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RESEARCH ARTICLE

# Epidemiological Study of the Co-infection of Immunosuppressive Poultry Pathogens in Tissue Samples of Chickens in Jiangsu Province, China from 2016 to 2022

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**ABSTRACT**

Chicken infectious anemia virus (CIAV), Marek's disease virus (MDV), reticuloendotheliosis virus (REV), and avian leukosis virus (ALV) are immunosuppressive pathogens of concern for poultry. To investigate the prevalence of immunosuppressive pathogens in diseased chickens and the interactions between different viral infections, 768 tissue samples collected from diseased chickens in Jiangsu Province, China were analyzed for CIAV, MDV, REV, and ALV using polymerase chain reaction. The detection rate of these four immunosuppressive pathogens was 55.99%, and the detection rates of CIAV, MDV, REV, and ALV were 29.95, 23.05, 9.90 and 23.44%, respectively. The detection rates of coinfection, dual infection, triple infection, and quadruple infection were 23.57, 17.45, 5.47 and 0.65%, respectively. The most common dual and triple infections were CIAV + ALV (detection rate: 5.08%) and CIAV + MDV + ALV (detection rate: 2.60%). The infection of chicken flocks with immunosuppressive pathogens in Jiangsu Province showed a decreasing trend from 2016 to 2022. There was a synergistic association between CIAV and REV or CIAV and ALV ($P < 0.01$). Therefore, infections and co-infections with immunosuppressive pathogens are prevalent in chicken flocks in Jiangsu Province, China, and CIAV plays a critical role in co-infection, providing an important guide for the future control of these diseases.

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## INTRODUCTION

The chicken infectious anemia virus (CIAV), Marek's disease virus (MDV), reticuloendotheliosis virus (REV), and avian leukosis virus (ALV) are common viruses that cause severe immunosuppression in poultry (Dong *et al*., 2014; Zhang *et al*., 2021; Zheng *et al*., 2023). CIAV, belonging to circoviridae, is an important pathogen of chickens causing significant immunosuppression and severe anaemia worldwide. MDV belongs to herpesviridae, REV and ALV belong to retroviridae, these three viruses are major pathogens that can cause avian tumor diseases. The four immunosuppressive pathogens can damage the immune system of poultry, interfere with the presentation of antigens during the immune response, and result in low levels of cellular and humoral immunity. They not only cause death in chickens, but also lead to complications or secondary infections. In clinical cases, these four immunosuppressive pathogens often appear as coinfections (Miles *et al*., 2001; Li *et al*., 2019; Shi *et al*., 2021; Li *et al*., 2021; Nishitha *et al*., 2021). Co-infection with different immunosuppressive pathogens can suppress the immune response, lead to a decrease or failure in the efficacy of vaccines (Zhang *et al*., 2017; Wang *et al*., 2020) and cause synergistic pathogenicity, resulting in enhanced pathogenicity of the viruses (Zhou *et al*., 2018; Zhang *et al*., 2021). Infection with immunosuppressive pathogens can significantly reduce the efficacy of avian influenza or Newcastle disease vaccines (Sun *et al*., 2009; Cui *et al*., 2006). Therefore, the infection of immunosuppressive poultry pathogens may cause the co-infection of chickens in parts of China, and it may also be one of the reasons for vaccination failure.

To investigate the prevalence trends of immunosuppressive pathogens in diseased chickens in

Jiangsu Province, China from 2016 to 2022, 768 tissue samples collected from nine cities in Jiangsu Province were analyzed for CIAV, MDV, REV and ALV using polymerase chain reaction (PCR). The immunosuppressive pathogen infection and co-infection statuses of chickens from Jiangsu Province were subjected to statistical analysis.

## MATERIALS AND METHODS

**Sample collection:** From January 2016 to December 2022, 768 tissue samples from diseased chickens were collected from Yangzhou, Taizhou, Nantong, Nanjing, Zhenjiang, Yancheng, Suqian, Huai'an and Lianyungang in Jiangsu Province. Diseased chickens exhibited emaciation, anemia, growth retardation, and increased mortality. The pathological lesions after necropsy included whitening of the pectoralis, enlarged liver or spleen with some tumor nodules, enlarged or atrophic thymuses, enlarged kidneys, pericarditis, perihepatitis, and airsacculitis. The detailed background information including sample collection date, area, breed, age, clinical signs and pathological lesions after necropsy was recorded, and samples including livers, spleens, thymuses, and bursas of Fabricius were collected from the diseased chickens and stored at -70°C.

**DNA/RNA extraction and complementary DNA synthesis:** Samples (0.1g of liver, spleen, thymus, and bursa of Fabricius) mixed with 1mL of phosphate-buffered saline were homogenized using a tissue homogenizer (BBY24M, Next Advance, Troy, NY, USA). DNA and RNA were extracted from the samples using an automated nucleic acid extraction system (KingFisher, Thermo Fisher Scientific, Waltham, MA, USA) according to the instructions of the magnetic-beads-based nucleic acid extraction kit (Ascend, Luoyang, China). RNA was transcribed into cDNA using the PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China).

**PCR for detection of CIAV, MDV, REV and ALV:** Synthesized primers for detecting CIAV, MDV-1, and REV were used as described previously (Todd *et al*., 1992; Silva, 1992; Davidson *et al*., 1995). The primers for detecting ALV were synthesized according to the Chinese standard SN/T 1172-2014 (quarantine protocol for avian leukosis) (Table 1). PCR was carried out in a 25 μL reaction volume containing 12.5 μL of 2× Rapid Taq Master Mix (Vazyme, Nanjing, China), 1 μL of forward primer (10 μM), 1 μL of reverse primer (10 μM), and 2.5 μL of DNA or cDNA template. Plasmids containing the target genes of CIAV, MDV, REV, and ALV were used as positive controls, and nuclease-free water was used as a negative control. The samples were amplified in a PCR System (9700, ABI, Foster, CA, USA) using the following procedures: CIAV: 94°C for 5 min, (94°C for 30 s, 58°C for 30 s, and 72°C for 45 s) × 30 cycles, and 72°C for 7 min; MDV/REV/ALV: 94°C for 5 min, (94°C for 30 s, 55°C for 30 s, and 72°C for 30 s) × 30 cycles, and 72°C for 7 min. The PCR products were analyzed by electrophoresis on 1% agarose gels with ethidium bromide and identified using a gel imaging analysis system (Gel Doc XR+, Bio-Rad, Hercules, CA, USA).

**Table 1:** Primers for PCR detection of CIAV, MDV, REV and ALV

| Name | Sequence | Amplicon Size (bp) | Target |
|---|---|---|---|
| CIAV-F | GACTGTAAGATGGCAAGACGAGCTC | 675 | capsid |
| CIAV-R | GGCTGAAGGATCCCTCATTC | | protein |
| MDV-F | ATGCGATGAAAGTGCTATGGAG | 314 | 132 bp |
| MDV-R | ATCCCTATGAGAAAGCGCTTGA | | repeats |
| REV-F | CATACTGGAGCCAATGGTT | 291 | LTR |
| REV-R | AATGTTGTACCGAAGTACT | | |
| ALV-F | CTAACGAGGCGAGGGAATG | 214 | pol |
| ALV-R | TTGGTGGGTTGGGTGGAGA | | |

CIAV, chicken infectious anemia virus; MDV, Marek's disease virus; REV, reticuloendotheliosis virus; ALV, avian leukosis virus

**Data analysis:** Statistical analyses were performed using SPSS statistical software package for Windows (version 17.0; SPSS Inc., Chicago, Illinois, USA). The chi-square test was used to analyze the detection rates of the different viruses. $P < 0.05$ was considered significantly different and $P < 0.01$ was considered extremely different.

## RESULTS

**Detection rates of the four immunosuppressive poultry pathogens:** PCR assays for CIAV, MDV, REV and ALV were used to detect infections. The expected target bands were amplified from the positive controls (Fig. 1). Samples with similar bands to those of the positive controls after PCR were identified as positive. The total number of positive samples was 430, accounting for 55.99% (430/768) of the samples. The number of samples positive for CIAV, MDV, REV, and ALV was 230, 177, 76, and 180, respectively, accounting for 29.95, 23.05, 9.90 and 23.44%, respectively.

**Single infections and co-infections with the four immunosuppressive poultry pathogens:** The detection rate for single infections was 32.42%, whereas the detection rate for coinfections was 23.57%. The detection rates for dual, triple, and quadruple infections were 17.45, 5.47 and 0.65%, respectively. For single infections, the detection rate was the highest for CIAV (12.63%). For co-infections, the most common types of dual and triple infections were CIAV + ALV (5.08%) and CIAV + MDV + ALV (2.60%) (Table 2).

**Age distribution for the four immunosuppressive poultry pathogens:** The detection rate of CIAV was significantly lower in chickens > 300 days of age than in chickens < 300 days of age. There was a higher detection rate of MDV in chickens in the 121–300 days age group, but there was no significant difference in the detection rates of REV in different age groups. The detection rate for ALV was significantly lower in chickens aged < 30 days than in those aged > 30 days. Co-infection occurred mostly in chickens aged 31–300 days, and there was a higher detection rate of immunosuppressive pathogens in chickens in the 121–300 days age group (Table 3).

**Breed distribution for the four immunosuppressive poultry pathogens:** The detection rate of these four pathogens in native chickens (63.10%) was significantly higher than that of three-yellow chickens (37.50%). The detection rate of CIAV in native chickens was the highest (36.90%), the detection rate of MDV in layers was the highest (27.72%), and the detection rate of REV in broilers was the lowest (5.96%) (Table 4).

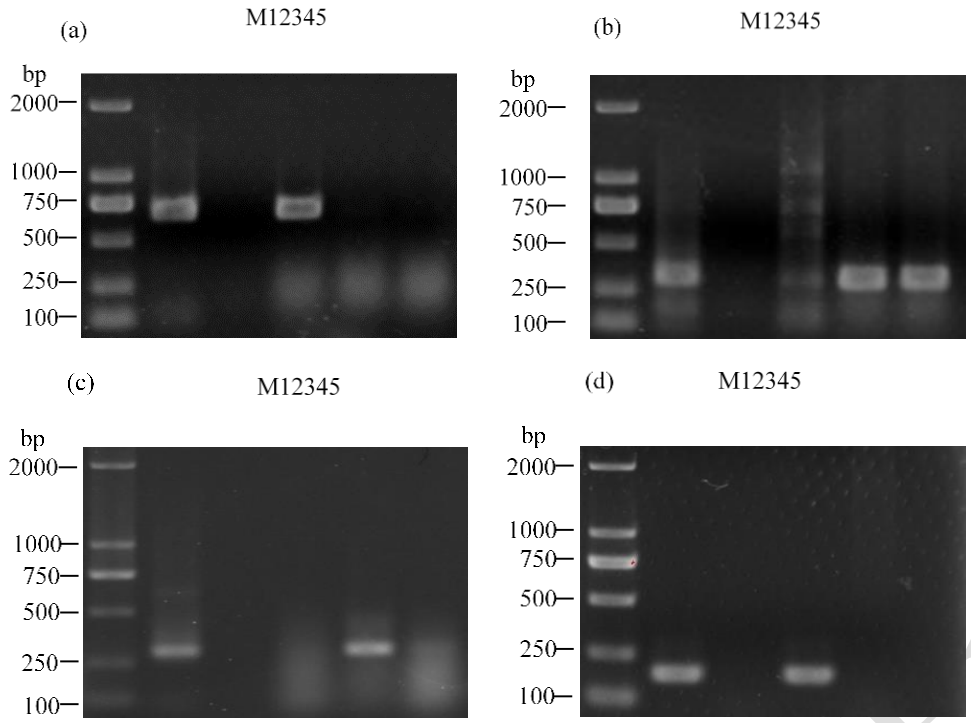


**Fig. 1:** PCR amplification genes specific for chicken infectious anemia virus (a, 675 bp), Marek's disease virus (b, 314 bp), reticuloendotheliosis virus (c, 291 bp), and avian leukosis virus (d, 214 bp). Lane M: Marker 2000 bp (TaKaRa); Lane 1: positive control; Lane 2: negative control; Lane 3-5: samples.

**Table 2:** Single infections and co-infections with the four immunosuppressive pathogens

| Infection type | Viruses | Number of positive samples | Detection rate |
|---|---|---|---|
| Single infection | CIAV | 97 | 12.63% |
| | MDV | 66 | 8.59% |
| | REV | 16 | 2.08% |
| | ALV | 70 | 9.11% |
| Dual infection | CIAV + MDV | 36 | 4.69% |
| | CIAV + REV | 16 | 2.08% |
| | CIAV + ALV | 39 | 5.08% |
| | MDV + REV | 12 | 1.56% |
| | MDV + ALV | 26 | 3.39% |
| | REV + ALV | 5 | 0.65% |
| Triple infection | CIAV + MDV + REV | 7 | 0.91% |
| | CIAV + MDV + ALV | 20 | 2.60% |
| | CIAV + REV + ALV | 10 | 1.30% |
| | MDV + REV + ALV | 5 | 0.65% |
| Quadruple infection | CIAV + MDV + REV + ALV | 5 | 0.65% |
| Co-infection | | 181 | 23.57% |
| Total infection | | 430 | 55.99% |

CIAV, chicken infectious anemia virus; MDV, Marek's disease virus; REV, reticuloendotheliosis virus; ALV, avian leukosis virus

**Table 3:** Distribution of the four immunosuppressive pathogens in chickens at different ages

| Virus | ≤ 30 days | 31–120 days | 121–300 days | ≥ 300 days |
|---|---|---|---|---|
| CIAV | 27/103 (26.21%) [b] | 129/367 (35.15%) [a] | 62/225 (27.56%) [b] | 12/73 (16.44%) [c] |
| MDV | 20/103 (19.42%) [b] | 74/367 (20.16%) [b] | 68/225 (30.22%) [a] | 15/73 (20.55%) [b] |
| REV | 12/103 (11.65%) [a] | 33/367 (8.99%) [a] | 23/225 (10.22%) [a] | 8/73 (10.96%) [a] |
| ALV | 9/103 (8.74%) [b] | 89/367 (24.25%) [a] | 67/225 (29.78%) [a] | 15/73 (20.55%) [a] |
| Co-infection | 18/103 (17.48%) [b] | 104/367 (28.34%) [a] | 50/225 (22.22%) [a] | 9/73 (12.33%) [b] |
| Total infection | 48/103 (46.60%) [b] | 195/367 (53.13%) [b] | 150/225 (66.67%) [a] | 37/73 (50.68%) [b] |

Different lowercase superscript letters indicate significant differences within a row ($P < 0.05$) based on the chi-square test. CIAV, chicken infectious anemia virus; MDV, Marek's disease virus; REV, reticuloendotheliosis virus; ALV, avian leukosis virus

**Table 4:** Distribution of the four immunosuppressive pathogens in different breeds of chickens

| Virus | Native chicken | Commercial layer | Broiler chicken | Three-yellow chicken |
|---|---|---|---|---|
| CIAV | 93/252 (36.90%) [a] | 78/285 (27.37%) [b] | 39/151 (25.83%) [b] | 20/80 (25.00%) [b] |
| MDV | 59/252 (23.41%) [a] | 79/285 (27.72%) [a] | 26/151 (17.22%) [b] | 13/80 (16.25%) [b] |
| REV | 28/252 (11.11%) [a] | 29/285 (10.18%) [a] | 9/151 (5.96%) [b] | 10/80 (12.50%) [a] |
| ALV | 69/252 (27.38%) [a] | 58/285 (20.35%) [a] | 35/151 (23.18%) [a] | 18/80 (22.50%) [a] |
| Co-infection | 73/252 (28.97%) [a] | 59/285 (20.70%) [b] | 29/151 (19.21%) [b] | 20/80 (25.00%) [a] |
| Total infection | 159/252 (63.10%) [a] | 164/285 (57.54%) [a] | 77/151 (50.99%) [b] | 30/80 (37.50%) [c] |

Different lowercase superscript letters indicate significant differences within a row ($P < 0.05$) based on the chi-square test. CIAV, chicken infectious anemia virus; MDV, Marek's disease virus; REV, reticuloendotheliosis virus; ALV, avian leukosis virus

**Year distribution for the four immunosuppressive poultry pathogens:** Infections with immunosuppressive pathogens were prevalent to different degrees in chicken flocks in Jiangsu Province from 2016 to 2022 (Table 5). The detection rates of CIAV, REV, and ALV were significantly higher in 2016 than in other years, whereas the detection rates of CIAV, MDV, REV, and ALV were significantly lower in 2022 than in other years.

**Table 5:** Distribution of the four immunosuppressive pathogens in chickens from 2016 to 2022

| Virus | 2016 | 2017 | 2018 | 2019 | 2020 | 2021 | 2022 |
|---|---|---|---|---|---|---|---|
| CIAV | 77/150 [a] (51.33%) | 56/192 [b] (29.17%) | 14/64 [b] (21.88%) | 34/116 [b] (29.31%) | 19/80 [b] (23.75%) | 14/65 [b] (21.54%) | 16/101[c] (15.84%) |
| MDV | 24/150 [a] (16.00%) | 61/192 [b] (31.77%) | 11/64 [a] (17.19%) | 31/116 [b] (26.72%) | 27/80 [b] (33.75%) | 10/65 [a] (15.38%) | 13/101[a] (12.87%) |
| REV | 27/150 [a] (18.00%) | 12/192 [b] (6.25%) | 9/64 [a] (14.06%) | 7/116 [b] (6.03%) | 11/80 [a] (13.75%) | 4/65 [b] (6.15%) | 6/101 [b] (5.94%) |
| ALV | 53/150 [a] (35.33%) | 57/192 [a] (29.69%) | 11/64 [b] (17.19%) | 36/116 [a] (31.03%) | 14/80 [b] (17.50%) | 5/65 [c] (7.69%) | 4/101 [c] (3.96%) |
| Co-infection | 54/150 [a] (36.00%) | 52/192 [b] (27.08%) | 12/64 [b] (18.75%) | 25/116 [b] (21.55%) | 23/80 [b] (28.75%) | 6/65 [c] (9.37%) | 9/101 [c] (8.91%) |
| Total infection | 111/150 [a] (74.00%) | 120/192 [b] (62.50%) | 24/64 [c] (37.50%) | 80/116 [b] (68.97%) | 37/80 [c] (46.25%) | 28/65 [c] (43.08%) | 30/101 [d] (29.70%) |

Different lowercase superscript letters indicate significant differences within a row ($P < 0.05$) based on the chi-square test. CIAV, chicken infectious anemia virus; MDV, Marek's disease virus; REV, reticuloendotheliosis virus; ALV, avian leukosis virus

**Table 6:** Comparison of interactions between dual infections

| | CIAV+ | MDV+ | REV+ | ALV+ |
|---|---|---|---|---|
| CIAV+ [a] | | 68/230 [b] * (29.60%) | 38/230 *** (16.52%) | 74/230 ** (32.17%) |
| CIAV- | | 109/538 (20.26%) | 38/538 (7.06%) | 106/538 (19.70%) |
| MDV+ | 68/177 * (38.42%) | | 29/177 ** (16.38%) | 56/177 * (31.64%) |
| MDV- | 162/591 (27.41%) | | 47/591 (7.95%) | 124/591 (20.98%) |
| REV+ | 38/76 ** (50.00%) | 29/76 * (38.16%) | | 25/76 (32.89%) |
| REV- | 192/692 (27.75%) | 148/692 (21.39%) | | 155/692 (22.40%) |
| ALV+ | 74/180 ** (41.11%) | 56/180 * (31.11%) | 25/180 (13.89%) | |
| ALV- | 156/588 (26.53%) | 121/588 (20.58%) | 51/588 (8.67%) | |

[a]: "+", positive samples; "-"negative samples; [b] Denominators represent the data from the corresponding column, whereas numerators represent the data from the corresponding row; *: The detection rates for dual infections were compared with those of single infections, $P < 0.05$, 0.01, and 0.001 are indicated with *, **, and ***, respectively.

The detection rates of CIAV, MDV, REV and ALV each year were 15.84%~51.33%, 12.87%~35.00%, 5.94%~18.00% and 3.96%~35.33%, respectively. The detection rates of coinfections and the total number of infections each year were 8.91%~36.00% and 29.70%~74.00%, respectively. Overall, the infection of chicken flocks with immunosuppressive pathogens in the Jiangsu area showed a decreasing trend from 2016 to 2022.

**Interactions among the four immunosuppressive poultry pathogens:** The detection rates of MDV, REV, and ALV were significantly higher in CIAV-positive samples than in CIAV-negative samples; the detection rates of CIAV, REV, and ALV were significantly higher in MDV-positive samples than in MDV-negative samples; the detection rates of CIAV and MDV were significantly higher in REV-positive samples than in REV-negative samples; and the detection rates of CIAV and MDV were significantly higher in ALV-positive samples than in ALV-negative samples. These results indicated a synergistic trend between most dual infections, except for REV and ALV (Table 6).

## DISCUSSION

CIAV is an important poultry pathogen, which is ubiquitous in many countries of the world. It has been associated with high immunosuppression in chickens and can cause heavy economic loss. MDV, REV and ALV are the major oncogenic viruses in poultry. They can cause immunosuppression, aggravation of co-infections, vaccination failures and mortality. A 314-bp band was amplified in the virulent strain of MDV-1 in this study, corresponding to two copies of a 132 bp tandem direct repeat; however, multiple bands were amplified in the attenuated vaccine strain (CVI988) (Fig. 1b, lane 3), corresponding to one and several copies of the 132 bp tandem direct repeat (Silva, 1992). PCR amplification of the ALV target gene, *pol*, can differentiate between exogenous and endogenous ALV. Recent epidemiological investigations have shown that immunosuppressive pathogens have caused epidemics in different areas of China (Meng *et al*., 2018; Su *et al*., 2019; Zheng *et al*., 2022). In this study, 768 tissue samples collected from diseased chickens in Jiangsu Province, China were tested for CIAV, MDV, REV, and ALV. The detection rate of these four immunosuppressive pathogens was 55.99%, and the detection rate of CIAV was relatively high, whereas the detection rate of REV was relatively low, indicating that the infection rates of immunosuppressive pathogens varied in different areas of China. At the same time, the detection rate of co-infection in Jiangsu was 23.57%, which was lower than the rate reported in central China (Zheng *et al*., 2022). This might be due to relatively developed economy in east China, and the biosecurity on farms is much better. We also found that chicks were more susceptible to CIAV infection, possibly because of their underdeveloped immune systems (Li *et al*., 2022). Conversely, the lowest positivity rate of ALV was observed in chickens younger than 30 days, which may be due to the protection provided by maternal antibodies (Dou *et al*., 2013). Native chickens were more susceptible to these four immunosuppressive pathogens, probably because most of the native chickens came from free-range facilities that lacked commercial breeding technologies. Based on limited samples collected from 2016 to 2022, the infection of chicken flocks with immunosuppressive pathogens in the Jiangsu area showed a decreasing trend, indicating that infections with these four pathogens in the Jiangsu area has been relatively restricted in recent years, which might also be related to the strengthening of biosecurity on farms.

The occurrence of one immunosuppressive disease can promote the occurrence of other immunosuppressive diseases, which may be one of the main reasons for the high detection rate of coinfections with immunosuppressive pathogens in chicken flocks. Co-infection with immunosuppressive pathogens can present a synergistic effect on immunosuppression, and the pathogens may also interact with each other to enhance their pathogenicity. A previous co-infection study demonstrated the synergistic pathogenesis of co-infection with CIAV and ALV-J and highlighted the positive effect

of CIAV on the pathogenesis of ALV-J (Zhang *et al*., 2021). Co-infection with Marek's disease and reticuloendotheliosis viruses can increase illness severity and reduce Marek's disease vaccine efficacy (Sun *et al*., 2017). Co-infection with CIAV and MDV can significantly enhance the tumorigenic effect of the RBIB strain of MDV (Miles *et al*., 2001). The synergistic replication of Marek's disease virus and avian leukosis virus subgroup J is responsible for the enhanced pathogenicity of superinfections in chickens (Zhou *et al*., 2018). Co-infection with ALV-J and REV causes more serious synergistic pathogenic effects, growth retardation, immunosuppression, and secondary *E. coli* infection in broiler chickens (Dong *et al*., 2015). In this study, a synergistic association between most dual infections could be found among the four pathogens. Unlike other viruses, CIAV as an immunosuppressive agent directly targets hemocytoblasts in bone marrow and precursor lymphocytes in thymus, and the lymphocyte depletion will increase susceptibility to various bacterial and viral infections (Ganar *et al*., 2017). Therefore, more attention should be paid to coinfections with immunosuppressive pathogens, especially for CIAV and other pathogens.

Marek's disease is a lymphoproliferative disease in chickens that causes devastating losses in commercial poultry flocks. MDV-infected chickens shed the virus for life. Although the highest detection rate of MDV in chickens in the 121–300 day age group, chickens older than 300 days also showed a high detection rate. Infectious cell-free virions survive in the environment for a long time and are horizontally transmitted to other chickens (Denesvre, 2013). CIAV, REV, and ALV can be transmitted both horizontally and vertically in chicken flocks, and the potential route of vaccine contamination should not be ignored (Zhao *et al*., 2014; Li *et al*., 2015; Wang *et al*., 2018; Li *et al*., 2022). The high detection rates of CIAV and ALV during the peak laying period in chicken flocks influence the progeny through vertical transmission. When REV contaminates live FPV and MDV vaccines, its basic components can be integrated into FPV and MDV (Singh *et al*., 2003; Zhang and Cui, 2005). The persistent detection rate of REV in chickens may have been due to REV contamination of live vaccines to some extent.

**Conclusions:** Infections and co-infections with CIAV, MDV, REV, and ALV are prevalent in chicken flocks in Jiangsu Province, China. Among the four immunosuppressive poultry pathogens, high detection rates of single CIAV infections and co-infections involving CIAV were found, providing accurate information for the prevention and control of these diseases.

**Conflict of interest:** The authors declare that they have no conflicts of interest.

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**Authors contribution:** DXP, CHY, WJJ, and ZQW designed the experiment. CHY and QZ executed the experiment and analyzed the data. YY and WJJ contributed reagents and materials. CHY and DXP wrote and revised the manuscript. All the authors have read and approved the final version of this manuscript.

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