Source Tracking of Chicken Infectious Anemia Virus in a SPF Chicken Population, the Latest Report in China

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Research Article

Keywords: SPF chicken, chicken infectious anemia virus, vertical transmission, genome analysis

Posted Date: September 27th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-929973/v1

Abstract

Background

Live attenuated vaccines have been extensively used to prevent infectious disease in poultry flocks. However, exogenous virus contamination in attenuated vaccines had been reported several times in the past, which brought enormous threat to poultry production and diseases prevention and control. Recently, an attenuated vaccine against Newcastle Disease produced in China was detected contamination with chicken infectious anemia virus (CIAV) in a routine inspection for exogenous virus. To understand the multiple routes of transmission of CIAV, and to better formulate correct prevention and control, it is necessary to find out the possible source of this contamination.

Methods

lood samples of SPF chickens that generated vaccines were collected to investigate CIAV antibody titers by ELISA test. Then, 14- to 18-day-old SPF chicken embryos (n=40) were randomly selected, DNA was extracted and detected by quantitative real-time PCR and nucleic acid dot hybridization assays. To further investigate the molecular features of the CIAV isolate, the complete genome of CIAV was amplified and analyzed.

Results

The results showed both SPF chickens and embryos for vaccine preparation were CIAV-positive. In addition, the full-length genome sequences of CIAV from vaccines and SPF chicken embryos were consisted of 2,298 nucleotides (nt) with 100% homology, named as SDSPF2020 (Genbank accession number: MW660821). It demonstrated 95.7%-99.6% homology with the complete nucleotide sequences of reference strains, and shared the closest evolution relationship with the Chinese strain HLJ15125.

Conclusion

This study illustrated that vertical transmission of CIAV from SPF chickens and embryos was an important way for exogenous virus contamination in vaccine production. As such, vaccine quality monitoring and health control are significant in the poultry industry from an environmental safety point of view.

Introduction

Chicken infectious anemia (CIA), caused by chicken infectious anemia virus (CIAV), is an economically immunosuppressive disease that brings huge economic loss in the poultry industry [1]. Since its first isolation from contaminated vaccines in Japan [2], it has been transmitted worldwide. Epidemiological investigation revealed that CIAV had also been sporadically distributed in different breeder flocks in China in recent years [3–6]. CIAV was a non-enveloped virus with a single-stranded circular DNA genome [7], which encodes three partially overlapping ORFs, namely VP1, VP2 and VP3. VP1 (51.6kDa) is a viral capsid protein associated with neutralizing antibody (NA) production. VP2 (24.0kDa) works as a scaffold protein which is thought to affect the conformation of VP1. VP3 (13.6kDa) is a non-structural protein also known as apoptin, which can induce apoptosis of susceptible cells [8]. Notably, amino acids (AA) of VP1 gene involved in the pathogenesis of CIAV are prone to variability, while VP2 and VP3 are relatively conserved. Furthermore, the noncoding region of the CIAV genome contains a series of conserved motifs which exhibit promoter activity and plays a crucial role in viral replication and transcription [9].

Thus, phylogenetic analysis of CIAV complete genome and VP1 sequences is a significant step to understand the evolutionary branches and reveal differences in amino acids and transcription elements between different strains.

Previous studies have shown that CIAV could spread through multiple routes and induce immune dysfunction in association with other pathogens [10]. It has been confirmed that CIAV can be transmitted vertically through eggs from infected breeder flocks, causing aplastic anemia, heterophil decrease, lymphoid depletion and atrophy of bone marrow hematopoietic tissue in young chicks [11]. The horizontal spread of CIAV, which was from feather shafts to infect chicks via the mucosal entries, is also a nonnegligible mode of viral dissemination [12]. The subclinical immunosuppression induced by horizontal transmission is ubiquitous, lasting up to 6 weeks in flocks. Despite a high prevalence in recent years, some investigations elucidated the CIAV horizontal spread relatively limited in commercial flocks[13]. However, many studies mentioned that commercial attenuated vaccines used in poultry production and SPF chickens were detected CIAV contamination [14–16]. This caused the use of CIAV-contaminated live-virus vaccines also becoming an important horizontal transmission route [17, 18].

At present, specific-pathogen-free (SPF) chicken embryo is the raw material to produce live poultry vaccine. Live poultry vaccines will be at risk of contamination with exogenous viruses including CIAV if SPF embryos with vertically transmitted viruses are used for production. In general, SPF chicken flocks should be screened for CIAV antibodies to ensure freedom from CIAV infection. However, several indirect evidences suggested that CIAV may exist in a latent state and transmit vertically independent of antibody status in SPF chickens [19, 20]. Furthermore, maintaining CIAV seronegative poultry population was extremely challenging due to the high resistance of CIAV in various extreme physical conditions or biosecurity filtered air houses [21].

Recently, an attenuated poultry vaccine was detected CIAV-positive during routine inspections at a poultry farm that produces commercial live poultry vaccines. To further reveal the possible source of this CIAV strain, a CIAV strain from a SPF chicken population was isolated and systemic experiments were performed in this study to show a complete chain of evidence for vertical transmission of CIAV from SPF chickens to SPF chicken embryos, and eventually, the attenuated live vaccine.

Materials And Methods

Background of CIAV contamination in vaccines

According to the requirements of Ministry of Agriculture and Rural Affairs of the People's Republic of China, all live poultry vaccines must be regularly detected for exogenous virus dissemination before marketing. To identify if the potential threat caused by live vaccines contaminated with any exogenous virus, we investigated commercial live poultry vaccines produced by a vaccine manufacturer with SPF poultry flocks. SPF chickens were inoculated with those different vaccines, and antibody responses were detected at 6wk post-inoculation using commercial enzyme-linked immunosorbent assay (ELISA) kits (Avian Leukosis Virus Antibody Test Kit-subgroup A /B, Avian Leukosis Virus Antibody Test Kit -subgroup J, Reticuloendotheliosis virus Antibody Test Kit, Chicken infectious anemia virus Antibody Test Kit, IDEXX, USA). The CIAV antibody was positive from these samples from SPF chickens inoculated with Newcastle disease virus (NDV)-attenuated vaccines, according to the manufacturer's instruction. Subsequently, DNA was extracted to detect the CIAV genome from both vaccines and immune organs of vaccinated chickens using DNA extraction kits (Tiangen, Beijing, China). According to published CIAV genome sequences (Additional file 1: Table S1), a pair of primers (Table 1) were designed and synthesized to amplify the

genome. To find the probable source of contamination, further analysis was conducted on the same batch of SPF chickens and embryos used in vaccine pre-production at the farm.

Table 1
Sequences of primers used for amplification in this study.

Primers	The sequences of the primers (5'→3')	Sizes
CIAV-F_1	5′- AAAGGCGAACAACCGATGA – 3′	533bp
CIAV-R_1	5′- TGCCTGTTACCCAGCTGC – 3′	
CIAV-F_2 [a]	5′- GCATTCCGAGTGGTTACTATTCC – 3′	942bp
CIAV-R_2	5′- TCTCCTCCGATGTCGAAATTTATA – 3′	
CIAV-com-F1[b]	5′- GCATTCCGAGTGGTTACTATTCC – 3′	842bp
CIAV-com-R1	5′- CGTCTTGCCATCTTACAGTCTTAT – 3′	
CIAV-com-F2[b]	5′- CGAGTACAGGGTAAGCGAGCTAAA – 3′	990bp
CIAV-com-R2	5′- TGCTATTCATGCAGCGGACTT – 3′	
CIAV-com-F3[b]	5′- ACGAGCAACAGTACCCTGCTAT – 3′	802bp
CIAV-com-R3	5′- CTGTACATGCTCCACTCGTT – 3′	

[a] Dot blot hybridization assay.

[b] Three pairs of primers were designed to amplify three overlapping fragments.

Detection of CIAV antibody in SPF Chickens

To determine whether chicken flocks were contaminated, serum samples were collected from SPF layer-type (LT) chickens (n = 1000) and tested by commercial ELISA assay according to the manufacturer's instructions (Chicken Infectious Anemia Virus Antibody Test Kit, IDEXX, USA). At the same time, yolks of SPF chicken embryos (n = 200) used for vaccine production were randomly collected, and maternal CIAV antibody was detected with the dilution ratio of 1:20 using commercial ELISA kit.

DNA extraction and CIAV detection in chicken embryos

14- to 18-day old SPF chicken embryos (n = 40) in the same production origin were randomly selected, and samples of pooled organs (liver, spleen and thymus) were excised from embryos. The tissues were homogenized in phosphate-buffered saline (PBS) for genomic DNA extraction, and then stored in TE buffer (10 mM Tris, pH 7.5; 1 mM EDTA, pH 8.0) at -20°C. DNA samples were amplified by conventional polymerase chain reaction (PCR) using the specific CIAV primers (please see the Table 1) and detected by dot blot hybridization assay as previously described [22, 23]. Briefly, 2 μl of PCR products were spotted on the nitrocellulose membrane as dots, then air-dried and hybridized with digoxigenin-labelled DNA probes. Meanwhile, high-sensitivity nested-PCR and quantitative real-time polymerase chain reactions (qPCR) were parallelly performed for CIAV detection according to published research [24].

Viral genome amplification and sequencing

The whole CIAV genome was amplified from SPF embryo DNA samples using three pairs of primers as mentioned previously (please see the Table 1) [15]. The PCRs were carried out under the following protocol: pre-denaturation at 95°C for 5min, followed by 34 cycles of 95°C for 30 s, 56°C for 45s, and 72°C for 1min, and a final elongation step at 72°C for 7min. The PCR products were purified by agarose gel electrophoresis and subcloned into the pMD-18T vector (TaKaRa Bio Inc., Japan), for sequencing by Sanger method (TSINGKE, Qingdao, China). Genomic DNA extracted from the attenuated vaccine was amplified and sequenced simultaneously.

Alignment and phylogenetic analysis of viral genome sequence

The complete genome sequences of the isolated strains were obtained by assembling separated overlapping fragments using DNAStar software (version 7.0), and multiple sequence alignment was performed with CIAV isolates and 59 reference CIAV genome sequences downloaded from GenBank (Additional file 1: Table S1) using clustal W method. Homology analysis was assembled by MegAlign and BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). To further investigate the genetic relationship of CIAV, phylogenetic tree was constructed on the basis of the full-length genomes, VP1, VP2, and VP3 regions using MEGA5.1 software by neighbor-joining (NJ) method, respectively [25]. In addition, transcriptional regulatory elements in non-coding region of the isolated strain were analyzed by the online service system of Nsite (Recognition of Regulatory motifs) of SoftBerry (http://www.softberry.com/ berry.phtml).

Results

Detection of CIAV infection in SPF chicken flocks

The results of antibodies detection showed that the positive rate of serum antibodies was 62% in the SPF poultry flocks, while the positive rate in egg yolks was 57% (Table 2), indicating the existence of CIAV infection in SPF flocks. To further investigate the infection status in this SPF flock, SPF chicken embryos were collected and analyzed for the presence of viral DNA. The positive rate of CIAV DNA in chicken embryos detected was 35% (14:40) by dot blot hybridization assay (Fig. 1), while other common viruses were negative. In addition, our data suggested all other detection methods were also CIAV positive (Figs. 2 and 3), which further confirmed vertical transmission of CIAV existed in SPF chicken population and SPF chicken embryos.

Table 2
Detection of chicken infectious anemia virus by antibody assays and molecular biological methods.

SPF flocks tested for CIAV			Antibody assays			Molecular biological methods			
Trial no.	No. of birds	No. of embryos	No. of positive	ELISA S/N ratios [a]	Flocks positive rate	No. of embryos positive			No. samples positive total [b] (%)
						Nucleic acid dot blots	Real-Time quantitative PCR	Nested PCR assay	
1	1000	/	622	≤ 0.6	62%	NA [c]	NA	NA	NA
				> 0.6					
2	/	200	94	≤ 0.6	47%	NT [c]	NT	NT	NT
				> 0.6					
3	/	40	NT	NT	NT	14	15	14	35.8%

[a] ELISA S/N ≤ 0.6 positive or high protective titers; S/N > 0.6 negative or low titer.

[b] The positive rate of the three methods was averaged.

[c] NT: not tested; NA: not applicable.

Genome amplification and phylogenetic analysis of CIAV

The full-length genome sequences of CIAV were obtained from vaccines and SPF chicken embryos by assembling three overlapping fragments. Both genomes consisted of 2,298 nucleotides (nt) with 100% homology. Therefore, the CIAV genomes from vaccines and SPF chicken embryos were confirmed to be the same strain namely SDSPF2020, and submitted to GenBank with accession NO.MW660821. The nucleotide sequence homology of SDSPF2020 and references was 95.7%-99.6%. The phylogenetic tree suggested that CIAV genomes could be divided into four major clusters [26], while SDSPF2020 fell within the A group and has the maximal nucleotide sequence identity (99.6%) with the Chinese wild strain HLJ15125 (KY486139), belonging to the same clades (Fig. 4).

Main coding protein sequence analysis of SDSPF2020

Phylogenetic analysis based on nucleotide and amino acid sequences of the VP1 showed that all the sequences fell into four gene clusters, which was consistent with the phylogenetic tree based on the complete nucleotide sequence. The nucleotide homology and amino acid homology of VP1 were 94.4%-99.6% and 97.1%-100% when compared with other reference strains. No significant difference was found between the reference sequences and SDSPF2020 in hypervariable region (AA139-157) of VP1. The amino acid sites of VP1 protein manifested the presence of lysine (K) at site 139, and glutamic acid (E) at site 144 in SDSPF2020 strain, which suggested that SDSPF2020 exhibited a virulent pathogenicity. The amino acid sequence changes of VP1 protein of SDSPF2020 strain mainly located in the C-terminal when compared with standard strain Cux-1, such as 251 (R), 287 (S), 370 (G) and 413 (S) (Fig. 5), among which one amino acid mutation (positions 447) with other references had never been found before. In addition, partial nucleobase substitution occurred at amino acid positions 75 (V), 89 (T) and 394

(Q) (Fig. 5). As for VP2 and VP3, the nucleotides and amino acids of SDSPF2020 were relatively conserved when compared with other reference strains. Sequence analysis showed that nucleotide homology of VP2 gene was 99.1%-99.8% and amino acid homology was 97.7%-100%, while the nucleic acid and amino acid homology of apoptin were 98.9%-100% and 96.7%-100%, respectively. In addition, no deletion or insertion was found in VP2 and VP3 protein. However, the nucleotides at sites 347 and 352 were different from the reference strain Cux-1, both of which were located in the NLS2 domain of VP3 protein.

Molecular characterization of non-coding region of SDSPF2020

The Clustal W method was used to analyze the homology of non-coding region fragments of 10 CIAV reference isolates. Compared with the reference strains, the results showed that the non-coding gene of SDSPF2020 contained a conservative region of DNA with high G + C content (nucleotide homology 97.0-99.4%). Most motifs in the untranslated region of SDSPF2020 were the same as those in reference strains, while there were several obvious differences in individual nucleobases existing in SDSPF2020. Transcription factor binding site analysis by NSITE demonstrated that four tandem array of DR regions were found in the non-coding region of SDSPF2020 and most of the reference strains except for Cux-1 isolate, which terminated 4nt upstream of the "CCAAT" box (Fig. 6). In addition, other sites like ATF/CREB binding sites ("ACGTCA" consensus sequence), SP1-binding sites, and other potential lymphoid specific transcription factor binding sites were all conserved and existed in the non-coding region of SDSPF2020.

Discussion

Since its first isolation, CIAV has been widely spread throughout the world. CIAV mainly caused aplastic anemia, immunosuppression, growth retardation and death, which brought significant losses to the poultry industry [27]. In China, the positive rate of CIAV antibodies in hens and broiler chickens has been increasing in recent years [28, 29]. According to the inspection data provided by Chinese epidemiological survey, the positive rate of CIAV in some unimmunized chickens was even as high as 87% in live bird markets, indicating that the chickens had been at high risk of infection [29]. Although CIAV vaccines are licensed in some countries, they are currently unavailable in China. CIAV could transmit both by contact transmission and horizontal transmission, among which horizontal transmission was recognized as a high risk of CIAV infection even in poultry farms with good biosafety conditions. Furthermore, usage of live attenuated avian vaccines contaminated with CIAV was another important way to transmit CIAV. In general, the contamination of attenuated vaccines was mainly caused by the use of CIAV-positive SPF chicken embryos during propagation of seed virus [30]. Although strict surveillance for the vaccine contamination had been taken, several cases of exogenous virus contamination in live poultry vaccine had been reported, such as avian leukosis virus (ALV) [31–33], reticuloendotheliosis virus (REV) [34–36], and fowl adenovirus (FAdV) [37, 38]. Therefore, it was of great significance to ensure the purity of SPF chicken embryos for vaccine manufacture.

Recently, a live attenuated vaccine against Newcastle Disease produced in China was detected to be contaminated with CIAV during a routine inspection. To further investigate the probable resource of CIAV contamination, serum samples and chicken embryos were collected from SPF chickens. Firstly, CIAV antibody titer was detected in the serum. Detection of CIAV antibody was the most immediate and effective measure to evaluate whether SPF chickens were infected with CIAV [39]. The results showed that the antibody positive rate was as high as 62%, which confirmed the existence of serious CIAV infection in this SPF chicken flock. Since the presence of CIAV antibodies in breeder flocks does not fully represent virus infection in SPF chicken embryos, therefore, 14- to 18-

day-old SPF chicken embryos were randomly selected for CIAV DNA detection by qPCR, dot-blot hybridization and nest-PCR assay. The results confirmed that SPF chickens were infected with CIAV, and the virus had been spread to embryos.

To further investigate the molecular characteristic of the CIAV in this SPF chicken flocks, full-length genome sequence was obtained by PCR amplification and aligned with other reference strains. Shockingly, the CIAV-SDSPF2020 strain showed the highest homology (99.6%) with HLJ15125 strain (accession number KY486139) isolated in China according to the database, and the whole nucleotide homology of the isolate was up to 99.5% with multiple wild strains (GX1804 and N8). According to the results of this study, we have reason to speculate on the possibility that vaccine contamination came from some wild strains. Some studies revealed that specific sites of VP1 protein contributed to viral pathogenicity and virulence by affecting functions of cellular receptor. For instance, residue 394 of VP1 was major genetic determinants for the pathogenicity of CIAV, VP1 residues 139 and 141 were associated with reduced transmission of the virus [40]. In this study, genomic sequences of SDSPF2020 and reference strains were compared to speculate the biological characteristics of SDSPF2020. The results showed that, residue 139,141 and 394 were lysine, glutamic acid and glutamine in SDSPF2020, indicating that SDSPF2020 exhibited a high-replicated ability and high-pathogenic phenotype [41]. Although SDSPF2020 has high homology with the wild CIAV strain HLJ15125, with only one amino acid mutation (positions 447), additional changes compared with the classical reference strain CUX-1 were found at residues 125 (L), 157 (M), 251 (R), 287 (S), 370 (G) and 413 (S) of VP1 protein. These amino acid mutations were universal and mainly concentrated in the C-terminal epitope dominant region, which contained abundant antigenic epitopes. In a word, the above regular variations lead to the formation of different evolutionary subbranches of CIAV strains during the transmission route, and also make isolated genetic evolution most similar to Chinese wild strains in recent years [5], and it has certain guiding significance to trace the origin of CIAV isolates clustering based on capsid protein in future research.

Interestingly, emerging evidence had proved that the non-coding region of CIAV genome with a length of 0.3kb contained dozens of conserved transcriptional regulatory elements which might contribute to viral replication and transcriptional regulation [9]. However, whether these conserved sequences were necessary for viral replication, apoptotic ability and cell adaptability was unclear and needed to be studied further. Therefore, clarifying the properties of transcription regulatory elements could provide a better understanding of the mechanisms of host transcriptional regulation and viral pathogenesis. It was found that the motifs of ATF/CREB binding sites and other potential lymphoid specific transcription factor binding sites were also retained in the SDSPF2020 genome. The ATF/CREB family of transcriptional regulators have diversified functions in controlling cell proliferation and apoptosis, affecting downstream transcription pathways to control programmed cell death [42]. Lymph-specific transcription factor binding site impacts globin gene expression, and SP1 binding site contributes to the cell specificity targeted by CIAV. Therefore, it was suggested that those conserved transcriptional regulatory elements might have an indispensable influence on pathogenicity and viral cytotropism under certain conditions. In addition, the non-coding region of SDSPF2020 contained a high G + C content region, which was considered to be associated with regulation of transcription or DNA replication.

Conclusions

In conclusion, the integrated evidence chain revealed a vertical transmission route of the exogenous CIAV contamination vaccine from SPF chicken embryos by detecting CIAV antibody in SPF chicken serum and nucleic acid in SPF chicken embryos and vaccine. These results remind us that the surveillance of vertically transmitted

viruses and biosecurity control of SPF chicken farm should be strengthened continuously to reduce the potential risks of exogenous virus contamination in the future.

Abbreviations

CIAV: Chicken infectious anemia virus; CIA: Chicken infectious anemia; SPF: specific-pathogen-free; ORF: Open reading frame; qPCR: quantitative real-time polymerase chain reactions; NCBI: National Center for Biotechnology Information; ELISA: commercial enzyme-linked immunosorbent assay; NDV: Newcastle disease virus; NJ: neighbor-joining; BLAST: Basic Local Alignment Search Tool.

Declarations

Acknowledgements

The authors would like to thank Wang Yixin for proofreading the draft and providing valuable feedback as well as Zhao Peng for guidance and insight into this article.

Authors' contributions

ZP designed the study. LY and WJJ performed the experiments. LY, WJJ, WYX, ZYW, ZH, CZN, MXY, WQ, CZZ and CS analyzed the data. LY drafted the manuscript. All authors read and approved the final manuscript.

Funding

This study was funded by the National Key Research and Development Program of China (grant numbers 2018YFD0500106).

Availability of data and materials

All the datasets used and/or analysis in this study are provided in figures, tables and supplementary files.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

Authors declared that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

1. McNulty MS. Chicken anaemia agent: a review. Avian Pathol. 1991; 20(2): 187-203.

2. Yuasa N, Taniguchi T, Yoshida I. Isolation and Some Characteristics of an Agent Inducing Anemia in Chicks. Avian Diseases. 1979; 23(2): 366.

3. Li Y, Yan N, Wang Y, Liu A, Liu C, Lan X, et al. Molecular evolution and pathogenicity of chicken anemia virus isolates in China. Arch Virol. 2021; 166(2): 439-449.

4. Zhang X, Liu Y, Wu B, Sun B, Chen F, Ji J, et al. Phylogenetic and molecular characterization of chicken anemia virus in southern China from 2011 to 2012. Sci Rep. 2013; 3: 3519.

5. Yao S, Tuo T, Gao X, Han C, Yan N, Liu A, et al. Molecular epidemiology of chicken anaemia virus in sick chickens in China from 2014 to 2015. PLoS One. 2019; 14(1): e0210696.

6. Tan C, Wang Z, Lei X, Lu J, Yan Z, Qin J, et al. Epidemiology, molecular characterization, and recombination analysis of chicken anemia virus in Guangdong province, China. Arch Virol. 2020; 165(6): 1409-1417.

7. Noteborn MH, de Boer GF, van Roozelaar DJ, Karreman C, Kranenburg O, Vos JG, et al. Characterization of cloned chicken anemia virus DNA that contains all elements for the infectious replication cycle. J Virol. 1991; 65(6): 3131-9.

8. Pringle CR. Virus taxonomy at the XIth International Congress of Virology, Sydney, Australia, 1999. Arch Virol. 1999; 144(10): 2065-70.

9. Meehan BM, Todd D, Creelan JL, Earle JA, Hoey EM, McNulty MS. Characterization of viral DNAs from cells infected with chicken anaemia agent: sequence analysis of the cloned replicative form and transfection capabilities of cloned genome fragments. Arch Virol. 1992; 124(3-4): 301-19.

10. Rosenberger JK,Cloud SS. Chicken anemia virus. Poult Sci. 1998; 77(8): 1190-2.

11. Taniguchi T, Yuasa N, Maeda M, Horiuchi T. Chronological observations on hemato-pathological changes in chicks inoculated with chicken anemia agent. Natl Inst Anim Health Q (Tokyo). 1983; 23(1): 1-12.

12. Davidson I, Artzi N, Shkoda I, Lublin A, Loeb E, Schat KA. The contribution of feathers in the spread of chicken anemia virus. Virus Res. 2008; 132(1-2): 152-9.

13. Tan J,Tannock GA. Role of viral load in the pathogenesis of chicken anemia virus. J Gen Virol. 2005; 86(Pt 5): 1327-1333.

14. Li Y, Hu Y, Cui S, Fu J, Wang Y, Cui Z, et al. Molecular characterization of chicken infectious anemia virus from contaminated live-virus vaccines. Poult Sci. 2017; 96(5): 1045-1051.

15. Li Y, Wang Y, Fang L, Fu J, Cui S, Zhao Y, et al. Genomic Analysis of the Chicken Infectious Anemia Virus in a Specific Pathogen-Free Chicken Population in China. BioMed research international. 2016; 2016: 4275718-

4275718.

16. Su Q, Li Y, Zhang Y, Zhang Z, Meng F, Cui Z, et al. Newcastle disease virus-attenuated vaccine LaSota played a key role in the pathogenicity of contaminated exogenous virus. Veterinary research. 2018; 49(1): 80-80.

17. Varela AP, Dos Santos HF, Cibulski SP, Scheffer CM, Schmidt C, Sales Lima FE, et al. Chicken anemia virus and avian gyrovirus 2 as contaminants in poultry vaccines. Biologicals. 2014; 42(6): 346-50.

18. Marin SY, Barrios PR, Rios RL, Resende M, Resende JS, Santos BM, et al. Molecular characterization of contaminating infectious anemia virus of chickens in live commercial vaccines produced in the 1990s. Avian Dis. 2013; 57(1): 15-21.

19. Miller MM,Schat KA. Chicken infectious anemia virus: an example of the ultimate host-parasite relationship. Avian Dis. 2004; 48(4): 734-45.

20. Cardona C, Lucio B, O'Connell P, Jagne J, Schat KA. Humoral immune responses to chicken infectious anemia virus in three strains of chickens in a closed flock. Avian Dis. 2000; 44(3): 661-7.

21. Cardona CJ, Oswald WB, Schat KA. Distribution of chicken anaemia virus in the reproductive tissues of specific-pathogen-free chickens. J Gen Virol. 2000; 81(Pt 8): 2067-2075.

22. Meng F, Dong G, Zhang Y, Tian S, Cui Z, Chang S, et al. Co-infection of fowl adenovirus with different immunosuppressive viruses in a chicken flock. Poult Sci. 2018; 97(5): 1699-1705.

23. Todd D, Creelan JL, McNulty MS. Dot blot hybridization assay for chicken anemia agent using a cloned DNA probe. J Clin Microbiol. 1991; 29(5): 933-9.

24. Li X, Fang L, Li Y, Cui Z, Chang S, Zhao P. Establishment of a nested PCR assay for detection of chicken infectious anemia virus in avian attenuated vaccine. China Poultry. 2017; 39(10): 18-22.

25. Stamatakis A. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics. 2006; 22(21): 2688-90.

26. Eltahir YM, Qian K, Jin W, Wang P, Qin A. Molecular epidemiology of chicken anemia virus in commercial farms in China. Virol J. 2011; 8: 145.

27. McNulty MS, McIlroy SG, Bruce DW, Todd D. Economic effects of subclinical chicken anemia agent infection in broiler chickens. Avian Dis. 1991; 35(2): 263-8.

28. Su Q, Zhang Y, Li Y, Cui Z, Chang S, Zhao P. Epidemiological investigation of the novel genotype avian hepatitis E virus and co-infected immunosuppressive viruses in farms with hepatic rupture haemorrhage syndrome, recently emerged in China. Transbound Emerg Dis. 2019; 66(2): 776-784.

29. Ducatez MF, Chen H, Guan Y, Muller CP. Molecular epidemiology of chicken anemia virus (CAV) in southeastern Chinese live birds markets. Avian Dis. 2008; 52(1): 68-73.

30. Miller MM, Ealey KA, Oswald WB, Schat KA. Detection of chicken anemia virus DNA in embryonal tissues and eggshell membranes. Avian Dis. 2003; 47(3): 662-71.

31. Wang P, Lin L, Li H, Shi M, Gu Z, Wei P. Full-length genome sequence analysis of an avian leukosis virus subgroup J (ALV-J) as contaminant in live poultry vaccine: The commercial live vaccines might be a potential route for ALV-J transmission. Transbound Emerg Dis. 2018; 65(4): 1103-1106.

32. Zhao P, Dong X, Cui Z. Isolation, identification, and gp85 characterization of a subgroup A avian leukosis virus from a contaminated live Newcastle Disease virus vaccine, first report in China. Poult Sci. 2014; 93(9): 2168-74.

33. Barbosa T, Zavala G, Cheng S. Molecular characterization of three recombinant isolates of avian leukosis virus obtained from contaminated Marek's disease vaccines. Avian Dis. 2008; 52(2): 245-52.

34. Biswas SK, Jana C, Chand K, Rehman W, Mondal B. Detection of fowl poxvirus integrated with reticuloendotheliosis virus sequences from an outbreak in backyard chickens in India. Vet Ital. 2011; 47(2): 147-53.

35. Li J, Dong X, Yang C, Li Q, Cui Z, Chang S, et al. Isolation, identification, and whole genome sequencing of reticuloendotheliosis virus from a vaccine against Marek's disease. Poult Sci. 2015; 94(4): 643-9.

36. Wei K, Sun Z, Zhu S, Guo W, Sheng P, Wang Z, et al. Probable congenital transmission of reticuloendotheliosis virus caused by vaccination with contaminated vaccines. PLoS One. 2012; 7(8): e43422.

37. Li Y, Fu J, Chang S, Fang L, Cui S, Wang Y, et al. Isolation, identification, and hexon gene characterization of fowl adenoviruses from a contaminated live Newcastle disease virus vaccine. Poult Sci. 2017; 96(5): 1094-1099.

38. Su Q, Hou L, Gao Y, Liu X, Cui Z, Chang S, et al. Research Note: Molecular relationship of the fowl adenovirus serotype 4 isolated from the contaminated live vaccine and wild strains isolated in China, 2013-2018. Poult Sci. 2020; 99(12): 6643-6646.

39. Imai K, Mase S, Tsukamoto K, Hihara H, Matsumura T, Yuasa N. A long term observation of antibody status to chicken anaemia virus in individual chickens of breeder flocks. Res Vet Sci. 1993; 54(3): 392-6.

40. Renshaw RW, Soine C, Weinkle T, O'Connell PH, Ohashi K, Watson S, et al. A hypervariable region in VP1 of chicken infectious anemia virus mediates rate of spread and cell tropism in tissue culture. J Virol. 1996; 70(12): 8872-8.

41. Yamaguchi S, Imada T, Kaji N, Mase M, Tsukamoto K, Tanimura N, et al. Identification of a genetic determinant of pathogenicity in chicken anaemia virus. J Gen Virol. 2001; 82(Pt 5): 1233-1238.

42. Persengiev SP,Green MR. The role of ATF/CREB family members in cell growth, survival and apoptosis. Apoptosis. 2003; 8(3): 225-8.

Figures

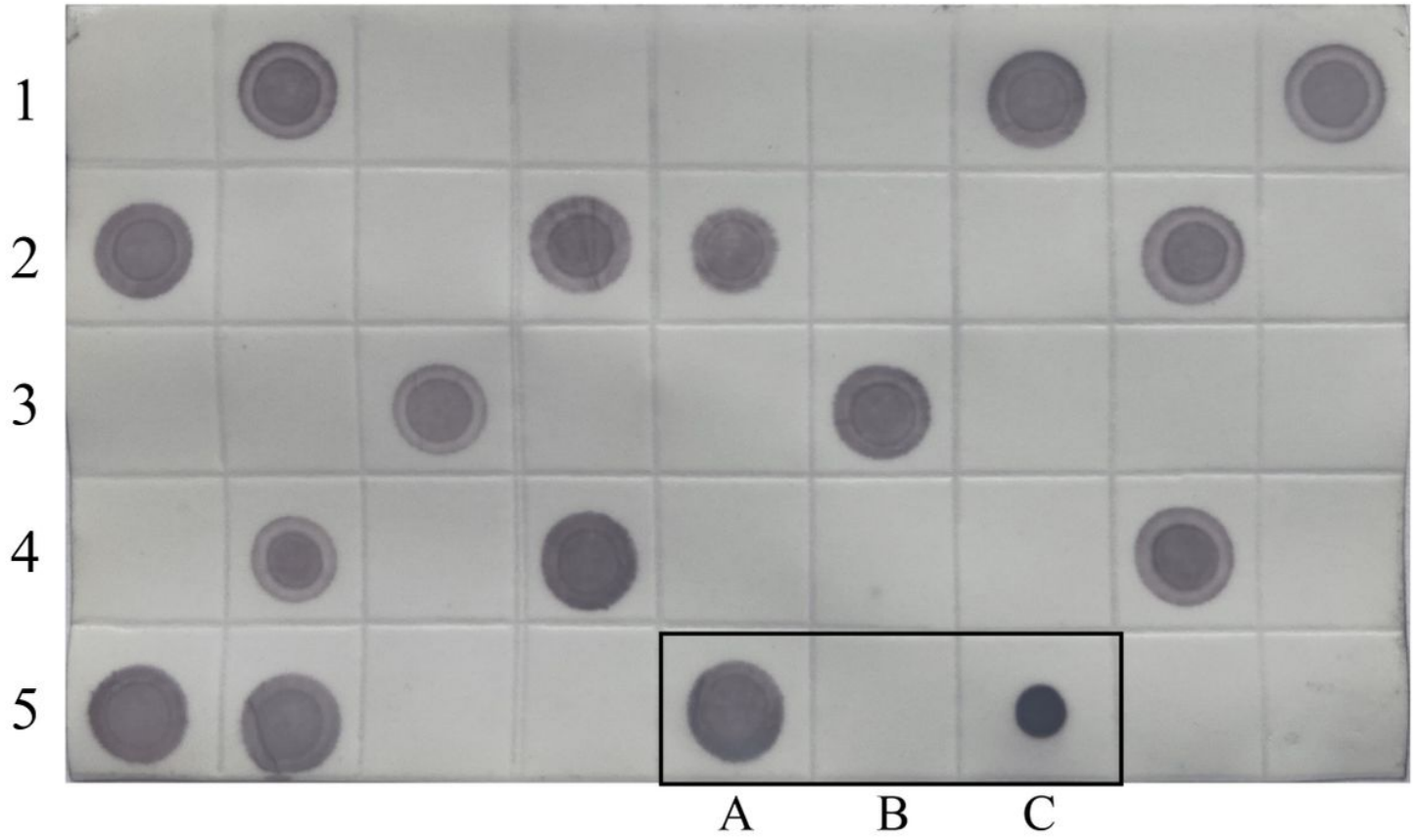

Figure 1

Dot-blot hybridization of chicken infectious anemia virus (CIAV). Dot-blot hybridization was performed to detect samples from SPF chicken embryos (A1-D5; n=40); E5: PCR positive control; F5: PCR negative control; G5: CIAV plasmid standard was used as positive control.

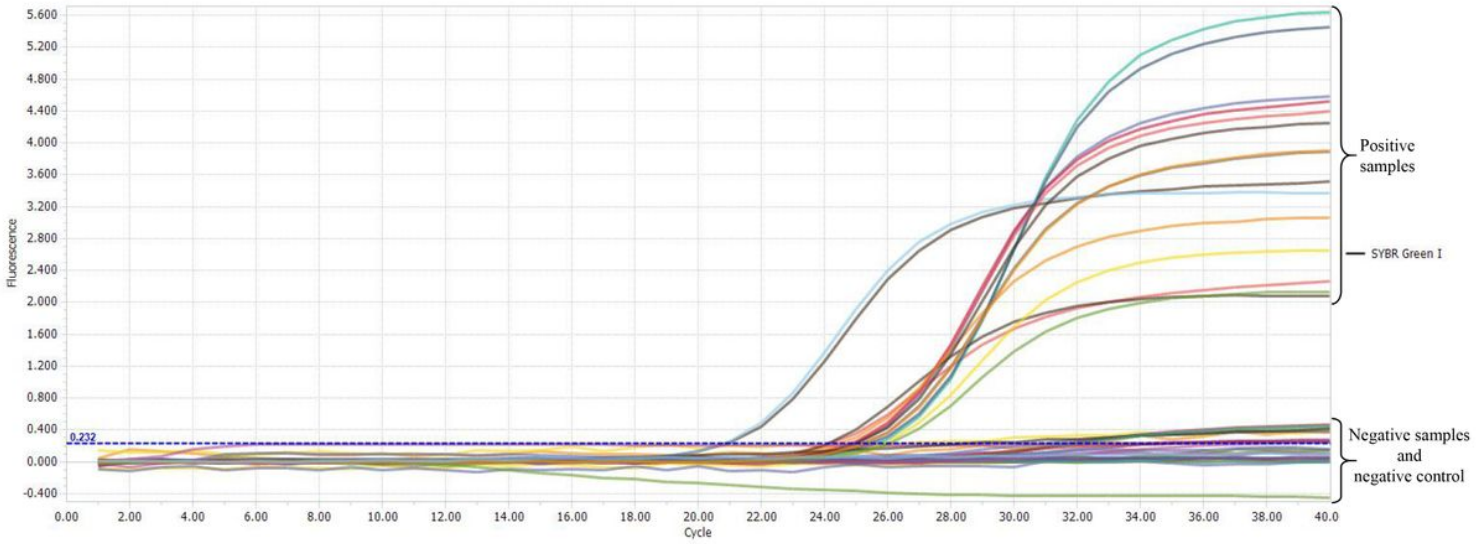

Figure 2

Detection of CIAV DNA in SPF chicken embryos using quantification real-time PCR. Data were distributed in an S-curve, and a negative standard curve wasn't obtained a Ct value peak. The infected the sample of CIAV positive are shown with a Ct value of 20 in the figure, which was detected in the infected SPF chicken embryos.

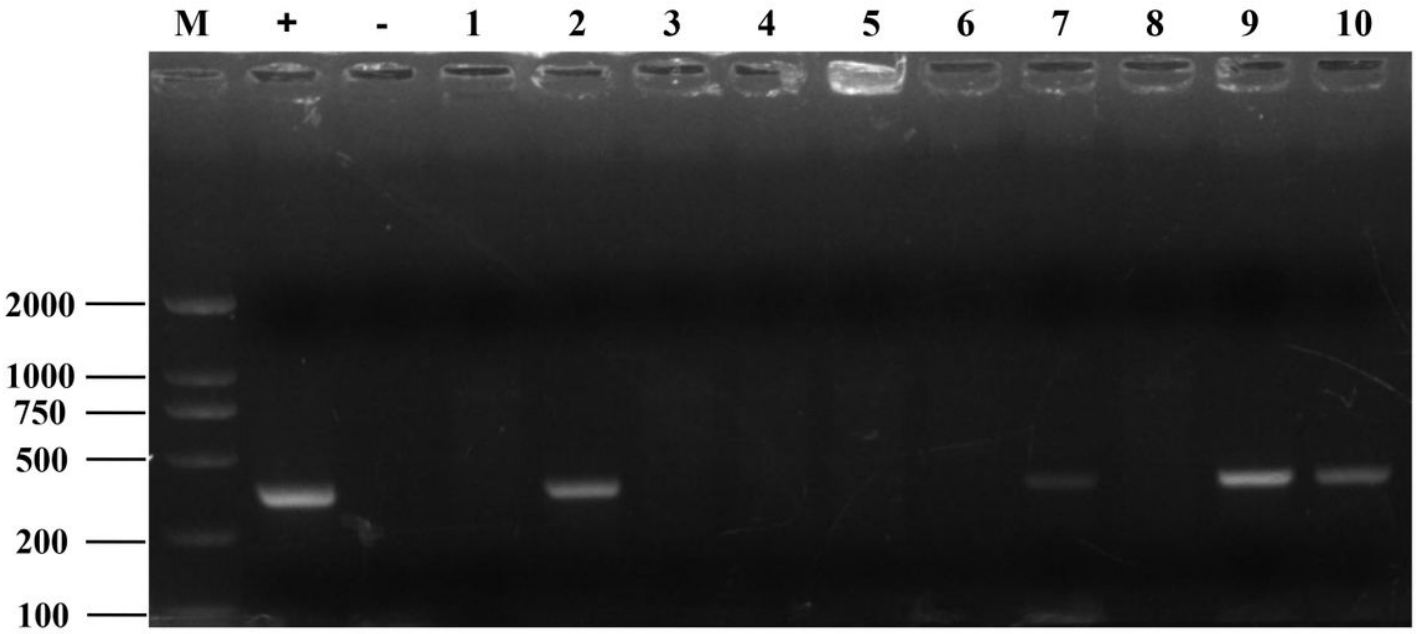

Figure 3

Agarose gel electrophoresis of nested-PCR products. Lane 1: 1,000 bp DNA ladder; lane 2: positive control; lane 3: negative control; lanes 4–13: DNA of SPF chicken embryo samples.

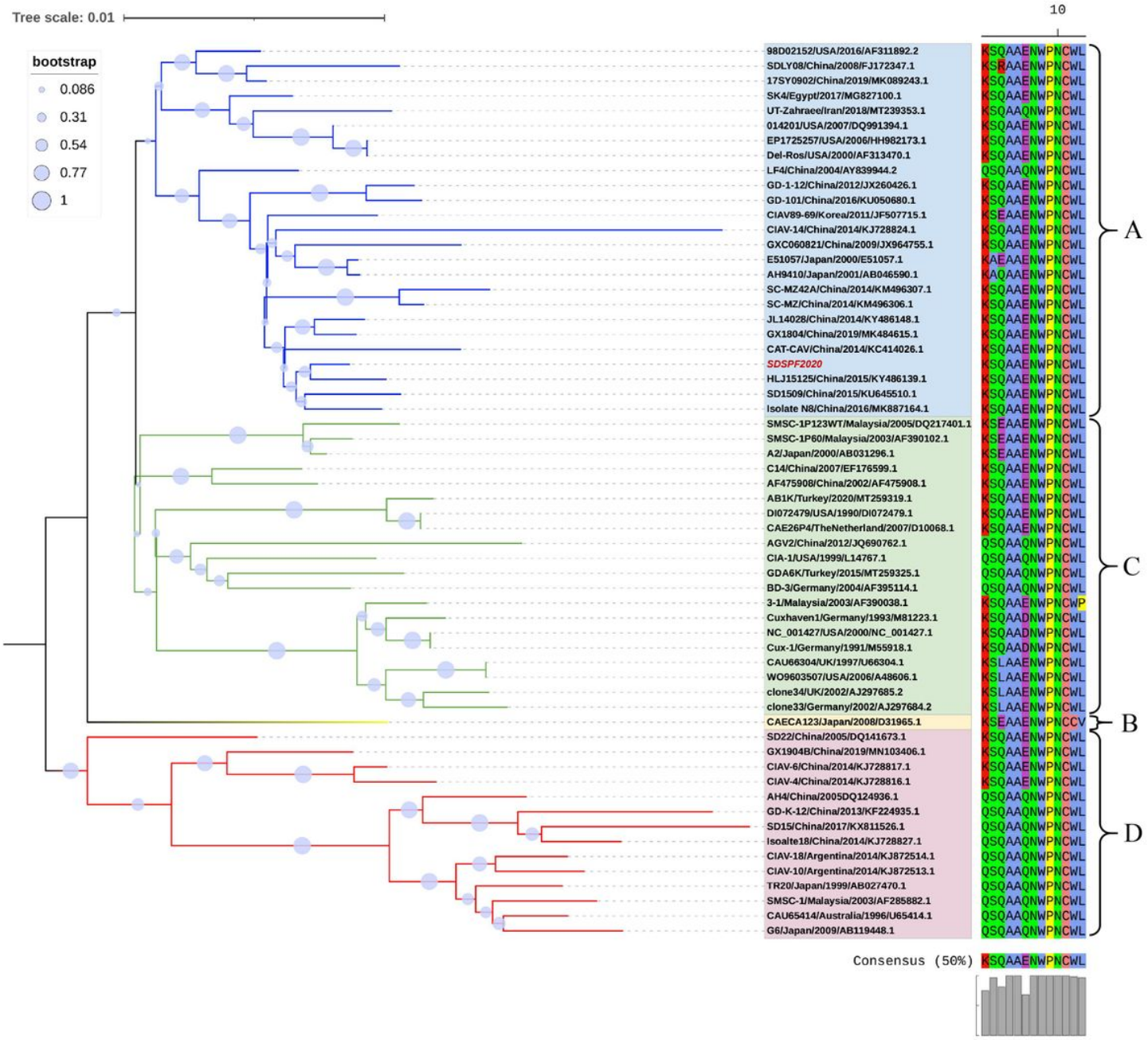

Figure 4

Phylogenetic analysis of SDSPF2020 complete genomes and other complete genomes available in GenBank, based on CIAV nucleotide sequences. The red label represents the isolated strain of the CIAV-SDSPF2020. Visualized multiple sequence alignments of VP1 protein hypervariable regions directly next to the tree (amino acid 139 to 151).

No.	Strain	75	89	97	125	139	144	157	251	287	370	394	413	447
1	01–4201	V	T	M	I	K	E	V	R	S	G	Q	S	G
2	98D02152	V	T	M	I	K	E	V	R	S	G	Q	S	T
3	3711	V	T	M	I	K	E	V	R	S	G	Q	S	S
4	A2	V	T	M	I	K	E	M	R	S	G	Q	A	T
5	AB1K	V	T	M	I	K	E	M	R	T	S	Q	A	T
6	AF475908	V	T	M	I	K	E	V	R	T	S	Q	A	T
7	AH4	I	T	L	I	Q	Q	V	R	T	S	Q	A	S
8	AGV2	V	T	M	I	Q	Q	V	R	A	S	Q	A	T
9	BD-3	I	T	L	I	Q	Q	V	R	A	T	Q	A	T
10	CAE26P4	V	T	M	I	K	E	M	R	T	S	Q	A	T
11	C14	V	T	M	I	K	E	V	R	T	S	Q	A	S
12	CAT-CAV	V	T	M	L	K	E	V	R	S	G	Q	S	S
13	CAV-10	I	T	L	I	Q	Q	V	R	T	S	Q	A	S
14	CIAV89-69	V	T	D	L	K	E	V	R	S	G	Q	S	S
15	Cux-1	V	T	M	I	K	D	V	Q	A	S	Q	A	T
16	clone 33	I	T	M	L	K	E	V	L	A	S	Q	A	T
17	HLJ15125	V	T	M	L	K	E	M	R	S	G	Q	S	T
18	Del-Ros	V	T	M	I	K	E	V	R	S	G	Q	S	G
19	G6	I	T	L	I	Q	Q	V	R	T	S	Q	A	S
20	GD-101	V	T	M	L	K	E	M	R	S	G	Q	S	S
21	GX1804	V	T	M	L	K	E	M	R	S	G	Q	S	S
22	LF4	I	T	M	L	Q	Q	V	R	S	S	Q	S	S
23	SD15	I	T	L	I	Q	Q	V	R	T	T	Q	A	S
24	SD22	V	T	M	L	K	E	V	R	S	S	Q	A	S
25	SDLY08	V	T	M	I	K	E	V	R	S	G	Q	S	T
26	SC-MZ	V	T	M	L	K	E	M	R	S	G	Q	S	S
27	Isoalte18	I	T	L	I	Q	Q	V	R	A	S	Q	A	P
28	N8	V	T	M	L	K	E	V	R	S	G	Q	S	S
29	TR20	I	T	L	I	Q	Q	V	R	T	S	Q	A	S
30	WO9603507	V	T	M	L	K	E	V	Q	A	S	Q	A	T
CIAV-SDSPF2020		V	T	M	L	K	E	M	R	S	G	Q	S	S

Figure 5

Amino acids at sites of common substitutions in VP1 protein coding sequences of different CIAVs. Each site differences are indicated by different color base box. The last row in the table shows the sites of the new isolate.

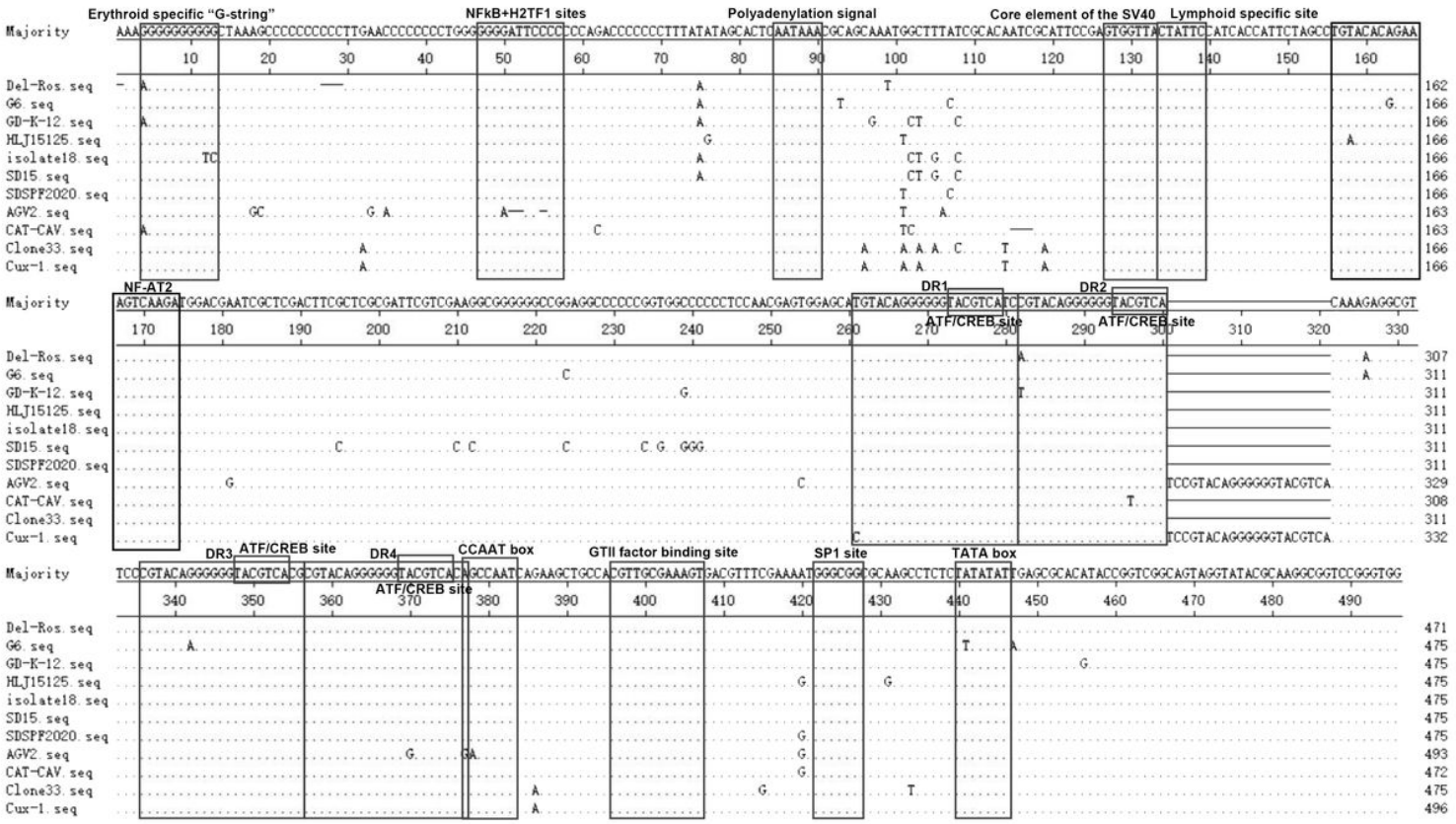

Figure 6

Sequence alignment of the non-coding region sequences of SDSPF2020 in contrast to 10 reference isolates. The sequences in black frames are the motifs of transcriptional regulatory elements in this study. Hide residues that match the consensus as dots and differences are indicated by the single-letter code.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.docx