Identification and Characterization of Hens Transmitting Avian Leukosis Virus (ALV) to Their Embryos by ELISAs for Detecting Infectious ALV, ALV Antigens and Antibodies to ALV

Kenji TSUKAMOTO, Makoto HASEBE[1)], Shin-ichiro KAKITA[1)], Hiroshi HIHARA, and Yuji KONO[2)]

Poultry Disease Laboratory, National Institute of Animal Health, 4909–58 Kurachi, Seki, Gifu 501–32, [1)]Goto Hatchery Inc., Research Center, 150 Nakakarayama, Kakamigahara, Gifu 504, and [2)]Department of Veterinary Microbiology, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Fuchu 183, Japan

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ABSTRACT. A total of 72 White Leghorn grandparent hens was examined by ELISA for avian leukosis virus (ALV), ALV antigens and anti-ALV antibodies to identify and characterize the hens transmitting ALV to their embryos (transmitters) by using fertilized eggs. These hens were divided into 3 groups as no antibody and non-viremic (NANV) (49 hens), antibody-positive and non-viremic (APNV) (21 hens) and no antibody and viremic (NAV) (2 hens) by testing the sera for the presence of ALV and anti-ALV antibody. Egg albumen and embryos were tested for the presence of ALV and ALV antigens. As a result, no ALV was detected in both albumen and embryos in the NANV group. On the other hand, all albumen samples collected repeatedly from 3 hens of the APNV group and 2 hens of the NAV group contained infectious ALV, although the infectivity differed with the individual. Also, these 5 hens produced infected embryos at varying frequencies. However, one AP hen which shed neither ALV nor ALV antigens into the albumen produced an infected embryo at a lower rate. These results indicate that testing for infectious ALV in albumen from a newly laid egg per hen is effective to identify the transmitters to some extent. When virus titers in each of 8 tissue samples from the 6 transmitting hens were determined, the highest virus titers were found in washing from the ampulla of the oviducts in most of the shedders, suggesting that embryo infection is closely correlated with ALV produced at the oviduct, but not with ALV transferred from the other parts of the body.—KEY WORDS: ALV, ELISA, eradication, transmission.

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Some chickens infected with avian leukosis virus (ALV) transmit the virus vertically to their offspring (transmitters), and the congenitally infected chicks are viremic throughout their lives (viremic hens) and shed large amounts of the virus into the environment (shedders). As a result, their hatchmates are infected horizontally early in life [22–24, 26] and most of them produce antibody to ALV (immune hens). Vertical transmission occurs not only in viremic hens at a high frequency but also occasionally in immune hens [23]. Therefore, detection of both transmitters will lead to progress in ALV eradication.

Viremic hens as the major transmitters can be detected by testing their sera for ALV. Immune transmitters which can be identified by detecting infected embryos are, however, difficult to identify since they produce infected progeny only infrequently [12, 23, 24]. Spencer *et al.* [27] and Payne *et al.* [19] found that there was a strong correlation between congenital transmitters and presence of ALV antigens in egg albumen or cloacal swabs. However, all of the transmitters could not be detected by testing albumen for ALV antigens in the field [6, 13, 14, 17, 19, 21, 26] or Payne and Howes [20] showed by repeated testing that it took 3 years to eradicate the virus in flocks. It is conceivable that some transmitters shedding a small amount of the virus into the albumen, which were not detectable by the test for ALV antigens, can be identified by testing the samples for infectious ALV.

Recently we established a rapid, simple and sensitive ALV-detection test by using ELISA that can be applicable for a number of test samples and showed that it could eliminate a false-positive reaction due to endogenous ALV antigens [29]. The purpose of the present study was to determine the efficacy of the ALV-detection test for detecting the transmitters in the field.

MATERIALS AND METHODS

Chickens: A grandparent White Leghorn flock reared in the Research Center of Goto Hatchery Inc. (Gifu, Japan) was used. Hens, about 2 years old, were selected for high egg production and were treated with force molting just before the experiment. A total of 72 hens was divided into 11 groups

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and each group of hens mated to a cock in a small room with trap nests to collect the eggs. All birds were vaccinated against Marek's disease, Newcastle disease, infectious bronchitis, avian encephalomyelitis, infectious bursal disease, fowl pox and infectious coryza.

Collection and preparation of samples from chickens: Serum and heparinized blood were collected from brachial vein and the blood was used as the whole blood samples.

An average of 3 eggs were collected from each hen on the day of lay and their watery albumen was taken through a hole near the obtuse end of the egg shell with a needle. On an average, 5 embryos of 10-day-old were collected per hen. Embryo extracts were prepared by expressing decapitated embryos through an 18-gauge needle, suspending each tissue in 0.5 m*l* of phosphate-buffered saline and clarifying the suspension by centrifugation at 13,000 × g for 5 minutes, and stored at −80°C before use.

Tissue samples were collected from 6 transmitters. Ten percent homogenates of the livers, kidneys, spleens, pancreas, and the ampulla and infundibulum of the oviducts were prepared with homogenizers and centrifuged at 6,000 × g for 5 minutes and the supernatants were collected. Lymphocytes obtained from 1 m*l* of heparinized blood by gradient centrifugation with Ficoll (Lymphoprep, Nyegaard & Co., AS, Torshov, N-0401 Oslo, Norway) were suspended in 1 m*l* of medium. Ampulla lavage specimens were collected from the oviduct by washing the duct repeatedly with a total of 4 m*l* of medium. These samples were tested for infectious ALV as described below.

Isolation and titration of ALV: Samples to be tested were inoculated onto chick embryo fibroblasts (CEF) of line 15I (C/CE or C/E) [11, 25] which were cultured in 24-well plates coated with collagen (MS-0024K, Sumitomo Bakelite Co., Ltd., Tokyo, Japan) and the culture fluid samples were examined for ALV antigens by the ELISA after 8 day-cultivation as described previously [29]. Sera, whole blood, albumen and tissue samples were tested soon after preparations of the samples, but embryo extracts were tested after storage at −80°C. ALV titers of albumen collected from the 6 transmitters were determined after storage of the albumen at −80°C. The inocula used were 0.1 m*l* of serum and albumen and 0.05 m*l* of whole blood, embryo extract and tissue sample. The RAV-1 strain of subgroup A of ALV was used as positive control.

Detection of ALV antigens: Albumen diluted (1:10) was examined for the presence of ALV antigens by ELISA as described previously [29]. A Nunc module plate (No. 4–69957, A/S Nunc, Roskilde, Denmark) coated with rabbit anti-ALV antiserum and horseradish peroxidase (HRPO)-conjugated rabbit anti-ALV antibodies were used. The culture fluid of RAV-1 infected and uninfected CEF of line 15I were used at 1:4 dilution as positive and negative controls, respectively.

Detection of antibodies to ALV: Sera were examined for the presence of antibodies to ALV by ELISA as described previously [30]. Subgroup A of ALV produced from LSCC-BK3 [10] and RAV-2 (subgroup B) produced from LSCC-RP9 [18] which was kindly provided by Dr. K. Nazerian (USDA, Regional Poultry Research Laboratory, East Lansing, Michigan, U.S.A.) were purified and used as ELISA antigens after treatment with Triton X-100. A Nunc module plate (No. 4–69957) coated with each subgroup of ALV antigens, and HRPO-conjugated rabbit anti-chicken IgG (No. 3204–0082, Cappel Laboratory., Cochranville, Pennsylvania, U.S.A.) were used. Sera from RAV-1 infected or RAV-2 infected chickens and from SPF chickens were used as positive and negative reference sera, respectively.

RESULTS

Grouping of hens and roosters according to infectious status: Table 1 shows the variation in the infectious status with ALV among the 72 hens and

Table 1. Grouping of hens and roosters according to the status of infection with ALV

Group[a]	Serum			Number of		%
	ALV	Antibody		hens	roosters	
NANV	−	−		49(43)[b]	8	68.7
APNV	−	+	10^{2}[c]	5(5)	1	28.9
			10^{3}	11(10)	1	
			10^{4}	5(3)	1	
NAV	+	−		2(2)	0	2.4
Total				72(63)	11	

a) NANV: no antibody and non-viremic; APNV: antibody-positive and non-viremic; NAV: no antibody and viremic.
b) Numbers in parentheses indicate the numbers of laying hens.
c) Antibody titer was shown by reciprocal of the serum dilution which was positive by ELISA with subgroup A of ALV antigens since all sera reacted more strongly with ALV of subgroup A than that of subgroup B.

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Table 2. Detection of ALV and/or ALV antigens in albumen and embryos obtained from the three groups of hens

Group[a]	No. tested	Serum		Albumen[c]		Embryo[d]	Transmitter[c]
		ALV	Ab[b]	ALV	Antigen	ALV	
NANV	43	−	$<10^2$	0 (0/104)	0 (0/105)	0 (0/133)	0/34
APNV	18 (5	−	10^2	2 (7/22)	1 (3/15)	3 (6/42)	3/5
	18 (10	−	10^3	1 (5/48)	1 (3/30)	1 (11/77)	1/10
	18 (3	−	10^4	0 (0/15)	0 (0/9)	0 (0/15)	0/3
NAV	2	+	$<10^2$	2 (5/5)	2 (4/4)	2 (3/9)	2/2

a) See the footnote a) of Table 1.
b) See the footnote c) of Table 1.
c) Number of parent hens laying eggs containing ALV or ALV antigens. (number of positive albumen/number tested)
d) Number of parent hens producing ALV-infected embryos. (number of infected embryos/number tested)
e) Number of transmitters/number of hens tested.

Table 3. Detection of ALV and/or ALV antigens in albumens and embryos obtained from the transmitters

Group[a]	Chicken	Serum		Albumen		Embryo
	No.	ALV	Ab[b]	ALV	Antigen	ALV
APNV	3306	−	10^2	0/5[c]	0/3[c]	1/8[c]
	3107	−	10^2	3/3	0/3	3/8
	3102	−	10^2	4/4	3/3	2/7
	3304	−	10^3	5/5	3/3	11/12
NAV	3103	+	$<10^2$	2/2	4/4	2/6
	3305	+	$<10^2$	2/2	1/1	1/3

a) See the footnote a) of Table 1.
b) See the footnote c) of Table 1.
c) Number of positive samples/number tested.

11 roosters. Forty-nine, 21 and 2 of the hens belonged to the no antibody and non-viremic (NANV), antibody-positive and non-viremic (APNV), and no antibody and viremic (NAV) groups, respectively.

Five, 11 and 5 of the 21 APNV hens, and 1, 1 and 1 of the 3 APNV roosters had anti-ALV antibody titers of 10^2, 10^3 and 10^4, respectively.

Whole blood samples from both 6 transmitters described below and 5 APNV non-transmitters selected randomly were tested for the presence of ALV. As a result, 1 APNV hen (No. 3102) and 2 NAV hens were positive.

Detection of ALV antigens and/or ALV in albumens and in embryos obtained from the 3 groups of hens: Eggs of all laying hens (43 NANV hens, 18 APNV hens and 2 NAV hens) were examined for ALV and ALV antigens in the albumen (Table 2). Neither ALV nor ALV antigens were detected in albumen from any hens of the NANV group. On the other hand, ALV was detected in albumen from 3 of the 18 APNV hens and ALV antigens in albumen from 2 of the 3 hens. Both ALV and ALV antigens were consistently found in albumen from 2 NAV hens.

Embryos of 34 hens of the NANV group, the 18 hens of the APNV group and the 2 hens of the NAV group were tested for transmission of the virus from their mothers (Table 2). No infected embryos were found in the NANV group. In the APNV group, some embryos from the 3 ALV-shedding hens were found to be infected with ALV. Moreover, one embryo from a hen which shed neither ALV nor ALV antigens into the albumen was positive for

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ALV. Two hens in the NAV group produced infected embryos. Thus, 6 transmitters were detected in this flock. Transmitters were found more frequently among the hens with antibody titer of 10^2 than among those with titers of 10^3 and 10^4.

Details of the 6 transmitters are shown in Table 3. ALV was found in all albumen samples from 5 of the 6 transmitters. ALV antigens were detected in all albumen specimens from 4 of those 5. No detectable ALV and ALV antigens were found in albumen from a hen (No. 3306) which produced an infected embryo at a ratio of 1:8 (designated as a sporadic transmitter). The infection rate of embryos was varying among individual transmitters.

Titers of ALV and ALV antigens in albumen collected from the transmitters: Albumen samples were titrated after storage at −80°C (Fig. 1). ALV and ALV antigen titers were definitely high in the albumen from the 2 NAV and one APNV transmitters (No. 3102) carrying infectious ALV in the whole blood, whereas both titers were low or undetectable in albumen from other 3 non-viremic transmitters. Of one transmitter (No. 3107), only 1 of the 5 stored albumen showed positive for ALV, however all 3 samples tested soon after sampling were positive for ALV, indicating that the infectivity was inactivated by freezing and thawing.

Besides, there was no tendency for hens laying eggs with high titers of infectious ALV in albumen to produce more infected embryos.

Virus distribution in tissues from the transmitters: Virus titers of tissue samples from the 6 transmitters are shown in Fig. 2. A high virus titer was detected in the ampulla and ampulla lavage of the oviduct.

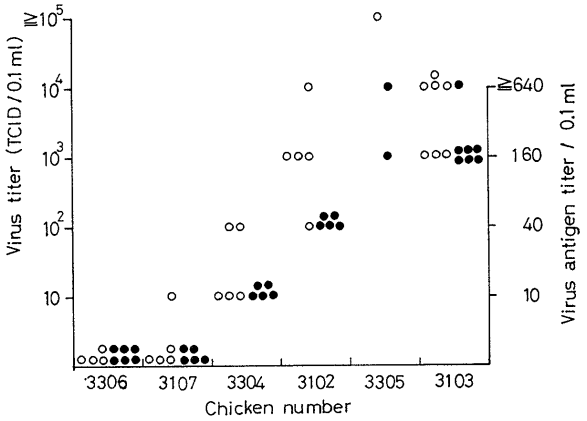


Fig. 1. ALV and ALV antigen titers in albumens collected from the six transmitters. Properties of the transmitters are shown in the lower part of the figure. Titers of infectious ALV (○) and viral antigens (●) of each egg are shown.

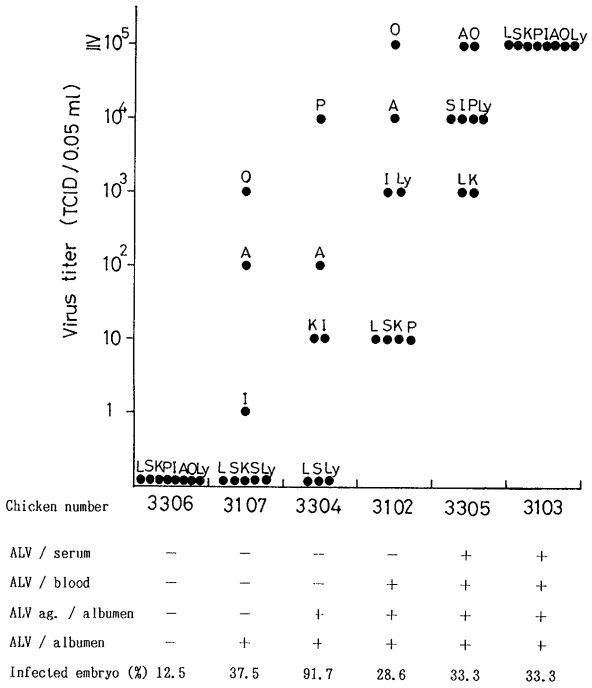


Fig. 2. Virus titers of samples collected from the transmitters. L=liver, S=spleen, K=kidney, P=pancreas, I=infundibulum of the oviduct, A=ampulla of the oviduct, Ly=lymphocyte, O=ampulla lavage of the oviduct.

The virus titer of the ampulla lavage was equal to or higher than those of other tissues in all the hens shedding ALV into albumen except for an immune transmitter (No. 3304), from which ampulla lavage was not collected because the ampulla was atrophied. Also, no virus was detected in any samples from the sporadic transmitter (No. 3306).

There was a tendency to high titers of infectious ALV in ampulla lavages in hens laying eggs with high titers of the virus (Figs. 1 and 2).

DISCUSSION

Development of the ELISAs for detecting infectious ALV by detecting ALV antigens in the cultured samples and for detecting antibodies to exogenous ALV [29] makes it possible to group large numbers of chickens rapidly according to the infectious status to ALV (Table 1). Selection of the only NANV group of hens is a reliable and practical method for the establishment of the ALV-free parent flocks (Table 2). Also, it is natural that the NANV roosters should be used for mating to prevent the horizontal transmission of ALV from the infected roosters to the NANV hens.

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However, in case that most of the parent hens were infected with ALV, selection of a small number of the NANV hens may lead to reduction in performance or genetic disorders in the ALV-free chickens. In such a case, it is necessary for the APNV non-trasmitting hens to be used for the parents in addition to the NANV hens. In this study, it was shown that the APNV hens were divided into 2 subgroups. One was the hens shedding ALV into albumen which produced infected embryos frequently and the other was the non-shedding APNV hens which produced infected embryos scarcely. Since chickens are infected with ALV persistently, growth of ALV in the body should be influenced by the immunosuppressive factors or some stress, and as a result of activation of ALV, the non-shedding APNV hens might produce infected chicks more frequently. It need further investigation to determine whether the non-shedding APNV hens can be selected for the parents as a second candidate.

For the detection of the transmitters from the APNV hens, the fresh albumen test for infectious ALV was shown to be more effective than that for ALV antigens (Table 3) and titers of the virus shed into the albumen seemed to be consistent in individual shedders (Fig. 1, Table 3). These results indicate that testing 1 albumen from a newly laid egg per hen for infectious ALV is effective to identify the congenitally transmitting hens to some extent. This ALV assay is especially useful for the identification of the hens, such as a hen of No. 3107, which shed amounts of ALV antigens undetectable by ELISA (Table 3). However, in such a case, it is important to test the samples for infectivity before freezing them, which inactivates the infectivity in some degree.

Characters of the transmitters were summarized in Fig. 2. Most of the transmitters were producing ALV in ampulla of the oviducts. The ampulla is hypothesized where the chicken embryos are infected congenitally with ALV [4]. Since virus titers of the ampulla lavage of the oviduct were higher than those of other tissue samples in 4 of the 5 transmitters (Fig. 2), chick embryos were considered to be infected not with ALV transferred from the other parts of the body but with ALV produced at the oviduct. Besides, virus titers of the ampulla lavages of the transmitters were about 10^2 infectious units higher than those of the stored fresh albumen (Figs. 1 and 2). The difference might be due to viral inactivation during the time between completion of albumen secretion and laying of the egg [5, 31]. Therefore, theoretically, the congenital transmitters might be detected more efficiently by testing ampulla lavages for ALV. Further experiments are under way to confirm a possibility that oviduct or vaginal swab tests for the virus can detect the transmitters not identified by testing albumen for ALV.

The existence of sporadic transmitters has been reported in a few papers [2, 3, 23]. In this study, we identified such a hen (No. 3306) from which only 1 of the 8 embryos examined was infected with ALV (Table 3). However, the virus was not detected in any tested area of the hen including the ampulla of the oviduct (Fig. 2). The mechanism of the sporadic transmission is unclear. However, practically, sporadic transmitters might be eliminated by selection of the non-shedding hens with high titers of antibody to ALV, since the transmitters tended to belong to the group of hens with low antibody titers to ALV (Table 2) as reported by Rubin *et al.* [24] .

Unexpectedly, the rate of infection of the grandparents (31.3%) was lower than that of commercial flocks [19, 22, 30]. It has been reported that ALV infection reduces egg production [7–9, 15] and that shedding of ALV antigens into the albumen is less frequent in chicken strains selected for high egg production than in random-bred control strains [28]. The grandparent flock has been selected for high egg production for 10 generations in this research center. Four of the 10 embryos examined were shown to be resistant to infection with subgroup A of ALV (data not shown), which is the most common subgroup of ALV in Japan [16, 30]. This rate seems to be higher than that for other commercial layers [1]. Therefore, this process may select the hens having a gene for resistance to infection with subgroup A of ALV.

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