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TESTING AND MANAGEMENT OF A SPECIFIC PATHOGEN FREE CHICKEN FLOCK WITH SPECIAL REFERENCE TO AVIAN LEUKOSIS VIRUS INFECTIONS*

By

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SANDELIN, K. and T. ESTOLA: *Testing and management of a specific pathogen free chicken flock with special reference to avian leukosis virus infections.* Acta vet. scand. 1975, *16*, 341—356. — A specific pathogen free (SPF) chicken flock was reared in isolation under laboratory conditions during five years and continuously tested for presence of specified avian pathogens. The potential occurrence of avian leukosis virus (ALV) was most thoroughly examined. The RIF and neutralization tests were unequivocally negative. Radioimmunoassay was used for detecting the presence of the major protein (gs-a) of the group-specific antigen of avian oncorna viruses. This test seemed to be well suited for checking ALV infections in chicken flocks whereas the COFAL (complement fixation avian leukosis) test was considered unreliable for this purpose. Yolk and serum from SPF chickens were negative for anti-gs-a antibodies measured by the radioimmunoassay; immunized or naturally infected birds showed anti-gs-a amounts correlating with the neutralizing titre. Besides, the flock was regularly tested for presence of seven other contagious avian pathogens. There was no evidence of infection.

SPF chicken flock; avian leukosis; laboratory diagnosis of avian leukosis virus infections.

Research laboratories and vaccine manufacturers continuously need embryonated chicken eggs. Because of the prevalence of various virus infections in commercial chicken flocks, e.g. avian leukosis, the use of eggs from such farms has many drawbacks.

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The present authors have indicated the difficulties in obtaining leukosis free eggs from commercial poultry flocks (*Sandelin & Estola* 1970). Both horizontal and vertical infection inside the flock have to be taken into account. To check or prevent the latter, e.g. transovarial transmission through the egg was considered impossible.

The first leukosis free flocks were described in 1963 by *Bang & Foard* and *Hughes et al.* Since that time many flocks of this kind have been bred in different parts of the world. In recent years the maintenance of two European SPF flocks has been described; one is kept in Huntingdon, England *(Cooper* 1970) and another in Cuxhaven, West Germany (*Vielitz & Landgraf* 1972).

Since 1969 an SPF flock has been maintained in laboratory conditions in the Institute of Virology, University of Helsinki. The present paper describes the management and testing of this flock. The suitability of different control methods for detecting ALV infections, including radioimmunoassy of the group-specific antigen, is discussed.

METHODS

Origin of birds

Brown Leghorn eggs were imported from Houghton Poultry Research Station, Houghton, Huntingdon, England, in December 1968. Genetically the stock is of C/O phenotype, i.e. the birds are susceptible to subgroups A, B, C, and D of avian leukosis sarcoma viruses.

Housing of birds

The chickens are kept in an isolated, windowless air-filtered unit situated in the Department of Virology, University of Helsinki. The unit consists of two separate rooms and one ante-room where protective clothes and boots are changed. The ante-room is equipped with ultraviolet light and a carpet soaked in disinfectant. Hands are disinfected by using chloramine. In the smaller room the egg incubator is kept and also pens for raising the chickens until they reach three-four months of age. In the chicken room proper, the birds are divided in groups of four-six hens and one rooster per pen. Each pen is equipped with trapnests. Here the temperature is maintained at 14—15 °C, the

humidity at 70 % and light is artificially produced during 15 hrs. of the day.

Only the attendant and two other persons (the authors) are allowed in the chicken premises. All birds are examined clinically once a week by a veterinarian (one of the authors).

New groups are bred about once every six months and the chickens are used as egg producers until two years of age. The laying percentage is estimated monthly.

Food and litter

Commercial chicken food in powder form is used as specified either for chicks, young chickens, or for laying hens. From the seventh week on, the diet is supplemented by a mixture of wheat, barley and oats. Food calcium and sand are always at hand. All the food components and the litter consisting of white wood shavings are sterilized in paper sacks by autoclaving at 120°C for 30 min.

Ordinary tap water is provided for drinking. It is, however, supplemented with a multivitamin preparation providing per l drinking water 5000 i.u. of vitamin A, 1000 i.u. of vitamin D_3, 2 mg of thiamine, 2 mg of riboflavin, 20 mg of nicotinic acid, and 1 mg of tocopheryl acetate.

Virus strains

The strains of Rous sarcoma virus (RSV) used when testing for presence of ALV are listed below:

RSV (RAV-1)	representing	subgroup	A
RSV (RAV-2)	„	„	B
RSV (RAV-7)	„	„	C
RSV (RAV-50)	„	„	D

These strains were kindly supplied by Dr. P. Vogt, University of Southern California, Los Angeles, Calif., USA. Stock consisting of partially purified virus was prepared from chick tumour extracts. Titres varied between 2 and 7×10^6 focus forming units (f.f.u.) per ml.

The Schmidt-Ruppin strain of RSV, subgroup D, used for producing COFAL positive hamster sera, was kindly provided by Dr. H. Hanafusa, Public Health Research Institute, New York, N.Y., USA.

RAV-1 and RAV-2 strains of ALV were also supplied by Dr. Hanafusa. These strains were used for experimental infection of chicken embryo cells and were maintained in cell cultures prepared from the leukosis free embryos.

The RIF test

For detecting congenital infection with ALV the RIF test (resistance inducing factor) was performed two-three times yearly on embryos of each laying hen. An adaptation of *Rubin*'s method of 1960 was used. Ten-day-old embryos to be tested were decapitated, eviscerated, and trypsinized. Fibroblast cultures were infected on first, second, and third cell transfer levels with about 100 f.f.u. of RSV representing subgroups A, B, C, and D except cultures of chicken groups 1, 2, and 3 that were challenged with RSV of subgroups A and B only. The foci were counted on the ninth day post infection. Evidence for a positive leukosis virus isolation was a 10-fold or greater decrease of focus count in tertiary cultures as compared to that in primary cultures.

As controls some field cases were included in the survey. Media from such cultures that were totally resistant were sampled and tested in an interference assay to ascertain whether the cells carried an ALV infection or were genetically resistant towards any of the subgroups. For such tests cultures from the leukosis free C/O embryos were used.

The COFAL test

The embryonic cell cultures were routinely tested for presence of the group-specific antigen of the avian leukosis sarcoma virus (ALSV) group by the COFAL test according to the method of *Sarma et al.* (1964). A total of $3—4 \times 10^7$ cells of the third transfer, were trypsinized, washed, and sedimented. The supernatant was removed leaving behind a volume equal to four times the cell pack in which the cells were resuspended. The antigen was prepared by freezing and thawing this cell suspension three times at —70°C. A two-fold dilution series of the suspension was used as antigen in the complement fixation test.

A COFAL positive hamster serum was produced by inoculation of four-week-old animals intramuscularly with 0.5 ml stock RSV, strain Schmidt-Ruppin, subgroup D. The hamsters carrying tumours were bled about four months post inoculation. The

microtechnique of the complement fixation test was employed, the incubation taking place at 4°C for 18 hrs. The 50 % hemolysis end point was estimated visually and taken as the COFAL antigen titre.

Assay of neutralizing antibody against ALV

The yolks to be tested for antibody content of the four subgroups of ALV were collected from the same eggs as were the embryos for the RIF and COFAL tests. Yolks from chicken groups 1, 2, and 3 were in each neutralization test examined for presence of antibodies against RSV strains of subgroups A and B only. Blood samples were taken from each bird before it was sent for necropsy. The extraction of yolk with chloroform, prior to the antibody test, was performed according to *Kottaridis et al.* (1967) and *Aulisio & Shelokov* (1967). This treatment did not seem to leave behind any residual chloroform interfering with the interpretation of results (*Sandelin*, to be published).

The yolks (and sera) were investigated by the focus neutralization test according to *Rubin et al.* (1961). Four-fold dilutions were incubated for 60 min. at room temperature with about 100 f.f.u. of the different RSV strains. Four-tenths ml of each mixture were then pipetted on duplicate Petri dishes (diam. 50 mm) of secondary chicken fibroblast cells prepared from the leukosis free C/O embryos. As neutralizing titre was considered the highest dilution of yolk (or serum) that inhibited 50 % of the focus count obtained in control dishes.

Immunization of chickens with RSV

Three to four-month-old SPF chickens were immunized with RSV strains representing subgroups A, B, C, and D. Four intramuscular injections of 1 ml stock virus were given at intervals of one week and were followed by one final intravenous injection of 1 ml. The birds were bled 10 days later and the sera used as controls in investigating the levels of neutralizing and group-specific antibodies.

Radioimmunoassay (RIA) of group-specific antigen and antibody of the ALSV group

A double antibody competitive-inhibition radioimmunoassay (RIA) was used for detection of both cell associated and extra-

cellular major gs protein of ALV. The assay and its application were recently described by *Suni et al.* (1973) and *Sandelin et al.* (1974). This protein will in the present text be referred to as gs-a (*Allen* 1969) as was the rule in our previous papers. The samples were examined at dilutions of 1:10, 1:100, and 1:1000.

Anti-gs-a antibodies in yolk and serum were detected by RIA according to the method of *Ruoslahti et al.* (1973) and *Estola et al.* (1974). The yolk or serum dilution which binds 10 % of the label precipitable in antibody excess is taken as the anti-gs-a titre.

Checking the occurrence of various avian pathogens

Avian encephalomyelitis (AE) infection was studied twice yearly on the laying stock by using an embryo susceptibility test according to *Sumner et al.* (1957). As test virus was used an AE strain that was egg adapted by Calnek. This strain was obtained in 1964 from Dr. K. Bakos, Stockholm, Sweden.

Presence of the following pathogens was studied serologically on blood samples:

— infectious bronchitis virus (IBV) by gel diffusion using a strain isolated in Finland (*Estola* 1966)

— infectious laryngotracheitis virus (ILTV) also by gel diffusion and using a strain isolated in Finland (*Estola* 1964)

— Newcastle disease virus (NDV) by hemagglutination inhibition using both a Hitchner Bl strain (*Hitchner & Johnson* 1948) and a Finnish strain (*Estola* 1974)

— chicken embryo lethal orphan virus (CELOV) by neutralization with a strain isolated in Sweden by *Bakos* (1963)

— Mycoplasma gallisepticum by plate agglutination using as test agent a commercial S6 antigen (supplied by Salisbury Lab., Charles City, Iowa, USA)

Necropsy

Every killed or succumbed bird of the flock was necropsied and examined especially for gross lesions of avian leukosis and Marek's disease (MD).

RESULTS

Chickens

Up till the present time a total of 68 hens and 15 roosters have been kept in order to continue the stock and to produce eggs.

The laying percentage of the flock is calculated once monthly, and has been 35 on an average including all adult groups. No main variations due to seasonal changes have been observed.

No clinical symptoms giving evidence of any contagious avian disease have been detected.

The mortality of the flock has so far been negligible; only one bird has succumbed during the whole period, the cause of death being internal hemorrhage due to fatty liver.

Necropsy has until now been performed on 75 birds including the surplus roosters. No gross lesions indicating infections with ALV and MDV or any other avian pathogen were observed.

Presence of specified avian pathogens

All 173 embryos representing the 68 chickens were negative in the RIF test (Table 1).

A total of 202 COFAL tests were made, either on cells identical to those of the third transfer of the RIF test or prepared specially for the COFAL test. Titres varying from $< 1:2$ to $1:8$ were obtained, 1:2 or lower occurring most frequently (Table 1). In the last reared chicken group (group 9), however, a COFAL titre of 1:8 seemed to be predominant. The age of the chicken did not influence the COFAL titre.

Table 1 also presents the results of the focus neutralization tests performed on yolks collected from the same eggs as were the embryos. One-hundred-and-fifty-eight yolks diluted 1:4 showed no sign of antibodies to any of the ALV subgroups investigated.

The serological tests performed on 75 necropsied birds for IBV, ILTV, NDV, and CELOV and for M. gallisepticum were all negative, as were the embryo susceptibility tests for detection of AEV.

Use of gs-a-RIA in detecting ALV in the SPF flock

Fibroblast cultures of 11 SPF embryos with COFAL titres of 1:4 or 1:8 were studied in the RIA for presence of gs-a antigen

Table 1. Results of tests performed in an attempt to detect inapparent avian leukosis virus.

Chicken group	Number of chickens	RIF test[1]	COFAL test[2]					Neutralization test[3]
			<1:2	1:2	1:4	1:8		
1	8	0/29	26	3	1	1	(31)	0/32
2	8	0/35	30	7	4		(41)	0/31
3	10	0/20	12	7	6		(25)	0/22
4	9	0/30	10	23	8		(41)	0/28
5	5	0/10		9	1		(10)	0/9
6	7	0/9	2	6	1		(9)	0/11
7	5	0/18	3	7	8	1	(19)	0/8
8	6	0/11		7	5		(12)	0/7
9	10	0/11	1		4	9	(14)	0/10
Total	68	173					202	158

[1] Results expressed as number of positive isolations per number of RIF tests performed. Chickens of groups 1, 2, and 3 are in each RIF test studied for presence of ALV belonging to subgroups A and B only, all the others for subgroups C and D as well.

[2] Results expressed as number of embryonic cell cultures giving the COFAL titre of the subheadings. Total number of performed COFAL tests in brackets.

[3] Results expressed as number of positive yolk reactions against RSV of different subgroups. Yolks of chicken groups 1, 2, and 3 are in each test studied for presence of antibodies against subgroups A and B only, all the other chickens against subgroups C and D as well. A titre of $< 1:4$ was considered negative.

(Table 2). As controls four cultures emanating from one and the same SPF cell pool were infected with RAV-1, RAV-2, RSV (RAV-1), and RSV (RAV-2), respectively. Data on four cell cultures prepared from naturally infected embryos were also included in the table. From these, ALV of subgroup A was isolated in the RIF test.

Cellular gs-a was not determined from the naturally infected embryos. Virus infection was shown to enhance the amount of gs-a in the cells. Levels of < 10 to 39 ng/ml cell protein were obtained from uninfected cells in contrast to 4840 to 11750 ng for experimentally infected ones.

The gs-a level of the medium collected from the above described cultures was determined from all cases of Table 2. Uninfected SPF cells shed minute amounts of gs-a, if at all (< 10—11 ng/ml), into the surrounding medium, whereas the

Table 2. Results of RIF and COFAL tests, and gs-a RIA of cultures of SPF embryos compared to experimentally infected ones and to cell cultures prepared from embryos carrying a natural leukosis infection.

Origin of cell cultures	RIF test	COFAL titre	ng gs-a/mg cell protein	ng gs-a/ml culture medium
SPF embryos				
1	neg.	1:8	<10	<10
2	neg.	1:4	11	<10
3	neg.	1:2	<10	<10
4	neg.	1:4	16	<10
5	neg.	1:4	<10	<10
6	neg.	1:8	13	<10
7	neg.	1:8	39	11
8	neg.	1:8	32	<10
9	neg.	1:8	28	<10
10	neg.	1:8	26	<10
11	neg.	1:8	37	<10
Experimentally infected SPF cell cultures[1]				
1 RAV-1			6840	1740
2 RAV-2			5050	1200
3 RSV (RAV-1)			4840	3200
4 RSV (RAV-2)			11750	4800
Naturally infected embryos from field flocks[2]				
1	pos.	1:4	n.d.	2900
2	pos.	1:16	n.d.	1800
3	pos.	1:4	n.d.	550
4	pos.	1:4	n.d.	148

[1] All cell cultures emanate from the same cell pool prepared from SPF embryos. The cultures were infected with the virus strains mentioned in the table.

[2] ALV belonging to subgroup A isolated.

levels for experimentally infected SPF cultures ranged from 1200 to 4800 ng/ml culture medium and for the field cases from 148 to 2900 ng.

In Fig. 1 are plotted both intracellular and extracellular amounts of gs-a, in both uninfected and infected chicken embryo cell cultures. Here are included results from further seven experimentally infected cultures to yield a total of 11 tests.

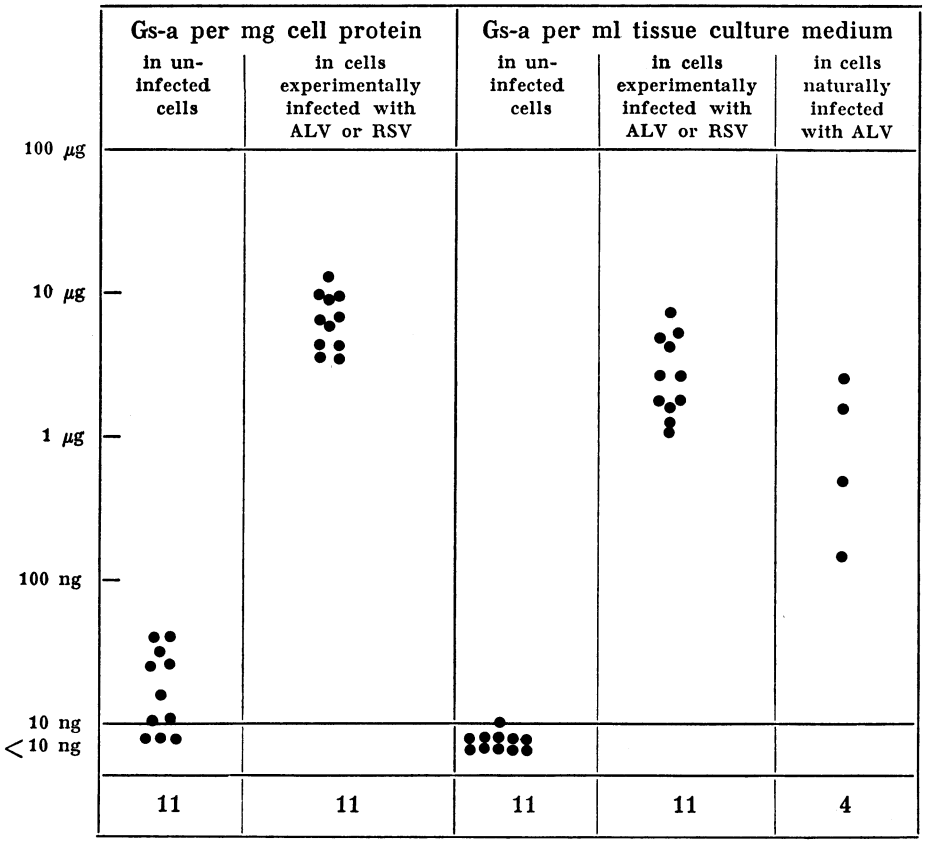

Figure 1. Intracellular and extracellular amounts of gs-a measured by RIA in chicken embryo fibroblasts from uninfected and experimentally or naturally infected cultures.

Use of RIA in detecting anti-gs-a antibodies in the SPF flock

The yolks from seven SPF chickens did not show binding of radiolabelled gs-a in RIA. Neither did the four sera tested (Table 3). Neutralizing antibodies were absent as already indicated in Table 1.

The sera of the four RSV immunized SPF chickens of Table 3 showed anti-gs-a activity. Titres between 1:600 and 1:10000 were obtained. Neutralizing antibody titres of the same sera ranged from 1:256 to > 1:1024.

Table 3. Gs-a and neutralizing antibody titres of yolk and serum of SPF, immunized, and naturally infected chickens.

Origin of chicken	Yolk		Serum	
	anti-gs-a titre[1]	neutralizing titre[2]	anti-gs-a titre[1]	neutralizing titre[2]
SPF chickens				
1	<1:10	<1:4	<1:10	<1:4[3]
2	<1:10	<1:4		<1:4
3	<1:10	<1:4	<1:10	<1:4
4	<1:10	<1:4		<1:4
5	<1:10	<1:4		<1:4
6	<1:10	<1:4	<1:10	<1:4
7	<1:10	<1:4	<1:10	<1:4
Immunized SPF chickens				
1 RSV (RAV-1)			1:10000	>1:1024
2 RSV (RAV-2)			1:5000	>1:1024
3 RSV (RAV-7)			1:500	1:256
4 RSV (RAV-50)			1:600	1:1024
Naturally infected chickens from field flocks[4]				
1	1:100	1:256 (A)	1:300	1:256 (A)
2	1:10000	1:256 (B)	1:10000	1:256 (B)
		1:64 (D)		1:256 (D)
3	1:1000	1:64 (B)	1:100	1:16 (B)
		1:64 (D)		1:16 (D)

[1] Yolk or serum dilution that in RIA binds 10 % of the label precipitable at antibody excess.
[2] Yolk or serum dilution that inhibits 50 % of focus count.
[3] Neutralizing antibodies investigated against serotypes A, B, C, and D.
[4] Subgroup of antibodies in brackets.

In yolks and sera from naturally ALV infected chickens, anti-gs-a antibody was also observed. The titres varied between 1:100 and 1:10000. Subgroup and titre of neutralizing antibody were studied parallelly; neutralizing titres ranged from 1:16 to 1:256.

DISCUSSION

Because of the ubiquitous presence of latent virus infections in commercial chicken flocks it is necessary to keep SPF birds for research purpose and vaccine production. The aim of the authors of the present work was to show that it is possible to

rear such a flock on a small scale under laboratory conditions as long as strickt precautions are maintained. Reliable, regular control tests must be performed to ensure that the material delivered from such an isolated chicken flock meets the demands of the researcher.

The laying percentage of the flock has so far been low, 35 on the average, during five years. This is a drawback which one must take into account when rearing SPF chickens. However, in this case the production of eggs was more than sufficient to satisfy the demands. The mortality of the chickens was negligible.

The diagnostic methods showed that the flock was avian pathogen free during the five years of regular testing. The status of Marek's disease was studied only clinically and at necropsy; however, no sign of infection was found.

The potential occurrence of avian leukosis virus infections was most thoroughly tested for. The reason for this was the need for strictly leukosis free chickens, embryos and cells to be used in research of various oncorna viruses. The results of the "classical" tests listed in Table 1 were unequivocally negative. A total of 173 RIF tests, each covering two or four antigens, was performed; infectious virus could not be isolated. Similarly, the serological tests showed absence of infection. A total of 158 yolk samples were assayed in the focus neutralization test, and again, each yolk was tested for antibodies against two or four subgroups of ALV.

When testing for presence of avian leukosis group-specific antigen, two methods were used. Inside the flock the COFAL titres varied to a great extent. Titres of $< 1:2$ to $1:8$ were obtained independent of the fact that the RIF tests on identical culture cells were all negative. The variability of the COFAL titres appears even more clearly in Table 2 where they are plotted next to the amounts of gs-a obtained by radioimmunoassay of identical cells. Whereas the results obtained in the RIF test and in the RIA always correlate, the results of the COFAL test do not always correspond with either. Thus, the COFAL test does not seem to offer a reliable method for detection of infectious virus. The various reasons for this discrepancy of correlation were discussed in a recent paper (*Sandelin et al.* 1974).

Small amounts of cell-bound gs-a have been detected in all cell cultures from this SPF flock. Some of the cases are listed in Table 2. That the endogenous gs-a of leukosis free cells is identical

with the viral, was shown by *Allen & Sarma* (1972) and *Vaheri & Ruoslahti* (1973). The amount of gs-a in cells free of infectious virus is, however, remarkably less than in infected cells (Table 2 and Fig. 1). It has to be kept in mind, though, that on these SPF cells no experiments have been performed to rescue subgroup E leukosis virus genome. However, our data are in agreement with results obtained by other authors (*Fritz & Qualtiere* 1973, *Stephenson et al.* 1973, *Chen & Hanafusa* 1974).

The expression of gs-a in culture medium surrounding infected or not infected cells gives an even clearer picture. Our experience in detecting avian leukosis infections using the shedding of gs-a into the medium as a criterium for an active infection was recently published (*Sandelin et al.*). Table 2 and Fig. 1 illustrate that out of the 11 SPF cultures tested, 10 contained less than 10 ng gs-a per ml cell culture fluid, one 11 ng. As comparison, two of the four naturally infected cultures, included as controls, showed considerably higher contents of extracellular gs-a, 2900 and 1800 ng. These values were as high as for the experimentally infected cultures. The two other RIF positive field cases also shed significant amounts of gs-a.

In conclusion, clearly elevated amounts of either intracellular or extracellular gs-a provide strong evidence for presence of infectious virus.

The results expressed in Table 3 on the antibody status of the SPF chickens are in full agreement with the before mentioned data. Neither neutralizing nor anti-gs-a antibodies were found in yolks or sera. Specimens from randomly chosen immunized or naturally infected chickens, included as controls, showed significant amounts of both neutralizing and anti-gs-a antibodies. It may also be seen that there is close correlation between the titres of these two specified antibodies both in yolks and sera of the field cases.

In summary, the sensitive radioimmunoassay of the group-specific avian leukosis virus antigen and of antibodies to this antigen, seems to offer a reliable method for checking for presence of ALV infections in chicken flocks. To our knowledge ours is the first SPF flock that has been screened by using this method.

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SAMMANFATTNING

Testning och skötsel av en hönsflock fri från specifika patogener med särskilt beaktande av infektioner förorsakade av hönsleukosvirus.

En specifikt patogenfri (SPF) hönsflock hölls isolerad i laboratorieförhållanden under fem år och testades fortlöpande för förekomst av specifika hönspatogener. Eventuell förekomst av hönsleukosvirus

(ALV) undersöktes i detalj. RIF- och neutralisationstesterna var entydigt negativa. För att påvisa förekomsten av det största proteinet (gs-a) i det grupp-specifika antigenet för höns-oncornavirus användes radioimmunitetsbestämning (RIA). Detta test visade sig lämpligt för kontroll av ALV-infektioner hos hönsflockar, då däremot COFAL- (complement fixation avian leukosis) reaktionen måste anses vara opålitlig för detta ändamål. Gulor och serum från SPF-höns reagerade negativt i RIA för anti-gs-a-antikroppar, medan immuniserade eller på fältet infekterade höns uppvisade kvantiteter i proportion till neutralisationstitern.

Hönsflocken undersöktes dessutom regelbundet för förekomst av sju andra smittosamma hönspatogener. Inga tecken på infektion förefanns.

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