

Article

Co-Infection and Phylogenetic Evolution of CIAV in Marek's Disease Tumour-Bearing Flocks in Central China

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Abstract

The avian immunosuppressive and neoplastic diseases are great threats to the poultry industry, causing huge economic losses worldwide. Most recently, the emerging hypervirulent variants of Marek's disease virus (HV-MDV), partially co-infected with avian leukosis virus (ALV) and/or reticuloendotheliosis virus (REV), have been identified as the key driver of tumour outbreaks in vaccinated chicken flocks, but the role of chicken infectious anemia virus (CIAV) remains unclear. Herein, we have investigated the prevalence and co-infection of CIAV in 71 clinical tumour-bearing flocks collected from central China during 2021–2023, which has shown a CIAV positivity rate of 59.2% (42/71). Notably, the incidence of CIAV mono-infection increased significantly from 0% (0/29) in 2021 to 23.7% (9/38) in 2023, whereas CIAV + MDV co-infection decreased from 65.5% (19/29) to 31.6% (12/38). A total of 20 viral genomes of epidemic CIAV isolates from diverse sources were obtained, and the phylogenetic analysis, including 91 reference isolates were clustered into four major lineages (A–D), with clade C further subdivided into subclades C1 and C2. Clade C1 consisted predominantly of Asian isolates, with 88.5% (46/52) of the isolates originating from mainland China. Among the 20 new isolates, 17 were clustered in subclade C1, two in C2, and one in B. The VP1 gene phylogeny showed a topology largely consistent with that of the whole-genome analysis. Moreover, all newly characterized isolates contained glutamine (Q) at VP1 residue 394, a molecular marker associated with high pathogenicity. Collectively, our data suggest that prevalent HV-MDV variants together with CIAV co-infections are the primary drivers of the ongoing tumour outbreaks in Chinese poultry flocks. Notably, the significantly increased CIAV mono-infections, possibly resulting from an independently evolving lineage among circulating Chinese strains, are likely to pose a new challenge for future control of disease.

Keywords: poultry; CIAV; MDV; co-infection; epidemiology; phylogenetic evolution



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1. Introduction

Avian immunosuppressive and neoplastic diseases, such as Marek's disease (MD), avian leukosis (AL), reticuloendotheliosis (RE), and chicken infectious anemia (CIA), are major epidemics in chicken flocks characterized by lymphoid hyperplasia, tumour formation, and severe immunosuppression, leading to vaccine failures, secondary infections and substantial economic losses to the poultry industry worldwide [1]. MD is caused by pathogenic Marek's disease virus (MDV) and induces serious immune organ atrophy, neural damage, and lymphoproliferative tumours that ultimately cause a large number of deaths. Globally, the direct economic losses resulting from MD outbreaks are estimated annually at 1–2 billion US dollars [2]. For decades, efficient control of MD has relied primarily on vaccination. However, the continuously expanded scale of poultry farming and the sustained high immune pressure imposed by long-term vaccination programmes have led to a significant increase in MDV virulence [3]. Some epidemic MDV strains, including the very virulent plus MDV (vv + MDV) and particularly the emerging hypervirulent variants of MDV (HV-MDV) [4–6], have significantly evaded the protection conferred by classical MD vaccines and caused frequent outbreaks of MD all over the world, especially in Asia countries [7], thereby posing a serious threat to the global poultry industry.

In addition to MDV, co-infections of avian leukosis virus (ALV), reticuloendotheliosis virus (REV), and even chicken infectious anemia virus (CIAV) further aggravate immunosuppression and tumourigenesis in chicken flocks, posing a major challenge to poultry health. To date, no commercial vaccine is available for the control of AL, and it completely relies on stringent biosecurity measures and continuous eradication programmes in breeding stocks to minimize viral prevalence. For REV co-infection, it rarely occurs as a primary etiological event but can exacerbate secondary immunopathological conditions in affected flocks. Nevertheless, its overall epidemiological impact on modern poultry production remains relatively limited [6]. However, for CIA, another major immunosuppressive disease caused by CIAV and typically affecting young chicks lacking maternal antibodies, it leads to lymphoid organ atrophy, aplastic anemia, and subcutaneous and muscular hemorrhages in hosts, displaying a high morbidity in infected flocks [8]. Most seriously, CIAV infection usually undermines the protection efficacy induced by other poultry vaccines and predisposes birds to potential secondary infections caused by diverse pathogenic agents, thereby resulting in considerable indirect economic losses [9].

CIAV belongs to the genus *Gyrovirus* within the family *Anelloviridae* [10] and has a small circular single-stranded DNA genome of approximately 2.3 kb that encodes three overlapping open reading frames (ORFs), designated VP1, VP2, and VP3 [11]. VP1, the sole capsid protein, is highly immunogenic and capable of eliciting protective neutralizing antibodies. VP2 acts as a multifunctional scaffolding protein with intrinsic phosphatase activity that facilitates the proper folding of VP1, thereby promoting the exposure of key antigenic epitopes and enhancing antibody recognition [12,13]. Moreover, VP2 has been reported to act as an apoptosis-inducing factor, suggesting a crucial role in CIAV-mediated cytopathogenicity [14]. VP3, also known as apoptin, serves as a principal virulence determinant of CIAV [15]. Both VP2 and VP3 are relatively conserved among CIAV isolates, whereas residues 139–151 of VP1 represent a hypervariable region that is closely associated with virulence and commonly used for molecular epidemiological and phylogenetic analysis [16].

Since the first report in 1979 [17], CIAV has spread globally to be one of the most common infectious diseases in poultry. China is the world's largest poultry producer that sustains more than 16 billion birds annually, including a population exceeding 1 billion laying hens. Since 2019, large-scale outbreaks of immunosuppressive and neoplastic diseases have re-emerged across the commercial poultry industry in China [4–6]. Our previous

work revealed that the prevalence of HV-MDV, partially co-infected with ALV and/or REV, was the predominant causative agent responsible for the frequent outbreaks of tumour cases in vaccinated chicken flocks during 2020–2021 [4–6]. However, since 2022, numerous clinical cases with tumour-bearing birds and potentially associated with CIAV co-infections have been reported by local veterinarians and farmers, resulting in substantial economic losses in poultry farms. To elucidate the current epidemiological landscape of CIAV and MDV co-infections in chickens, we have conducted a systematic investigation on clinically suspected tumour cases across 71 affected poultry farms distributed in central China during 2021–2023. Furthermore, genetic and phylogenetic analyses were performed based on the viral genomes and VP1 genes of currently circulating CIAV viruses to characterize the molecular evolution and provide a scientific basis for future effective prevention and control of the disease.

2. Materials and Methods

2.1. Ethics Statement

The diseased chickens collected from poultry farms were provided with free access to water and feed, and then humanely euthanized for sample collection. The experimental protocol was reviewed and approved by the Laboratory Animal Management Committee of Institute of Animal Health (IAH), Henan Academy of Agricultural Sciences (HAAS, Zhengzhou, China), following the protocols of the Laboratory Animal Guidelines for the Ethical Review of Animal Welfare permitted by State Administration for the Market Regulation and Standardization Administration of China (permit no. GB/T 35892-2018; *Laboratory animal—Guideline for ethical review of animal welfare*, Standards Press of China: Beijing, China, 2018).

2.2. Sample Collection

The liver and spleen samples were collected from 217 chickens of different breeds and ages exhibiting suspected tumour lesions across 71 commercial poultry farms located in Henan, Shandong, and Shanxi Provinces in central China. Samples from 29 cases obtained in 2021 were derived from our previous investigations [6], while additional clinical specimens collected from 42 tumour-bearing cases reported by local veterinarians or farmers and collected during 2022–2023 were newly included in this study (sampling sites are shown in Figure 1). Detailed background information on the recently collected 42 clinical cases from poultry farms is summarized in Table S1.

2.3. DNA Extraction

Approximately 10 mg of each sample of livers (for CIAV) or spleens (for MDV) was suspended in 500 µL of sterile phosphate-buffered saline (PBS, pH 7.4) in a 1.5 mL Eppendorf tube containing two autoclaved stainless-steel beads. The tissues were homogenized at 50 Hz for 5 min at 4 °C using an automated tissue grinder (SCIENTZ-48L; Xinzhi Biologicals, Ningbo, China), followed by three freeze–thaw cycles at −20 °C for 30 min each. The homogenates were then centrifuged at 8000 rpm for 5 min, and the resulting supernatants were collected and stored at −80 °C until further processing. Genomic DNA was extracted using the TIANamp Genomic DNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's protocols. DNA concentrations were determined, standardized to 300 ng/µL, and stored at −20 °C until use.

2.4. PCR Amplification of MDV

The meq gene, specific to MDV-1 and with a length of 1020 bp, was amplified using the primer pair MDV-meq-F/R [6], with primer sequences listed in Table S2. DNA

extracted from 77 samples collected across 42 farms during 2022–2023 was selected for initial screening. Each of 20 μ L PCR reaction mixture contained 10 μ L 2 $\times$ EasyTaq PCR SuperMix (+dye; TransGen Biotech, Beijing, China), 8 μ L of ddH₂O, 0.5 μ L of each primer (10 μ M), and 1 μ L of template DNA (100 ng). The thermal cycling programme was set as follows: initial denaturation at 95 $^{\circ}$ C for 5 min, 30 cycles of 95 $^{\circ}$ C for 30 s, 58 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 1 min, followed by a final extension at 72 $^{\circ}$ C for 10 min. Amplified products were separated by electrophoresis on a 1% agarose gel and visualized using a gel documentation system under UV illumination. If the positive band was detected from any of the spleen samples from a bird, the chicken was designated as an MDV-infected individual, and the corresponding chicken flock/poultry farm was identified as a confirmed MDV-positive flock/farm.

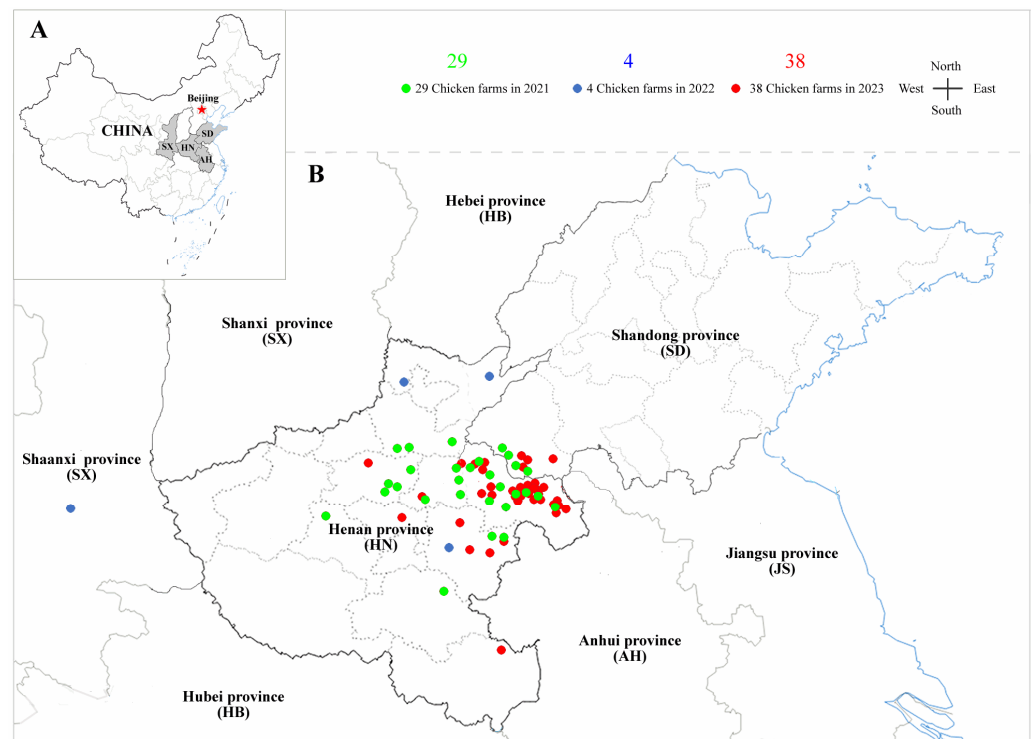


Figure 1. Geographic distribution of poultry farms with neoplastic disease cases in central China during 2021–2023. (A) Geographic location of the central provinces in China. (B) Distribution of poultry farms with case reports in central China. Cases sampled in 2021, 2022, and 2023 are shown by green, blue, and red spots, respectively.

2.5. PCR Amplification of CIAV

Three pairs of CIAV-specific primers, CIAV-1F/1R, CIAV-2F/2R, and CIAV-3F/3R, covering the entire viral genome [18], were synthesized by Sangong Biotechnology Co., Ltd. (Shanghai, China). The primer sequences are listed in Table S2, and the amplicons are expected in size of 842 bp, 990 bp, and 737 bp, respectively. The most efficient primer pair, CIAV-2F/2R, was used for the first round of screening of all the clinical samples collected during 2021–2023. Each of the 20 μ L PCR reactions contained 10 μ L of 2 $\times$ EasyTaq PCR SuperMix (+dye; TransGen Biotech, China), 8 μ L of ddH₂O, 0.5 μ L of each primer (10 μ M), and 1 μ L of template DNA (300 ng). Cycling parameters were set as follows: initial denaturation at 95 $^{\circ}$ C for 5 min, followed by 30 cycles of 95 $^{\circ}$ C for 30 s, 55 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 1 min, with a final extension at 72 $^{\circ}$ C for 10 min. Amplified products were separated by 1% agarose gel electrophoresis and visualized using a gel documentation system under UV illumination. Once the positive band was detected in any of the liver samples from a bird, the chicken was designated as a CIAV-infected individual,

and the corresponding chicken flock/poultry farm was identified as a confirmed CIAV-positive flock/farm.

2.6. Cloning and Sequencing of CIAV Genome

Twenty CIAV-positive liver samples collected from different poultry farms were selected for full-genome amplification using the three primer pairs listed in Table S2 [18]. PCR amplifications for VP1, VP2, and VP3 genes were performed as previously described, yielding amplicons of 842 bp, 990 bp, and 737 bp, respectively. The PCR products were verified by 1% agarose gel electrophoresis, purified using a gel extraction kit (Tiangen Biotechnology, China), and cloned into the pMD18-T vector (TaKaRa Biotechnology, Dalian, China). The recombinant plasmids were then transformed into *Escherichia coli* DH5 α . Positive clones were identified and subjected to Sanger sequencing (General Biosystems, Chuzhou, China). The resulting sequences were validated using BLAST v2.16.0 and assembled into complete genomes with DNASTAR software v7.1 (DNASTAR Inc., Madison, WI, USA). All newly obtained CIAV genome sequences were deposited in the GenBank database, and accession numbers are provided in Table S3.

2.7. Phylogenetic Analysis

The whole genome sequences of 91 CIAV reference strains were collected from the GenBank database (Table S3). The complete viral genome and VP1 genes of 111 CIAV isolates, including 20 viruses newly obtained in this study, were separately aligned and analyzed using MEGA v11 [19]. Phylogenetic trees, based on whole viral genomes or VP1 genes, were constructed using the Neighbour-Joining method. The evolutionary distances were computed using the Maximum Composite Likelihood model, assuming uniform rates among sites. Gaps and missing data were treated using the pairwise deletion option. The reliability of the trees was estimated with 1000 bootstrap replicates and further refined in iTOL v6 [20].

2.8. Sequence Analysis of the VP1 Gene and UTRs of CIAV Isolates

The homology of VP1 amino acid (aa) sequences between 20 newly obtained CIAV isolates and 91 reference strains was analyzed using DNASTAR MegAlign (DNASTAR Inc., Madison, WI, USA). Key mutations in the VP1 hypervariable region (aa 139–151) were examined for potential associations with pathogenicity among epidemic CIAV Chinese strains. Additionally, the untranslated regions (UTRs) of 20 newly obtained CIAV isolates were analyzed and characterized to evaluate possible regulatory variations.

2.9. Whole Gene Recombination Analysis of CIAV

Recombination events in the viral genomes of 20 newly obtained CIAV isolates and 91 reference strains were analyzed using RDP5 software v5.0 [21], which utilizes seven algorithms, including RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan, and 3Seq. A Bonferroni-corrected *p*-value cutoff of 0.05 was used as the threshold for statistical significance. Events supported by at least five algorithms were considered significant.

2.10. Statistical Analysis

Data compilation and visualization were performed using GraphPad Prism 10.0 (GraphPad Software, Boston, MA, USA). The 95% confidence intervals (CIs) for prevalence rates at both individual and flock levels were calculated using the Wilson/Brown method. Comparisons were performed between 2021 and 2023 in Central China using Fisher's exact test. Differences in the distribution of onset age of disease across different years (2021, 2022, and 2023) were analyzed using the Kruskal–Wallis test, followed by Dunn's multiple comparisons test. A *p*-value of <0.05 was considered statistically significant.

3. Results

3.1. CIAV Infection in MD Tumour-Bearing Chicken Flocks in 2021

To evaluate the prevalence of CIAV and co-infections in poultry farms with tumour-bearing cases collected in 2021, liver samples collected from 29 chicken flocks across central China [6] were retrospectively screened for CIAV infection. As shown in Table 1, the overall positive rate of CIAV infection in individual birds was 40.7% (57/140), and for detected chicken flocks, co-infection of CIAV + MDV reached a positive rate of 65.5% (19/29). Regionally, as listed in Table S4, Henan province ($n = 25$ flocks) exhibited individual- and flock-level positive rates of 37.5% (45/120) and 64.0% (16/25), respectively, whereas Shandong province ($n = 4$ flocks) showed correspondingly higher rates of 60.0% (12/20) and 75.0% (3/4). Interestingly, CIAV co-infection was confirmed in 65.5% (19/29) of detected flocks, while none of the CIAV mono-infections (0/29) were observed in these MD tumour-bearing chicken flocks. For these 29 cases, based on the collected background data [6], the onset ages of disease distribution for MDV and CIAV infections were both concentrated to 60–120 days, with a median age of 90 days (Figure 2A,B, green bars).

Table 1. Co-infections of CIAV in suspected tumour-bearing chicken flocks collected in central China in 2021.

No.	Poultry Farms	Breeds	Category	Positive Rates of CIAV		Diagnosis Results for Chicken Flocks #		
						Mono-Infection		Co-Infection
				Nos.	Percent	CIAV	MDV	CIAV + MDV
1	HNXZ1	Partridge chicken	Layer	0/2	0%		*	
2	HNZM	Partridge chicken	Broiler	0/5	0%		*	
3	HNXZ2	Liangfenghua	Breeder	0/5	0%			
4	HNYY1	Jinghong	Layer	3/5	60%			*
5	HNLK1	Hyline Brown	Layer	2/5	40%			*
6	HNLK2	Jinghong	Layer	2/4	50%			*
7	HNZC1	Jinghong	Layer	5/5	100%			*
8	HNSQ1	Jinghong	Layer	1/5	20%			*
9	HNLY1	Jinghong	Layer	3/4	75%			*
10	HNYY1	Jinghong	Layer	0/5	0%		*	
11	SDCX1	Jinghong	Layer	0/5	0%		*	
12	SDSX	Jinghong	Layer	4/5	80%			*
13	SDCW	Hyline Brown	Layer	4/5	80%			*
14	SDCX2	Jinghong	Layer	4/5	80%			*
15	HNSQ2	Jinghong	Layer	4/5	80%			*
16	HNSC	Hyline Brown	Layer	0/5	0%		*	
17	HNYY2	Jinghong	Layer	2/5	40.0%			*
18	HNZC2	Jinghong	Layer	5/5	100%			*
19	HNXZ3	Partridge chicken	Breeder	1/5	20%			*
20	HNQX	Jinghong	Layer	3/5	60.0%			*
21	HNZC3	Jinghong	Layer	3/5	60.0%			*
22	HNYY2	Jinghong	Layer	2/5	40.0%			*
23	HNPDS	Hyline Brown	Layer	0/5	0%		*	
24	HNLY2	Jinghong	Layer	4/5	80.0%			*
25	HNZC4	Hyline Brown	Layer	0/5	0%		*	
26	HNSX	Jinghong	Layer	4/5	80.0%			*
27	HNZC5	Jinghong	Layer	1/5	20.0%			*
28	HNFQ	Hyline Brown	Layer	0/5	0%		*	
29	HNWS	Muyuan Red	Layer	0/5	0%		*	
Total	NA	NA	NA	57/140	40.7%	0% (0/29)	31.0% (9/29)	65.5% (19/29)

Data of MDV infection was collected from our previous work [6]. * The star indicates positive pathogens detected in chicken flocks. NA, not applicable.

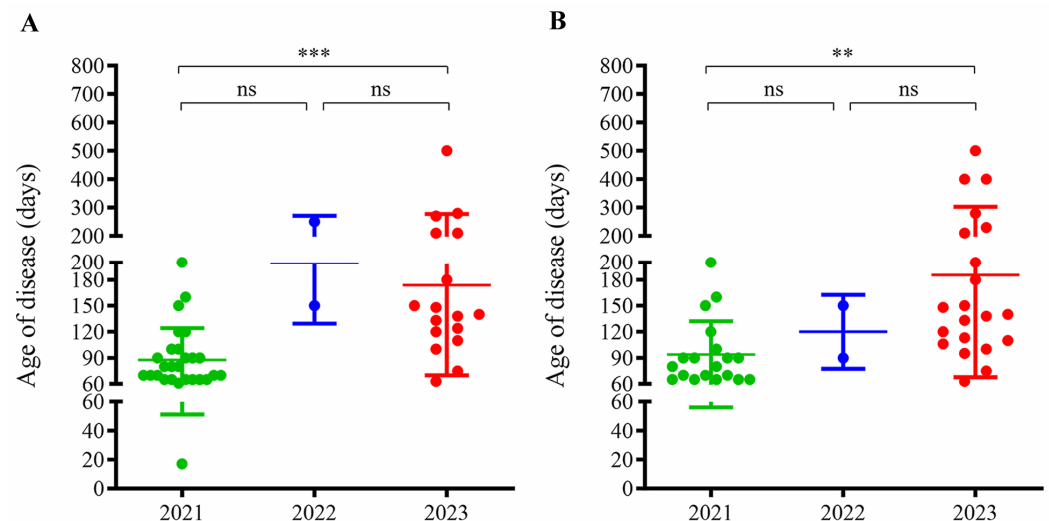


Figure 2. Distribution of the onset ages of diseases. (A) Distribution of the onset age for MD cases. A total of 28, 2, and 17 MD cases collected in 2021, 2022 and 2023 are shown by green, blue, and red bars, respectively. (B) Distribution of the onset age for CIA cases. A total of 19, 2, and 21 CIA cases collected in 2021, 2022 and 2023 are shown by green, blue, and red bars, respectively. Statistical significance was analyzed using the Kruskal–Wallis test followed by Dunn’s multiple comparisons test. Asterisks indicate statistically significant differences between groups (** $p < 0.01$ and *** $p < 0.001$); ns, no significance.

3.2. Prevalence and Infections of MDV and CIAV in Poultry Farms During 2022–2023

To investigate the current infection dynamics of CIAV and MDV in poultry farms with tumour outbreaks, a comprehensive molecular survey was conducted on 41 tumour-bearing chicken flocks collected from Henan, Shandong, and Shanxi provinces in central China during 2022–2023 (Figure 1). Regionally in 2023, as shown in Table S4, Henan province ($n = 34$ flocks) showed a CIAV-positive rate of 52.1% (25/48) at the individual level and 52.9% (18/34) at the flock level, while in Shandong province ($n = 4$ flocks), both the individual and flock-level rates reached to 75.0% (3/4). As shown in Tables 2 and 3, the overall CIAV-positive rates detected in 2022 and 2023 were 14.3% (2/14) and 53.8% (28/52) at the individual level, and 50% (2/4) and 55.3% (21/38) at the flock level, respectively. The corresponding individual MDV-positive rates were 68.2% (15/22) and 49.1% (27/55), accompanied by the flock-positive rates of 50% (2/4) and 44.7% (17/38), respectively. However, in 2023, the mono-infections of CIAV or MDV were detected in 23.7% (9/38) and 13.2% (5/38) of flocks, respectively, whereas co-infections involving both viruses occurred in 31.6% (12/38) of flocks. Unexpectedly, based on the collected data of the onset age of disease in layers, as shown in Table S1 and Figure 2A,B (red bars), the onset days of MD and CIA cases outbreak in 2023 with tumours mainly ranged from 60 to 300 days, with an increased median age of 180 days.

Table 2. Co-infections of CIAV in suspected tumour-bearing chicken flocks in central China in 2022.

No.	Poultry Farms	Breeds	Category	Positive Rates of Two Pathogens				Diagnosis Results for Chicken Flocks		
				CIAV		MDV		Mono-Infection		Co-Infection
				Nos.	Percent	Nos.	Percent	CIAV	MDV	CIAV + MDV
1	SXXA	Liangfenghua	Breeder	0/5	0%	11/12	91.7%		*	
2	HNPY	Hyline White	Layer	1/2	50.0%	0/2	0%	*		
3	HNNH	Roman laying hens	Layer	0/5	0%	4/5	80.0%		*	
4	HNNY	Hyline Brown	Layer	1/2	50.0%	0/3	0%	*		
Total	NA	NA	NA	2/14	14.3%	15/22	68.2%	50.0% (2/4)	50.0% (2/4)	0% (0/4)

* The star indicates positive pathogens detected in chicken flocks. NA, not applicable.

Table 3. Co-infections of CIAV in suspected tumour-bearing chicken flocks in central China in 2023.

No.	Poultry Farms	Breeds	Category	Positive Rates of Two Pathogens				Diagnosis Results for Chicken Flocks		
				CIAV		MDV		Monoinfection		Co-Infection
				Nos.	Percent	Nos.	Percent	CIAV	MDV	CIAV + MDV
1	HNXY	Partridge chicken	Breeder	1/4	25.0%	3/4	75.0%			*
2	HNXYC3	UA	Layer	0/1	0%	0/1	0%			
3	HNXY1	Jingfen No.6	Layer	0/1	0%	0/1	0%			
4	HNXYC4	UA	Layer	0/1	0%	1/1	100%		*	
5	SDCX4	UA	Layer	1/1	100%	1/1	100%			*
6	HNMQ1	UA	Layer	0/1	0%	0/1	0%			
7	HNSQ3	UA	Layer	0/1	0%	0/1	0%			
8	SDSX2	UA	Layer	0/1	0%	1/1	100%		*	
9	HNMQ2	UA	Layer	0/1	0%	0/1	0%			
10	HNSQ4	Hyline Brown	Layer	0/1	0%	0/1	0%			
11	HNHB	Hyline Brown	Layer	1/1	100%	2/2	100%			*
12	HNXY2	UA	Layer	0/1	0%	0/1	0%			
13	HNXYC5	Green eggshell chicken	Layer	0/1	0%	0/1	0%			
14	HNXYC6	Hyline Brown	Layer	1/1	100%	0/1	0%	*		
15	HNHL2	Jinghong	Layer	1/1	100%	0/1	0%	*		
16	HNSQ5	Nongda No.3 laying hens	Layer	0/1	0%	0/1	0%			
17	HNSQ6	Local Chicken	Layer	0/1	0%	1/1	100%		*	
18	HNXY3	UA	Layer	0/1	0%	1/1	100%		*	
19	HNLK3	UA	Layer	0/1	0%	0/1	0%			
20	HNLY	Partridge chicken	Layer	0/5	0%	6/7	85.7%		*	
21	HNXYC7	UA	Layer	0/1	0%	0/1	0%			
22	HNXYC8	Green eggshell chicken	Layer	0/1	0%	0/1	0%			
23	HNXYC9	Game cock	Breeder	1/1	100%	0/1	0%	*		
24	HNSQ7	UA	Layer	1/1	100%	1/1	100%			*
25	HNWS2	Xinyang Black chicken	Layer	8/8	100%	3/8	37.5%			*
26	HNHL3	Jinghong	Layer	1/1	100%	1/1	100%			*
27	SDCX5	Xinlian Black chicken	Layer	1/1	100%	0/1	0%	*		
28	HNDC	Xinlian Black chicken	Layer	1/1	100%	0/1	0%	*		
29	HNSX2	Hyline Brown	Layer	1/1	100%	0/1	0%	*		
30	HNSQ8	UA	Layer	1/1	100%	1/1	100%			*
31	HNTK	UA	Layer	1/1	100%	1/1	100%			*
32	HNSQ9	Green eggshell chicken	Layer	1/1	100%	1/1	100%			*
33	HNXY4	UA	Layer	1/1	100%	1/1	100%			*
34	HNZK	UA	Layer	1/1	100%	0/1	0%	*		
35	HNXY5	UA	Layer	1/1	100%	0/1	0%	*		
36	HNLY3	Black-pumage chicken	Layer	1/1	100%	1/1	100%			*
37	SDCX6	UA	Layer	1/1	100%	1/1	100%			*
38	HNMQ3	UA	Layer	1/1	100%	0/1	0%	*		
Total	NA	NA	NA	28/52	53.8%	27/55	49.1%	23.7% (9/38)	13.2% (5/38)	31.6% (12/38)

* The star indicates positive pathogens detected in chicken flocks. UA, unavailable chicken species; NA, not applicable.

3.3. Whole-Genome Sequencing and Recombination Analysis of CIAV Isolates

A total of 20 strongly CIAV-positive samples confirmed by PCR amplification, as demonstrated in Figure 3, were subjected to full-genome amplification using three primer pairs covering the entire viral genome (Table S2). Successfully, the amplified products were cloned, sequenced, and assembled into 20 complete circular genomes, with GenBank Acc. Nos. listed in Table S3. All isolates possessed the canonical 2298 bp genome without insertions or deletions. Pairwise nucleotide identities among the 20 isolates ranged from 96.6% (e.g., CIAV-HNLK2 vs. CIAV-HNLY1-1) to 99.7% (e.g., CIAV-HNYY1 vs. CIAV-SDCX2). Compared to the vaccine strains Cux-1 and Del-Ros, the highest sequence identities were observed in CIAV-HNAY (98.0% and 98.6%, respectively), whereas CIAV-HNLY1-1 exhibited the lowest identities (96.6% and 96.9%, respectively). When compared with the virulent field strain YN04, nucleotide identities ranged from 96.7% (CIAV-HNLY1-1) to 99.5% (CIAV-HNPY), indicating a close evolutionary relationship between the new isolates and previous circulating virulent lineages. The recombination analysis performed in RDP5 using seven algorithms did not find any significant recombination events in the newly sequenced genomes of 20 CIAV isolates.

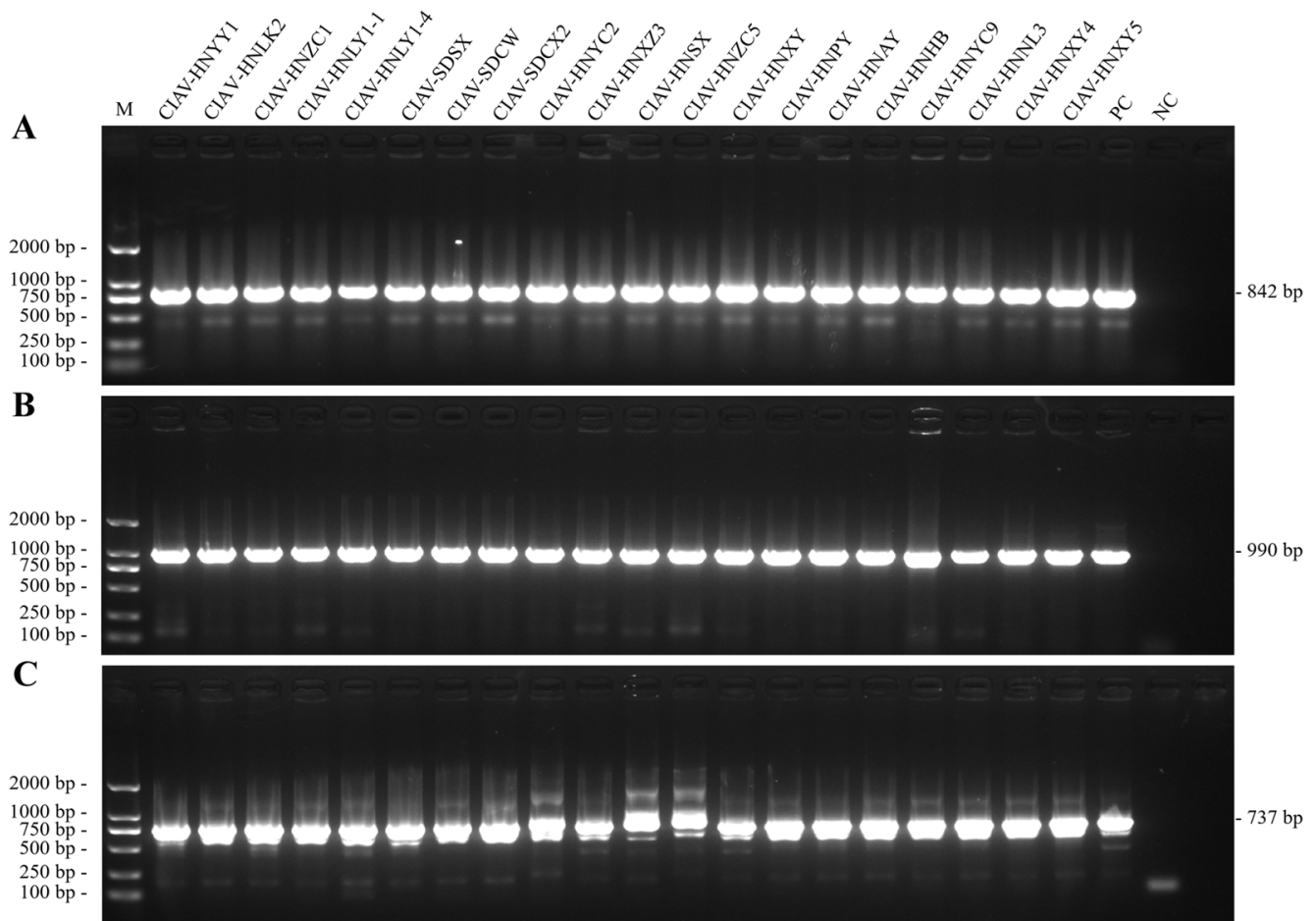


Figure 3. PCR amplification of CIAV from 20 clinical samples collected from diseased poultry flocks. (A) Amplification of fragment 1 using primers CIAV-1F and CIAV-1R. (B) Amplification of fragment 2 using primers CIAV-2F and CIAV-2R. (C) Amplification of fragment 3 using primers CIAV-3F and CIAV-3R. M, DNA marker; PC, positive control; and NC, negative control.

3.4. Phylogenetic Analysis of Current Circulating CIAV Viruses

The whole-genome and VP1 gene sequences from 20 new CIAV isolates and 91 reference strains, as listed in Table S3, were aligned and analyzed for phylogenetic reconstruction. Based on the whole-genome phylogeny, all strains were resolved into four distinct clades A, B, C and D (Figure 4A). Clade A comprised two isolates exclusively from Australia, whereas clade B contained three early Chinese strains. Clade C represented a dominant global lineage that included isolates from Asia, Europe, North and South America, and Africa. It was further subdivided into two subclades: C1 (predominantly Asian isolates—88.5% (46/52) from China, along with South Korea, India, Vietnam, and Japan) and C2 (vaccine strains Cux-1, Del-Ros, and 26P4, together with diverse global isolates). Clade D comprised geographically diverse isolates from multiple continents, including Asia, Europe, Africa, the Americas, and Australia. Among the newly obtained Chinese CIAV isolates, 17 clustered within clade C1, two within C2, and one within B, while none were assigned to clades A or D. The VP1 gene-based phylogenetic tree exhibited an almost identical topology to the whole-genome phylogenetic tree (Figure 4B), confirming consistent clustering patterns across both datasets. Overall, the current prevalent Chinese CIAV isolates clustered primarily in clade C1, forming a geographically distinct lineage that exhibited spatial clustering but no evident temporal differentiation.

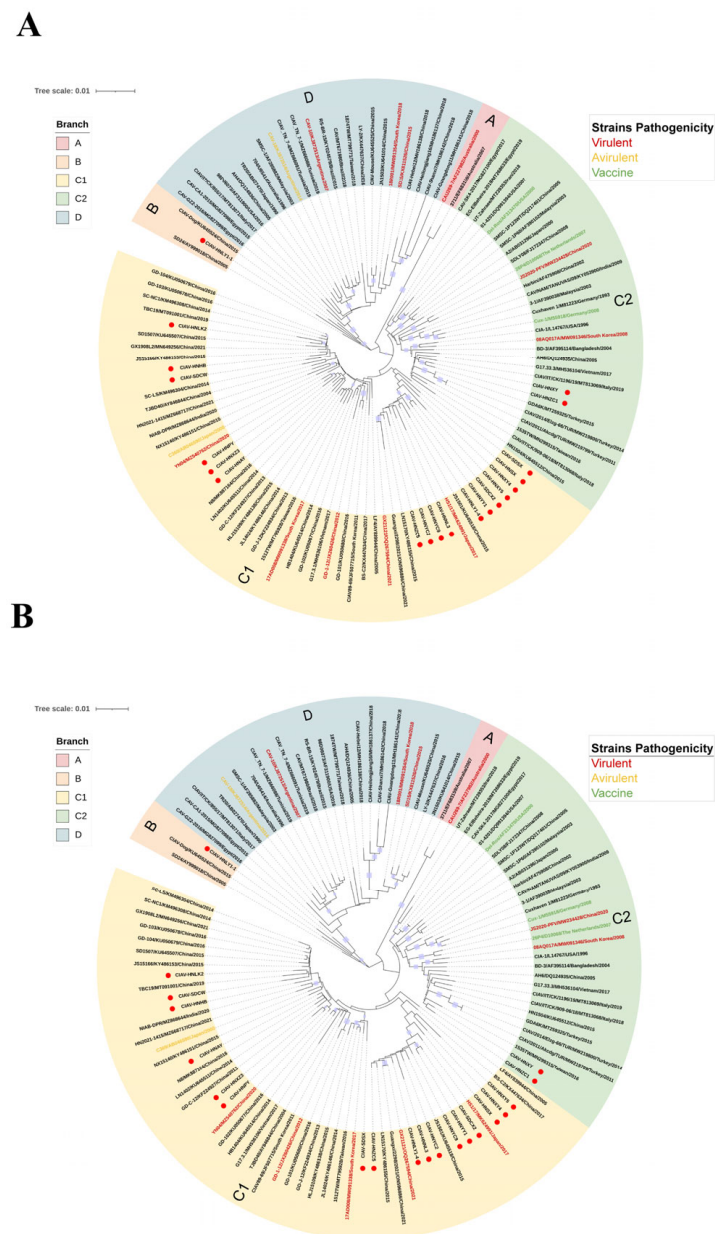


Figure 4. Phylogenetic analysis of CIAV isolates. (A) Phylogenetic tree analyzed based on the complete viral genomic sequences; (B) phylogenetic tree analyzed based on the VP1 nucleotide sequences. The 20 CIAV isolates newly identified in this study were indicated by red dots. Strains names colored in red, yellow, and green represent virulent, avirulent, and vaccine strains, respectively.

3.5. Mutations of Amino Acid Residues in VP1 Proteins of Circulating CIAV Viruses

Alignment of the 450-amino acid VP1 proteins from 20 new CIAV isolates and 16 reference strains with known virulence listed in Table 4 [22–31] revealed 30 amino acid substitutions, corresponding to an overall variability of 6.7% (Table 4). Notably, residue 394, a principal molecular determinant of CIAV virulence, was found to be consistently encoded as glutamine (Q) in all 20 isolates, whereas histidine (H), indicative of attenuated virulence, was not detected in any of the new isolates. Further analysis of the hypervariable region (amino acids 139–151) revealed distinct sequence heterogeneity among these isolates. Four isolates (CIAV-HNZC1, CIAV-HNLY1-1, CIAV-HNXY, and CIAV-HNXY5) possessed glutamine (Q) at both positions 139 and 144, whereas the remaining isolates encoded lysine (K) and glutamic acid (E) at these respective positions.

Table 4. Amino acid mutations in the VP1 proteins of CIAV isolates.

Category	No.	Strain	Acc. No.	Year for Isolation	Country	Amino Acid Site of VP1															Virulence	Citation	
						75	89	97	125	139	141	144	157	287	290	370	376	394	413	436			447
Reference	1	Del Ros	AF313470	2000	USA	V	T	M	I	K	Q	E	V	S	A	G	L	Q	S	V	G	Vaccine	
	2	Cux-1	M55918	2008	Germany							D		A		S			A		T	Vaccine	
	3	26P4	D10068	2007	The Netherlands								M	T		S			A		T	Vaccine	
	4	CAU269-7	AF227982	2000	Australia									T		R					S	Virulent	[22]
	5	HS1 /17	MK624991	2017	Japan				L	L			M	T	P				A	I	S	Virulent	[23]
	6	GX21121	OQ267594	2021	China				L	L			M	N	P	A			A	I	S	Virulent	[24]
	7	GD-1-12	JX260426	2012	China					L			M					I			S	Virulent	[25]
	8	17AD008	MW091338	2017	South Korea					L			M					I			S	Virulent	[26]
	9	YN04	MZ540762	2020	China					L			M					I			S	Virulent	[27]
	10	JS2020-PFV	MW234428	2020	China		K						M	T		S			A		T	Virulent	[28]
	11	08AQ017A	MW091346	2008	South Korea	I		L		Q		Q		A		S			A		S	Virulent	[26]
	12	CAV-10	KJ872513	2007	Argentina	I		L		Q		Q		T	P	S			A		S	Virulent	[29]
	13	18R001	MW091354	2018	South Korea	I		L		Q		Q		T	P	S			A		S	Virulent	[26]
	14	SD15	KX811526	2015	China	I		L		Q		Q		T	P	T			A		S	Virulent	[30]
	15	C369	AB046590	2000	Japan				L		A							I	H		S	Attenuated	[31]
	New isolates	16	CAV-18	KJ872514	2014	Argentina	I		L		Q		Q		T	P	T			A		S	Attenuated
1		CIAV-HNYY1		2021	China			L	L				M	N	P	A			A	I	S	/	
2		CIAV-HNLK2		2021	China				L								I				S	/	
3		CIAV-HNZC1		2021	China	I		L		Q		Q		A	P	T			A		S	/	
4		CIAV-HNLY1-1		2021	China			L		Q		Q		T	P	T			A		S	/	
5		CIAV-HNLY1-4		2021	China			L	L				M	T	P	A			A	I	S	/	
6		CIAV-SDSX		2021	China				L										A		S	/	
7		CIAV-SDCW		2021	China			L	L				M				I				S	/	
8		CIAV-SDCX2		2021	China			L	L				M	N	P	A			A	I	S	/	
9		CIAV-HNYC2		2021	China			L	L				M	N	P	A			A	I	S	/	
10		CIAV-HNXZ3		2021	China				L				M				I				S	/	
11		CIAV-HNSX		2021	China			L	L				M	T	P	S			A		S	/	
12		CIAV-HNZC5		2021	China			L	L				M	T	P	A			A	I	S	/	
13		CIAV-HNXY		2023	China	I		L		Q		Q		A	P	T			A		S	/	
14		CIAV-HNPY		2022	China				L				M				I				S	/	
15		CIAV-HNAY		2022	China				L								I				S	/	
16		CIAV-HNHB		2023	China				L								I				S	/	
17		CIAV-HNYC9		2023	China			L	L				M	N	P	A			A	I	S	/	
18		CIAV-HNNL3		2023	China			L	L				M	T	P	A			A	I	S	/	
19		CIAV-HNXY4		2023	China				L				M	N	P	A			A	I	S	/	
20	CIAV-HNXY5		2023	China			L		Q		Q		N	P	A			A	I	S	/		

"/" means undefined.

3.6. Sequence Analysis of the UTRs of Circulating CIAV Isolates

Alignment of the UTR sequences of 20 new CIAV isolates revealed that the nucleotide identities ranged from 96.6% to 100%, with the lowest similarity observed between CIAV-HNLY1-1 and CIAV-HNPY (96.6%), and a complete identity between CIAV-HNSX and CIAV-HNXY (100%). Comparative analysis of the conserved regulatory motifs demonstrated nucleotide substitutions within the NF-AT2, ATF/CREB, and DR3 elements, whereas no variation was observed in the lymphoid-specific site, SV40 enhancer core element, polyadenylation signal, NFκB + H2TF1 sites, and erythroid-specific G-string (Figure 5). Four direct repeat (DR) regions were consistently detected within the UTRs, among which DR3 exhibited substitutions in 40% (8/20) of isolates. Additionally, single-nucleotide polymorphisms (SNPs) were also observed in both NF-AT2 and ATF/CREB regulatory motifs (Figure 5).

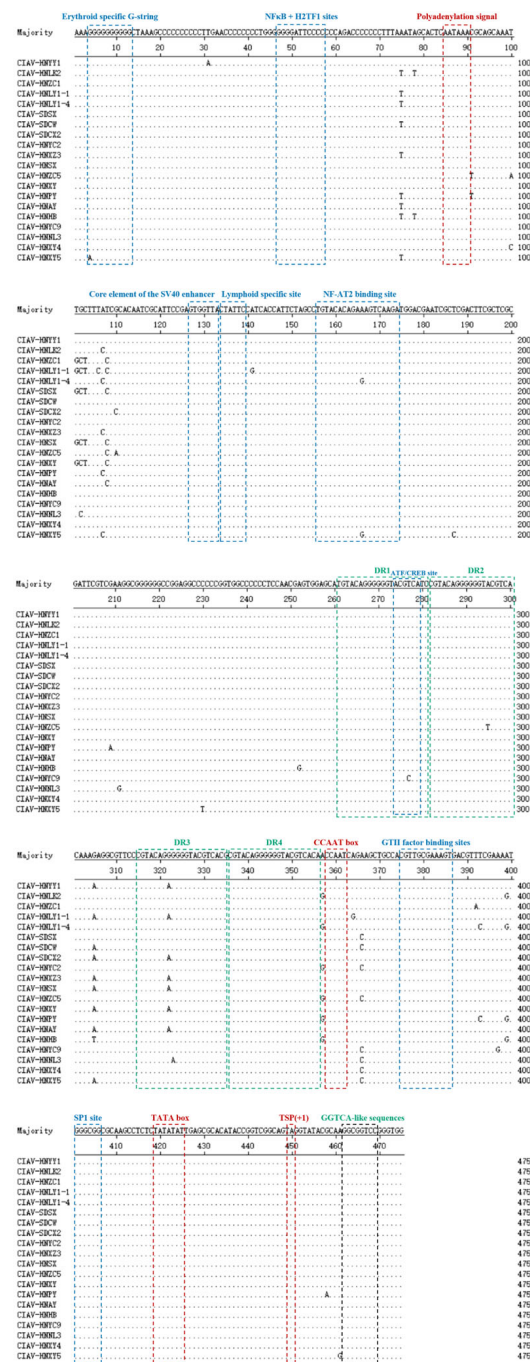


Figure 5. Sequence alignment of the UTR nucleotide sequences of 20 new CIAV isolates. Conserved nucleotide sequences were indicated with dots, and variable sites were indicated with letters. Putative

cis-acting regulatory elements are highlighted and colour-coded according to their functions: core promoter and mRNA processing elements are shown in red dashed boxes, including the TATA box, CCAAT box, Transcription Start Point (TSP), and polyadenylation signal; transcription factor binding sites and enhancers are shown in blue dashed boxes, including NF- κ B, Sp1, ATF/CREB, NF-AT2, GII, SV40 enhancer core element, and lymphoid/erythroid specific sites; and repeat sequences and specific motifs are shown in green dashed boxes, including direct repeats (DR1–DR4) and GGTC-like sequences.

4. Discussion

CIAV is a major immunosuppressive pathogen in poultry and has achieved world-wide dissemination since it was first isolated and identified in Japan in 1979 [17]. The virus causes severe immunosuppression in young chicks by impairing both humoral and cellular immune responses. Furthermore, co-infection with other avian immunosuppressive and neoplastic pathogens, such as MDV, ALV, and REV, can further synergistically exacerbate disease severity, leading to markedly increased morbidity and mortality in flocks [6,32,33]. Our previous work has demonstrated that recent outbreaks of avian neoplastic diseases in China are primarily driven by epidemic HV-MDV viruses, partially co-infected with ALV and/or REV [6]. However, the potential role of CIAV and its co-infection under these cases remains insufficiently understood. To clarify this question, an overall epidemiological investigation was conducted across 71 poultry farms in central China from 2021 to 2023, focusing on CIAV prevalence in MD tumour-bearing flocks and suspected clinical cases. Surprisingly, in 2023, our data revealed highly individual and flock levels of CIAV positive rates reached to 53.8% (28/52) and 55.3% (21/38), respectively, markedly exceeding the previously reported infection rates varying from 13.3% to 37.5% [34–38]. Such a discrepancy may be partly due to the sampling strategies performed in different studies. Previous studies primarily focused on general clinical cases and samples (e.g., dead birds exhibiting anemia or growth retardation) [34–38], while our present work mainly focused on tumour-bearing chicken flocks. Usually, the tumour-associated viruses induce immunosuppression, which may facilitate the co-infections of CIAV in hosts, which is likely to contribute to a higher infection rate. In the year 2021, co-infection of MDV + CIAV was dominant in 65.5% (19/29) of flocks, but mono-infections of MDV or CIAV were only observed in 31.0% (9/29) and 0% (0/29) of the flocks, respectively. It means that all CIAV infections during this period occurred almost exclusively as a secondary infection. By contrast, data obtained from 2023 revealed a significant epidemiological shift, as the co-infections of MDV + CIAV declined to 31.6% (12/38), while CIAV mono-infections significantly increased to 23.7% (9/38). Our data suggest that CIAV mono-infection is now more frequently detected in tumour-bearing flocks and suspected cases, which may act as a primary contributor in a subset of cases, although additional untested co-factors cannot be excluded. In future works, more potential causative agents, such as ALV and REV, need to be simultaneously investigated.

When compared with the previously reported MDV and CIAV co-infection rates of 11.1% and 4.69% [39,40], the substantially higher values detected in this study underscore the escalating severity of both secondary and primary infections of CIAV in present poultry production, especially for the poultry farms suffering immunosuppressive and neoplastic diseases. Furthermore, previous investigations have demonstrated that CIAV infection reduces the protective efficacy of the CVI988/Rispens vaccine against MDV, thereby enhancing the virulence and pathogenic potential of both vMDV and vvMDV strains [41,42]. Over the past decade, immunosuppressive and neoplastic diseases, such as MD, have

continuously affected commercial poultry populations despite widespread vaccination efforts [6,33,43]. The present findings indicate that co-infection of MDV + CIAV represents a major etiological factor in the recurrent outbreaks of MD cases in vaccinated chicken flocks. Such co-infections likely exacerbate immune dysfunction and promote tumorigenesis, highlighting an urgent need to incorporate CIAV surveillance and control into integrated immunization and biosecurity measures. In a recent work, it has demonstrated that a recombinant MDV vaccine strain rMS-ΔMeq expressing CIAV VP1 and VP2 showed promise in protecting against CIAV [44], suggesting that bivalent vaccines could potentially control the co-infection of both immunosuppressive agents.

Phylogenetic analyses of both whole viral genomes and VP1 genes from 20 newly obtained CIAV isolates and 91 reference strains produced consistent topologies, resolving four major clades A, B, C and D. Clade C represented the most widely distributed lineage and was further subdivided into subclades C1 and C2. Subclade C1 consisted almost exclusively of Asian strains, with 88.5% (46/52) of CIAV isolates originating in China. Seventeen newly identified isolates, such as CIAV-HNXZ3, CIAV-HNPY and CIAV-HNAY, were clustered tightly within C1 along with the highly pathogenic YN04, indicating an ongoing circulation among contemporary Chinese field strains. In contrast, subclade C2 encompassed strains from geographically diverse regions (e.g., the United States, Iran, Germany, New Zealand, India, Malaysia, and Italy) with only a few representatives from China. Two novel isolates, CIAV-HNZC1 and CIAV-HNXY, were positioned within subclade C2 but remained phylogenetically distant from the classical vaccine strains 26P4, Cux-1, and Del-Ros that also reside in this subclade. This distant clustering pattern suggests that these viruses might represent foreign-introduced lineages that have undergone a local adaptive evolution, but further phylogeographic analysis is required to confirm this hypothesis. No novel isolate was assigned to clade D, which contained a mixture of global strains from multiple continents in the world. Notably, one isolate (CIAV-HNLY1-1) was clustered within clade B along with a previously reported canine-derived CIAV strain [45], showing a high genetic homology. Overall, Chinese CIAV isolates predominantly cluster into a distinct lineage within subclade C1, demonstrating geographical clustering and regional genetic specificity. Analysis of the whole viral genomes had not revealed evidence for recombination among the 20 newly CIAV isolates, indicating a relative genetic stability of the currently circulating viruses. Nevertheless, the presence of isolates in clades C2 and B, including more lineages showing distinct genetic diversity, underscores the increasing epidemiological and genetic complexity of CIAV. These findings highlight the importance of continuous molecular surveillance, phylogenetic monitoring, and early-warning systems to track the emergence and spread of novel CIAV variants.

The VP1 protein, the sole structural protein of CIAV, exhibits the highest sequence variability among viral genes, underscoring the significance of amino acid substitution analysis in understanding viral evolution and pathogenicity [16]. Previous studies have identified glutamine (Q) at position 394 of VP1 as a critical molecular determinant of high pathogenicity in CIAV isolates [31,45]. Consistently, all 20 new isolates analyzed in this study contained Q at this position, suggesting a potentially pathogenic capacity, as previously reported that the molecular signatures associated with high pathogenic potential, consistent with most of the field isolates [22–31]. Furthermore, amino acid residues at positions 139 and 144 have been shown to affect viral replication and propagation efficiency in host cells, with strains harbouring Q at both positions exhibiting markedly reduced replication rates [16]. Four double-Q variants, such as CIAV-HNZC1, CIAV-HNLY1-1, CIAV-HNXY, and CIAV-HNXY5, have been presently identified, but the impact on replication kinetics and pathogenicity warrants further experimental evaluation. The UTR of

CIAV contains critical regulatory elements, including putative NF-AT2 and ATF/CREB-like motifs within the DRs, which act as potential binding sites for host transcription factors and are essential for viral transcription and replication [46–48]. In addition, nucleotide polymorphisms were identified within the DR3 and ATF/CREB regulatory motifs in the UTRs of several CIAV isolates; these mutations could hypothetically affect viral transcriptional regulation and/or replication efficiency, although further experimental validation is required. Furthermore, no significant correlation between the specific motif variants and viral clades or CIAV infection status was observed in this study.

In summary, this study comprehensively investigated CIAV + MDV co-infections in tumour-bearing chicken flocks across central China during 2021–2023, together with a molecular evolutionary analysis of circulating CIAV viruses. Our data have further confirmed that the epidemic HV-MDV viruses, in combination with CIAV co-infections, are primarily responsible for the resurgence of immunosuppressive and neoplastic diseases in vaccinated poultry populations. Notably, the significant increases in both CIAV co-infection and mono-infection compared with previous reports have highlighted the emerging challenges for future control of the disease. Most circulating Chinese CIAV isolates are clustered in subclade C1 and form a unique regional genotype, whereas the presence of a few isolates in subclade C2 and clade B suggests an increasing genetic and epidemiological diversification. Amino acid profiling of VP1 identified molecular signatures associated with high pathogenic potential, consistent with previous studies on CIAV virulence in mono-infections and supported by recent field observations from clinical veterinarians. These findings characterize the current molecular epidemiological landscape of CIAV in tumour-bearing chicken flocks in central China and provide an important basis for the development of targeted diagnostic and control measures against this re-emerging pathogen.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/v18020227/s1>, Table S1: Background information of suspected clinical cases of avian neoplastic diseases collected from poultry farms in central China during 2022–2023; Table S2: Primers used for PCR amplification of MDV or CIAV genes in this study; Table S3: Background information of the newly obtained and reference CIAV isolates; Table S4. Positive rates of CIAV and/or MDV infections in tumour-bearing diseased chicken flocks in central China collected in 2021 and 2023.

Author Contributions: Conceptualization, J.L. and Z.-H.Y.; methodology, F.H. and B.S.; software, F.H. and B.S.; validation, L.-P.Z., M.T., S.-G.W. and W.-K.Z.; formal analysis, F.H. and B.S.; investigation, F.H., G.-X.L., Y.-X.Z., Z.Y., Z.-F.P. and Q.L.; resources, J.L., Z.-H.Y., G.-X.L., Y.-X.Z. and Z.Y.; data curation, F.H. and B.S.; writing—original draft preparation, F.H. and B.S.; writing—review and editing, J.L., S.-G.W. and Y.Y.; visualization, F.H. and B.S.; supervision, J.L. and Z.-H.Y.; project administration, J.L.; funding acquisition, J.L. and Z.-H.Y. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The experimental protocol was reviewed and approved by the Laboratory Animal Management Committee of Institute of Animal Health (IAH), Henan Academy of Agricultural Sciences (HAAS, Zhengzhou, China), following the protocols of the Laboratory Animal Guidelines for the Ethical Review of Animal Welfare permitted by State Administration for the Market Regulation and Standardization Administration of China (permit no. GB/T 35892-2018).

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article and Supplementary Materials. Further inquiries can be directed to the corresponding author.

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Conflicts of Interest: Author Zhen Yang was employed by the company Yangzhou Youbang Biological Pharmaceutical Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial.

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