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RESEARCH ARTICLE

PCR Detection and Histopathological Analysis of Avian Leukemia Virus Subgroup E Type in Chicken

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ABSTRACT

Chicken leukemia caused by avian leukemia virus has been troubling the global chicken industry, and its efficient and accurate detection and prevention can effectively reduce economic losses. This article aims to conduct pathogen analysis and identify a batch of suspected cases of chicken leukemia in a free-range chicken farm in Zhangshu City, Jiangxi Province, providing a basis for the disease diagnosis and treatment. Suspected chicken leukemia in broiler chickens was screened through clinical, pathological observations and H&E staining, then confirmed by PCR. According to clinical and anatomical diagnosis, the diseased chicken was weaker and unable to stand than the healthier broiler chicken of the same age. H&E staining showed heart, liver, spleen, lungs, and kidney lesions. The PCR and electrophoresis analysis detected a specific band of 405bp, which was consistent with the size of the target gene fragment. Therefore, these suspected cases were diagnosed as avian leukemia virus infection. The results indicated that the PCR is simple, direct, and highly accurate and can diagnose suspected cases of viral leukemia infection in farm chickens.

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INTRODUCTION

Chicken leukemia is a chronic infectious disease caused by avian leukemia virus (ALV), one of the most critical diseases endangering the poultry industry (Chen *et al.,* 2018; Li and Chen, 2023). ALV is an enveloped RNA virus belonging to the Alpha retrovirus genus of the retroviridae family. Based on the characteristics of the virus envelope protein. ALVs isolated from chickens are divided into seven subgroups: A, B, C, D, E, J, and K (Payne *et al.,* 1991; Cui *et al.,* 2014). The ALV genome consists of two copies of ssRNA, containing three genes encoding essential structural proteins: gag, pol, and env (Ballandras *et al.,* 2011). Prevention of the disease is greatly hampered by its commonness and the wide variety of viral subgroups.

In general, chickens are only infected under natural conditions, and the primary source of infection is infected chickens. There are two forms of dissemination: horizontal and vertical (Cuperus *et al.,* 2013; Zeghdoudi *et al.,* 2017). Usually, breeders infected with chicken leukemia virus to the next generation of uninfected chickens or infect the same chickens through daily feed, drinking water, and feces (Wang *et al.,* 2023; Feng *et al.,* 2024). Due to different varieties and ages, the incidence rate also varies greatly. It usually occurs in chickens aged 3-8 months (Feng *et al.,* 2017; Wang *et al.,* 2019). Once a disease occurs, chickens lose economic value, which is a massive blow to the poultry industry (Wu *et al.,* 2022b; Tan *et al.,* 2024). So far, the rapid spread of the disease has been controlled by strengthening feeding management and sorting of sick flocks. However, the lack of fast and accurate diagnostic methods of ALV still hinders the development of the chicken industry.

The biotechnology emergence has improved sensitivity and specificity, as well as the speed of obtaining results, significantly improving the diagnosis of diseases. In terms of disease detection and diagnosis, many methods have been established such as immunofluorescence technology, ELISA, PCR, complement

binding analysis, etc. Among these, PCR has the characteristics of fast and high specificity and has become one of the most commonly used and sensitive methods for detecting pathogens (Khan *et al.,* 2019; Radwan *et al.,* 2022; Suharsono *et al.,* 2023; Ascanio *et al.,* 2024). PCR, one of the most convenient and commonly used biotechnologies, is often used for disease diagnosis due to its high specificity (Sancha *et al.,* 2024) and is frequently reported as a diagnostic tool. In published literature, there many reports on PCR diagnosis of infectious and non-infectious diseases of poultry (Wu *et al.,* 2022a; Gentile *et al.,* 2023; Shibanuma *et al.,* 2023; Walker *et al.,* 2024; Sancha *et al.,* 2024; Mukanov *et al.,* 2024). As a high-precision diagnostic tool, PCR is very convenient and cost-effective, making it suitable for clinical disease diagnosis, especially ALV (Wu *et al.,* 2022b).

With this background, we aimed to provide new insights into the diagnosis of ALV. In this experiment nucleic acids were extracted from serum samples of diseased chickens. PCR-specific amplification, nucleic acid electrophoresis, and HE stains were used to diagnose chicken leukemia infection, assess the damage caused by the avian leukemia virus to chickens, and provide theoretical references and implications for diagnosing avian leukemia virus-in infected chickens.

MATERIALS AND METHODS

Materials: A chicken farm in Zhangshu City, Jiangxi Province, China, provided 30 healthy and 30 sick chickens. The farm mainly raises Qingyuan leprosy tree chickens, and we have collected immunization and medication records (Tables 1 and 2). Frequent infection of diseased chickens appeared without noticeable symptoms. Even if they repeatedly medicated before sending them for examination, the situation did not improve. After clinical diagnosis, we suspected that these chickens could have been infected by avian leukemia virus.

Table 1: Vaccine history of sick chickens

Age in Days	Vaccine
1	Marek's vaccine
7	New bifid vaccine
14	Imported bursa vaccine
21	Newcastle disease vaccine 4

All vaccines were produced by Harbin Veterinary Research Institute, China.

Table 2: Medication history of sick chickens

Age and condition	Drugs used
When the chickens begin to open their mouths	Ciprofloxacin
More than ten days after the chickens	Sulfanilamide, Chlorpromazine
Chickens 22-23 days	Doxycycline
Preventive medicine for 30-day castration	Cephalosporin, Levofloxacin, Metronidazole
Treatment of intractable death, diarrhea, and peritonitis in chickens in recent 40 days	Mequindox Florfenicol + Doxycycline Amikacin + Azithromycin Florfenicol + Tavanillin Mequindox + Colistin

The dosage of veterinary medicines was followed of Zoetis, New Jersey, USA.

Testing methods

Clinical diagnosis: Poultry farmers were asked about the disease history and feeding management of the sick chickens and observations about the abnormal performance of the sick chickens, such as their mental state and fecal state. Sick chickens were palpated for any abnormal changes in body temperature and any abnormal odor in excretions and secretions. Lesions in the skin, subcutaneous tissue, muscle, and internal organs of the sick chickens were noted. Caseous exudate, if oozing out, was also noted.

Collection of disease materials: All the sick chickens were weighed with empty stomachs, and about 5mL blood was drawn from the wing vein from each sick chicken and allowed to stand at room temperature for about one hour. Then, it was centrifuged at 3500rpm for 15min. Serum was collected and stored at -80℃.

Nucleic acid Extraction and Primer design: Serum samples were taken at -80°C, and viral nucleic acid was extracted using the virus genome DNA/RNA Extraction Kit (Takara, Japan) following the manufacturer's instructions. A pair of primers was designed based on the gene sequence of chicken white blood cell disease virus in GenBank: forward primer: 5'-tatgctttggatggacgctt-3', reverse primer: 5'-attctggctgatgatgatgatggctta-3'. The PCR primer and program were used for the first time, and primers were synthesized by Qingke Biotechnology Co., Ltd.

Clinical Diagnosis of ALV and PCR Specificity: The PCR was carried out according to TransGen's instructions in Beijing, China. The PCR amplification products were electrophoresed using a 1% agarose gel at 120V and 80mA for 20 min, and then the gel was observed under a gel imager. With ALV as the reference strain and MDV and CIAV as the control, PCR detection was carried out to determine the specificity of chicken leukemia virus nucleic acid without mutation and failure. A standardized PCR was used to detect the nucleic acid in the serum of suspected chicken leukemia cases and compared with the identification results of traditional virology to analyze ALV infected the sick chicken.

Sequencing and Evolutionary tree analysis and homology analysis: PCR-purified products were sent to Qingke Biotechnology Co for sequencing results with Snapgene. Search for virus sequences of ALV-A, B, C, D, E, J, and K subgroups on NCBI, construct an evolutionary tree, analyze the relationships between subgroups, and compared the homology of virus nucleic acid between other subgroups.

Hematoxylin and Eosin staining: Organs such as the heart, kidneys, lungs, and spleen from healthy and diseased chickens were preserved in 10% buffered formalin and processed for histopathological studies using the routine method of dehydration and embedding in paraffin. Sections of 4–5μm thick were cut and stained with hematoxylin and eosin. Then differentiated with hydrochloric alcohol. Dehydration was performed through an alcohol gradient and cleared in xylene. The tissue sections were embedded with neutral gum and observed and recorded under an inverted microscope (Kleczek *et al.* 2020).

RESULTS

Clinical diagnosis results: We observed healthy and sick chickens and found that sick chickens were thinner, had less energy, and had difficulty in walking compared to their peers (Fig. 1). They coughed incessantly and have open mouth breathing. There was no unique, abnormal odor in secretions and excretions. Diseased chickens had subcutaneous bleeding and pale skin and muscles. The heart, kidneys, and spleen were enlarged which is a typical pathological change of chicken leukemia virus (Fig. 1). Morbid chickens also had mucus and bleeding in the trachea and throat, visceral adhesions, cheesy exudates in the abdominal and intestinal cavities, swelling of leg joints, skeletal deformities, and sciatic nerve bleeding.

PCR, Specificity of ALV Identification and Clinical Application: PCR successfully amplified chicken leukemia virus-positive nucleic acid amplification, a fragment of approximately 405bp (Fig. 2). As a negative control, no fragments were amplified. At the same time, CIAV, MDV, and the blank control did not amplify fragments, which proved the specificity of the primers we designed and used (Fig. 3) and consistently a single band of 405bp was observed in both the ALV-positive nucleic acid and serum nucleic acid of a suspected chicken with leukemia (Fig. 4).

Sequencing results: The sequencing results showed a fragment size of 405 bp. The similarity between the blast sequence of NCBI and the gag gene of the avian leukocyte disease virus was 100%. The ALV we detected belongs to the ALV subgroup E type (Fig. 5A) indicating that sick chickens were infected with ALV, and evolutionary tree analysis showed that there was a significant difference in homology between ALV-E and other subgroups (Fig. 5B). The nucleic acid homology comparison results of the virus subgroup indicated that ALV-E has more mutation regions compared to other subgroups (Fig. 5C).

Hematoxylin and Eosin staining: The hearts, liver, spleen, lungs, and kidneys of sick chickens showed significant pathologic changes compared to healthy chickens. Compared with the H&E staining results of normal organs, the heart of the diseased chickens showed disorganized and loose muscle fibers along with degenerated nuclei (Fig. 6). The liver showed vacuolar degeneration, apoptotic changes and pyknotic nuclei. The spleen was infiltrated with inflammatory cells. The lungs showed marked atelectasis and congestion as compared to healthy chickens. Some of the renal tubules in kidneys were having condensed and necrosed nuclei, and urinary space at some places increased in glomerulus (Fig. 6).

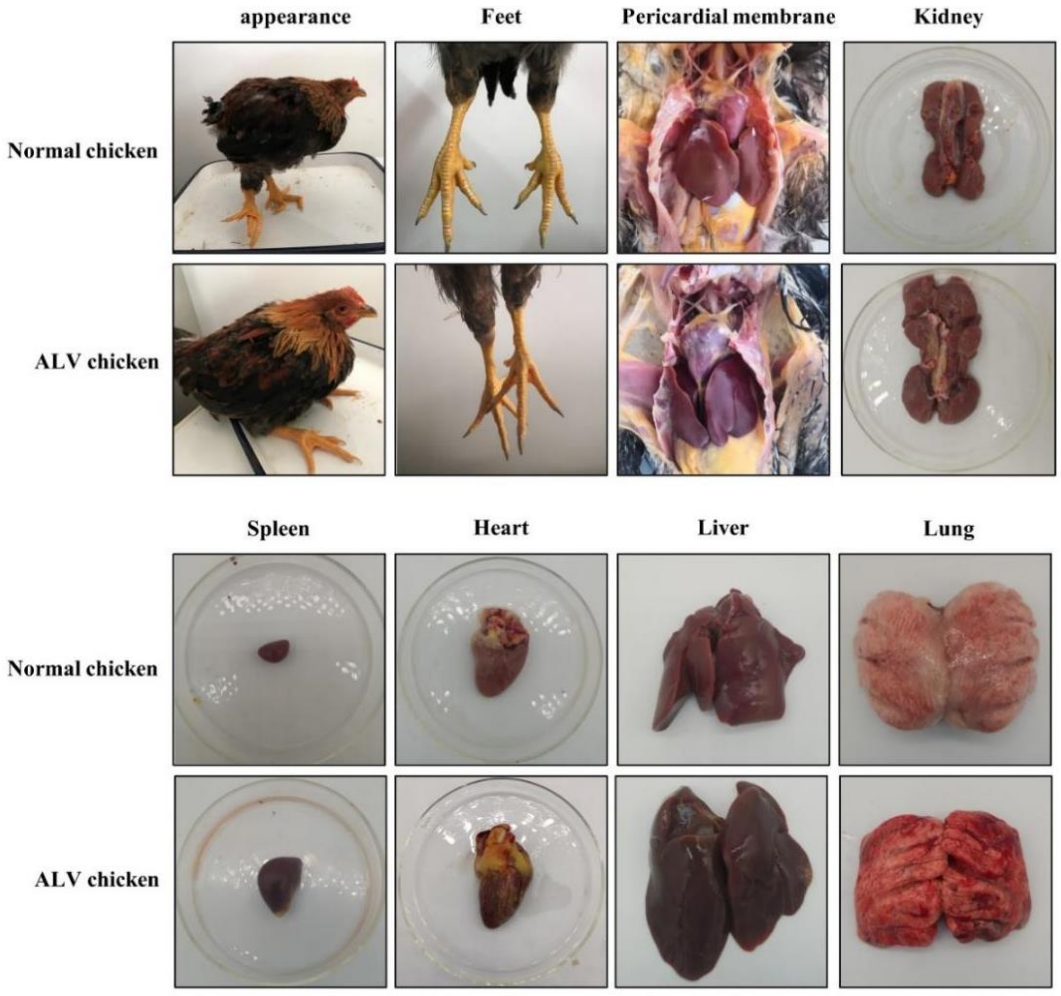

Fig. 1: The anatomical structure of healthy and sick chickens. We observed that the sick chickens were depressed, with slender limbs, pericardial exudation, enlarged kidneys, spleens, hearts, and livers, and obvious hemorrhages in the lungs.

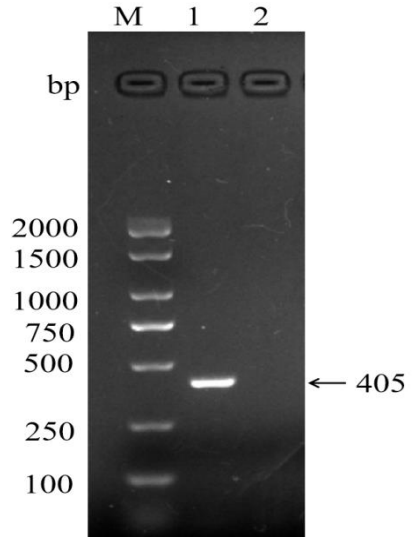

Fig. 2: PCR amplification products of chicken leukemia virus nucleic acid. Lane M = DNA Marker (DL 2000); 1 = ALV, and 2 = blank control.

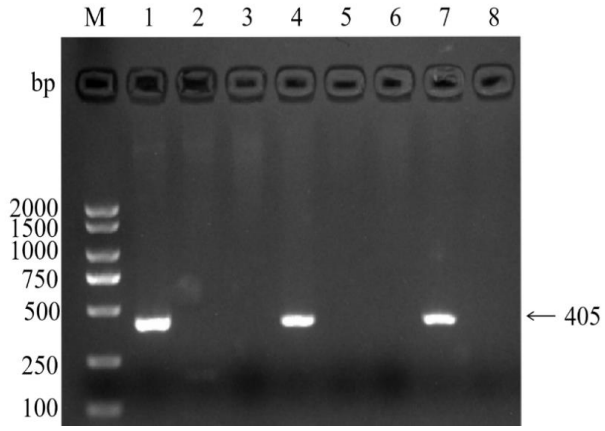

Fig. 3: PCR-specific amplification products of chicken leukemia nucleic acid and control virus DNA. Lane M = DNA Marker DL 2000; 1, 4, and 7 = ALV; 2 and 5 = CIAV, and 3 and 6 = blank controls.

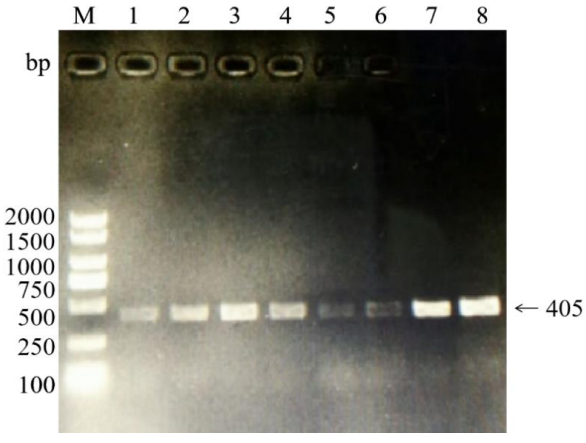

Fig. 4: PCR amplification products of ALV-positive nucleic acid and serum nucleic acid of sick chicken. Lane M = DNA Marker DL 2000; 1-7 = sick chicken serum nucleic acid, and 8 = ALV-positive nucleic acid.

DISCUSSION

Accurate and rapid diagnosis of chicken leukemia is crucial for its prevention and control. Our experiment successfully detected ALV infection using PCR and organ lesions caused by ALV in chicken leukemia using H&E staining.

Since its first discovery in 1868, ALV was divided into seven subgroups: A, B, C, D, E, J, and K. Among them, ALV-A, B, C, D, J, and K are exogenous, while ALV-E seems to be endogenous (Chang *et al.,* 2013). From the sequencing BLAST results, it can be inferred that the nucleic acid we obtained belongs to the ALV-E subgroup. According to the evolutionary tree and homology comparison, it can be seen that ALV-E has a significant difference in homology with ALV-A, B, C, D, J, and K, and there are many mutations in ALV-E in the genome. Compared with other subgroups of ALV, this also explains why only ALV-E is endogenous in ALV. Endogenous retroviruses contain all the necessary genes to integrate their RNA genome into the host genome, which can be transmitted to offspring and is difficult to eradicate (da Silva *et al.,* 2024). According to the literature, endogenous retroviruses (ERVs) account for approximately 3% of the chicken genome, and ALV-E has a high degree of homology with exogenous ALVs, making it the first endogenous retrovirus to be found in chickens (Hu *et al.,* 2017).

ALV-E has a high homology with exogenous ALVs, making it the first endogenous retrovirus discovered in chickens (Hu *et al.* 2017). This also explains why ALV is difficult to purify, as ALV-E integrates its nucleic acid into the host genome and spreads it to offspring. There are published reports that ALV-E interference can lead to a decrease in the production performance of broiler chickens, such as weight loss and slow weight gain, seriously affecting the development of the broiler breeding industry (Kuhnlein *et al.,* 1981; Gavora *et al.,* 1991; Fulton *et al.,* 2021). Our clinical observations exhibited that chickens infected with ALV-E are slimmer than healthy chickens, supporting our diagnosis.

ALV is an immunosuppressive virus that can inhibit the body's immune function by affecting innate and adaptive immune responses and the function of immune cells and phagocytes (Zheng *et al.,* 2022; Wang *et al.,* 2022). Congenital and adaptive immune responses can affect the function of monocytes, macrophages, dendritic cells (DC), B cells, and T cells (Feng *et al.,* 2016; Wang *et al.,* 2022; Du *et al.,* 2022). As one of the most important immune organs in the body, the spleen has the function of carrying out immune responses. Under normal metabolism, the spleen can produce immune factors and immune cells, which can promote the normal metabolism of immune cells in the body and affect the immune function of the body (Munga *et al.,* 2023; Huang *et al.,* 2024; Sha *et al.,* 2024; Samborska *et al.,* 2024).

Histopathological diagnosis showed significantly enlargement of the spleen, with a large amount of infiltrating inflammatory cells, indicating that ALV infection significantly affected the morphology and function of the chicken spleen. Our results also demonstrated that ALV infection can lead to spleen damage. Significant pathological changes in the heart, liver, lungs, and kidneys of chickens infected with ALV were observed. However, there is currently no detailed literature reporting on ALV infection causing heart, liver, lungs, and kidneys in chickens, which seems to be our new findings.

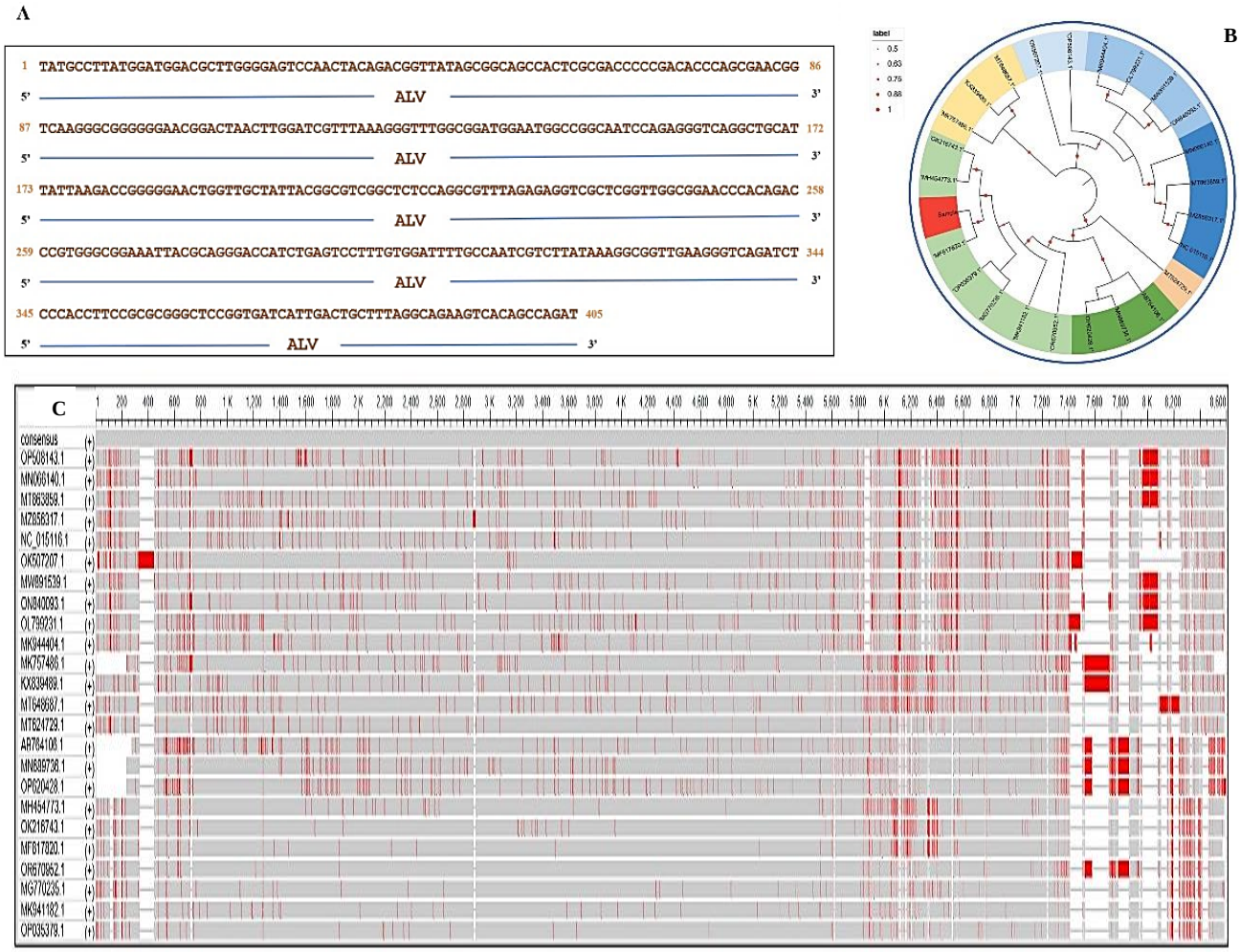

Fig. 5: Sequencing and evolutionary tree analysis of avian leukemia. A) The sequencing results of ALV were identified as the ALV-E subgroup through NCBI BLAST comparison, B) Evolutionary tree analysis of ALV, and C) Nucleic acid comparison between ALV subgroups, with red indicating the mutation region.

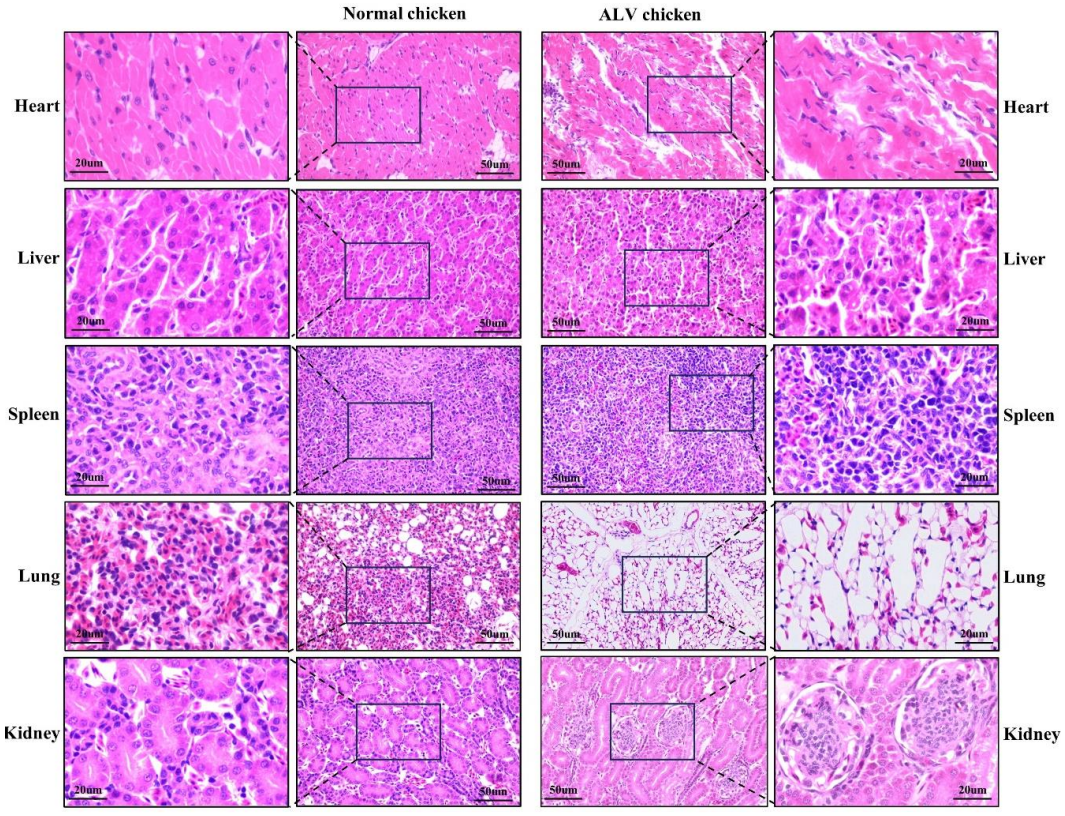

Fig. 6: Photomicrographs of the heart, liver, spleen, and kidneys of healthy and ALV-infected chickens. We observed disorganized and loose muscle fibers in heart. There was vacuolar degeneration and apoptotic changes in liver of the diseased chickens. Marked inflammatory cells infiltration in the spleen. Lungs showed marked atelectasis while renal tubules showed condensed and necrosed nuclei, and urinary space at some places was increased in glomerulus. B = 50µm and 20µm; Stain: H and E.

Since the same pathological features and clinical symptoms may exist in different diseases, the PCR reaction has high specificity properties in laboratory tests, which helps in the diagnosis and treat chicken leukemia virus. In the present study, PCR detected specific bands consistent with avian leukemia target genes in the serum nucleic acid of diseased chickens. In recent years, many studies have shown the application of PCR in diagnosing infectious diseases. For example, PCR is used to detect pathogens to determine whether the disease belongs to subclinical or recessive status (Di Azevedo *et al.*, 2022). The number of pathogens is also related to the disease's treatment effectiveness, severity, and infectivity (Vesper, 2024). PCR has also been widely used for the diagnosis of novel coronavirus (Shirato *et al.*, 2020; Zhang *et al.*, 2023; Almulla *et al.*, 2024). However, PCR has also certain limitations. For example, PCR is prone to false positives, and diagnostic errors can cause significant losses. This is why along with PCR, we also used H&E staining for the diagnosis of chicken leukemia virus. In summary, by combining clinical diagnosis, PCR and H&E staining results, diagnosis of chicken leukemia virus becomes easy and leading to better future prevention and treatment of ALV and reducing the waste of human and material resources caused by misdiagnosis and mistreatment.

Conclusions: In summary, we obtained 405bp specific bands from diseased chicken serum virus samples through PCR detection, which was consistent in size with the target gene bands. Based on the clinical symptoms and anatomical changes of the diseased chicken and the results of PCR detection and H&E staining, it was diagnosed that the diseased chicken has been infected with the avian leukemia virus. At the same time, combination of PCR and H&E staining techniques has provided new theoretical references for clinical diagnosis of avian leukemia virus-induced avian leukemia. This study has provided basis to further explore more efficient and accurate diagnostic methods.

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Abbreviations

ALV:	Avian leukemia virus
bp:	Base pairs
CIAV:	Chicken Anemia Infectious virus
H&E staining:	Hematoxylin-eosin staining
MDA5:	Melanoma differentiation-associated gene 5
MDV:	Marek's disease virus
NCBI:	National Center for Biotechnology Information
PCR:	Polymerase Chain Reaction
TLR7:	Toll-like receptors7

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