BARD

FINAL REPORT

PROJECT NO. IS-3008-98

**The Epidemiology of Avian Leukosis Virus-subtype J:
Infection in Broiler Breeder Flocks of Poultry and its
Eradication from Pedigree Breeding Stock**

M. Malkinson, R.L. Witter, I. Davidson, M. Kotler

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The Epidemiology of Avian Leukosis Virus-subtype J: Infection in Broiler Breeder Flocks of Poultry and Its Eradication from Pedigree Breeding Stock

Investigators

**Principal Investigator (PI):
Dr.M.Malkinson**

**Co-Principal Investigator (Co-PI):
Dr. R L Witter**

**Collaborating Investigators:
Dr Irit Davidson
Prof.M.Kotler**

Institutions

**Kimron Veterinary Institute
Israel**

A D O L, East Lansing, USA

**Kimron Veterinary Institute
Hebrew University of
Jerusalem**

Abbreviations:

ALV-J; ELISA; ML; PCR

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Principal Investigator

B.A. Yakobson, D.V.M.

**Director
Kimron Veterinary Institute**

Authorizing Official, Principal Institution

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BARD Final Scientific Report Cover Page

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	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)		1		1
Submitted, in review, in preparation	2	1	1	4
Invited review papers				
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Books				
Master theses				
Ph.D. theses				
Abstracts				
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1	1	1	3
Longer Visits (Sabbaticals)				

Description of Cooperation:

KVI and HUI received reference ALU-I strains from ADOL primers for PCR and DF-1 cells, C/E embryonated eggs. Dr. Caroline Benet was trained in cloning and sequencing by Dr. Bob Silva.

Patent Summary (numbers)

	Israeli inventor (s) only	US inventor (s) only	Joint IS/US inventors	Total
Submitted				
Issued (allowed)				
Licensed				

Table of Contents

- I **Abstract**
 Achievements
 Cooperation
 List of Publications

- II **R L Witter (ADOL) Final Report**

- III **M Kotler (HUJ) Final Report**

Section B Appendix

- IV **M Malkinson (KVI) 3rd Annual Report**

- V **ADOL Appendix 1- Reprint**
 2- 3rd Annual report
 3- Draft manuscript

- VI **KVI Draft manuscript**

Abstract

Objectives

1. Establish diagnostic procedures to identify tolerant carrier birds based on
 - a) Isolation of ALV-J from blood, b) Detection of group-specific antigen in cloacal swabs and egg albumen. Application of these procedures to broiler breeder flocks with the purpose of removing virus positive birds from the breeding program.
2. Survey the ALV-J infection status of foundation lines to estimate the feasibility of the eradication program
3. Investigate virus transmission through the embryonated egg (vertical) and between chicks in the early post-hatch period (horizontal). Establish a model for limiting horizontal spread by analyzing parameters operative in the hatchery and brooder house.
4. Compare the pathogenicity of ALV-J isolates for broiler chickens.
5. Determine whether ALV-J poses a human health hazard by examining its replication in mammalian and human cells.

Revisions. The eradication objective had to be terminated in the second year following the closing down of the Poultry Breeders Union (PBU) in Israel. This meant that their foundation flocks ceased to be available for selection. Instead, the following topics were investigated:

- a) Comparison of commercial breeding flocks with and without myeloid leukosis (matched controls) for viremia and serum antibody levels.
- b) Pathogenicity of Israeli isolates for turkey poults.
- c) Improvement of a diagnostic ELISA kit for measuring ALV-J antibodies

Background. ALV-J, a novel subgroup of the avian leukosis virus family, was first isolated in 1988 from broiler breeders presenting myeloid leukosis (ML). The extent of its spread among commercial breeding flocks was not appreciated until the disease appeared in the USA in 1994 when it affected several major breeding companies almost simultaneously. In Israel, ML was diagnosed in 1996 and was traced to grandparent flocks imported in 1994-5, and by 1997-8, ML was present in one third of the commercial breeding flocks. It was then realized that ALV-J transmission was following a similar pattern to that of other exogenous ALVs, but because of its unusual genetic composition, the virus was able to establish an extended tolerant state in infected birds. Although losses from ML in affected flocks were somewhat higher than normal, both immunosuppression and depressed growth rates were encountered in affected broiler flocks and affected their profitability.

Conclusions. As a result of the contraction in the number of international primary broiler breeders and exchange of male and female lines among them, ALV-J contamination of broiler breeder flocks affected the broiler industry worldwide within a short time span. The Israeli national breeding company (PBU) played out this scenario and presented us with an opportunity to apply existing information to contain the virus. This BARD project, based on the Israeli experience and with the aid of the ADOL collaborative effort, has managed to offer solutions for identifying and eliminating infected birds based on exhaustive virological and serological tests. The analysis of factors that determine the efficiency of horizontal transmission of virus in the hatchery resulted in the workable solution of raising young chicks in small groups through the brooder period. These results were made available to primary breeders as a strategy for reducing viral transmission. Based on phylogenetic analysis of selected Israeli ALV-J isolates, these could be divided into two groups that reflected the countries of origin of the grandparent stock.

Implications. The availability of a simple and reliable means of screening day old chicks for vertical transmission is highly desirable in countries that rely on imported breeding stock for their broiler industry. The possibility that ALV-J may be transmitted to human consumers of broiler meat was discounted experimentally.

Achievements

Significance of achievements listed according to objectives.

A. Identification of individual tolerant birds

The practice of housing premium genetic lines of birds in individual laying cages facilitated easy access to bleeding, cloacal swabbing and egg sampling. Based on the comparison of two diagnostic methods for detecting the virus, viz., isolation in cell culture and detection of viral antigen in cloacal swabs and egg albumen, the examination of cloacal swabs offered a reliable indication of the shedder status of laying flocks.

B. Survey of foundation lines

Cloacal swab analysis enabled the rapid screening of large flocks of birds and offered a means of reducing infection within one generation. In Israel, a comparison of more than 2000 birds belonging to two foundation female lines housed on three separate farms yielded ALV-J shedding levels that were similar to those detected by the more rigorous approach based on virus isolation in a sample of 60 birds of each line. Some differences in the incidence of infection between tolerant and tolerant-immune birds among male and female lines were recorded. These were attributed to the practice of housing male line birds on litter rather than in cages thus catalyzing long term virus transmission. F1 generation birds were not available for investigation, however an alternative strategy of selection and reduction based on elimination of antibody-positive birds by ELISA testing was found to effectively decrease the number of positive birds. Unfortunately, none of these birds were used for breeding.

C. Effect of hatchery and brooder house interventions on the reduction of horizontal transmission.

Commercial broiler chickens were inoculated as embryos in order to mimic vertical transmission. After hatching they served as donors for hatchmates reared or hatched in the same compartment. Cloacal swab transfer, needle transfer and ingestion of meconium were also investigated as the means for establishing experimental exposure to the virus. Experimental data showed that viral transmission was most effectively interrupted by placing simple wire partitions in the hatcher or by rearing the chickens in small groups for at least 4 weeks.

This experimental design contributed much to our understanding of the factors affecting the kinetics of horizontal spread of ALV-J. The simplicity of such

interventions that resulted in an effective reduction in contact transmission led to their ready adoption by major breeding companies.

D. Pathogenicity of field isolates for broiler chickens.

Differences in transmissibility of six Israeli ALV-J isolates were measured over a six weeks period in small groups of chickens housed in isolators. The viruses had been isolated from broiler breeding flocks suffering from ML. The numbers of positive cloacal swabs from the inoculated groups ranged from 6 to 30% while the levels of viremia were from 40% to 60%. Only two of the six contact groups had birds that shed virus whereas five of them became viremic (range:5%-35%). Besides emphasizing variations in pathogenicity, these results show that cloacal shedding is a less sensitive indication of experimental infection.

E. Is ALV-J a human health hazard?

In the past avian tumor viruses have been examined for their ability to cross over the species barrier and become carcinogenic in mammals. In this BARD project exhaustive attempts to cultivate ALV-J in human and mammalian cell lines proved unsuccessful. It became apparent that the virus scarcely adsorbed to human CEM, H9, HeLa and human foreskin cells nor to BHK and CHO cells. In those experiments where evidence of penetration occurred, amplification of cellular (proviral) DNA by PCR yielded bands of aberrant molecular weight. These products were not increased in quantity after prolonged incubation. When C/E chick embryo fibroblasts were either fused or co-cultivated with the infected human cell lines to amplify the infectivity of the virus, but neither approach resulted in virus production. It was therefore concluded that human and mammalian cells were not permissive for ALV-J infection.

F. Comparison of ML-positive and negative commercial broiler breeder flocks regarding viremia, antigenemia and circulating antibody response.

The available immunological techniques were compared with PCR to identify the clinically infected status of commercial flocks as well as to define their immune response. Thus, serum samples were taken from birds in flocks affected with clinical ML and from non-ML flocks and examined for group-specific antigen (ELISA), viremia (propagation in c.e.f.) and ALV-J antibody (ELISA). Virus was demonstrated in 64.1% of sera of ML positive birds compared with 16.7% of sera from non-ML flocks. Antibodies were present in 41.5% of ML birds and in 24.1% of non-ML birds. Antigenemia was demonstrated in 58.1% of ML birds and 46.4% of non-ML birds. Envelope primers were selected for PCR amplification of some of the isolates and yielded products of approximately 1kb that differed somewhat in size.

The env gene of seven randomly selected isolates was sequenced and a phylogenetic tree constructed with US and UK isolates included for comparison. The Israeli isolates appeared to fall into two sub-groups – United Kingdom-like and USA-like. It was determined that the flocks yielding these isolates came from two separate foreign breeder companies.

G. Pathogenicity of ALV-J for turkey flocks.

There is one publication that ALV-J can induce tumors in young turkeys following experimental infection. An infectivity trial lasting 22 days was conducted using groups of infected and in-contact groups of young poults given six Israeli isolates. Infection as measured by viremia and cloacal swabs was established in some of the inoculated groups but no evidence for contact transmission was found. Moreover, none of the birds shed virus in their feathers throughout the experiment.

H. Improvement of commercial ELISA tests for detecting ALV-J antibodies.

There are two ELISA kits for measuring ALV-J antibodies available commercially at present. Both appear to give a high proportion of false positive results in commercial flocks including breedingstock not affected clinically with ML. Components of the kits were analyzed with panels of sera from experimentally infected and field-exposed birds that were also examined by serum neutralization and immunofluorescence. The most important variables included the dilution of the test serum, the cut-off values for the negative and positive sera, and the incorporation of multiple substrains in the capture antigen. These recommendations will result in a kit that should distinguish between ALV-J infected and non-infected breeding flocks and their progeny. It should be possible to employ the new kit for screening day-old chicks marketed worldwide.

Cooperation.

1. ADOL supplied the prototype ALV-J viruses and embryonated eggs (C/E) and access to DF-1 cell line essential for establishing and standardizing the diagnostic procedures at KVI. These in turn were transferred to HUJ for the preparation of virus stocks. On several occasions consultation between the partners took place in regard to test protocols, especially relating to antigen assays and the use of rearing cubicles for small groups.
2. Dr Caroline Banet-Noach was instructed in the cloning of DNA of Israeli strains in Dr. Silva's laboratory (ADOL). Although not a partner of this BARD, Dr.Silva's help is particularly appreciated since Dr Noach learnt the rudiments of cloning, sequencing and the construction of phylogenetic trees during her brief stay at ADOL.

3. The contributions of Dr Aly Fadly, Director of ADOL, and Dr Henry Hunt in enabling the transfer of reagents and methods developed in their laboratories are gratefully acknowledged.

List of publications.

A. Reduction of horizontal transmission of avian leukosis virus subgroup J in broiler breeder chickens hatched and reared in small groups. *Avian Pathology*, (2001) vol.30: 641-654.

December 25, 2002

BARD IS-3008-98
Agreement 58-3635-9-F148
Final Report
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Contributions by Avian Disease and Oncology Laboratory
East Lansing, Michigan
Prepared by R. L. Witter

The progress during the duration of the subject project by the East Lansing group (USA) is listed in 3 research units of work.

Research Unit 1 – Reduction of horizontal transmission of avian leukosis virus subgroup J in broiler breeder chickens hatched and reared in small groups (Witter and Fadly) – 1998 to 2000

Abstract: Transmission of avian leukosis virus, subgroup J (ALV-J), from donor chickens inoculated as embryos to simulate congenital infection to uninfected hatchmates was studied in two strains of commercial broiler breeder chickens. Chicks of two commercial lines free of ALV-J became infected when hatched (1/2 lots positive) or reared (8/8 lots positive) in direct physical contact with ALV-J-infected donors. Infection also occurred when chicks were exposed in the hatchery to ALV-J-infected donors by cloacal swab transfer (2/2 lots positive), needle transfer during subcutaneous inoculation (2/2 lots positive), or ingestion of infected meconium (2/2 lots positive). However, transmission was delayed or prevented by wire partitions in the hatcher and rearing of small groups in cubicles, and rarely (1/10 lots positive) resulted from short-term direct or indirect contact. In a simulated field test, a flock of 503 broiler breeder chickens with an initial embryo infection rate of 4.6% was hatched and reared as 48 small groups through 4 weeks of age. Groups were tested at hatch and at 3 weeks, and 14 infected groups were eliminated. This flock tested negative for ALV-J infection from 4 through 32 weeks and did not transmit infection to progeny or develop tumors. A control group of 377 chickens with a similar initial infection rate was hatched and reared as a single group. This control flock transmitted virus to 5.7% of its progeny and about 5% of the hens developed tumors. The small group hatching and rearing practices employed in these studies allowed for the accurate identification and removal of groups containing chickens infected prior to hatching and prevented horizontal transmission of ALV-J between uninfected and infected groups for at least 4 weeks. More importantly, application of these procedures successfully eradicated ALV-J in a single generation under laboratory conditions. This suggests that similar procedures could be a valuable adjunct to virus eradication programs in the field.

Full report is published: Witter, R.L. and A.M. Fadly. Reduction of horizontal transmission of avian leukosis virus subgroup J in broiler breeder chickens hatched and reared in small groups. Avian Pathol. 30:641-654, 2001 (reprint attached with complete tabular data, Appendix 1)

Research Unit 2 – Transmission rates and viremia induction following low dose exposure to avian leukosis virus subgroup J (Witter) – 1999 to 2001

During earlier trials, we observed evidence of delayed viremias in at least two incidents. One situation involved embryos inoculated at 7d in the yolk sac with ALV-J (for use as donors in a contact exposure trial) where the donor chicks tested virus negative at 13, 20 and 27 days after hatch whereas another groups tested positive at 35 days. The virtual absence of contact transmission in this trial confirmed the poor infection level in the donors. The second situation involved chicks exposed by contact to graded doses (exposures) of ALV-J where viremias were not detected until 28 days. In both cases, it appeared that the chicks or embryos were exposed to minimal doses of virus. The possibility was considered, therefore, that low dose exposure of chicks or embryos to ALV-

J may result in a rather different type of infection profile, characterized by delayed viremias and probably resulting in transient infections. If so, this might be important to the understanding of infection profiles under field conditions.

On the other hand, traditional experience suggests that in embryos once infection becomes established the virus replicates to high titer and chicks hatch with persistent infection. Thus, this trial was a preliminary probe to determine whether with commercial broiler breeder strains and ALV-J infection, there was any evidence of unconventional or unexpected infection patterns with very low dose exposures. Two trials were conducted to evaluate these possibilities:

Trial 5A: Graded doses of ALV-J strain Hc-1 was given to commercial broiler breeder chicks from two sources. The exposure was by the combined cloacal-nasal route, as we have used in previous trials. The inoculum volume of 50ul was divided and half delivered to the nares and the remainder inserted into the cloaca, using an automatic pipette. The chickens were then placed in HB isolators. Blood samples were obtained at 2, 4, 6, 8 and 12 weeks. Each isolator also contained 2 contact control chicks from the same strain which were bled at weeks 2, 4, 6, and 8 to monitor the speed of contact transmission within the isolator. Antibody tests (indirect FA) were done on sera from the 8 and 12 week bleeds.

Results showed that the minimal dose of 10^2 infectious doses /ml (=about 5 infectious doses per chick) was beneath the threshold needed to establish infections. Thus, the series of doses produced an endpoint for both chicken strains and assured that some chickens had truly been exposed to a minimal dose. Although 1 chicken of strain B inoculated with 50 TCID became viremic at 8 wks, this was the only chicken in the entire experiment that appeared to demonstrate a delayed onset of viremia. However, it should be noted that most of the chickens that became viremic in the study developed persistent viremias and only 8 (total) appeared to have transient infections. There was very limited contact spread through 8 weeks, which was surprising but may have been related to the use of relatively low doses. Interestingly, strain B appeared more susceptible than strain A by a factor of nearly 100-fold.

Trial 5B: This trial was to identify parameters related to virus transmission and onset of viremia or antibody following low dose exposures in ovo. Graded doses of Hc-1 were inoculated into the yolk sac of 7 day embryos of two commercial strains, using 100ul per embryo. Chicks were hatched in pedigree baskets, kept separate by dose group. A group of hatcher controls, uninoculated, were hatched in the same incubator in separate pedigree baskets. Other controls were hatched in a separate incubator. After hatch, each dose group was placed in a separate HB isolator. Two contact controls were added to monitor horizontal transmission within the isolator. Blood samples were collected at weeks 4, 8 and 12 and tested for viremia. Antibody tests were done at 12 weeks only (indirect FA).

In this trial, an endpoint was not reached. Even the minimal dose induced persistent infections in most chicks of both strains (all except one). There were no birds that developed late viremias. Hatcher contacts and normal controls remained free of infection during the trial. Viremia data from the contact birds at 4 and 8 weeks indicated that the amount of contact transmission may have been less in the lowest dose groups compared to higher doses. No difference in susceptibility was noted between strains A and B, although the absence of a clear endpoint precludes a critical comparison.

Several conclusions may be made:

- The experiments provided no evidence that low dose exposures may induce late onset viremias. The results obtained fit well with published data on other ALV strains. The null hypothesis, therefore, is rejected.
- There is some indication that chickens exposed to high doses are less likely to develop antibodies and are more likely to infect contact control chickens, compared to chickens infected with lower doses.
- In one of two trials, strain B appeared more susceptible than strain A. However, differences between these same strains in several previous experiments were inconsistent and definitive conclusions on relative susceptibility of strains are not warranted.

This work is not scheduled for publication. A full copy of an internal ADOL report with tables is attached (Appendix 2)

Research Unit 3 – Serological tests to detect antibodies to avian leukosis virus subgroup J (Hunt, Witter and others) – 2000 to 2002

Comparison of the different techniques (ELISA, Fluorescent Antibody, and Virus Neutralization) for detecting serum antibody to the subgroup J Avian Leukosis Virus (ALV-J).

Summary

Broiler breeder chickens have recently developed a problem with tumors attributable to a virus termed avian leukosis virus J (ALV-J). Eradication of ALV-J is the method for reducing these tumors. Chickens infected with ALV-J generally produce an antibody to ALV-J. One technique used to identify infected chickens detects the antibody response to the virus. Thus the serum antibody status of a chicken is an indication of the success or failure of the eradication process. This paper compares five methods to detect antibody to ALV-J. The results indicate that the strain of ALV-J infecting the chickens plays an important role in the reliability of the tests used to detect antibody to ALV-J. The paper concludes that the current tests available to the industry do not detect multiple strains of ALV-J and thus need modification.

Abstract

Two commercial enzyme linked immunosorbent assays (ELISA), serum neutralization (SN), and fluorescent antibody (FA) based techniques were compared for their ability to detect serum antibodies to the avian leukosis virus subgroup J (ALV-J). In titration experiments using positive control antibody to the ADOL-HcI strain of ALV-J, the results of the four different techniques were highly correlative (correlation coefficient >0.9). The sensitivity of the two different commercial ELISA tests varied depending on the positive control serum used. Two of the positive control sera were negative when tested at the manufactures recommended dilution with one of the ELISA kits. The strong correlation in results breaks down (correlation coefficient <0.7) when the tests are compared using experimental and commercial broiler serum samples. The KPL kit was more accurate when detecting sero-conversion in the experimentally infected broiler samples while the IDEXX kit was better when commercial broiler samples were analyzed. The substrain (ADOL-HcI or HPRS-103) of the ALV-J envelope used in the test was an important variable with some sera responding to both envelopes, other sera positive using only the ADOL-HcI strain and still other sera only responding to the HPRS-103 strain. The data show that reliable results depend on the dilution of the experimental sera, the criteria chosen for positive or negative results, and the use of multiple substrains for the capture antigen.

This work is being prepared for publication. A draft copy is enclosed (**Appendix 3**), but should be considered a privileged communication until accepted.

Other Information:

- The US principal investigator did not charge the grant funds for travel to Israel. However, this does not mean that the US principal investigator did not have appropriate contacts with his Israeli principal investigator.
 - March 2000 – Witter visited Malkinson in Bet Dagan in conjunction with another meeting held in Neve Ilan. Project discussions were fruitful. Also, a site visit to a broiler breeder farm was conducted.
 - May 2002 – Malkinson visited Witter in East Lansing to discuss project results and consult with other laboratory scientists on current issues in retrovirus and herpesvirus research.
 - October 2002 – Witter visited Malkinson in Limassol, Cyprus in conjunction with another meeting. The overall results of the collaboration were discussed and plans made for the final report.

List of Appendices:

- Appendix 1 – Research unit #1 (reprint)
- Appendix 2 – ADOL annual report 2001, Research unit #2
- Appendix 3 – Draft manuscript by H.H. Hunt - **Privileged communication** (Note – identity of ELISA kits should be kept confidential), Research unit #3

BARD – Final Report contributed by Prof. M. Kotler, HUJ

The overall goal of our project was to determine whether ALV-J is capable of infecting mammalian cells.

In effect our results confirmed and extended those of Payne *et al.*, (J.Gen.Virol 1991, 72:801-807) who showed that ALV-J and its RSV pseudotype did not replicate in or transform several mammalian cell lines of mouse, monkey, bovine and pig origin.

Aim A

Initially we estimated the absorption rate of ALV-J (HPRS – 103) for several human and mammalian cells. The lines used in these experiments included human CEM, H9, HeLa and human foreskin cells. BHK, CHO and chick C/E cells were also used as controls.

Cell monolayers plated on cover slips or in suspension (H9 and CEM) were infected with concentrated virus. Cells were washed in PBS and then fixed with formaldehyde. An estimate of the amount of virus absorbed by the cells was determined using rabbit anti ALV-J serum prepared in our laboratory. The serum was absorbed with chick (C/E) cell powder to remove non-specific activity. In conjunction with fluorescent goat anti-rabbit serum, the amount of absorbed virus was determined using C/E cells as a control. These experiments clearly showed that ALV-J is actively absorbed to chick cells whereas all the mammalian cell lines absorbed only small amounts of virus.

We concluded that the absorption rate of ALV-J to mammalian cells is lower than that to C/E cells.

Aim B

To determine whether ALV-J penetrates mammalian cell membranes we followed the production of viral DNA at 2 and 6 hrs post-infection. In several experiments preparations of CHO and HeLa cells infected with ALV-J showed weak bands at 550 and 2000 bp, indicating that a few cells or few viruses had penetrated the cells and that their genome was reversely transcribed. In contrast, when C/E cells were used as the positive control, clear bands of similar sizes were present. These were absent in non-infected C/E cell extracts.

These results indicate, therefore, that only small amounts of viruses penetrated into mammalian cells and nonintegrated viral DNA was produced in small, insignificant amounts.

The PCR system was applied to determine whether infected mammalian cells contain proviruses. Cellular DNAs were extracted at 48 and 96 hrs post infection and were subjected to PCR amplification. The results of these experiments are not clear or inconclusive so far: Several CHO, HeLa and human foreskin preparations infected with ALV-J showed weak bands at 550 and 2000 bp, suggesting that a few cells were indeed infected. However, several additional bands appeared in infected but not in uninfected cells. Moreover, in one CHO infected culture we identified a PCR band of 700 bp (see 2nd Year Report) instead of the expected 545 bp band, which was not seen in the uninfected cells. One interpretation of these results is to assume that mutated-recombinant viral DNA became integrated occasionally into CHO cells in culture. However, when these experiments were repeated at 10-14 days post-infection, ALV-J specific bands were not identified. At this stage we cannot exclude the possibility that some cells (CHO and HeLa) became infected with ALV-J. However, since these cells have no selective advantage, or because the viral DNA may have been rejected from these cells, it was impossible to recover proviral DNA after 10-14 days of infection. These results were supported by biological experiments (see below).

Aim C

Much effort was invested in attempts to release ALV-J from infected mammalian cells. CHO, HeLa and human foreskin cells were infected with ALV-J, using various methods to increase the level of infectivity (see 1st and 2nd Year Annual Reports). In one series of experiments, C/E cells were co-cultivated 7-10 days post-infection with the infected mammalian cells. In other experiments, UV-irradiated Sendai virus was used to fuse the avian cells with the infected mammalian cells and the media harvested from these cultures were used to infect fresh C/E cells. The C/E cells were transferred 1-2 times to increase the infectivity of a virus, if it existed.

All these experiments yielded negative results. We were unable to demonstrate production of ALV-J from infected cells, nor could we release ALV-J virus by fusion.

Conclusions:

- 1) The absorption of ALV-J to mammalian cells is weaker than to avian cells.
- 2) At present we cannot exclude the possibility that the virus penetrates a very small percentage of the cells and results in the production of viral DNA
- 3) Proviruses and/or aberrant proviruses **may be** produced in a very small number of cells.
- 4) During cell propagation these viral DNAs disappear from the cultured cells.
- 5) No virus production could be detected in any of the mammalian cells so far examined.

BARD Project No. IS-3008-98

3rd Annual Report

M.Malkinson, Beit Dagan

A. Detailed description of research activities.

Contents

1 Identification of ALV-J strains isolated from Israeli flocks

2. Infectivity trial of ALV-J isolates in turkey poults.

Project 1: Identification and characterization of ALV-J strains isolated from Israeli flocks

Introduction

During the course of the BARD project, the following commercial broiler breeder flocks were examined for viremia:

Two great-grandparent (GGP) flocks and two clinically affected grandparent (GP) flocks, one of which was a flock of roosters (male line) and the other was at the point of lay. Both flocks had been imported at one day old. Seven parent flocks presenting ALV-J lesions were examined. Their ages ranged from 14 to 34 weeks. In addition one broiler flock, aged 45 days with uneven growth was investigated.

Virology

Plasma from individual birds was inoculated in to chick embryo fibroblasts(c/e) or DF1 cells growing in plates containing glass cover slips. 5-7 days later, the growth medium was removed and the cover slip washed in PBS, immersed in cold acetone and examined by immunofluorescence with the aid of a monoclonal antibody to the ALV-J envelope protein. The remaining cells were lysed and examined for group antigen (gag) by ELISA. Viral isolation was determined to be positive according to IF and ELISA results.

PCR

Plasma was inoculated into a second plate without a cover slip. After one week the medium was removed and the monolayer was scraped off the plate and pelleted by centrifugation. The pellet was then treated with lysis buffer to release the cell nuclei. DNA was extracted with phenol/chloroform and following purification, PCR was performed with various ALV-J primers. These included.....

Sequencing

DNA was amplified using Dr.R.Silva's primer pair 6+2 to yield a 2.4kb fragment comprising the *env* protein gene, and the R, D, E and 3'LTR elements.

Cloning

The PCR product was cloned into E.coli using the PCR-Blunt vector kit (Invitrogen). The size of the insert was checked by EcoR1 restriction. DNA was sequenced on an ABI Model 373A automatic DNA sequencer using several sequencing primers such as T7, TA, F6 etc.. The *env* sequences of the Israeli isolates were compared with US isolates Hc1, 4817, 5701, 6803, 0661, 1696, 8827, and the UK isolate HPRS-103.

A phylogenetic tree was constructed using the *env* glycoprotein sequences aligned using Clustal V.

Results

ALV-J isolates

A total of 30 viruses were isolated and characterized by IF and PCR (Table 1). Of these 8 were isolated from GGP flocks, 5 from GP flocks, 18 from P flocks and 1 from a broiler flock (Table 2). Seven isolates were selected from this cluster for sequencing based on cloning analysis

Table 3 shows that of these seven viruses, 4 originated from 4 separate farms while the other 3 were from the same flock. The stretches of sequenced fragments varied in length from 51 to 675 nts according to the particular isolate (Table 4). An example of a sequencing map is shown in figure 1. This represents the isolate KVI 57 for which a total of 1581 nts were sequenced.

The phylogenetic tree based on the *presumptive amino acid alignments employing Sequencer version 3.1 (Gene Codes Corporation)* is shown in figure 2. Based on the flock of origin of the KVI isolates it was found that six of the seven viruses originated from breeding stocks imported from either the USA or UK, while KVI 57 was isolated from a parent flock derived from an Israeli breeding company. Figure 2 shows that the Israeli isolates could be divided into two cohorts as follows; KVI 53, 55 and 57. These were more closely related to HPRS 103, ADOL Hc1, 4817 and 5301. KVI 53 and 55 were shown to be isolates from a UK-based breeding company. The second cohort comprised KVI 61, 64, and 67-three isolates from the same P flock, and KVI 83 that was isolated from a GP flock imported as day olds from the same US breeder. These were closely related phylogenetically to ADOL 1696, 6827 and 6803, three more recently isolated US viruses.

Table 1: Israeli isolates of ALV-J: DNA examined by PCR at KVI and ADOL

FLOCK	No.	age (wks)	PCR						
			ELISA	IF	Israeli		USA		
					H5-H7	5+5	6+2	R5-F5	H5-AD1
C/E	51		Neg	Neg	Neg	Neg	Neg	Neg	Neg
ADOL	52		Pos	Pos	Pos	Pos	Pos	Pos	Pos
A	53	36	Pos	Neg	Pos	Neg	Pos	Pos	Neg
B	54	6.5	Neg	Neg	Neg	Neg	Neg		
C	55	28	Neg	Neg	Pos	Pos	Pos	Pos	Neg
	56		Pos	Pos	Pos	Pos	Pos		
D	57	34	Pos	Pos	Pos	Pos	Pos		
	58		Neg	Neg	Neg	Neg	Neg		
	59		Neg	Neg	Neg	Neg	Neg		
	60		Neg	Neg	Neg	Neg	Neg		
E	61	14	Pos	Pos	Pos	Pos	Pos		
	62		Pos	Pos	Pos	Pos	Pos		
	63		Pos	Pos	Pos	Pos	Pos		
	64		Pos	Pos	Pos	Pos	Pos		
	65		Neg	Neg	Neg	Neg	Neg		
	66		Neg	Neg	Neg	Neg	Neg		
	67		Pos	Pos	Pos	Pos	Pos		
	68		Neg	Neg	Neg	Neg	Neg		
F	69	50	Neg	Neg	Pos	Neg	Neg	Neg	Neg
	70		Neg	Neg	Neg	Neg	Neg		
	71		Neg	Neg	Neg	Neg	Neg		
	72		Neg	Neg	Neg	Neg	Neg		
G	73	18	Neg	Neg	Neg	Neg	Neg		
	74		Neg	Neg	Neg	Neg	Neg		
	75		Neg	Neg	Neg	Neg	Neg		
	76		Neg	Neg	Neg	Neg	Neg		
	77		Neg	Neg	Pos	Neg	Neg	Neg	Neg
	78		Neg	Neg	Pos	Neg	Pos	Pos	Neg
H	79	28	Pos	Pos	Pos	Pos	Pos		
	80		Pos	Pos	Pos	Pos	Pos		
	81		Pos	Pos	Pos	Pos	Pos		
	82		Neg	Neg	Pos	Neg	Neg	Neg	Neg
	83		Pos	Pos	Pos	Pos	Pos		
I	84	30	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	85		Neg	Neg	Pos	Neg	Neg	Neg	Neg
	86		Neg	Neg	Pos	Neg	Neg	Neg	Neg
	87		Neg	Neg	Pos	Neg	Neg	Neg	Neg
	88		Neg	Neg	Pos	Neg	Neg	Neg	Neg
	89		Neg	Neg	Pos	Neg	Neg	Neg	Neg
	90		Neg	Neg	Pos	Neg	Neg	Neg	Neg

Table 2: Origins of the ALV-J viruses isolated at KVI

Type of flock	No of flocks	No of isolates	No characterized	Strain
GGP	2	8	0	KVI 83 KVI 53, 55, 57, 61, 64, 67
GP	2	5	1	
P	7	18	6	
Broiler	1	1	0	

GGP: great grand-parent

GP: grand-parent

P: parent

Table 3: Identity of partially sequenced ALV-J isolates

KVI	Farm	Country of origin
53	A	UK
55	B	UK
57	C	Isr
61	D	USA
64	D	USA
67	D	USA
83	E	USA

Table 4: Israeli isolates of ALV-J: env-DNA cloned and sequenced

FLOCK	No. of sample	ELISA IF		PCR			clone No.	Eco R1	Sequencing primer		
		p-27		H5-H7	5+5	6+2			T7	TA	F6
A	53	Pos	Neg	Pos	Neg	Pos	1	Pos	446*	621	223
							2	Neg			
							3	Pos	NT	NT	NT
C	55	Neg	Neg	Pos	Pos	Pos	1	Pos	577	507	601
							2	Neg			
							3	Pos	NT	NT	NT
C	56	Pos	Pos	Pos	Pos	Pos	a	Neg			
							b	Pos			
							c	Pos			
							d	Neg			
							e	Pos	NT	NT	NT
D	57	Pos	Pos	Pos	Pos	Pos	1	Pos	426	630	525
							2	Neg			
							3	Pos	NT	NT	NT
E	61	Pos	Pos	Pos	Pos	Pos	1	Pos	581	198+269+135	513
							2	Pos	NT	NT	NT
							3	Pos	NT	NT	NT
E	62	Pos	Pos	Pos	Pos	Pos	1	Neg			
							2	Pos	NT	NT	NT
							3	Neg			
E	63	Pos	Pos	Pos	Pos	Pos	1	Pos	NT	NT	NT
							2	Neg			
							3	Pos	NT	NT	NT
E	64	Pos	Pos	Pos	Pos	Pos	1	Neg			
							2	Pos	274+51+107	675	501
							3	Pos	NT	NT	NT
E	67	Pos	Pos	Pos	Pos	Pos	1	Neg			
							2	Pos	570	222+155+118	570
							3	Neg			
G	78	Neg	Neg	Pos	Neg	Pos	1	Neg			
							2	Neg			
							3	Pos			
H	79	Pos	Pos	Pos	Pos	Pos	1	Neg			
							2	Neg			
							3	Neg			
H	80	Pos	Pos	Pos	Pos	Pos	4	Pos	NT	NT	NT
							5	Pos	Neg	Neg	Neg
							6	Neg			
H	81	Pos	Pos	Pos	Pos	Pos	4	Neg			
							5	Pos	Neg	Neg	Neg
							6	Neg			
H	83	Pos	Pos	Pos	Pos	Pos	1	Pos	542	467	576
							2	Pos	NT	NT	NT
							3	Pos	NT	NT	NT

*: number of nucleotide sequenced

Fig 1. Sequencing map KVI 55

Figure 2: Phylogenetic relationships of KVI and ADOL isolates.

Phylogenetic relationship of the isolates was calculated with the maximum parsimony method (MEGA program). The numbers at the branch points represents the result of bootstrap analysis of 1000 replicates.

Project 2: Infectivity trial of 6 Israeli ALV-J isolates in young turkey poults.

Following a publication on rapid tumor induction by ALV-J in young turkeys, we have inoculated groups of 3-4 day old poultry with previously characterized isolates (see 2nd Annual Report). Each group included non-injected birds to serve as monitors of contact transmission. The birds were weighed, bled and wing feathers detached at seven days intervals.

The experiment was terminated after 21 days.

Table 1 shows that all the except KVI-55 induced viremia in the inoculated groups, one week later, while KVI-53, 57 and 61-inoculated poults were viremic at two weeks post-inoculation. None of the in-contact poults became viremic throughout nor was ALV-J detected by PCR in the feathers of any of the birds during 3 weeks post-infection. Splenomegaly was observed at the termination of the experiment in the poults inoculated only with KVI-57.

Table 2 shows that the average weights of the two sub-groups did not differ markedly from each other and the weight gains of all six isolates were similar.

Table : Body weights of infected and contact groups of turkey poult.

Age (d)		1	7	14	21
Isolate					
control		66.1	128.7	230.3	319.1
53	injected	61.6	134.3	228.8	281.0
	contact	66.9	140.3	238.0	308.5
55	injected	65.6	127.6	223.6	312.3
	contact	62.3	123.3	222.3	294.8
57	injected	68.4	137.9	252.7	351.3
	contact	68.2	125.9	222.6	318.0
61	injected	68.9	146.1	256.3	377.5
	contact	71.2	139.8	258.3	348.7
64	injected	67.4	133.4	239.3	333.8
	contact	52.8	103.0	193.4	279.9
83	injected	59.6	116.3	207.4	342.3
	contact	65.5	122.2	227.8	321.3

Appendix 2
ADOL report – research unit #2

105-14 AR01

Project: 109 (105)
Phase: 02 (14)
CRIS: 32000-013
Date: 11/28/01
By: RLW

ANNUAL REPORT

1. Leaders: R.L. Witter (cooperators: A. M. Fadly, M. Malkinson)

2. Title: Horizontal transmission of ALV-J

3. Objectives:

- study factors related to transmission rates and time to viremia following low dose exposure

4. Background:

During earlier trials, we observed evidence of delayed viremias in at least two incidents. One situation involved embryos inoculated at 7d in the yolk sac with ALV-J (for use as donors in a contact exposure trial) where the donor chicks tested virus negative at 13, 20 and 27 days after hatch whereas another groups tested positive at 35 days. The virtual absence of contact transmission in this trial confirmed the poor infection level in the donors. The second situation involved chicks exposed by contact to graded doses (exposures) of ALV-J where viremias were not detected until 28 days. In both cases, it appeared that the chicks or embryos were exposed to minimal doses of virus. The possibility was considered, therefore, that low dose exposure of chicks or embryos to ALV-J may result in a rather different type of infection profile, characterized by delayed viremias and probably resulting in transient infections. If so, this might be important to the understanding of infection profiles under field conditions.

On the other hand, traditional experience suggests that in embryos once infection becomes established the virus replicates to high titer and chicks hatch with persistent infection. Thus, this trial was a preliminary probe to determine whether with commercial broiler breeder strains and ALV-J infection, there was any evidence of unconventional or unexpected infection patterns with very low dose exposures.

5. Results:

Trial 5A: Graded doses of ALV-J strain Hc-1 was given to commercial broiler breeder chicks from two sources. The exposure was by the combined cloacal-nasal route, as we have used in previous trials. The inoculum volume of 50ul was divided and half delivered to the nares and the remainder inserted into the cloaca, using an automatic pipette. The chickens were then placed in HB isolators. Blood samples were obtained at 2, 4, 6, 8 and 12 weeks. Each isolator also contained 2 contact control chicks from the same strain which were bled at weeks 2, 4, 6, and 8 to monitor the speed of contact transmission within the isolator. Antibody tests (indirect FA) were done on sera from the 8 and 12 week bleeds.

- **Table 1** – Results showed that the minimal dose of 10^2 infectious doses /ml (=about 5 infectious doses per chick) was beneath the threshold needed to establish infections. Thus, the series of doses produced an endpoint for both chicken strains and assured that some chickens had truly been exposed to a minimal dose. Although 1 chickens of strain B inoculated with 50 TCID became viremic at 8 wks, this was the only chicken in the entire experiment that appeared to demonstrate a delayed onset of viremia. However, it should be noted that most of the chickens that became viremic in the study developed persistent viremias and only 8 (total) appeared to have transient infections. There was very limited contact spread through 8 weeks, which was surprising but may have been related to the use of relatively low doses. Interestingly, strain B appeared more susceptible than strain A by a factor of nearly 100-fold.

Trial 5B: This trial was to identify parameters related to virus transmission and onset of viremia or antibody following low dose exposures in ovo. Graded doses of Hc-1 were inoculated into the yolk sac of 7 day embryos of two commercial strains, using 100ul per embryo. Chicks were hatched in pedigree baskets, kept separate by dose group. A group of hatcher controls, uninoculated, were hatched in the same incubator in separate pedigree baskets. Other controls were hatched in a separate incubator. After hatch, each dose group was placed in a separate HB isolator. Two contact controls were added to monitor horizontal transmission within the isolator. Blood samples were collected at weeks 4, 8 and 12 and tested for viremia. Antibody tests were done at 12 weeks only (indirect FA).

- **Table 2** – In this trial, an endpoint was not reached. Even the minimal dose induced persistent infections in most chicks of both strains (all except one). There were no birds that developed late viremias. Hatcher contacts and normal controls remained free of infection during the trial. Viremia data from the contact birds at 4 and 8 weeks indicated that the amount of contact transmission may have been less in the lowest dose groups compared to higher doses. No difference in susceptibility was noted between strains A and B, although the absence of a clear endpoint precludes a critical comparison.

6. Summary and conclusions:

- The experiments provided no evidence that low dose exposures may induce late onset viremias. The results obtained fit well with published data on other ALV strains. The null hypothesis, therefore, is rejected.
- There is some indication that chickens exposed to high doses are less likely to develop antibodies and are more likely to infect contact control chickens, compared to chickens infected with lower doses.
- In one of two trials, strain B appeared more susceptible than strain A. However, differences between these same strains in several previous experiments were inconsistent and definitive conclusions on relative susceptibility of strains are not warranted.

7. Recommendations:

- The data do not appear sufficient for publication as is, and do not warrant additional work.
- File the report.
- Offer the data to M. Malkinson (collaborator on the underlying BARD grant) or ADOL colleagues in case any of these persons can utilize it in future publications.

Table 1. Experiment 5A - Effect of low dose exposure to ALV-J at hatching

Strain	Treatment	TCID Dose	Inoculates											late	contact spread	
			viremia					aby			v class			viremia	thru 8 wks	
			2	4	6	8	12	8	12	per	tra	neg	>4wk	vir	aby	
A	cloacal-nasal	5	0/4	0/4	0/4	0/3	0/2	0/3	0/2	0	0	4	0	0/1	0/1	
		50	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0	0	4	0	0/2	0/2	
		500	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0	0	4	0	0/2	0/2	
		5000	1/4	2/4	1/4	1/4	1/4	2/4	2/3	1	1	2	0	0/2	1/2	
		50000	4/4	3/4	3/4	2/4	2/4	1/4	1/4	2	2	0	0	0/2	0/1	
	none	none	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0	0	4	0			
B	cloacal-nasal	5	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0	0	4	0	0/2	0/2	
		50	0/4	1/4	1/4	2/4	1/4	0/4	2/3	1	1	2	1	0/2	0/2	
		500	1/4	2/4	2/4	2/4	2/4	1/4	0/3	2	0	0	0	0/2	0/2	
		5000	3/4	4/4	3/4	3/4	3/4	1/4	1/4	3	1	0	0	1/2	1/2	
		50000	3/3	2/3	2/3	1/3	0/3	2/3	3/3	0	3	0	0	0/2	1/2	
	none	none	0/4	0/4	0/4	0/4	0/4	0/4	0/3	0	0	0	0			

repeats on some virus tests still to be completed
some aby tests still to be completed

Principal findings:

- minimal infectious doses, in this trial, did NOT result in late onset of viremia
- there was very limited contact spread through 8 wks as measured by virus and antibody – unusual?
- infections induced by cloacal-nasal exposure were mostly persistent
- strain B appeared more susceptible than strain A (by nearly 100-fold)

Table 2. Experiment 5B - Effect of low dose exposure to ALV-J in ovo

Strain	Treatment	Dose	per bird TCID ₅₀	Inoculates								v class			late viremia >4wk	contact spread		
				viremia				aby		per	tra	neg	viremia 4	viremia 8		aby 8		
				0	4	8	12	8	12									
A	yolk sac/7d	10 ² /ml	10	nd	4/4	4/4	3/4		2/4	3	1	0	0	0/2	0/2	2/2		
		10 ³ /ml	100	nd	4/4	3/3	3/3		0/3	3	0	0	0	1/2	0/2	2/2		
		10 ⁴ /ml	1,000	nd	4/4	4/4	4/4		0/4	4	0	0	0	2/2	0/2	2/2		
		10 ⁵ /ml	10,000	nd	3/3	4/4	4/4		0/4	4	0	0	0	2/2	2/2	1/2		
		10 ⁶ /ml	100,000	nd	4/4	4/4	4/4		0/4	4	0	0	0	2/2	2/2	0/2		
	hatcher contact	none		nd	0/6	0/6	0/6		0/6	0	0	6	0					
	none	none		nd	0/4	0/4	0/2		0/2	0	0	4	0					
B	yolk sac/7d	10 ² /ml	10	nd	4/4	4/4	4/4		1/4	4	0	0	0	2/2	1/2	1/2		
		10 ³ /ml	100	nd	4/4	3/4	4/4		0/4	4	0	0	0	1/2	1/2	2/2		
		10 ⁴ /ml	1,000															
		10 ⁵ /ml	10,000	nd	3/3	2/2	2/2		0/2	2	0	0	0	1/1	0/1	0/2		
		10 ⁶ /ml	100,000	nd	3/3	3/3	2/2		0/2	3	0	0	0	2/2	2/2	0/2		
	hatcher contact			nd	0/6	0/6	0/5		0/5	0	0	6	0					
	none	none		nd	0/4	0/4	0/4		0/4	0	0	4	0					

repeats on some virus tests still to be completed done T6031
 some aby tests still to be completed done T6035

Principal findings:

- minimal infectious doses, in this trial, did NOT result in late onset of viremia
- minimal infectious doses, in all but one case, resulted in persistent viremias
- contact spread through 8 wks as measured by virus and antibody was robust, but may have occurred less frequently in the low dose groups
- no difference in relative susceptibility was detected between strains A and B.

Appendix 3

Research unit #3. Draft manuscript prepared by H.D. Hunt – Privileged communication
Note – identity of ELISA kits should be kept confidential

Comparison of the different techniques (ELISA, Fluorescent Antibody, and Virus
Neutralization) for detecting serum antibody to the subgroup J Avian Leukosis Virus
(ALV-J).

H.D. Hunt^{AE}, G. Rosales^B, E. Jensen^B, J. McKay^B, V. Leathers^C, R. Shoberg^C, C.
Lamichhane^D, L. D. Bacon^A, R. L. Witter^A, and A. F. Fadly^A

^AUSDA-Agricultural Research Service, Avian Disease and Oncology Laboratory, 3606
East Mount Hope Road, East Lansing, MI 48823. ^BAvigen Group, Ross Breeders, 5015
Bradford Drive, Huntsville, Alabama 35805. ^CIDEXX Laboratories, Inc., One IDEXX
Drive, Westbrook, Maine 04092. ^DSynbiotics, Kirkegaard & Perry Laboratories, Inc., 2
Cessna Court, Gaithersburg, MD 20879-4174

^E Corresponding author.

SUMMARY

Three enzyme linked immunosorbent assays (ELISA), serum neutralization (SN), and fluorescent antibody (FA) based techniques were compared for their ability to detect serum antibodies to the avian leukosis virus subgroup J (ALV-J). In titration experiments using positive control antibody to the ADOL-Hc1 strain of ALV-J, the results of the three different techniques were highly correlated (correlation coefficient >0.9). The sensitivity of the two different commercial ELISA tests varied depending on the positive control serum used. Two of the positive control sera were negative when tested at the manufactures recommended dilution using one of the ELISA kits. The strong correlation in the sample to positive ratio (S/P) results decreased (correlation coefficient <0.7) when the tests are compared using experimental and commercial broiler serum samples. Commercial kit A (KPL) was more accurate when detecting antibody-conversion in the broiler samples experimentally infected with Hc1 while commercial kit B (IDEXX) performed better when analyzing field broiler samples. Using the immunofluorescent based techniques, the sub-strain (ADOL-Hc1 or HPRS-103) of the ALV-J envelope used in the test was an important variable. A portion of the experimental plasma samples responded to both envelopes while other plasma samples were positive using only the ADOL-Hc1 or HPRS-103 sub-strains. The sub-strain of virus was also an important consideration using the ELISA kits. The data show that reliable results depend on the dilution of the experimental plasma/sera, the criteria chosen for positive or negative results, and the use of multiple ALV-J substrains for the capture antigen.

1 INTRODUCTION

2 The detection of serum antibody to the avian leukosis virus subgroup J (ALV-J) is
3 an important screening tool used to monitor the effectiveness of ALV-J eradication and
4 bio-security procedures. The three methods used to monitor serum antibodies for ALV
5 are the serum neutralization (SN) test; fluorescent antibody (FA) detection and the
6 enzyme linked immunosorbent assay (ELISA) [Smith, 1986 #167][Hunt, 1999
7 #575][Tsukamoto, 1985 #176]. The poultry industry uses the commercial ELISA test
8 kits because of their high throughput and convenience instead of the other two methods
9 to monitor sero-conversion [Venugopal, 1997 #468].

10 In experiments in which the analysis of serum antibodies to ALV-J is required we
11 have observed a small but significant percentage of samples lacking a clear-cut positive
12 or negative result. This small percentage of plasma with ambiguous results has also
13 been observed in the commercial setting. These ambiguous serum samples fall into
14 two main categories: 1) samples with results that are borderline negative or positive
15 using the established test criteria and 2) samples with clear results using one method
16 but the opposite or borderline result using another method. In this report, we compare
17 the ELISA, SN, and FA techniques to determine the most accurate method and/or
18 criteria for analyzing ambiguous serum samples.

20 MATERIALS AND METHODS

21 Plasma and Sera Samples

22 One set of samples used for this analysis came from experiments at ADOL
23 designed to test the use of small group rearing to reduce horizontal transmission of
24 ALV-J in commercial broiler lines of birds. These experimental plasma samples were
25 selected from two flocks of broiler birds as described by [Witter, 2001 #713]. One group,
26 T5890, came from a flock in which embryo-infected chicks were allowed to remain in
27 contact with non-infected birds resulting in an infected flock. The other was similar
28 except that the flock was hatched and reared in small groups to 4 weeks, during which
29 time all infected birds and their small groups were detected and removed. The
30 remaining chicks were considered clean, based on the failure to detect viremias or
31 antibodies (FA) at any subsequent time through 32 weeks. Four types of plasma were

1 chosen for this analysis based on their virus neutralization results: 1) antibody positive
2 plasma with 90% or greater virus neutralization, 2) suspicious plasma with 80 to 90%
3 virus neutralization, 3) low negative plasma with 70 to 80% virus neutralization and 4)
4 high negative plasma with only 10 to 20% virus neutralization.

5 In addition to the plasma obtained from the controlled experiments described
6 above, broiler plasma from both virus positive and virus negative commercial breeder
7 flocks was sent to ADOL for analysis. The samples selected for further analysis
8 displayed ambiguous results for antibody to ALV-J using an ELISA assay developed
9 and performed by the primary commercial Breeder Company. Thirty samples from the
10 virus negative flock and fifty samples from the virus positive flocks were selected for
11 analysis.

12 The positive control antisera used in this analysis was produced in adult 15I₅
13 (sera #712A), 15.15I₅ (sera #787B) or 15.P-19 (sera #1442) white leghorn chickens by
14 five twice-weekly immunizations using 1×10^7 to 1×10^8 Line 0 chick embryo fibroblasts
15 (CEFs) infected with the ADOL-Hc1 strain of ALV-J [Bacon, 2000 #715]. The negative
16 control serum was obtained from virus free broiler birds housed in isolation.

17 ELISA

18 All samples were analyzed, as per the manufacturer instructions, at ADOL using
19 two different commercial ELISA kits for ALV-J antibody supplied by Synbiotics,
20 Kirkegaard & Perry Laboratories (Gaithersburg, MD) and IDEXX Laboratories, Inc.,
21 (One IDEXX Drive, Westbrook, MA). Additionally, the commercial broiler samples, but
22 not the experimental broiler samples, were split and sent to representatives of KPL and
23 IDEXX for independent testing in their laboratories.

25 Fluorescent Antibody Analysis

26 For flow cytometry (FC), DF1 cells (negative control) and DF1 cells expressing either
27 the ADOL-Hc1 or the HPRS-103 envelope (DF1/J) was used to test the plasma and
28 sera for ALV-J antibodies [Hunt, 1999 #575]. The cells were labeled with a 1:40 dilution
29 of test plasma/sera for 20 minutes. In some experiments, different dilutions of
30 plasma/sera were analyzed as indicated in the tables of results. The cells were then
31 washed with phosphate buffered saline, and counter labeled with FITC conjugated goat

1 anti chicken immunoglobulin (Bethyl Laboratories Inc., P.O. Box 850, Montgomery, TX).
2 After a second wash, five to ten thousand cells were analyzed for fluorescence intensity
3 using a FACSsort flow cytometer (Becton Dickinson, San Jose, CA). The fluorescence
4 intensity for each sample was calculated as the mean channel fluorescence (MCF)
5 which was then used to calculate a sample to positive (S/P) ratio. The positive controls
6 consisted of convalescent sera to ADOL-Hc1 and HPRS-103 produced in Line 0
7 chickens. The negative control consisted of the MCF of the DF1 cells labeled with the
8 positive control or experimental sample plasma/sera.

9 The S/P ratio for flow cytometry was calculated using the formula:

$$\frac{(\text{MCF of Sample Plasma/sera on DF1/J}) - (\text{MCF of Sample Plasma/sera on DF1})}{(\text{MCF of Positive Plasma/sera on DF1/J}) - (\text{MCF of Positive Plasma/sera on DF1})}$$

14 A S/P ratio greater than or equal to 0.100 was considered positive.

16 Some plasma were analyzed by fluorescent microscopy. For this assay, Line 0
17 chick embryo fibroblasts (CEFs) were infected with the ADOL-Hc1 strain of ALV-J and
18 plated in 96 well flat bottom microtiter plates. Uninfected CEFs were included in the
19 same well and served as an internal negative control. The confluent microtiter plates
20 were fixed with a 60% alcohol/40% acetone mixture for 10 minutes, washed, rehydrated
21 in PBS and stored at -20°C until use. The test plasma/sera was diluted 1:20 into the
22 test wells of the microtiter plates and incubated at room temperature for 30 minutes.
23 The microtiter plates were then washed and a 1:20 dilution of FITC labeled goat anti
24 chicken immunoglobulin was added for an additional 30 minutes. The test wells were
25 then washed and evaluated as positive or negative using a Leica DMIRB fluorescent
26 microscope.

28 Virus Neutralization

29 Virus neutralization (VN) assays were performed using Line 0 chick embryo
30 fibroblasts (C/E). Virus isolates ADOL-Hc1 [Fadly, 1999 #578] and HPRS-103 [Bai,
31 1995 #263] were used as indicator viruses for the presence of ALV-J neutralizing
32 antibodies by a virus neutralization test as described by [Fadly, 1998 #583]. Briefly,
33 ALV-J virus and a 1:5 dilution of serum were incubated for 30 minutes. The virus/serum

1 mixture was then used to infect Line 0 CEFs. The next day the CEFs were washed with
2 media and incubated at 37°C. After 10 days of incubation a standard p27 ELISA assay
3 was performed[Smith, 1979 #462]. The optical density (OD) of the p27 assay was used
4 to calculate a S/P ratio. Positive and negative control sera for each virus consisted of
5 convalescent sera (see above) and normal chicken sera respectively.

6 The S/P ratios were calculated using the formula:

$$\frac{\text{OD cultures with virus \& negative control plasma/sera} - \text{OD cultures with virus \& experiment Plasma/sera}}{\text{OD cultures with virus \& negative control plasma/sera} - \text{OD cultures with virus \& positive control plasma/sera}}$$

11 For this comparison, plasma with 50% or greater inhibition of the p27 specific activity
12 ($S/P \leq 0.50$) was considered positive. The reciprocal of the VN S/P ratio was used for
13 calculating the correlation coefficients between the ELISA and flow cytometry S/P
14 results.

15 RESULTS

16 Sensitivity of the Tests.

17 A comparison of the sensitivities of the different assays is presented in Table 1.
18 For this comparison, titration of three hyper-immune sera from chickens immunized with
19 the ADOL-Hc1 strain of ALV-J was used. It should be noted that the manufactures
20 recommended final sera dilution for the IDEXX and KPL kits is 1:500 and 1:100
21 respectively. Using positive control serum 712A, the IDEXX kit was positive (S/P
22 >0.600) for all dilutions tested including the recommended 1:500. This same serum was
23 positive ($S/P >0.399$) at the 1:80 and 1:100 dilutions but negative for larger dilutions
24 using the KPL kit. This situation was reversed using positive control serum 787B. With
25 this serum, all but the 1:640 dilution was considered positive using the KPL kit but only
26 the 1:40 and 1:80 dilutions were considered positive using the IDEXX kit. The end
27 points for positive control serum 1442 was 1:100 and 1:160 for the KPL and IDEXX kits
28 respectively.

29 Using flow cytometry, all three positive control sera had prozone effects at the
30 lower dilutions. Nevertheless, all three sera were clearly positive at the recommended
31 final dilution of 1:40. By flow cytometry, positive sera 712A had the highest titer
32 followed by 1442 and 787B.

1 In the virus neutralization test, all three positive control sera were positive at the
2 recommended 1:5 dilution and 712A had the highest titer followed by 787B and 1442.

3 Although the sensitivity varied between the tests and the positive test sera used,
4 the correlation coefficients of the S/P ratios were highly correlated. The correlation
5 coefficients of the ELISA S/P values were equal to or greater than 0.95 using all three
6 positive control sera (see Table 2). The worst correlation coefficients occurred between
7 VN and the KPL ELISA data, which fell slightly below 0.8.

8 Analysis of Ambiguous Plasma Reactivity to ADOL-Hc1

9 Four types of plasma selected from a group rearing experiment were analyzed
10 and the results presented in Table 3. The four types of plasma were selected was
11 based on virus neutralization test results and included clear positive (VN O.D. <0.100)
12 and negative (high negative, VN O.D. >0.7) samples. The ambiguous samples
13 analyzed were labeled suspicious (VN O.D.s between 0.1 to 0.2) and low negative (VN
14 O.D.s between 0.2 and 0.3). The fluorescence analysis by microscopy and flow
15 cytometry are highly correlated when low dilutions of the plasma are used with only one
16 plasma (#4291) out of twenty-four differing between the two techniques. The flow
17 cytometry results using a 1:200 dilution of plasma are less dependable with
18 disagreement on six of the twenty-four plasma including two of the highly positive
19 plasma. Three of the suspicious samples and three of the low negative samples were
20 positive by VN but negative by either fluorescent technique. In addition, one sample
21 (#4289) was negative by virus neutralization but clearly positive using both fluorescent
22 tests including flow cytometry at the 1:200 dilution.

23 The results using the KPL kit were similar to the fluorescence results except for
24 three samples. The KPL ELISA called one of the three positive serums (#4361)
25 negative. Two of the high negative plasma, numbers 4291 and 4289, showed
26 conflicting results with the former being positive by ELISA and microscopy but negative
27 by flow cytometry and the latter being positive using both microscopy and flow
28 cytometry but negative by ELISA. The ELISA results using the IDEXX kit clearly differed
29 from the VN, fluorescence, and KPL results. The IDEXX kit called only two of the
30 plasma positive.

1 Although the results differed between the two ELISA kits, Table 4 shows that the
2 correlation between their S/P results are very high (>0.8). There was also good
3 correlation between the results of the ELISA and flow cytometry S/P results but less
4 correlation between VN and the other tests.

5 Analysis of Ambiguous Commercial Broiler Plasma.

6 All the samples from the virus negative flock tested negative for ALV-J antibody
7 by virus neutralization and flow cytometry (Table 5). On the other hand, 11 of the 25
8 plasma coming from the ALV-J negative flock were positive for ALV-J antibody by one
9 or more of the ELISA assays.

10 Using the IDEXX kit, one sample was highly positive when the assay was
11 performed at IDEXX Laboratories but negative in a second analysis performed at
12 ADOL. The ELISA performed by the primary breeder also identified this sample as
13 highly positive. This sample was negative using the KPL kit.

14 The results using KPL kit identified four positive samples when the assay was
15 performed at KPL laboratories and five positive samples in a second analysis performed
16 at ADOL. Of the positive samples using the KPL kit, three tested positive at both the
17 KPL and ADOL laboratories. All of the samples identified as positive by the KPL kit
18 were low positive with S/P ratios in the 0.4 to 0.6 range (positive = > 0.399).
19 Interestingly, there was no agreement between the positive samples identified by the
20 primary breeder with the IDEXX or the KPL ELISA kit.

21 The analyses of the samples from the virus positive flocks are presented in Table
22 6. Five of the samples in this group came from broilers testing negative for ALV-J by
23 virus isolation (VI-), the virus status of the other 45 samples is unknown.

24 The samples in Table 6 are grouped by consensus among the nine different tests
25 used in this analysis. Group one consists of samples identified as either positive ($n=1$)
26 or negative ($n=7$) by all tests used, group two are samples in which eight of nine tests
27 returned either a positive ($n=5$) or negative ($n=9$) result, in group three seven of nine
28 tests agree and so on. The number of samples in each test differing from consensus is
29 tabulated at the bottom the table. This grouping shows that the Ross ELISA and virus
30 neutralization using the ADOL-Hc1 strain have more outliers than the other tests. The
31 virus neutralization test using ADOL-Hc1 is consistently negative even when the other

1 tests indicate a consensus positive result. All of the consensus positive samples in
2 groups 2 through 5 (n=15) were negative using the ADOL-Hc1 VN test. The Ross
3 ELISA test identifies 21 positive samples within the consensus negative samples. This
4 type of analysis gives some measure of each tests reliability when compared in the
5 context of all the tests performed. A different comparison is using correlation
6 coefficients presented in Table 7. Although this analysis is useful when comparing
7 different repetitions of the same test, its value in comparing between different types of
8 tests is limited (see discussion). With this limitation in mind, the Ross ELISA correlated
9 well with the IDEXX results (0.730 and 0.966) and FC using HPRS-103 (0.814) but
10 poorly with the other tests. The IDEXX and KPL results correlated poorly as did the
11 other tests in this group.

12 DISCUSSION

13 Three groups of samples ranging from well-defined positive control sera to highly
14 undefined plasma from commercial flocks were used to compare the common methods
15 for analyzing serum antibodies to ALV-J. The first group of samples consisted of
16 positive control sera generated by intraperitoneal challenge using a cloned strain of
17 ALV-J (ADOL-Hc1). Samples in this group came from inbred birds of three 15.B
18 congenic lines in which the virus status was clearly defined throughout the experiment.
19 These B genotypes may have affected the ability of antibody to recognize different ALV-
20 J epitopes in the different tests or kits (Bacon 2000). The second group of samples came
21 from a well controlled experiment set up to mimic the commercial setting with regard to
22 ALV-J exposure (Witter and Fadly 2001). The commercial broiler birds in this group
23 were hatched at ADOL and exposed to ALV-J by seeder birds infected with the cloned
24 ADOL-Hc1 strain. The viremia of these birds was monitored by virus isolation at hatch
25 and then approximately every 6 weeks until 32 weeks of age. The samples used in this
26 experiment were from birds that tested virus negative at hatch however, because of the
27 contact nature of the ALV-J exposure; the exact virus status for each bird was not
28 determined. We do know that the samples used in this experiment came from birds that
29 were viremia negative from 12 weeks on. Thus, either the samples came from birds
30 that were never infected with the virus or birds that had cleared the virus, presumably by
31 converting to ALV-J antibody positive. The third group of samples came from a

1 commercial source of broiler birds naturally infected with an unknown strain or strains of
2 ALV-J. The samples in this group came from both virus positive and virus negative
3 flocks, however again, the exact virus exposure is unknown. The samples from the
4 virus positive flocks are thought to represent both vertically and horizontally infected
5 birds.

6 In titration experiments, all of the tests were capable of detecting antibody to
7 ALV-J present in the positive control sera (Table 1). Not surprisingly the results of each
8 test were dilution dependent. At the recommended dilution of 1:500 the results from two
9 of the three sera using the IDEXX kit fell into the negative range ($S/P < .600$). This
10 shows that using one serum dilution for the test and establishing one criterion to
11 distinguish positive and negative results may lead to erroneous results. Since these
12 sera came from inbred birds responding to cloned virus, one would expect general
13 agreement in antibody titers among the various tests however this was not the case.
14 Serum #712A (from a B15/B15 MHC haplotype) was the highest titer serum using the
15 IDEXX, flow cytometry and virus neutralization tests but #787B (from a B5/B15 MHC
16 haplotype) was highest using the KPL test. It is possible that some of the variability in
17 the positive control sera could be due to the B haplotype of the chicken however more
18 research is needed to verify this.

19 The established criteria used for the KPL kit worked well for the commercial
20 broiler plasma experimentally exposed to the Hc1 strain of ALV-J (Table 3). The IDEXX
21 kit was less effective on these samples but more effective on the commercial plasma
22 exposed naturally (Table 6). It must be pointed out that both the positive and negative
23 results as well as the correlation between the S/P ratios varied between the two kits.
24 Thus, simply adjusting the criteria for positive and negative results would not solve
25 these differences. There is a fundamental difference between the kits in that the Ross
26 and IDEXX kits use an envelope derived from the HPRS-103 strain of ALV-J while the
27 KPL kit uses the envelope derived from ADOL-Hc1. This may be the most important
28 difference in the accuracy of the IDEXX and KPL kits since the control and one group of
29 experimental samples was infected using the ADOL-Hc1 virus and the other set of
30 experimental samples came from unknown or mixed infections. Since there is clear
31 serologic differences between these two strains [Fadly, 1999 #578], each kit may be

1 better suited to substrains of the ALV-J virus. This is also clear when the results using
2 HPRS-103 and ADOL-Hc1 and flow cytometry or virus neutralization are compared.
3 Virus neutralization using ADOL-Hc1 worked well when using samples from ADOL-Hc1
4 infected birds but was ineffective in detecting antibody in the samples infected with
5 unknown strains. Flow cytometry shows that the naturally infected commercial samples
6 contained mixed serological reactivities suggesting the flock was infected with at least
7 two different serologic subtypes of ALV-J (Table 6). One subtype is represented by
8 sample 6426 in which a strong signal is obtained using HPRS-103 infected cells but a
9 weak signal occurs using ADOL-Hc1 infected cells. In contrast, sample number 6457 is
10 highly positive using ADOL-Hc1 infected cells but negative using HPRS-103 infected
11 cells.

12 The accurate analysis of serum antibodies to ALV-J depends on several
13 important factors. The dilution of sample used in the test is very important and more
14 than one dilution may be necessary for some samples. The criteria used to define
15 positive and negative samples may need adjusting, especially for the ambiguous
16 samples examined here. Perhaps the most important variable however, is the substrain
17 of virus envelope used in the test. There is a high probability that more than one
18 substrain of ALV-J will be circulating in a flock and it would appear prudent to use more
19 than one subtype of ALV-J envelope in the assay used to monitor antibody to ALV-J.

20 Bacon, L. D. (2000). Production of neutralizing antibody to ALV-J differs in aged hens
21 from seven B-congenic white leghorn lines. International Symposium on ALV-J and
Other Avian Retroviruses, Rauschholzhausen, Germany, World Veterinary Poultry

Table 1. Sensitivity of the Elisa, Flow Cytometry and Virus Neutralization (VN) Tests Using Positive Control Sera¹

IDEXX (C) ²		KPL (B) ³		Flow Cytometry ADOL-Hc1		VN ADOL-Hc1	
Dilution	S/P ²	Dilution	S/P ³	Dilution	S/P ⁴	Dilution	S/P ⁵
Positive Control Serum 712A ¹							
1:40	1.987⁷	1:80	0.588	1:40	0.366	1:4	0.012
1:80	1.423	1:100	0.562	1:80	1.150	1:8	0.051
1:160	1.080	1:160	0.259	1:160	0.647	1:16	0.064
1:320	0.760	1:320	0.181	1:320	0.352	1:32	0.377
1:500	0.614	1:640	0.134	1:640	0.113	1:64	0.800
Positive Control Serum 787B ¹							
1:40	1.267	1:80	1.107	1:40	0.217	1:2	0.007
1:80	0.765	1:100	1.011	1:80	0.425	1:4	0.089
1:160	0.448	1:160	0.638	1:160	0.242	1:8	0.248
1:320	0.248	1:320	0.452	1:320	0.158	1:16	0.543
1:500	0.168	1:640	0.301			1:32	0.662
Positive Control Serum 1442 ¹							
				1:10	0.044	1:0	0.014
				1:20	0.444	1:2	0.008
1:40	2.103	1:80	0.843	1:40	0.482	1:4	0.098
1:80	1.564	1:100	0.773	1:80	0.642	1:8	0.440
1:160	1.005	1:160	0.347	1:160	0.355	1:16	0.659
1:320	0.592	1:320	0.218				
1:500	0.387	1:640	0.138				
Negative Control Serum ⁶ 34028, 27787 & 32050							
1:500	-0.024	1:100	0.085	1:40	-0.033	1:20	0.786
1:500	0.001	1:100	0.187	1:40	-0.002	1:20	0.840
1:500	-0.022	1:100	0.108	1:40	-0.001	1:20	1.000

¹Chickens (151₅×7₂) were immunized repeatedly with CEFs infected with ADOL-Hc1.

²IDEXX Kit. A positive S/P value is greater than 0.600 at final serum dilution of 1:500.

³KPL Kit. A positive S/P value is greater than 0.399 at a final serum dilution of 1:100.

⁴A S/P value greater than 0.1 is considered positive by flow cytometry.

⁵A S/P value less than 0.5 is considered positive by virus neutralization.

⁶Negative control sera was obtained from hatch mates of the experimental broiler chickens (see materials and methods).

⁷ Bold S/P numbers are considered a positive result and bold dilution are manufactures

Table 2. Correlation Coefficients Between ELISA, Virus Neutralization, and Flow Cytometry S/P Ratios using Positive Control Sera¹ to ALV-J

	IDEXX (C) ²	KPL (B) ²	Flow Cytometry ADOL-Hc1 ³	VN ADOL-Hc1 ⁴
Serum 712A ⁵				
IDEXX (C) ²	-	0.955	0.995	0.877
KPL (B) ²		-	0.971	0.760
Flow Cytometry ADOL-Hc1 ³			-	0.864
Serum 787B ⁵				
IDEXX (C) ²	-	0.947	0.997	0.910
KPL (B) ²		-	0.857	0.975
Flow Cytometry ADOL-Hc1 ³			-	0.928
Serum 1442 ⁵				
IDEXX (C) ²	-	0.976	0.936	0.876
KPL (B) ²		-	0.812	0.869
Flow Cytometry ADOL-Hc1 ³			-	0.707

¹Positive control sera was obtained from chickens (15I₅x7₂) that were immunized weekly using ADOL-Hc1 infected CEFs (1x10⁷/immunization).

² S/P values as per manufactures instruction used for correlation.

³ S/P values for flow cytometry were calculated as in materials and methods.

⁴ The reciprocal of correlation coefficient is reported.

⁵ To avoid prozone effects the dilutions used to calculate correlation coefficients were 712A 1:80

Table 3. Analysis of Commercial Broiler Plasma¹ Reactivity to the Hc1 strain of ALV-J

Serum Sample		Test Procedure																
		Virus Neutralization					Fluorescence				ELISA							
		Virus Neutralization					icroscopy		Flow Cytometry ³				KPL (B) ⁴		IDEXX (C) ⁴			
							Dilution	Result	Dilution	S/P ratio	S/P > 0.1=+	Dilution	S/P ratio	S/P > 0.1=+	Dilution	S/P RATIO	Dilution	S/P RATIO
Sample #	Description	Dilution	O.D.	S/P <0.500=+	Dilution	Result	Dilution	S/P ratio	S/P > 0.1=+	Dilution	S/P ratio	S/P > 0.1=+	Dilution	S/P RATIO	Dilution	S/P RATIO	S/P > .6	
16	Neg. control serum 34028	1:20	0.786	-	1:20	-	1:40	-0.033	-	:200	0.015	-	1:100	0.085	-	1:500	-0.024	-
17	Neg. control serum 27787	1:20	0.840	-	1:20	-	1:40	-0.002	-	:200	-0.003	-	1:100	0.187	-	1:500	0.001	-
18	Neg. control serum 32050	1:20	1.000	-	1:20	-	1:40	0.000	-	:200	-0.004	-	1:100	0.108	-	1:500	-0.022	-
19	T5890 positive 4250	1:20	0.002	+	1:20	+	1:40	1.152	+	:200	0.293	+	1:100	0.571	+	1:500	0.250	-
20	T5890 positive 4361	1:20	0.026	+	1:20	+	1:40	0.329	+	:200	0.089	-	1:100	0.343	-	1:500	0.123	-
21	T5890 positive 4267	1:20	0.062	+	1:20	+	1:40	0.281	+	:200	0.071	-	1:100	0.498	+	1:500	0.087	-
22	T5890 positive 4385	1:20	0.085	+	1:20	+	1:40	0.174	+	:200	0.067	-	1:100	0.517	+	1:500	0.055	-
23	T5890 suspicious 4351	1:20	0.132	+	1:20	+	1:40	1.073	+	:200	0.266	+	1:100	1.373	+	1:500	1.266	+
24	T5890 suspicious J685	1:20	0.164	+	1:20	+/-	1:40	0.600	+	:200	0.106	+	1:100	0.463	+	1:500	0.071	-
25	T5891 suspicious 4110	1:20	0.114	+	1:20	-	1:40	0.005	-	:200	0.013	-	1:100	0.193	-	1:500	-0.025	-
26	T5891 suspicious 4108	1:20	0.160	+	1:20	-	1:40	0.006	-	:200	0.010	-	1:100	0.192	-	1:500	-0.003	-
27	T5891 suspicious 4190	1:20	0.184	+	1:20	-	1:40	0.002	-	:200	0.008	-	1:100	0.205	-	1:500	-0.008	-
28	T5890 low negative 4248	1:20	0.209	+	1:20	+	1:40	0.210	+	:200	0.041	-	1:100	0.584	+	1:500	0.054	-
29	T5890 low negative 4295	1:20	0.262	+	1:20	+	1:40	0.235	+	:200	0.050	-	1:100	0.439	+	1:500	0.269	-
30	T5890 low negative 4299	1:20	0.304	+	1:20	+	1:40	0.517	+	:200	0.152	+	1:100	0.698	+	1:500	1.174	+
31	T5891 low negative 4109	1:20	0.236	+	1:20	-	1:40	0.009	-	:200	0.010	-	1:100	0.301	-	1:500	-0.005	-
32	T5891 low negative 4084	1:20	0.260	+	1:20	-	1:40	0.003	-	:200	0.014	-	1:100	0.190	-	1:500	-0.014	-
33	T5891 low negative 4044	1:20	0.288	+	1:20	-	1:40	0.002	-	:200	-0.003	-	1:100	0.184	-	1:500	-0.004	-
37	T5891 high negativ 4059	1:20	0.770	-	1:20	-	1:40	0.009	-	:200	0.018	-	1:100	0.292	-	1:500	0.012	-
38	T5891 high negativ 4200	1:20	0.780	-	1:20	-	1:40	0.004	-	:200	0.008	-	1:100	0.174	-	1:500	-0.019	-
39	T5891 high negativ 4007	1:20	0.814	-	1:20	-	1:40	0.002	-	:200	0.012	-	1:100	0.248	-	1:500	-0.003	-
34	T5890 high negativ 4291	1:20	0.703	-	1:20	+	1:40	0.065	-	:200	0.036	-	1:100	0.499	+	1:500	0.050	-
35	T5890 high negativ 4333	1:20	0.719	-	1:20	-	1:40	0.024	-	:200	0.034	-	1:100	0.366	-	1:500	0.042	-
36	T5890 high negativ 4289	1:20	0.905	-	1:20	+	1:40	0.355	+	:200	0.122	+	1:100	0.242	-	1:500	0.250	-

¹Chickens (Commercial Broilers) were exposed at hatch using embryo infected (ADOL-Hc1) chicks. The T5890 group were virus (Hc1) positive, the T5891 group was virus (Hc1) negative by virus isolation 4 weeks post exposure.

² Infected and uninfected CEFs were mixed, plated into 96 well plates and fixed with 70% alcohol/30% acetone. The fixed cells were incubated with the experimental serum samples, washed with PBS.

³ labeled with goat anti chicken antibody labeled with fluorescein, washed with PBS and viewed using a fluorescent microscope.

Table 4. Correlation Coefficients Between ELISA, Virus Neutralization, and Flow Cytometry S/P Ratios using the Ambiguous Commercial Broiler Sera¹ Experimentally Exposed to ALV-J

	IDEXX (C) ²	KPL (B) ²	Flow Ccytometry ADOL-Hc1 ³	VN ADOL-Hc1 ⁴
IDEXX (C) ²	-	0.820	0.690	0.772
KPL (B) ²		-	0.777	0.579
Flow Cytometry ADOL-Hc1 ⁴			-	0.552

¹Chickens (Commercial Broilers) were exposed at hatch using chicks infected with ADOL-Hc1 at day 4 of embryonation.

² S/P values as per manufactures instruction used for correlation.

³ S/P values for flow cytometry were calculated as in materials and methods using the 1:40

dilution.

⁴

Table 5. Analysis of Ambiguous Commercial Broiler Sera from Virus Negative Flocks

Serum	ELISA				Flow Cytometry		Virus Neutralization	
	Ross (A) ¹	IDEXX (B) ²	IDEXX (C) ³	KPL (A) ⁴	KPL (B) ⁵	ADOL-Hc1 HPRS-103	HPRS-103 ADOL-Hc1	ADOL-Hc1 HPRS-103
Number	Result				Result		Result	
VI Status ⁶	S/P	S/P	S/P	S/P	S/P	S/P	S/P	S/P
6380	0.000	0.113	-0.009	0.714	0.523	+	1.081	1.044
6381	0.452	0.209	0.022	0.122	0.194	-	1.032	1.050
6382	0.038	0.018	0.001	0.082	0.164	-	1.014	0.984
6383	0.106	0.017	0.056	0.571	0.605	+	0.932	0.966
6384	0.179	0.000	0.016	0.041	0.230	-	0.968	0.960
6385	0.135	0.000	-0.012	0.020	0.051	-	0.975	0.948
6386	0.187	NT	NT	0.061	NT	NT	0.868	0.910
6387	0.072	0.013	-0.009	0.082	0.274	-	0.975	0.938
6388	0.084	0.226	0.009	0.082	0.105	-	0.977	0.973
6389	0.917	2.274	0.097	0.143	0.139	-	0.972	1.005
6390	0.130	0.231	0.132	0.102	0.128	-	1.040	0.964
6391	0.384	0.578	0.129	0.143	0.408	+	1.015	1.054
6392	0.232	0.107	0.005	0.286	0.158	-	0.920	0.965
6393	0.384	0.052	0.000	0.122	0.180	-	0.855	0.915
6394	0.060	0.128	0.004	0.265	0.523	+	0.966	0.868
6395	0.031	0.032	-0.004	0.143	0.158	-	0.930	0.887
6396	0.235	0.124	0.007	0.163	0.137	-	0.904	0.915
6397	0.729	0.283	0.009	0.122	0.144	-	0.928	0.881
6398	0.791	0.491	0.054	0.184	0.276	-	0.932	0.891
6399	0.118	0.000	-0.007	0.061	0.064	-	0.953	0.913
6400	0.145	0.126	0.011	0.408	0.438	+	1.019	0.946
6401	0.174	0.330	0.014	0.245	0.298	-	1.034	1.002
6402	0.183	0.092	0.056	0.184	0.162	-	0.973	0.961
6403	0.041	0.000	-0.023	0.041	0.138	-	0.980	0.911
6404	0.476	0.021	-0.013	0.102	0.159	-	0.916	0.892
6405	-0.008	0.189	-0.014	0.531	0.260	-	0.924	0.881
6406	0.043	0.111	-0.008	0.163	0.160	-	0.903	0.846
6407	0.138	0.000	0.005	0.041	0.076	-	0.876	0.862
6408	0.074	0.111	0.022	0.224	0.257	-	0.934	0.898
6409	0.214	0.209	0.021	0.204	0.302	-	0.941	0.891

¹ ELISA data from Ross Breeders using their own preparations (1/11/4/89)

² Data from IDEX Laboratories

³ Data from ADOL using IDEX Kit (10/24/01)

⁴ Data from KPL laboratories (2/14/00)

⁵ Data from ADOL using KPL laboratories (1/12/01)

Table 6. Analysis of Ambiguous Commercial Broiler Sera from Virus Positive Flocks

Consensus	Serum		ELISA										Flow Cytometry				Virus Neutralization									
			Ross (A) ¹		IDEXX (B) ²		IDEXX (C) ³		KPL (A) ⁴		KPL (B) ⁵		ADOL-Hc1		HPRS-103		HPRS-103		ADOL-Hc1							
	Result																									
	Number	VI Status ⁶	S/P	(+ = >.419)	S/P	(+ = >.599)	S/P	(+ = >.599)	S/P	(+ = >.399)	S/P	(+ = >.399)	S/P	(+ = >.10)	S/P	(+ = >.10)	S/P	(+ = <.50)	S/P	(+ = <.50)						
Group 1 (all 9 tests agree)																										
+	6435	NT	2.458	+	1.525	+	1.093	+	0.429	+	0.410	+	0.598	+	0.850	+	-0.005	+	0.467	+						
-	6420	NT	0.120	-	0.020	-	0.005	-	0.102	-	0.160	-	0.009	-	0.006	-	0.989	-	0.925	-						
-	6421	-	0.050	-	0.000	-	-0.043	-	0.041	-	0.024	-	-0.002	-	-0.006	-	1.045	-	1.012	-						
-	6422	NT	0.360	-	0.000	-	-0.034	-	0.163	-	0.118	-	0.057	-	0.081	-	0.975	-	1.006	-						
-	6438	-	0.202	-	0.000	-	0.014	-	0.102	-	0.158	-	0.014	-	0.008	-	0.987	-	0.942	-						
-	6443	NT	0.262	-	0.003	-	NT	NT	0.102	-	NT	NT	-0.003	-	0.002	-	1.042	-	1.158	-						
-	6451	-	0.351	-	0.055	-	-0.004	-	0.061	-	0.079	-	0.005	-	-0.005	-	1.006	-	1.136	-						
-	6459	NT	0.103	-	0.165	-	0.010	-	0.082	-	0.172	-	-0.010	-	0.001	-	0.969	-	1.079	-						
Group 2 (8 of 9 test agree)																										
+	6418	NT	2.130	+	1.647	+	1.194	+	0.531	+	1.178	+	1.091	+	0.777	+	0.009	+	0.827	-						
+	6436	NT	2.015	+	1.346	+	0.669	+	0.408	+	0.684	+	0.732	+	0.479	+	-0.005	+	0.895	-						
+	6442	NT	3.902	+	3.319	+	3.233	+	0.673	+	1.236	+	0.134	+	1.170	+	0.007	+	1.079	-						
+	6448	NT	4.055	+	3.179	+	3.760	+	0.673	+	1.027	+	1.361	+	1.160	+	0.062	+	1.061	-						
+	6449	NT	1.464	+	0.898	+	0.940	+	0.531	+	0.710	+	0.485	+	0.964	+	0.020	+	1.111	-						
-	6411	NT	0.397	-	0.386	-	0.092	-	0.245	-	0.550	+	0.026	-	-0.001	-	1.067	-	1.006	-						
-	6413	NT	0.395	-	0.000	-	0.003	-	0.102	-	0.088	-	0.017	-	0.127	+	0.939	-	0.913	-						
-	6427	-	0.429	+	0.355	-	-0.001	-	0.143	-	0.163	-	0.030	-	0.003	-	0.900	-	0.887	-						
-	6428	NT	0.469	+	0.073	-	0.019	-	0.224	-	0.351	-	0.014	-	-0.002	-	0.916	-	0.872	-						
-	6431	NT	0.544	+	0.427	-	0.222	-	0.286	-	0.203	-	0.009	-	0.046	-	1.086	-	1.067	-						
-	6434	-	0.244	-	0.028	-	-0.006	-	0.102	-	0.578	+	0.017	-	-0.003	-	0.930	-	0.924	-						
-	6440	NT	0.519	+	0.096	-	0.014	-	0.122	-	0.171	-	0.012	-	0.043	-	0.899	-	0.968	-						
-	6450	NT	0.741	+	0.066	-	0.102	-	0.163	-	0.185	-	-0.006	-	0.031	-	1.032	-	1.116	-						
-	6452	NT	0.576	+	0.144	-	0.000	-	0.184	-	0.157	-	0.028	-	0.002	-	1.026	-	1.056	-						
Group 3 (7 of 9 test agree)																										
+	6410	NT	1.556	+	0.622	+	1.104	+	0.204	-	0.999	+	0.976	+	0.670	+	-0.005	+	0.868	-						
+	6416	NT	2.705	+	1.735	+	2.205	+	0.265	-	0.940	+	0.165	+	0.450	+	0.542	+	0.863	-						
+	6426	NT	2.812	+	2.306	+	2.186	+	0.612	+	0.660	+	0.097	+	0.714	+	0.002	+	0.894	-						
-	6415	NT	0.282	-	0.090	-	0.025	-	0.429	+	0.481	+	0.058	-	0.023	-	0.878	-	0.859	-						
-	6417	NT	0.950	+	0.427	-	NT	NT	0.429	+	NT	NT	0.045	-	0.021	-	0.878	-	0.819	-						
-	6424	NT	0.489	+	0.168	-	0.175	-	0.204	-	0.280	-	0.021	-	0.142	+	0.840	-	0.885	-						
-	6432	NT	0.464	+	0.039	-	0.037	-	0.143	-	0.172	-	0.020	-	0.171	+	1.043	-	1.038	-						
-	6444	NT	0.259	-	0.004	-	0.006	-	0.571	+	0.470	+	0.016	-	0.097	-	0.993	-	1.122	-						
-	6447	NT	0.218	-	0.035	-	0.011	-	3.224	+	3.313	+	0.011	-	-0.007	-	1.020	-	1.075	-						
-	6453	NT	0.650	+	1.035	+	0.077	-	0.245	-	0.306	-	0.011	-	0.007	-	0.981	-	1.068	-						
Group 4 (6 of 9 test agree)																										
+	6419	NT	1.448	+	1.100	+	0.704	+	0.306	-	0.388	-	0.164	+	0.714	+	-0.005	+	0.842	-						
+	6446	NT	2.022	+	1.623	+	1.284	+	0.265	-	0.288	-	0.663	+	0.359	+	0.043	+	1.039	-						
+	6458	NT	3.702	+	0.004	-	2.865	+	0.306	-	0.812	+	0.613	+	0.725	+	0.000	+	0.865	-						
-	6412	NT	0.711	+	0.313	-	0.160	-	0.163	-	0.247	-	0.696	+	0.160	+	0.858	-	0.901	-						
-	6425	NT	0.754	+	0.402	-	0.311	-	0.245	-	0.416	+	0.017	-	0.370	+	0.817	-	0.892	-						
-	6429	NT	0.498	+	0.382	-	0.225	-	1.306	+	0.512	+	0.007	-	0.028	-	0.950	-	0.919	-						
-	6430	NT	0.455	+	0.320	-	0.113	-	0.265	-	0.299	-	0.234	+	0.206	+	0.987	-	0.933	-						
-	6445	NT	0.520	+	0.210	-	0.208	-	0.306	-	0.433	+	0.025	-	0.109	+	1.017	-	1.098	-						
-	6455	NT	0.618	+	0.639	+	0.107	-	0.204	-	0.246	-	0.139	+	0.057	-	0.991	-	1.040	-						
-	6456	NT	0.769	+	0.382	-	0.187	-	0.204	-	0.284	-	0.004	-	0.310	+	0.420	+	0.969	-						
Group 5 (5 of 9 test agree)																										
+	6414	NT	1.590	+	0.649	+	0.541	-	0.122	-	0.663	+	0.465	+	0.226	+	0.893	-	0.821	-						
+	6437	NT	1.199	+	0.592	-	0.528	-	0.245	-	0.452	+	0.222	+	1.165	+	0.002	+	0.869	-						
+	6454	NT	1.036	+	0.317	-	0.564	-	0.306	-	0.516	+	0.144	+	0.220	+	0.471	+	1.047	-						
+	6457	NT	0.464	+	3.010	+	0.075	-	0.429	+	0.417	+	0.780	+	0.026	-	0.985	-	1.007	-						
-	6423	NT	0.969	+	0.422	-	0.567	-	0.837	+	0.390	-	0.078	-	0.657	+	-0.002	+	0.921	-						
-	6433	NT	0.734	+	0.116	-	0.067	-	0.898	+	0.411	+	0.031	-	0.237	+	0.992	-	0.986	-						
-	6439	NT	1.158	+	0.520	-	0.625	+	0.327	-	0.307	-	0.017	-	0.122	+	0.474	+	0.934	-						
-	6441	NT	0.851	+	0.293	-	0.211	-	0.286	-	0.314	-	0.130	+	0.384	+	0.077	+	1.189	-						
Number of negative samples in the consensus positive groups																										
Number of positive samples in the consensus negative groups																										
0			3			0			8			2			1			1			2			17		
21			2			1			7			9			4			12			4			60		

¹ ELISA data from Ross Breeders using their own preparations (11/14/99)

² Data from IDEX Laboratories

³ Data from ADOL using IDEX Kit (10/24/01)

⁴ Data from KPL laboratories (2/14/00)

⁵ Data from ADOL using KPL laboratories (11/2/01)

Table 7. Correlation coefficients between S/P ratios of the tests used to detect ALV-J antibody in the virus positive flocks.

	Ross ELISA (A)	IDEXX (B)	IDEXX (C)	KPL (A)	KPL (B)	FC HPRS-103	FC ADOL-Hc1	VN HPRS-103	VN ADOL-Hc1
Ross ELISA (A) ¹	-	0.730	0.966	0.077	0.336	0.814	0.617	0.767	0.262
IDEXX (B) ²		-	0.721	0.109	0.270	0.618	0.591	0.542	0.110
IDEXX (C) ³			-	0.101	0.363	0.777	0.562	0.690	0.138
KPL (A) ⁴				-	0.842	0.092	0.021	0.065	-0.066
KPL (B) ⁵					-	0.291	0.268	0.243	-0.005
FC HPRS-103						-	0.577	0.884	0.263
FC ADOL-Hc1							-	0.576	0.254
VN HPRS-103 ⁶								-	0.299
VN ADOL-Hc1 ⁶									-

¹ ELISA data from Ross Breeders using their own preparations (11/14/99)

² Data from IDEX Laboratories

³ Data from ADOL using IDEX Kit (10/24/01)

⁴ Data from KPL laboratories (2/14/00)

⁵ Data from ADOL using KPL laboratories (11/2/01)

⁶

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Serological and virological findings in normal broiler breeder flocks and those clinically affected with avian leukosis virus subtype J.

M.Malkinson^A, C.Noach^A, I.Davidson^A, A.F.Fadly^B and R.L.Witter^B

^A Kimron Veterinary Institute, Beit Dagan, Israel

^B USDA-ARS, ADOL, 3606 East Mount Hope Rd., East Lansing USA

SEROLOGICAL AND VIROLOGICAL FINDINGS IN NORMAL BROILER BREEDER FLOCKS AND THOSE CLINICALLY AFFECTED WITH AVIAN LEUKOSIS VIRUS J

SUMMARY:

Nine broiler breeder flocks comprising 5 flocks clinically affected with myeloid leukosis tumors (ML+) and 4 tumor-free flocks (ML-) were compared. Serologically, each flock was examined for avian leukosis subgroup J (ALV-J) serum antibodies by ELISA and for antigenemia (group-specific GS antigen- *gsa*) by antigen-trapping ELISA. GS antigen were detected in 25 out of 43 sera (58.1%) of ML+ birds compared with 13 out of 28 ML- birds (46.4%; $p < 0.08$), while 20 out of 44 ML+ birds (45.5%) had ALV-J antibodies compared with 7 out of 29 ML- birds (24.1%; $p = n.s.$) For virus assay, sera from 39 ML+ birds and 30 sera from non-ML flocks were inoculated on C/E chick embryo fibroblasts and 7 days later the harvested medium was examined for *gsa* content. In the ML+ group, 25 (64.1%) were GSA positive while only 5 (16.7%; $p < 0.05$) of the ML- sera had *gsa*. Similar differences between the 2 groups were found regarding detection of ALV-J in C/E cells by immunofluorescence (16/27 vs. 4/22), and of viral RNA by PCR using 2 different sets of env-gene primers H5-H7 (17/28 vs. 3/23) and R5-F5 (14/18 vs. 2/23). Using the primer pair R5-F5 the product size was approximately 1000 bp, while some heterogeneity in size among isolates was discernable.

MATERIALS and METHODS

Birds

In the study, nine broiler breeder flocks were selected for laboratory investigation. Five of them presented clinical myeloid leukosis and the other four experienced non-specific losses. The ages of the birds ranged from 6.5 weeks to 38 weeks. The nine flocks included one broiler, one rooster, one replacement, and six pedigree breeding flocks

Serology

Blood was collected from birds at the farm or the laboratory. Serum was examined by ELISA for ALV-J antibody (Idexx kit) (Venugopal et al., 1997).

ALV group specific antigen (gsa) in serum was measured by ELISA (Crittenden and Smith, 1984). The plate was coated with rabbit anti-p27 IgG (SPAFAS #583301) diluted 1:5000 in carbonate coating buffer and incubated overnight at room temperature. The plate was washed once with PBS – Tween 20. Blotto (DIFCO #0032173) was then added for 20 minutes at room temperature, then washed once more. Serum samples were added undiluted to the plate which was then incubated at 37°C for 30 minutes. The plate was then washed three times. Rabbit anti-p27 IgG HRP conjugate (SPAFAS #573301) was diluted 1:1000 in blotto and incubated at 37°C for 30 minutes, then washed five times (Smith et al., 1979). Finally, ABTS substrate was added, the absorbance was read at 405nm after 15 minutes.

Virus isolation

Commercial SPF and line O embryos (ADOL East Lansing) were trypsinized to prepare chick embryo fibroblasts (cef). Secondary cef were inoculated in suspension and grown for 7 to 9 days (Fadly & Witter, 1999) in Leibovitz – McCoy medium (Biological Industries Ltd., Israel) with 0.5% fetal calf serum (Biological Industries Ltd., Israel). For each serum, two 60 mm plates of cells, with and without a glass coverslip were inoculated. The coverslip was used for immunofluorescent staining while the remaining cells on the plate were assayed for GSA by ELISA. DNA was extracted from the

cells in the other plate for PCR. The ELISA was performed on cef extracts as described above. To prepare the antigen, the supernatant was removed and the cells were lysed with PBS – Tween 20 (2%), then frozen and thawed three times. Fibroblasts were also infected with tenfold serial dilutions (10^{-1} to 10^{-7}) of three reference ALV-J isolates (ADOL 5701, ADOL 6803 and HC1) supplied by Dr A. Fadly, East Lansing (Fadly & Smith, 1999).

Immunofluorescence

After removal from the plate, the coverslip was washed briefly in PBS and fixed with cold acetone, then air-dried for a few minutes. Virus was identified with a monoclonal antibody supplied by Dr. Venugopal (AHT, Compton U.K.). It was diluted 1/100 in PBS and incubated on the coverslip for 1 hour at room temperature, followed by 3 washes with PBS of 5 minutes each. The anti-mouse FITC conjugate (Sigma #F0257) was diluted 1/30 in PBS and incubated for 1 hour at room temperature. The first wash included 1% Evans blue for counter-staining followed by 2 more washes with PBS. The slide was air-dried for one minute. Mounting buffer and a coverslip were attached and the slide examined by UV microscopy.

PCR

The medium was removed from the second plate and the cells were scraped off and pelleted by centrifugation. The pellet was treated with lysis buffer (SDS 0.5%, NaCl 0.1M, Tris pH 8.0 10mM, EDTA 1mM, proteinase K 200ng/ml) for 1 hour at 56°C. DNA was extracted by phenol-chloroform method (Sambrook et al.). The ALV-J env gene was amplified by three separate PCR systems (Table 1).

1. Primers H5-H7 (Smith et al., 1998)

DNA was amplified in a 25 μ l reaction with 10mM Tris HCl (pH 8.8), 50mM KCL, 2.0 mM MgCl₂, dATP, dCTP, dGTP, dTTP each at 400 mM, 1.2mM of each primer, 2% formamide and 0.5 unit Taq polymerase (Advanced Biotechnologies Ltd., U.K.). Cycling program: one cycle of 93°C for 1 min, 13 cycles of N°C for 1 min and 72°C for 1.5 min (N°C starting at 60°C,

decreasing by 1°C at each cycle) then 30 cycles of 93°C for 1 min, 48°C for 1 min and 72°C for 1.5 min, followed by final elongation at 72°C for 10 min.

II. Primers H5-AD1 (Smith et al., 1998)

DNA was amplified in a 25µl reaction with 10mM Tris HCl (pH 8.8), 50mM KCL, 2.0mM MgCl₂, dATP, dCTP, dGTP, dTTP each at 200mM, 0.6mM of each primer, 1% formamide and 0.5 unit Taq polymerase (Advanced Biotechnologies Ltd., U.K.), using the H5-H7 cycling program.

III. Primers R5-F5 (R. Silva ADOL, East Lansing, MI., U.S.A., personal communication)

DNA was amplified in a 25µl reaction with 10mM Tris HCl (pH 8.5), 50mM KCL, 1.5mM MgCl₂, dATP, dCTP, dGTP, dTTP each at 40mM, 1.2mM of each primer and 0.5 unit Taq polymerase (Advanced Biotechnologies Ltd., U.K.). The first step of the cycling program was 3 min at 95°C, then 30 cycles of 95°C for 1 min, 57°C for 1 min and 72°C for 2 min, followed by a final elongation at 72°C for 10 min.

RT-PCR

RNA was extracted directly from the serum using a Qiagen kit (QIAamp Viral RNA kit #52904). RNA (5µl) was heated with 0.5µl of the direct primer (H5 for PCR with primers H5-H7 and H5-AD1, F5 for PCR with primers F5-R5) at 60°C for 2 min. The reaction mix: buffer: 0.5µl dNTP (each at 200mM), 0.5µl RNAsin, 0.12µl super AMV reverse transcriptase (CHIMERX, Wisconsin, USA #1372-01) complete to 10µl with water, was then added. The reaction mixture was incubated at 39°C for 90 min. The PCR was performed as described above.

Sequencing

2µl of DNA was amplified in a 50µl reaction with 10mM Tris HCl (pH 8.3), 50mM KCL, 1.5mM MgCl₂, 0.001% gelatin, dATP, dCTP, dGTP, dTTP each at 25mM, 25pmoles of each primer (6 and 2 of Silva) and 0.5 unit Taq polymerase (Sigma #D1806). The first step was incubation for 3 min at

95°C, then 30 cycles of 95°C for 1 min, 57°C for 1 min and 72°C for 2 min, followed by a final elongation at 72°C for 5 min.

The PCR product was then cloned with the original TA cloning Kit (Invitrogen #K2040, Carlsbad, CA, USA). The insert size was checked by restriction with EcoR1 (Gibco). The selected clones were amplified and the plasmid DNA was extracted with the S.N.A.P. miniprep Kit (Invitrogen #K1900, Carlsbad, CA, USA).

RESULTS

Titration of ALV-J viruses in chicken embryo fibroblast culture as detected by ELISA, IF and PCR with primers H5-H7 and R5-F5

To establish the sensitivity of the three assays to detect ALV-J, we initially tested them with each of the three ALV-J prototype strains: HC1, ADOL 6803 and ADOL 5701. The sensitivity of each of the three assays is shown in Table 2 and the electropherogram of the PCR is shown in Fig 1.

With the ELISA, the replication of the ALV-J strains in cef yielded end points of 10^{-4} for ADOL 6803 and HC1, and 10^{-6} for ADOL 5701.

Immunofluorescence was observed in those dilutions that were also gsa positive except for isolate ADOL 6803 that was tenfold less sensitive by IF giving an end point of 10^{-3} .

PCR performed with two primer pairs, H5-H7 and R5-F5 were equally sensitive in detecting proviral DNA after 7 days in culture. Overall, the ELISA and PCRs were tenfold more sensitive than IF for ADOL 6803 in yielding an end point of 10^{-4} . The two PCRs were tenfold less sensitive than IF and ELISA for ADOL 5701 giving an end point of 10^{-6} . For HC1, the two PCRs detected virus at a dilution of 10^{-5} that was tenfold more sensitive than detected by ELISA and IF.

Comparison of the sensitivity of PCR with H5-H7 and R5-F5 primers using the DNA extracted from HC1-infected cef

ALV-J strain, HC1 DNA template was employed to compare the sensitivities of two primer pairs, H5-H7 and R5-F5. The DNA was diluted tenfold through 10^{-5} .

Primers H5-H7 appeared to be ten times more sensitive than R5-F5 (Fig 2 and Table 3). However, the optimal PCR conditions were different since the volume of DNA template for the R5-F5 primers in the mixture was 1µl and 3µl for H5-H7. Thus, the actual difference in sensitivity was only 3-fold, which is not significant.

The sensitivity of the two PCR systems for ALV-J field isolates.

C/E cells were inoculated with sera from clinically affected birds and virus replication was assayed by PCR. The DNAs of the isolates that were detected by H5-H7 primers but not by R5-F5 primers, were diluted 1/10 and the two PCRs were repeated for undiluted and diluted viral DNA with both sets of primers. (Table 4)

The results show that one isolate (Z 2) was undetected by R5-F5, suggesting a sequence change at the site amplified by the R5-F5 primers while the other three isolates (KM 7, KY 1 and KY 5) differed by one tenfold dilution, indicating a low level of proviral DNA.

Comparison of two PCR systems for detecting field isolates of ALV-J.

Nine flocks that were positive by ELISA or by immunofluorescence were further examined by PCR. The sizes of the PCR product with the R5-F5 primers were around 1000 bp (denoted +low and +high in Table 5). Amplification revealed that within individual flocks, the size of the PCR product with the primers R5-F5 was constant, while some differences in product size were observed between flocks. This finding indicates the clonality of the isolates on an individual flock, and also the heterogeneity of ALV-J strains circulating in Israeli farms (Fig 3 and Table 5).

Farm G

A flock of 18 weeks old roosters was further investigated because it presented clinical signs of myeloid leukosis, whereas tissue culture propagated virus was negative by PCR. The sera were therefore tested by RT-PCR (Fig 4 and Table 6).

On serological examination, high antibody levels were recorded but only a relatively low antigenemia. RT-PCR was performed with primers H5-H7

and with H5-AD1 and yielded three positive ALV-J and seven positive ALV A-E samples. ELISA detected gsa in cef inoculated with 4 serum samples (all four were RT-PCR positive with H5-AD1 while two were positive with H5-H7). None of the four samples was ALV-J PCR positive. Thus the serum antigen detected by ELISA may belong to another ALV subgroup that was detected in the serum by RT-PCR.

The negative ALV-J PCR from chick embryo fibroblast DNA may be explained by technical reasons such as interference with the replication of ALV-J in cef, non-vital virus in serum or an undetectable level of virus.

Comparison of detection of gsa and ALV-J antibody, by ELISA, IF and PCR in flocks with clinical myeloid leukosis and in flocks experiencing non-specific losses.

Nine commercial broiler breeder flocks of different ages and suffering from elevated losses and presenting a wide spectrum of clinical findings ranging from non-specific lesions e.g., stunted growth, egg peritonitis to clinical ML were examined. The flocks were divided into two groups based on the presence or absence of ML tumors notably on the inner aspect of the sternum at necropsy (Tables 7 and 8)

A significant difference ($p < 0.08$) between the numbers of gsa-positive serum samples in the ML and non-ML flocks (68.4% vs. 46.4%) was found, but not in the numbers of ALV-J antibody containing birds (41.6% vs. 34.5%)

The presence of infectious virus in some of the surveyed flocks was established by IF and gsa production in cell culture rather than by PCR. A high level of viremia was detected in ML flocks with 60.5% of the sera were gsa positive after 7 days in culture. Of these isolates, more than half was confirmed by PCR as ALV-J. This may be due either to failure of the primers to detect all field isolates or the possibility that other ALV sub-groups were circulating in the flocks. This alternative is less likely if the difference in the gsa ratios between the two groups is real (60.5% vs. 10%; $p < 0.001$), although 3 out of 4 were identified as ALV-J positive. Moreover, there was a close agreement between the ratios of positive ALV-J samples in the ML-positive flocks by each method (PCR = 54.2%, IF = 53.8%, GSA = 60.5%). In the

ML-negative flocks, the absolute number of birds that were positive by each method was almost the same (PCR = 3, IF = 4, GSA = 3 birds).

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Sambronde et al.

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Table 1: The primer pairs used in the present study.

subgroups	Product size	Primers	Sequence 5'-3'
ALV A-E	295-326 bp	H5 (dir.)	GGA TGA GGT GAC TAA GAA AG
		AD1 (rev.)	GGG AGG TGG CTG ACT GTG T
ALV J	545 bp	H5 (dir.)	GGA TGA GGT GAC TAA GAA AG
		H7 (rev.)	CGA ACC AAA GGT AAC ACA CG
ALV J	1 000 bp	F5 (dir.)	GGT ATT TTC TTG ATT TGT GGG G
		R5 (rev.)	GAT TCG CAA GTT TCA TAC CCC T
ALV-J	2 300 bp	6 (dir.)	CTT GCT GCC ATC GAG AGG TTA CT
		2 (rev.)	AGT TGT CAG GCA ATC GAC

Table 2: Comparison of three methods to detect ALV-J strains ADOL 6803, ADOL 5701 and HC1 replication after 7 days in cell culture.

ADOL 6803 Dilution	ELISA gs antigen	IF	PCR	
			H5-H7	R5-F5
10 ⁻¹	NT*	+	+	+
10 ⁻²	NT	+	+	+
10 ⁻³	+	+	+	+
10 ⁻⁴	+	-	+	+
10 ⁻⁵	-	-	-	-
10 ⁻⁶	-	-	-	-
10 ⁻⁷	-	-	-	-

ADOL 5701 Dilution	ELISA gs antigen	IF	PCR	
			H5-H7	R5-F5
10 ⁻¹	NT	+	+	+
10 ⁻²	NT	+	+	+
10 ⁻³	+	+	+	+
10 ⁻⁴	+	+	+	+
10 ⁻⁵	+	+	+	+
10 ⁻⁶	+	+	-	-
10 ⁻⁷	-	-	-	-

HC1 Dilution	ELISA gs antigen	IF	PCR	
			H5-H7	R5-F5
10 ⁻¹	NT	+	+	+
10 ⁻²	NT	+	+	+
10 ⁻³	+	+	+	+
10 ⁻⁴	+	+	+	+
10 ⁻⁵	-	-	+	+
10 ⁻⁶	-	-	-	-
10 ⁻⁷	-	-	-	-

*NT: not-tested

Table 3: Level of detection of DNA with two sets of primers.

Template DNA dilution	ALV-J primers	
	H5-H7	R5-F5
Undiluted	NT	+
10 ⁻¹	+	+
10 ⁻²	+	+
10 ⁻³	+	+
10 ⁻⁴	+	-
10 ⁻⁵	-	NT

Table 4: Comparison of sensitivity of two sets of primers with various field isolates.

Field isolates	Dilution	H5-H7	R5-F5
Z 2	undiluted	+	-
	10 ⁻¹	+	-
KM 7	undiluted	+	-
	10 ⁻¹	-	-
KY 1	undiluted	+	-
	10 ⁻¹	-	-
KY 5	undiluted	+	-
	10 ⁻¹	-	-

Table 5: PCR of laboratory strains and field isolates with the two sets of primers.

No.	Name	Age of flock (wks)	H5-H7	R5-F5
1	c/e cells		-	-
2	Z	34	+	+ high
3			+	-
4			-	-
5			-	-
6			+	+ high
7			-	-
8			+	+ high
9			-	-
10	S	38	+	+ low
11	B	6.5	+	+ high
12	KM	36	+	-
13	KY	30	+	-
14			+	+ high
15			+	-
16			-	-
17	G	18	-	-
18			-	-
19			-	-
20	SB	14	+	+ low
21	Ca		+	+ high
22	An		+	+ high
23			+	+ high
24			+	+ high
25			+	+ high
26			+	+ high
27	Hc1		+	+ high
28	ADOL 6803		+	+ low
29	ADOL 5701		+	+ low

Legend to figures:

Fig 1: Comparison of PCR detection of ALV-J DNA prepared from C/E cells inoculated with tenfold dilutions (10^{-1} to 10^{-7} noted 1 to 7) of three prototype viruses: ADOL 6803, ADOL 5701 and HC1 and cultured for 7 days.

M: molecular weight (1Kb DNA ladder, Gibco BRL U.S.A., # 15615-016).

-: negative control.

1018bp and 517bp: molecular weight of the marker.

“high”: PCR product and “low”: PCR product.

Fig 2: Sensitivity of two PCR systems: H5-H7 and R5-F5 applied to one DNA template undiluted (noted 0) and diluted from 10^{-1} to 10^{-5} (noted 1 to 5).

1000: molecular weight of the R5-F5 product.

545: molecular weight of the H5-H7 product.

Fig 3: Comparison of field isolates with the primers R5-F5. Number of the isolates: see Table 5.

Fig 4: RT-PCR of flock G sera amplified with the primers H5-H7 and H5-AD1. Product of H5-H7: 545 bp, Product of H5-AD1: 295-326 bp.

Fig. 1

Fig 2

Fig. 2

Fig. 3

H5-H7

H5-AD1

[25.4]