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RESEARCH ARTICLE

Co-infection of ALV-J and *Salmonella pullorum* in Laying Hens

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ABSTRACT

A subgroup J avian leukosis virus (ALV-J), designated as SD1306, which was isolated and identified from inspection of suspected infection, a *Salmonella pullorum* (SP) strain was isolated at the same time. Sixty Hy-line brown chickens were used for the study. Detected the body weight, the immune organs index, histopathology, quantity change of the intestinal flora, the viremia and anti-ALV-J antibody after being inoculated the two pathogens. Results showed that the indicators had no significant change during the latent period, while at the 6th and 7th week, compared with other two groups inoculated separately, the body weight of co-infected group was significant lower; the thymus index was below the group infected with SD1306, while the spleen index was significant higher all the time. No evident tumour nodules and swelling in any tissues were seen at necropsy. Histopathological findings revealed large islands of leukomonocytes or myelocytes in tissues containing tumors. *Salmonella* in rectal feces of co-infected group was much higher than the group infected with *Salmonella pullorum* from the 3rd week, the number of *E. coli* significantly exceeded the group *infected* with *Salmonella pullorum* at the 7th week. The co-infected group positive rate of ALV-p27 antigen was 69.23%, while the group infected with SD1306 was 38.46% at the 5th week. The positive rate of both groups declined at the 6th week, rose again at the 7th week. Correspondingly the positive rate of anti-ALV-J antibody continued to decrease from the 6th week to the 7th week. It showed that ALV-p27 antigen and antibody level fluctuated strongly in some time and there was a negative correlation between them. The damage to the chicken was more serious in the co-infected chicken than the chicken infected separately.

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INTRODUCTION

Subgroup J avian leukosis virus (ALV-J) mainly infected broiler chickens and also had been found in laying hens in succession (Xu *et al.*, 2002; Sun *et al.*, 2007; Yu *et al.*, 2012). The disease caused tumors in laying hens and the tumors' symptoms were more and more diversified, such as medullary tumor, myeloblastome and aneurysm. Pullorum disease did great harm to poultry husbandry for years (Hui *et al.*, 2012; Zhang *et al.*, 2011), chicks' cardinal symptom mainly included systemic infection and high mortality. The adult chickens' clinical symptoms were mild, it caused laying drop and malformed reproductive organs. Many pathogens co-infected with ALV-J (Qin *et al.*, 2010; Wang *et al.*, 2011), that made diseases control and purification more difficult in chicken flocks and caused more people's attention. In our epidemiological survey the positive rate of ALV and SP presented a certain degree of correlation (Huang *et al.*, 2013). The two pathogens were vertical propagation, they caused the decline of production performance and even death. We did the animal regression test to investigate the co-infection of ALV and SP may have synergy pathogenic effect.

MATERIALS AND METHODS

Separation and identification of the pathogens: Took the liver, kidney and spleen from one sick laying hen, added four times sterile PBS, filtered by 0.2μm filter after centrifugation, added green streptomycin mixture. When 70%~80% of the cell culture flask covered with DF-1

cells, added the filtered solution, set the negative control at the same time. Detected P27 antigen in the cell culture supernatant via ELISA, identified cell DNA by PCR method, compared and analyzed with other 14 reference strains home and abroad when the gene had be sequenced. The ALV-J specific primers had been reported (Cheng *et al.*, 2010) and the target fragment was 924bp.

Isolated and cultured *salmonella* from rectum, picked several typical bacterial colonies, sequenced 16SrRNA of PCR product, measured its LD50 by improved koushi method after the SP was determined.

Chicken-weight and Immune organ index: Sixty Hy-line brown chickens were divided into four groups randomly at 1 day of age. Chickens of Groups I and II were inoculated 0.2 ml SD1306 strain through abdomen, while Groups III and IV were inoculated the same amount of normal saline at 1 day of age. At 10 days of age, chickens of Groups I and III were inoculated doubled LD50 through abdomen, while those in Groups II and IV were inoculated equal normal saline at the same time.

Observed the chicken flocks' growing status every day, measured the weight at set intervals. Chose a chicken which closed to the average weight from Groups of I, II and IV respectively, weighed the thymus and spleen after anatomical observation lesions. The calculation method of immune organ index was as followed:

The immune organ index=the immune organ weight (g)/ chickens weight (kg)

Pathological observations and ALV-J detection: Selected a chicken which closed to the average weight from Group I at the 6th week and 7th week did the tissue section of internal organs after visual inspection and anatomical observation lesions.

The vaginal swab samples and the serum samples were tested for ALV-P27 and ALV-J via ELISA kits produced by IDEXX Company. The operational approach followed the instruction of kit strictly.

Quantification of *E. coli* and *salmonella*: At the 3rd, 5th and 7th week picked 5 chickens from Groups I, III and IV randomly, took 0.1g feces to the centrifuge tube by using sterile cotton swab from the rectum, mixed thoroughly after adding 1ml normal saline, added 0.1ml diluent to the sterile centrifuge tube which was filled with normal saline. Calculated the quantity of *salmonella* and *E. coli* in per gram of feces and then analyzed results. The results were showed by "lgCFU/g".

The bacteria quantity in per gram of feces (CFU/g)=the average quantity of bacteria×200×dilution multiple

Statistical analysis: The data of body-weight-gain of the chickens at different age and the quantity (lg CFU/g) of *Salmonella* and *E. coli* were presented as mean±SE. Difference were considered statistically significant at $P<0.05$ using Student's *t*-test.

RESULTS

Isolation and identification of pathogens: There was no any cytopathic effect during the blindly 3 generation of DF-1 cells that infected with inspected samples, but the ALV-p27 antigen in the cell culture supernatant was tested to be positive in the 3rd generation. Extracted cDNA from cells, through specific primers amplified target fragment by PCR, the amplified fragments obtained from the liver, spleen and kidney were all 924bp (Fig. 1). Sequenced the positive results and the homology was 86.1%~96.5% when compared with the 14 strains ALV-J gene sequence which were reported home and abroad. The homology was up to 96.5% compared with HPRS-103. The relations also were showed in genetic evolutionary tree (Fig. 2).

The PCR product sequencing compared with the reported SP 16SrRNA was 99%, they were the same species. The LD50 of this strain was 4.4×10^7CFU/ml.

Impact on the co-infected chicken body weight and immune organ index: Within 10 days of age after the chicken flocks infected with ALV-J, there were no significant differences among the groups ($P>0.05$).The body weight of Group I, II and III were greatly significant lower than Group IV ($P<0.01$) from the 6th week. Groups I and II had significant difference ($P<0.05$) at the 3rd week, but there was no significant difference between Groups II and IV ($P>0.05$), chickens' weight gain were reduced significantly. There was significant difference between Group I and the Group II at the 4th week ($P<0.05$), while Groups I and III had significant difference at the 5th week ($P<0.05$), the body weight loss of co-infected group decreased significantly compared with the groups infected separately. At the 6th, 7th week, the body weight loss of Group I greatly significant decreased compared with Groups II and III ($P<0.01$), the co-infection caused significant weight loss with the increase of age (Table 1).

The thymus index of Group I was always lower than Group IV (Fig. 3), the thymus became smaller after co-infection. The spleen index of Group I was higher than Group IV, the spleen increased. The spleen index was relatively stable with the increase of age, it meant the enlargement of spleen was not obvious (Fig. 3).

Gross and histological lesions: From the 2nd week, cases occurred in co-infected chickens such as exhaustion, diarrhea and emaciation (Fig. 4A). At the 6th week, the thymus of co-infected chickens had mild swelling, other internal organs had no obvious changes yet (Fig. 4B, 4C). The tumors of different sizes that formed by medullary tumor cells and lymphocyte tumor cells occurred in the liver (Fig. 5A), most of them distributed around the blood vessels. Most tumors and the surrounding liver cells had apparent boundaries, the liver cytomembrane near the boundary was destroyed and occurred serious degeneration and necrosis (Fig. 5B). The focal and sporadic medullary tumor cells were observed in the tumor simultaneously (Fig. 5C). The tumors' infiltrating hyperplasia occurred in the glandular stomach (Fig. 5D), some were between the two glands, made the muscular layer thinner (Fig. 5E), the hyperplastic medullary tumor cells and lymphocyte tumor cells were observed in the glandular simultaneously (Fig. 5F).

Changes of antigen and antibody: At the 5th week, the percentages of ALV-p27 antigen-positive of Groups I and

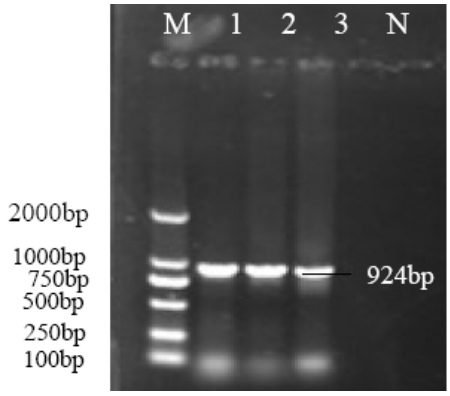

Fig. 1: Detection of ALV-J (924bp) by PCR from livers, spleens and kidneys samples of layer hens. N: Negative control. M. Marker. No.1: Liver. No.2: Spleen. No.3: kidney.

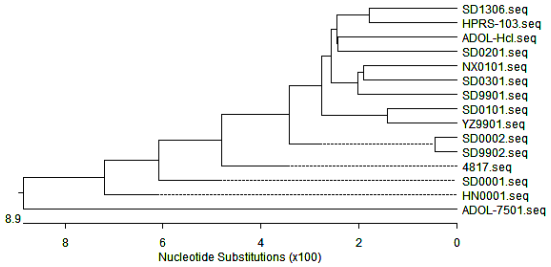

Fig. 2: Evolutionary tree of the gp85 gene of ALV-J strains.

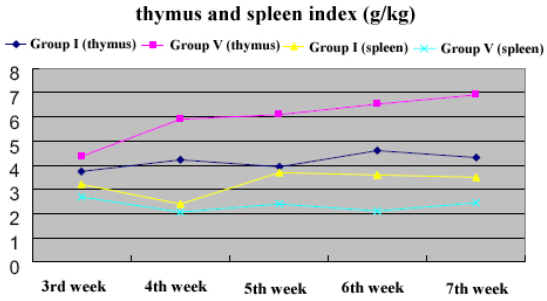

Fig. 3: Effects of thymus and spleen index in immunosuppressive chickens induced with the co-infection.

II were the highest, for Group I was 69.23% and Group II was 38.46%. At the 6th week, both of the percentage of ALV-P27 antigen-positive decreased, but at the 7th week, both increased (Table 2). In this stage, the percentage of ALV-P27 antigen-positive in Group I was higher than that of Group II, the percentage of ALV-P27 antigen-positive in the same group performed in difference in different week. From the 6th week to the 7th week, the percentage of anti-ALV-J antibody-positive decreased (Table 3), precisely contrary to the variation of P27. So, the titer of ALV-P27 antigen and the anti-ALV-J antibody had a negative correlation in some degree.

Quantity of *E.coli* and *Salmonella* in the rectum: The quantity of *Salmonella* in rectum of chickens in Groups I and II had significant difference (P<0.05) and it declared that the quantity of *Salmonella* in group I increased apparently. The results obtained at the 5th and the 7th week showed that the quantity of *Salmonella* in Groups I and III had significant difference (P<0.05), when compared with Group IV, and it indicated that the quantity of *Salmonella* in chickens of Group I increased (Table 3). The quantity of *E. coli* showed that, from the 3rd week, chickens in group I were great significantly different compared with Group IV (P<0.01), and, from the 5th week, chickens in Group III were also great significantly different from Group IV (P<0.01). The results indicated that the quantity of *E. coli* in the experiment group increased. The quantity of *E. coli* in Groups I and III was different from the 3rd week to the 5th week (P<0.05) and at the 7th week, the difference of *E. coli* in Groups I and III was extremely significant (P<0.01) (Table 4). It indicated that the quantity of *E. coli* in rectum of birds with co-infection increased obviously.

DISCUSSION

In the latent period of infected flocks, there was no obvious change in the immune function indicator, the productivity of the flocks were not affected significantly. But at the later stage of infection, chickens infected with ALV-J caused lymphocyte apoptosis and necrosis directly, and then led to severe lesions in bone marrow. Bone marrow was where lymphoid stem cells produced, it affected the growth of thymus, and then the thymus atrophied, thus the thymus index decreased. As these central immune organs were damaged, the mature lymphocytes in peripheral immune organs, such as spleen, were decreased. Spleen was the major place where the organism produced antibody and where immune response happened, so, if the lymphocytes in spleen were decreased, the humoral immunity, the cell-mediated immunity and other nonspecific immunities would be cut down. This was the real reason of immunosuppression Zhang *et al*., 2010. Immunosuppression made the ability of anti-infection of the organism declined, so it made it easy for the pathogenic bacteria such as *Salmonella* and *E. coli* to infect the body and the balance of the bacteria in intestinal canal was destroyed. This was the reason that the number of *Salmonella* and *E. coli* in the rectum of co-infected group increased significantly than the group infected with SP separately. SP also caused inflammation, and led to the spleen edema in co-infected chickens, and the spleen index increased. SP was a bacteria that led to disease rapidly, loss of weight in infected chickens due to dehydration and diarrhea, the growth inhibition of chickens was quicker and more obvious when SP infected with ALV-J.

Table 1: Effects of the co-infection on body weight gain of the chickens at different age

Groups	10	21	28	35	42	49
Group I	59.52±4.31	145.95±18.87^{a}	221.31±34.92^{Aa}	304.33±43.58^{Aa}	361±36.84A^{a}	437.4±57.67^{Aa}
Group II	58.93±5.27	159.43±9.90	245.92±19.11^{b}	354±25.51^{b}	463±19.19B^{b}	527±30.79^{Bb}
Group III	59.17±7.87	146±7.22^{a}	234.58±22.10^{a}	331.09±25.71^{Ac}	428±33.42B^{c}	485±39.50^{Bc}
Group IV	59.07±6.10	166.77±12.47^{c}	263.42±17.99^{Bb}	409.91±27.37^{B}	567.33±33.08^{C}	681.25±39.11^{C}

Values (Mean±SE) in the same column bearing same superscript indicates no significant difference (P>0.05), while different lowercase and uppercase superscript show significant difference at P<0.05 and P<0.01, respectively.

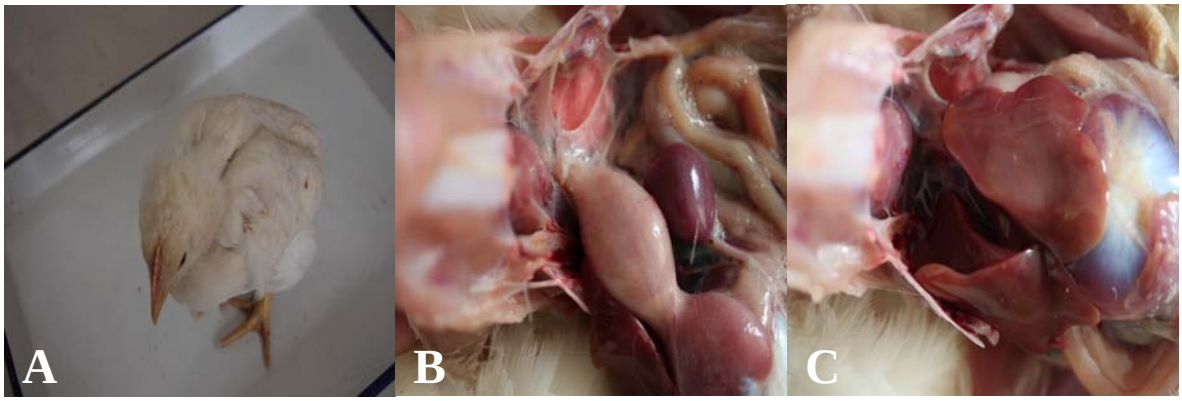

Fig. 4: Gross lesions of sick chicken induced by the co-infection. A: The sick chicken skeletization. B. Thymus swelling slightly. C: The internal organs swelling were not obvious.

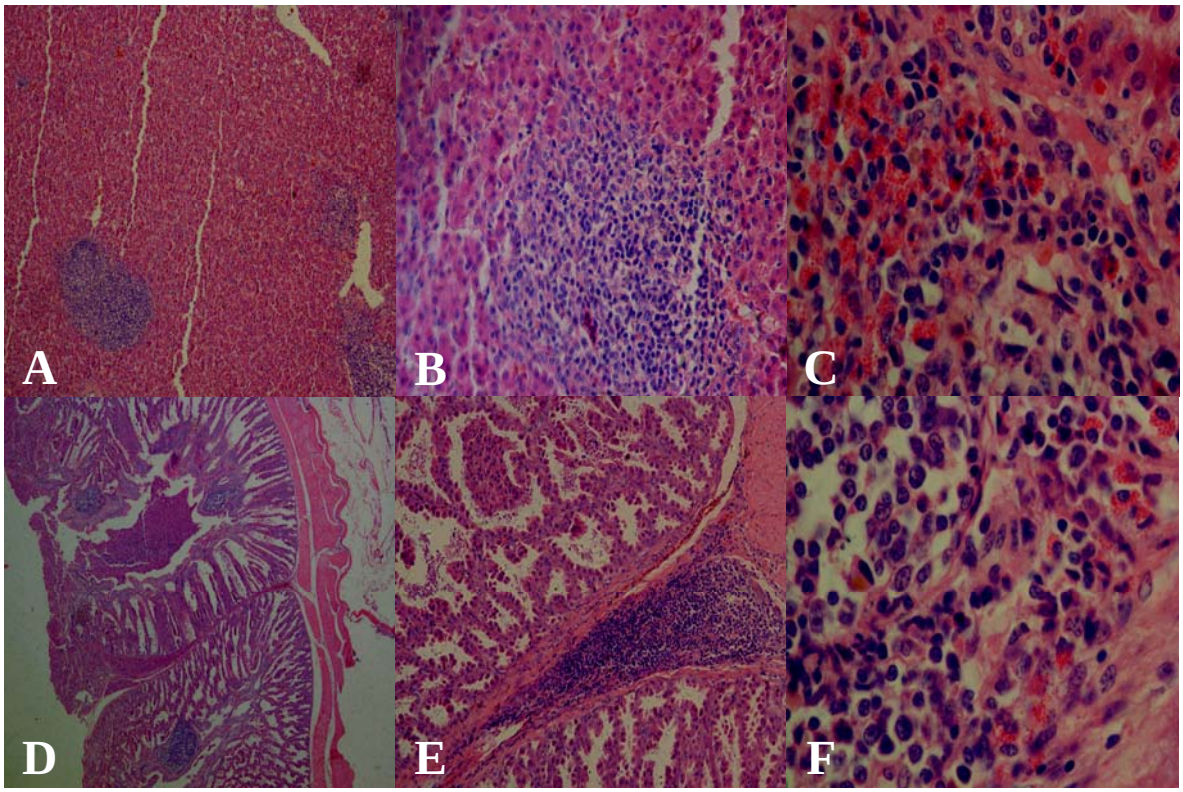

Fig. 5: Histopathology of the chicken (HE staining). A: lots of lymphocyte tumor in the liver. B: Hepatocyte degeneration and necrosis. C: Myelocytomatosis. D: The lymphocyte proliferation among the glandular stomach. E: lymphocyte proliferation was between two glands. F: The lymphocyte tumor cells and myelocytomatosis both in the lymphocyte proliferation.

Table 2: The percentage of ALV-p27 antigen-positive in the co-infected and ALV-J-infected chickens

Groups	[a]Sixth (%)	Seventh (%)
I	54.55	30
II	36.36	20

[a]The chickens at the age of six weeks, the same as below

Table 3: The percentage of anti-ALV-J antibody in the co-infected and ALV-J-infected chickens

Groups	Forth (%)	Fifth (%)	Sixth (%)	Seventh (%)
I	14.29	69.23	27.27	50
II	14.29	38.46	18.18	30

ALV-J mainly infected the bone marrow cells and its precursor cells, and thus formed myelocytome and lymphocytoma (Chai *et al.*, 2010; Deng *et al.*, 2011). This variant cells grew faster than the normal bone marrow cell, and they also could enter in blood sinus, and then to every organ, to grow to tumor metastases (Cheng *et al.*, 2010; Pandiri *et al.*, 2009). As spleen, liver and glandular stomach had affluent blood, when infected, tumor metastases appeared at the 1st time, and most was located surround the vessel. Generally, tumors caused by ALV-J in chickens appear earlier, but in this study the tumors appeared much later. It needed further study to find whether it was determined by the species and the quantity of hormone in bodies. 6 weeks after the chickens were infected, no visible tumor nodules appeared in visceral organs, but we could find tumors in the slices made by pathological tissue. We should pay more attention to diagnose it in the primary. After SP infection, lymphocytes would have infiltrative growth in some tissues such as

Table 4: Quantity (lg CFU/g) of *Salmonella* and *E.coli* detected from rectal feces of the tested chickens

Groups	*Salmonella*			*E. coli*		
	Third	Fifth	Seventh	Third	Fifth	Seventh
I	8.48 ± 0.47^{B}	9.26 ± 0.13^{Ba}	8.79 ± 0.19^{Ba}	9.34 ± 0.75^{Bb}	9.47 ± 0.28^{Aa}	9.39 ± 0.34^{A}
III	8.33 ± 0.56^{B}	8.57 ± 0.09^{Bb}	8.34 ± 0.35^{Bb}	8.87 ± 0.50^{a}	9.12 ± 0.47^{Ab}	8.68 ± 0.21^{Bb}
IV	6.96 ± 0.46^{Aa}	6.88 ± 0.27^{Aa}	6.71 ± 0.15^{Aa}	8.56 ± 0.23^{Aa}	8.47 ± 0.15^{B}	8.23 ± 0.09^{C}

Values (Mean±SE) in the same column bearing same superscript indicates no significant difference (P>0.05), while different lowercase and uppercase superscript show significant difference at P<0.05 and P<0.01, respectively.

liver (Chen *et al.*, 2006), and we also found some scattered grown lymphocytes in lesion tissues in the chickens of co-infected group.

The ALV-p27 antigen and anti-ALV-J antibody of the chickens in co-infected group were higher than each separately infected group all the time. Obviously, the two pathogens could occur synergistic effect, and this explained why chickens in co-infected group could get a higher percentage of morbidity. This was in line with the results of epidemiological investigation carried out before (Huang *et al.*, 2013). How SP caused the change of the ALV-p27 antigen and anti-ALV-J antibody was needed further investigation. ALV-J and SP spread widely in the flocks, and both were vertical spread. In this study, we preliminary found that ALV-J and SP could infect chickens synergistically, so the purification of the both pathogens should be considered in production at the same time.

Contributions of Authors: YY Jing designed the experiment and completed most of the works. YS Li and JK Xin analyzed some test results and collected materials. JQ Chai and ZZ Cui gave experiment instruction. Thank all the authors' contribution to the experiment.

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