (Figure 1B). In the livers of the clinical infected chickens, the cytoplasm of the lymphoid tumor cells underwent multifocal proliferation. They were stained brown by IHC (Figure 2A). Positive staining was also detected in the tumor cells in the spleen and the bursa of Fabricius tissues (Figure 2B; Figure 2C). In contrast, no positive stained cells were observed in the control group (Figure 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) were detected in the p53, which was consistent with the results reported in previous studies [17]. 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.

P53 antigen and P53 antibody levels for MD

The Figure 3 showed the P53 antigen and antibody levels of different groups. The OD values were as the Y-axis and the different groups of samples were as the X-axis. The result showed that the concentration of P53 of the clinical and experimental MDV-infected group was significantly lower than control group. However, the P53 antibody in experimental infection group was significantly higher than control group.

Discussion

It is widely acknowledged that P53 of human is an intra-nuclear phosphorylated protein. The wide type P53 exists in the nucleus for an extremely short time [18]. Meanwhile mutated P53 is not digested quickly and prolongs half-life to 20-40 hours, and therefore they accumulate inside tumor cells [19]. In addition, as IHC has become an important indicator of various biomarker detection, P53 IHC is an effective substitute marker for TP53 mutation status [10]. At present, P53 IHC is mainly applied for human tumor diseases. It is 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 was worth noticed that P53 are almost expressed in the cytoplasm. The potential reason was that the mutated p53 lost nuclear localization function and eventually accumulated 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 [20]. In this study, p53 was shown multiple types of mutations in natural and experimental infections of MDV. The five most frequent mutation sites were 651, 786, 828, 864, 879 respectively. The altered codons compared with wild p53 were 217 (ACG-ACA), 262 (GCA-GCC), 276 (CGC-CGG), 288 (GCC-GCA), 293 (ACC-ACA), but they were all synonymous mutations.

At the same time, a short form of p53 transcript was detected in a clinical case. The deleted sequence was located at 631-677 bp in the open reading frame of the reference sequence, resulting in missense mutations from the position 210 to the termination. The short form of p53 transcript was also found at the cases infected avian leukosis virus [17]. A series of missense mutations from the position 163 to the termination due to a base deletion were found in an experimentally infected chicken. In the present research, it was found that the p53 has a 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 [21]. Once the p53 is deleted or 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 in human. The accumulated mutant P53 acts as a target antigen to elicit an autoimmune response [22]. However, the serum P53 antigen in clinical and experimental MDV-infected groups was significantly lower than control group, which was different from cancer research in human. Only the serum P53 antibody concentrations of experimental infected group was significantly higher than the control group. 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.

Conclusion

Our results indicated that the mutation and expression of p53 were detected in MDV infection, and suggested that these mutations were relevant with the development of MD. The mutant P53 was expressed in the tumor cells of various tissues of the diseased chicken. They lost its nuclear localization function and transferred from the nucleus to cytoplasm. The p53 in MDV infected chicken trend to mutate, which was consistent with cancer patients. However, unlike the researches on human cancer, the concentration of P53 showed a significant decrease in the groups of natural and experimental MDV infection. It might provide a new idea for the diagnosis and monitoring of MD.

Methods

Experimental infection of chickens with MDV

Twenty 1-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 as an infection group and a control group. Two groups of chickens were raised separately in isolators with filtered air under positive pressure. At the first day of the experiment, 2,000 plaque forming units (PFU) of vvMDV (GX0101), which normally cause tumors at the tenth week, was inoculated intra-abdominally (i.a.) into each chicken in the infection group. The control group was inoculated with PBS. At the tenth week, serum was collected from seven chickens each group. The experiment ended after ten weeks. All chickens were carbon dioxide euthanized and their liver and spleen were collected.

Natural infection of chickens with MDV

Seven 160-days-old laying hens were gotten from a local flock. They were confirmed as MDV natural infection and absence of other common viral diseases by PCR detection. Their serum was collected for detecting P53 antigen and antibody. Their tissue samples, including liver, spleen, pancreas and bursa of Fabricius, were collected and fixed with 4% formaldehyde for 48h for histopathological and immunohistochemical studies.

Histopathology and Immunohistochemical

The fixed liver, spleen, pancreas and bursa of Fabricius were dehydrated, waxed, and cut into 3μm slices, followed by Haematoxylin and eosin (H-E) staining for histopathology observation. In immunohistochemical (IHC) processing, tissues were cut into sections of 3μm thickness and 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 (0.01mol/L; pH 6.0; 100°C) for antigen retrieval by microwaving and cooled at room temperature for 20 min [15]. Endogenous alkaline peroxidase was quenched with 3% hydrogen peroxide solution in methanol for 30 min [16]. 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 reverse transcription

Total RNA of liver and spleen tissue samples (50mg/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 were amplified. The PCR reaction was performed by using thermal cycler (Takara, Japan) under the following conditions: 95℃ for 5min; followed by 35 cycles of 95℃ for 30s, 56℃ for 30s, 72℃ for 45s, and then a final 72℃ for 10min extension cycle. DNA templates that were acquired from the MDV positive chickens were amplified using primer pair p53-1/-2. The SPF chicken were regarded as negative control. DNA fragments were successfully amplified with sizes of 687bp. 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.

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

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 ** $p < 0.01$.

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.

Declaration

Acknowledgments
Not applicable.

Funding

The work was supported by grants from the National natural science foundation project (NO. 31672518). The funding body was solely involved in funding and had no role in the design of the study, the collection, analysis, and interpretation of the data, or in writing the manuscript.

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.

Ethics approval and consent to participate

The sample were collected and handled in accordance with the good animal practices required by the Animal Ethics Procedures and Guidelines of the People's Republic of China. Informed consent to participate was obtained from the chicken farmer owner. All animal protocols and procedures were performed according to the Chinese Regulations of Laboratory Animals and were approved by the Animal Ethics Committee of Shandong Agricultural University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Primers used to amplify chicken p53 cDNA

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

Table 2. Mutations of p53 genes in MD

Sample Info	Mutation analysis			
	Base mutation sites	amino acid mutations		Mutation area
		Site from to		
NC	ND	ND		
MD (C)	611 T-C; 786 A-C; 828 C-G; 1026 A-G	204	Y A	Core domain
		329	E G	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 delete; 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	Core domain
		293	T I	C-terminal domain
		365	G D	
MD (E)	786 A-C; 828 C-G; 864 C-A	ND		
MD (E)				Core domain
	651 G-A; 701 G-T; 804 C-T; 828 C-G; 864 C-A;	234	R L	C-terminal domain
	907 G-A	303	V M	
		152	E Y	
		153	H Y	

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

Figures

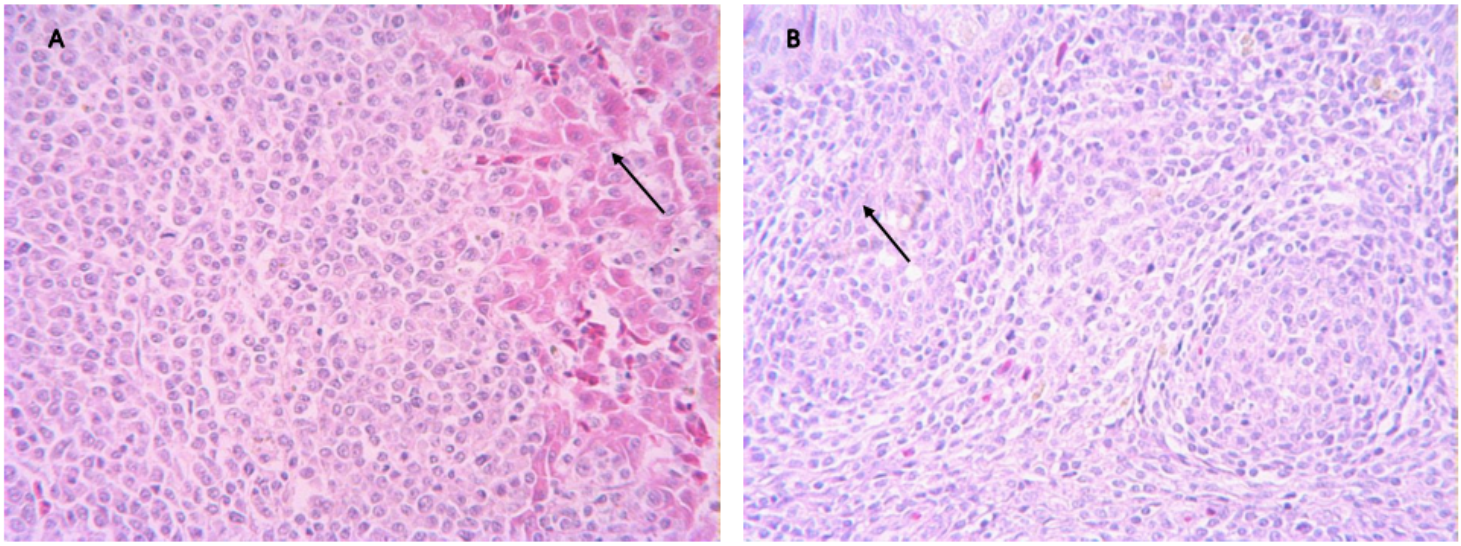

Figure 1

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 in the bursa of Fabricius (HE, 400×).

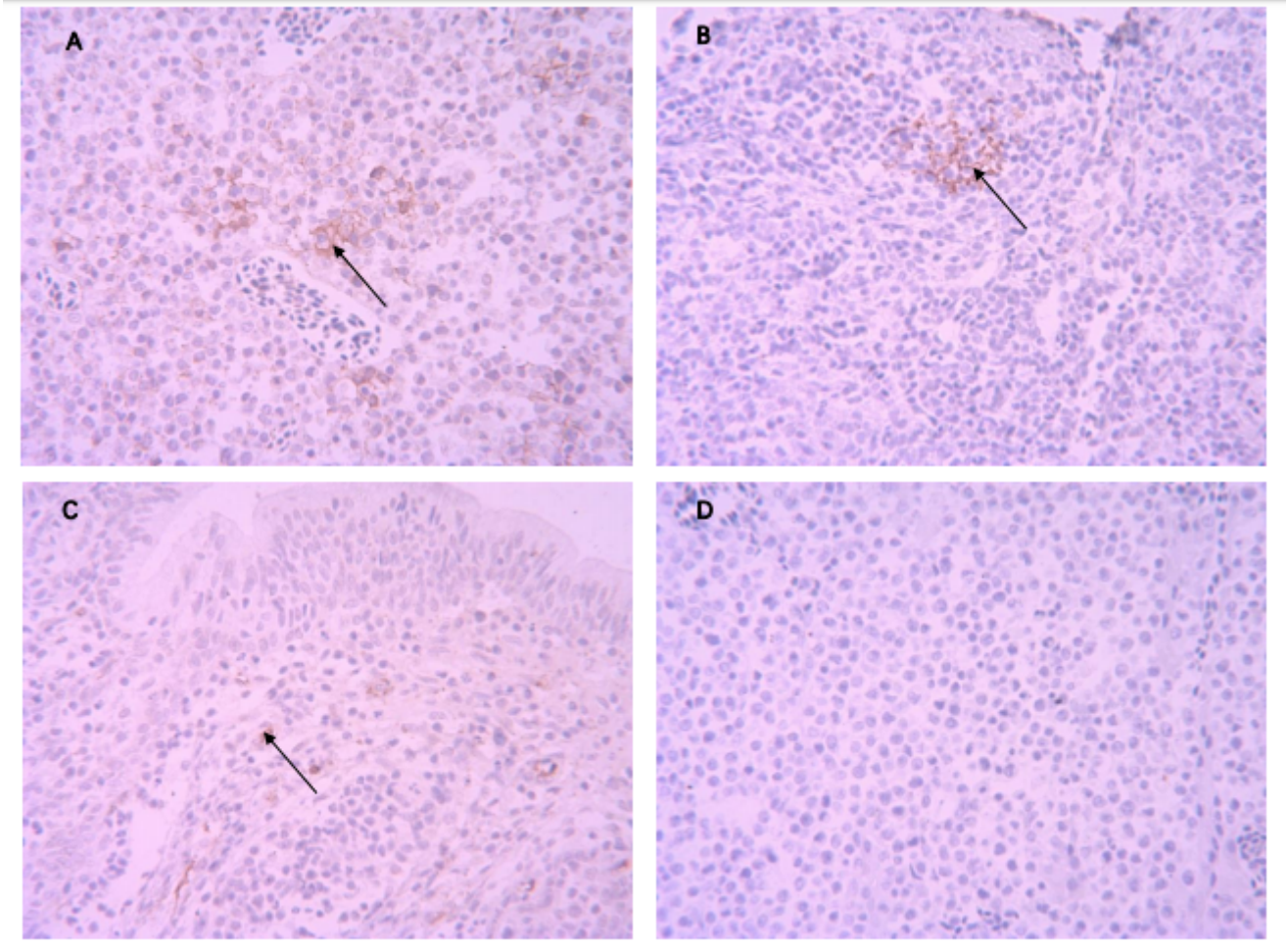

Figure 2

Immunohistochemical staining of p53 in infected chickens. (A) Liver. lymphoid tumor cells with cytoplasm expression (HE, 400×). (B) Spleen. lymphoid tumor cells with cytoplasm expression (HE, 400×). (C) Bursa of Fabricius. lymphoid tumor cells with cytoplasm expression (HE, 400×). (D) Liver. Negative controls were incubated with PBS (HE, 400×).

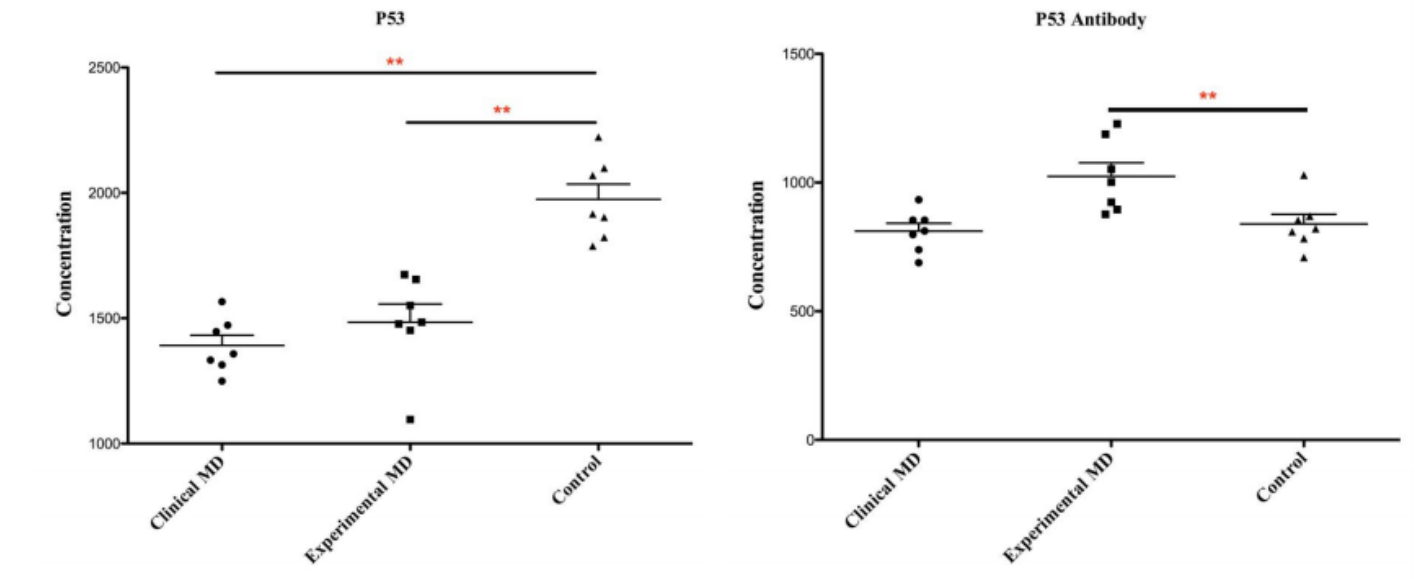

Figure 3

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

Supplementary Files

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- p53inMDARRIVEChecklist.pdf