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RESEARCH ARTICLE

Co-infection of Avian Leukosis Virus and *Salmonella pullorum* with the Preliminary Eradication in Breeders of Chinese Local "ShouGuang" Chickens

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ABSTRACT

The study was designed to investigate the infection status and to finish the preliminary eradication of avian leukosis virus (ALV) and *Salmonella pullorum* (SP) in breeders of Chinese local "ShouGuang" chickens. ALV antigen and antibody was tested via ELISA, and SP antibody was detected by serum plate agglutination test (SPAT). The etiology and pathology was also studied. The ALV-P27 antigen, ALV-A/B and SP antibody positive chickens were eliminated in turn, and then the negative were retained as the breeder flocks. The results showed that the positive rate of antigen to ALV-P27, antibody to ALV-A/B, ALV-J and SP was 57.8, 6.7, 0 and 17.8% in this breeder farm, respectively. The co-infection of ALV and SP was confirmed and the positive rate of both SP and ALV-P27 or ALV-A/B was 10 and 1%, respectively. There were obvious tumor nodules and lymphoid tumor cells in the comb, liver and spleen of the co-infected chickens. The degenerative and atrophic ovarian follicles, inflammatory cell infiltration in muscle biopsies were also found. The elimination rate of ALV-p27, ALV-A/B and SP positive chickens was 55.4, 13 and 6.1%, respectively. The final amount of the breeder conservation was 309 chickens. In conclusion, the co-infection of ALV-B and SP was found and more emphasis should be given on its prevention; the preliminary eradication of "ShouGuang" breeder chickens was finished.

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INTRODUCTION

Avian leukosis virus (ALV) infection of chickens has been widespread around the world (Naz *et al*., 1990; Spencer *et al*., 2003; Lupiani *et al*., 2006). The infection of ALV is also very common in Chinese flocks and the case has been reported in 80% of the provinces (Gao *et al*., 2012), including many local varieties (Sun and Cui, 2007; Pan *et al*., 2011; Li *et al*., 2012). The harm of avian leukosis includes three aspects: tumor-associated deaths (Bacon *et al*., 2004; Payne and Nair, 2012), reduced productivity and immunosupression (Spackman *et al*., 2003). The pullorum disease also occurred in most part of the world (Johnson *et al*., 1992; Salem *et al*., 1992; Sato *et al*., 1997). It mainly hazards the chicks and turkeys, and shows hepatosplenomegaly and peritonitis (Khan *et al*., 2004; Setta *et al*., 2012). The adult chickens often show invisible infection and long-term carriers of the bacteria. The breeder-chickens with salmonella usually show the degradation of productivity and the decrease in survival rate of their offspring (Barrow, 1993; Barrow and Freitas-Neto, 2011). Both of ALV and *Salmonella pullorum* (SP) can be transmitted vertically (Witter *et al*., 2000; Berchieri *et al*., 2001; Akhtar *et al*., 2011; Shahzad *et al*., 2012; Feng *et al*., 2012) and the main measures of the prevention and control includes the eradication of pathogen (Fadly, 2000; Anderson *et al*., 2006). Many large international breeders-companies have inducted a lot of manpower, financial and material resources to complete the purification of the core progenitor flocks. However, in China, many varieties of local chickens have rarely performed the purification due to the lack of capital, technology and other aspects so that the original species flocks remained threatened.

"ShouGuang" chicken is one of the most excellent breeds of local chickens in China, dual-purpose of eggs and meat with a long history, and listed as a National Breeds and Local Varieties Protection List of Shandong

Province. This is the only breeder chicken farm of "ShouGuang" chickens, but the eradication of ALV and SP has never been conducted. Recently, the pathological symptoms of tumor, decline of egg production and the peritonitis can be found sometimes. In the present study, the infection status of ALV and SP were first investigated in "ShouGuang" breeders and the preliminary eradication was carried out, too.

MATERIALS AND METHODS

Investigation of ALV and SP infection status: In the "ShouGuang" breeder chickens farm, the samples of vaginal swab and serum were collected from 90 "ShouGuang" chickens including 30 original cocks, 20 original hens, 20 improved cocks and 20 improved hens, which were selected randomly. The vaginal swab samples were stored at -20°C and thawed before using and the blood samples were placed in the 4°C around the night to precipitate the serum for further use. The vaginal swab samples and the serum samples were tested for ALV-P27 (the P27 antigen of avian leukosis virus), ALV-A/B (the antibody of avian leukosis virus of subgroup A and B) and ALV-J (the antibody of avian leukosis virus of subgroup J) via ELISA kits produced by IDEXX Company. The SP antibodies of the serum were tested by SPAT. The positive and negative standard serum and diagnostic antigen were all produced by the Chinese Institute of Veterinary Drugs Control.

Pathological observations and pathogen detection: The chickens infected by both ALV and SP were selectively separated. The sick chickens were necropsied and recorded for the gross lesions. The liver, spleen, kidney and intestinal tissues were collected and fixed in the 10% neutral formalin solution to make the HE staining slice for the optical microscope observation.

The vaginal swab samples were collected by aseptic manipulation for the isolation of *Salmonella pullorum* using the SS (Salmonella-Shigella Mediun) agar plates. The isolated bacteria were identified by Gram's staining examination and biochemical test. The SS agar and the biochemical tube were produced by Qingdao Haibo and Hangzhou Tianhe. The DNA samples from liver tumors were extracted using DNAiso reagent (produced by Takara Company) for the detection of ALV by PCR. The primers were synthesized by Sangon Company.

Epidemic strains of ALV: The vaginal swab and serum samples of different days and different kinds of chickens were collected for the epidemiological investigation of ALV in the farm to make sure the epidemic strains and reasonable program of purification.

Eradication of ALV and SP: The eradication program was performed by four steps. The positive chickens of ALV-P27 antigen, ALV-A/B and salmonella antibody were eliminated in turn. The details were showed in the following.

Step 1: the ALV-P27 antigen of all chickens in the farm was tested and the positive was eliminated.

Step 2: the ALV-A/B antibody of the rest of chickens was tested and the positive was eliminated.

Step 3: the salmonella antibody of the rest of chickens was tested and the positive was eliminated.

Step 4: the negative chickens in the three indices were retained for the breeder flocks after the examination of the productivity.

RESULTS

Positive rate of ALV and SP: The results showed that the infection of ALV and SP had existed in the farm (Table 1). The positive rate of antigen was higher than the antibody, so it seemed that it was easier to check the antigen. In different flocks, it was easier to test antigen in cocks and antibody in hens. The positive rate of antigen in cocks was the highest, reaching 66.7%. The ALV-J antibody was not detected.

The co-infection of the two pathogens had also existed, especially in the hens. The positive rate (10%) of both ALV-P27 and SP was higher than the rate (2.2%) of ALV-A/B and SP. The detail was showed in the Table 2.

Table 1: Positive rate of avian leukosis virus and *Salmonella pullorum* in the random-sampled flock

	Original chickens (%)		Improved chickens (%)		Sum total (%)
	Cocks	Hens	Cocks	Hens	
ALV-P27	66.7	50	60	50	57.8
ALV-A/B	6.7	15	5	0	6.7
ALV-J	0	0	0	0	0
SP	6.7	15	45	10	17.8

Table 2: Positive rate of the co-infection of avian leukosis virus and *Salmonella pullorum*

	Original chickens (%)		Improved chickens (%)		Sum total (%)
	cocks	hens	cocks	Hens	
ALV-p27 and SP	0	15	20	10	10
ALV-A/B and SP	0	5	0	0	1.1
ALV-J and SP	0	0	0	0	0

Gross lesions of the co-infected chickens: The typical symptom was easy to observe in most of the co-infection cases. The sick chicken showed emaciation, messy matt feather (Fig. 1A), and malodorous manure. The granulation-like protrusions (Fig. 1B) and hemorrhagic spots could be seen on the surface of some atrophy pale combs.

The gray tumors nodules were observed on the surface (Fig. 1C) and ventral surface (Fig. 1D) of the enlarged livers. The similar lesions were also seen on the spleens (Fig. 1F). The significant lesions hadn't been found on the kidneys.

The ovarian follicles were degenerative and atrophic. Some of them turned from pale or deep yellow to gray or leaden in color and shaped cystic or pear (Fig. 1E), which may be caused by *Salmonella pullorum*.

Histological lesions of the co-infected chickens: The hyperplasia of tumor cells was observed in the portal and parenchyma area of liver and the normal liver lines were squeezed to destruction (Fig. 2A). These tumor cells had the similar morphology with a round large cell body, slightly basophilic cytoplasm and pathological mitotic such as asymmetry, irregular and cyclic nucleus (Fig. 2B). The similar lesions were also observed in the spleen section (Fig. 2C).

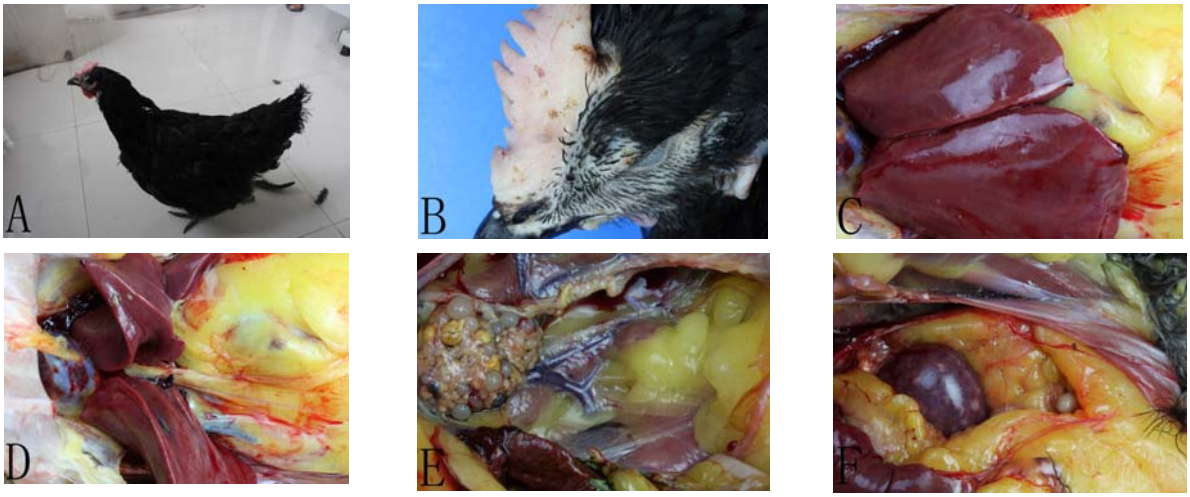

Fig. 1: A: The sick chicken with messy matt feather. B: The pale comb with granulation-like protrusions. C-D: The enlarged liver with nodules on the surface and ventral surface. E: The degenerative follicles and some of them were leaden. F: The spleen with tumor nodules on the surface.

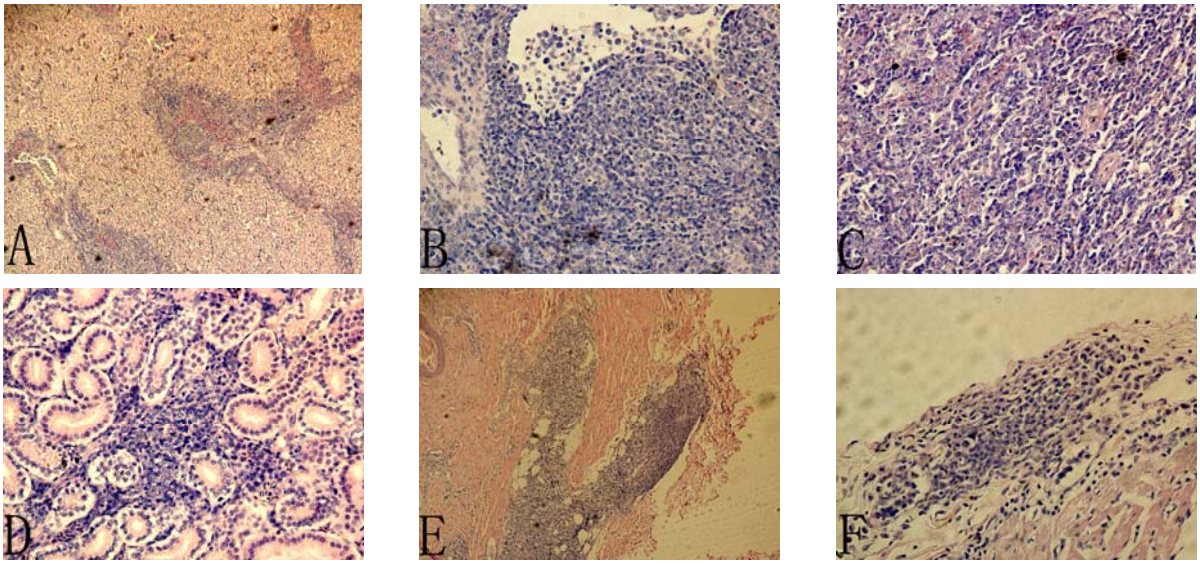

Fig. 2: A: The hyperplasia of tumor cells in liver (100×). B: The typical lymphoid tumor cells in liver (400×). C: The tumor cells in the spleen (400×). D: The tumor cells in the kidney (400×). E: The tumor cells in the comb (400×). F: The histiocytic infiltration in the myocardial tissue (400×).

Some tubular epithelial cells were swollen and degenerated. Lymphocyte-like tumor cells gathered and spread between the renal tubules and corpuscles, which caused the normal tissue destruction (Fig. 2D). The proliferation and dissemination of tumor cells in the comb sarcoma also observed (Fig. 2E). The histiocytic infiltration could be observed in some myocardial tissue (Fig. 2F), which may be caused by *Salmonella pullorum*.

Table 3: Epidemic strains of avian leukosis virus before the eradication

Strains	Original chickens (%)		Improved chickens (%)		Sum Total (%)
	145d	340d	145d	340d	1d
ALV-p27	75	25	45.2	15.9	62.8
ALV-A/B	0	6.7	0	2.3	1.3
ALV-J	0	0	0	0	0

Table 4: Eradication rate of avian leukosis virus and *Salmonella pullorum*

Strains	Original chickens (%)		Improved chickens (%)		Sum
	Cocks	Hens	Cocks	Hens	Total (%)
ALV-P27	71.2	51.6	68.6	53.6	55.4
ALV-A/B	0	13.2	6.5	17.5	13
SP	4.6	3.2	10.3	10.6	6.1

Detection of ALV: Taking the cDNA as the template, the genome band of the ALV-A/B primer presented in 253bp, which was identical with the expected molecular weight and the primer for ALV-A and ALV-J had not shown the expected bands. So, the chickens had infected avian leukosis virus of subgroup B.

Isolation and identification of SP: The colorless translucent small colony with a black center had grown up after the target sample was inoculated into the SS agar plate. The result of Gram's staining examination and biochemical test showed that the bacteria was *Salmonella pullorum*.

Epidemic strain of ALV: The investigation result (Table 3) showed that the avian leukosis had occurred in the farm. The positive rate was greatly influenced by the age. The ALV-P27 antigen can be detected in every group and the positive rate of 145 days-group was higher than the 340 days-group. However, the positive rate of ALV-A/B antibody of both the original and improved was 0% in the

145 days, and lower than the 340 days-group. The positive rate of ALV-J antibody was still 0% in every group. Combined with the investigation result of the random-sampled flocks (the 90 chickens), it was sure that the epidemic strain of ALV in this farm was subgroup B. The main job of the late program was the eradication of ALV-P27, ALV-A/B and SP.

Eradication results: The details of the eradication were showed in Table 4. The positive rate of ALV-P27 antigen was up to 55.4%, with the cocks higher than the hens. 415 chickens, including 19 original cocks, 233 original hens, 32 improved cocks and 131 improved hens, was retained from the 931 chickens after the eradication of ALV-P27 positive chickens.

The positive rate of ALV-A/B antibody was up to 13%, with the hens higher than the cocks. 294 chickens, including 22 original cocks, 158 original hens, 29 improved cocks and 85 improved hens, was retained from the 338 chickens after the eradication of ALV-A/B positive chickens.

The positive rate of SP antibody was up to 13%, with the hens higher than the cocks, the improved higher than the original. 276 chickens, including 21 original cocks, 153 original hens, 26 improved cocks and 76 improved hens, was retained from the 276 chickens after the eradication of SP positive chickens.

Finally, 309 chickens had been remained for the breeder flocks, including the 276 chickens remained from the breeders after the three steps eradications and the 33 negative chickens remained from the random-sampled-flocks.

DISCUSSION

The co-infection of avian leukosis and other disease had been very prevalent in China, but most of the studies in this field were focused on the different subgroup of ALV (Fenton *et al*., 2005; Zhang *et al*., 2008), the immunosuppressive or oncogenic virus such as REV (Cui *et al*., 2009; Ongor and Bulut, 2011), MDV and CAV (Lütticken, 1997; Qin *et al*., 2010; Williams and Sellers, 2012). This was the first report of the co-infection of ALV-B and SP by the methods of serology, pathology and identifying of pathogen.

The typical tumor nodules were found in lots of the co-infected chickens and the proliferation of tumor cell also observed, all of which were indicating the typical symptom of avian leukosis disease. The degenerative and atrophic ovarian follicles, inflammatory cell infiltration in muscle biopsies were found in parts of the flock, which was considered as the symptom of pullorum disease. The typical peritonitis was not found, which may because of that salmonella mainly did harm to the chicks and the adult chickens often showed inapparent infection (Shivaprasad, 2000). At the same time, the moist environment of the breeding birds was better than the commercial birds, so that the peritonitis rarely occurred.

The positive rate of SP after the ALV-eradication (6.1%) was obviously lower than before (17.8%). It seems that the chickens, infected ALV, were easier to be infected by SP. The collaborative pathogenic mechanism of ALV and salmonella maybe existed. Both of the two pathogens could transmit vertically to reduce the rate of fertilization, hatching and survival of the chick (Stedman and Brown, 1999; Barrow and Freitas-Neto, 2011). In another hand, the chickens infected ALV could make immuno-suppression (Wang *et al*., 2011), which makes a chance for the infection of *Salmonella pullorum*. This mechanism corresponded with the phenomenon in Table 5. However, the pathogenetic mechanism required more evidences.

The eradication program was designed according to the biological characteristic of ALV. The sick chickens usually appeared as persistent infection. The positive rate of antigen was higher than the rate of antibody and the detection rate was influenced by the days of the birds. Therefore, the ALV-P27 antigen-positive-chickens were eliminated firstly, and then the positive chickens of ALV-A/B and pullorum. In this study, the eradication of avian leukosis virus and SP was eliminated simultaneously to avoid the repeated tests because of the co-infection. Most of these new methods did favor to the improvements of purification efficiency, the reduction of workload and economical cost. The eradication of ALV and SP had been finished preliminarily in the Chinese local breeding "ShouGuang" flock, which has great significance to the breeder conservation of "ShouGuang" chickens. The experience and methods of this study had also provided important reference for the eradication of other breeds of local breeder flocks in the world.

Conclusion: The co-infection of ALV and SP existed in the "ShouGuang" chickens, and the preliminary eradication was finished. However, more work must be done generation by generation to eradicate them completely. ALV and SP would be monitored continuously in the following studies.

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