AVIAN LEUKOSIS VIRUS AS A RISK FACTOR FOR OVARIAN ADENOCARCINOMA IN LAYER HENS

BY

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THESIS

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Abstract

Background: Ovarian cancer has the highest mortality of all the gynecological cancers in women. It is the fifth leading cause of cancer death among women due to late stage clinical diagnosis when treatment options are less effective. An important limiting factor in the development of new and effective treatments is identification of a suitable animal model. Ovarian adenocarcinoma (OAC) occurs spontaneously in hens, as in women, and increases in prevalence with age. Ovarian adenocarcinoma in women and hens have similar histologic features, biomarker staining and epidemiological characteristics. Recently, human endogenous retroviruses (HERVs) have been shown to be associated with ovarian cancer in women. Hens also have endogenous retroviruses, notably Avian Leukosis Virus E (ALV-E). The link between ALV-E and OAC in hens has not been adequately investigated. ALV infection in hens can be diagnosed by detecting ALV antigen in serum using an antigen-capture ELISA. However, this test does not distinguish endogenous ALV-E from exogenous ALV subgroups. ALV-E can be specifically identified by detecting expression of Eenv in RNA isolated from tissues.

Hypotheses*:* 1.The prevalence of ALV, Eenv mRNA expression, and OAC in aged birds in the University of Illinois Urbana-Champaign (UIUC) Poultry Research Laboratory laying flock is > 5%. 2. Birds that have OAC are at increased risk of being positive for ALV by antigen capture ELISA than those that do not have OAC. 3. Birds that have OAC are at increased risk of manifesting expression of Eenv mRNA in their spleen than those that do not have OAC.

Animals*:* 177 White Leghorn hens of three different age groups: 104 weeks old, 130 weeks old and ≥ 165 weeks old

Methods*:* Hens were stratified by age and then randomly selected. Blood was withdrawn via jugular or cardiac venipuncture and the hens were humanely euthanized in a carbon dioxide

chamber and immediately necropsied. Tissues harvested included ovaries, spleens and any gross lesions. Tissues were fixed in 10% buffered formalin for histopathology. Serum was used to conduct the ALV ELISA and the spleen was used to extract RNA and perform RT-PCR to detect ALV-Eenv mRNA expression.

Results*:* The overall prevalence of OAC was 22.6% with significant associations between OAC and age, and ALV and age. Hens with OAC were 5.2 times more likely to be ALV positive than hens without OAC, and hens with OAC in the 165-week age stratum were also 5.2 times more likely to be ALV positive then hens without OAC in this age stratum. ALV-Eenv mRNA expression was not uniformly expressed across the three age strata; there was a tendency for older hens and hens with OAC to be more likely to express ALV-Eenv mRNA. ALV-Eenv mRNA expression was associated with an increased risk of being ALV positive. Older hens and hens with OAC were more likely to express ALV-Eenv mRNA.

Conclusions*:* This is the first time a viral infection has been associated with OAC in hens. Endogenous ALV-E in hens may be analogous to the HERV's, which have been associated with OAC in women. Since the risk of ALV, Eenv mRNA expression, and OAC all increased with age, additional studies are needed to determine causal relationships.

Dedication

To my parents, Sarran and Marie Ramoutar, whose love and unwavering support of my dreams have taken me to heights I would never have achieved on my own.

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List of Abbreviations

ALV-	Avian Leukosis Virus
ALV-E-	Avian Leukosis Virus subgroup E
BLV-	Bovine Leukemia Virus
BRAF-	B-Raf proto-oncogene serine/threonine-protein kinase
CA125-	Cancer antigen 125
CO_2-	Carbon dioxide
COFAL-	Complement Fixation Test for Avian Leukosis
C.I.-	Confidence interval
DEPC-	Diethylpyrocarbonate
dNTP-	Deoxyribonucleotide triphosphate
EF1α-	Eukaryotic elongation factor 1 alpha
EGFR-	Epidermal growth factor receptor
ELISA-	Enzyme-linked Immunosorbent Assay
FeLV-	Feline Leukemia Virus
FIGO-	International Federation of Gynecology and Obstetrics
FSH-	Follicle stimulating hormone
GnRH-	Gonadotropin releasing hormone
HERVs-	Human endogenous retroviruses
HRT-	Hormone replacement therapy
KRAS-	Kirsten rat sarcoma viral oncogene homolog
LH-	Luteinizing hormone
LTR-	Long terminal repeat
MLV-	Murine Leukemia Virus
Muc-16-	Mucin 16
OAC-	Ovarian adenocarcinoma
OSE-	Ovarian surface epithelium
PCNA-	Proliferating Cell Nuclear Antigen
PR-	Progesterone receptors
PTEN-	Phosphatase and tensin homolog
RIA-	Radioimmunoassay
RIF-	Resistance-inducing factor interference procedure
RSV-	Rous Sarcoma Virus
RT-PCR-	Reverse transcription polymerase chain reaction
SPF-	Specific pathogen free
TAE buffer-	Tris-acetate EDTA buffer
TE buffer-	Tris-EDTA buffer
TGFα-	Tumor growth factor alpha
UIUC-	University of Illinois at Urbana Champaign
USA-	United States of America

Chapter 1. Introduction

Ovarian cancer has had a devastating effect on women. In 2009, ovarian cancer was responsible for approximately 14,600 deaths and 21,550 new cases[1] in the United States of America (USA). It is estimated that one in every 71 women will develop ovarian cancer with an overall lifetime risk of 1.5%[2]. Although ovarian cancer accounts for only 5% of all cancers in women in the USA[3], it has the highest mortality of all cancers of the female reproductive system[4], and is the 5th leading cause of cancer death among women[3], with a case fatality rate of approximately 68%[3]. Annual treatment costs for ovarian cancer in the USA are estimated to be $ 2.2 billion[1].

An important limiting factor in the development of new and effective treatments for ovarian cancer is the identification of a suitable animal model. Women develop ovarian adenocarcinoma (OAC) spontaneously, which rarely occurs in most species except for rodents and chickens. Various strains of mice and rats will spontaneously develop ovarian tumors of a wide variety of histological subtypes[5], but do so at a very low prevalence and late in life. For this reason, these species are most often induced to develop OAC for research[5]. The laying hen develops OAC at a much higher prevalence with similar histologic features, biomarker staining and epidemiological characteristics to OAC in women and therefore can be used as a practical model without a need for artificial induction of the tumor. Recently, human endogenous retroviruses (HERVs) have been shown to be associated with ovarian cancer in women[6]. No viral risk factor thus far has been shown in hens to cause OAC. Avian Leukosis Virus subgroup E (ALV-E) is an endogenous retrovirus of chickens that is considered to be non-pathogenic. However, with the discoveries of HERV's and their association with OAC in women, we

hypothesize that ALV-E may be analogous to HERVs, in that HERVs can be considered potential risk factors for ovarian oncogenesis. The Avian Leukosis group of viruses is well documented as being associated with a variety of oncogenic disorders, such as B-cell lymphoma/leukemia, erythroleukemia (erythroblastosis), myeloid leukemia (myeloblastosis or myelocytomatosis) and connective tissue tumors (fibrosarcoma, nephroblastoma, etc.)[7-9]. Recently, the emergence of exogenous infectious ALV-J has been shown to actually be a re-emergence of an endogenous strain of ALV-J (ev/J 4.1 Rb)[10]. These findings, coupled with the finding that 2% of the chicken's genome is comprised of endogenous retroviral DNA, with 11 new families of these viruses recently discovered[11], indicates that further investigation is needed to determine the relationship between ALV-E (and other ERV) and OAC. If there is a relationship, then this relationship will further augment the application of the hen as a model for OAC in humans.

The specific objectives of this thesis research project were:

1. To determine the prevalence of OAC in aged birds in the UIUC poultry research laboratory laying flock.
2. To determine the association between ALV and OAC in aged birds in the UIUC poultry research laboratory laying flock
3. To determine the association between expression of ALV-E encoded mRNA and OAC in aged birds in the UIUC poultry research laboratory laying flock.

<u>Hypotheses</u>

1. The prevalence of ALV, ALV-Eenv mRNA expression, and OAC in aged birds in the UIUC poultry research laboratory laying flock is > 5%.

2. Birds with OAC are at increased risk of being positive for ALV
3. Birds with OAC are at increased risk of manifesting expression of ALV- Eenv mRNA in their spleen than those that do not have OAC.

Chapter 2. Literature Review

Introduction

Ovarian cancer has had a devastating effect on women. The National Cancer Institute estimated that in the USA in 2009 there were 21,550 new cases of ovarian cancer diagnosed and 14,600 deaths due to ovarian cancer[1]. One in every 71 women will develop ovarian cancer during their lifetime[2]. Although ovarian cancer accounts for only 5% of all cancers in women in the USA[3], it has the highest mortality of all cancers of the female reproductive system[4]. With a case fatality rate of approximately 68%[3], ovarian cancer is the 5th leading cause of cancer death among women[3]. Annual treatment costs for ovarian cancer in the US are estimated to be $ 2.2 billion[1].

One important limitation for the development of new and effective treatments for ovarian cancer is the identification of a suitable animal model. Spontaneous occurrence of OAC is rarely seen in most species. Various strains of mice and rats will spontaneously develop ovarian tumors of a wide variety of histological subtypes[5]. Spontaneous onset of OAC in rodents typically begins at a later age and the rate of occurrence is very low[5] making them suboptimal as models for experimental study of ovarian cancer in humans. One proposed explanation for this stems from the fact that ovarian carcinoma is associated with frequent ovulation[12]. Adult female animals of most species, whether domestic or wild, are either persistently pregnant, lactating or seasonally anestrus. These physiological states are not associated with ovulation and ovarian cancer[13]. The domestic hen is an exception.

Hens show many similarities to humans related to the development of OAC. Hens, like women, are persistent ovulators and ovulate repeatedly for many years. Previous researchers have reported a 4% prevalence of spontaneous development of OAC in 2-yr-old hens[14]. This prevalence increases with age[15-17]. Both women and hens normally have simple, squamous to cuboidal epithelium covering the ovarian surface, with epithelial cells demonstrating nuclear staining for progesterone receptors (PR)[13]. In both species, cells of ovarian tumors stain strongly for cytokeratin (marker for epithelial cells) and proliferating cell nuclear antigen (PCNA) (an indicator of mitotic activity)[13]. Epidemiologic data suggest that progesterone may confer some protection from ovarian cancer in women[18], and treatment with progestin in hens has been correlated with a reduced incidence of the disease[14].

It is because of these similarities to women that several studies have used domestic hens as a research model for human OAC[13,15,17,19,20]. Human endogenous retroviruses have recently been associated with OAC in women[6] and raise the possibility that endogenous retroviruses may also be associated with OAC in hens. This literature review will compare and contrast the pathology and pathogenesis of OAC in women and hens, with particular attention to the current literature on the potential role of reverse transcribing retroviruses in OAC in both women and hens.

Ovarian Adenocarcinoma: Pathology and Pathogenesis in Hens: Laying hens possess only one functional ovary – the left ovary (Figure 1), with the right ovary and oviduct regressing during embryonic development. In the normal laying hen, the ovary contains a hierarchy of pre-ovulatory follicles with the largest yolk- filled follicle (F1) destined to

ovulate first, and the second largest (F2) to ovulate the following day[22]. Also observed on the normal ovary will be a number of small white and large yellow follicles.

A normal healthy hen's ovary has a layer of squamous to cuboidal epithelial cells on the surface[13]. The earliest histologically detectable structures of OAC, according to Fredrickson[15], are small groups of cells forming round acini, either in the ovarian stroma or growing near or within the theca externa. In some cases, early acini somewhat resemble thecal gland cells. Individual acini often enlarge to line slit-like spaces, or a cribriform pattern may predominate. In some tumors, individual acini are separated by wide bands of dense fibrous connective tissue. A characteristic of all forms of OAC is a single layer of low columnar or cuboidal cells surrounding lumens. Giles[19] reported that ovarian tumors in hens were often composed of columnar or high cuboidal epithelial cells with basally situated nuclei and abundant eosinophilic cytoplasm. The nuclei were vesicular with prominent nucleoli in some areas. These cells tended to form nests as well as glands of various sizes, often accompanied by a desmoplastic reaction. There were also atypical cells resembling squamous epithelial cells.

Ultrastructurally, the layer of cells surrounding the lumens of tubules and acini forms a tight adherent ring of cells joined along their apical borders by prominent desmosomes. A prominent feature is the presence of short microvilli projecting into the acinar lumen, which contains a variable amount of moderately electron-dense material. The tumor may also contain groups of darkly staining cells within the ovarian stroma or loose clusters of cells attached to the cortical surface. Such cells appear to have undergone degeneration, with formation of cytoplasmic vacuoles and densely osmophilic, irregularly shaped cytoplasmic inclusions. Glandular forms of the tumor are also seen.

Infrequently nests of cells appear to have undergone some degree of squamous metaplasia, with intervening areas containing structures similar to acini with transitional forms in adjacent areas[15]. Hens may also develop granulosa cell tumors and Sertoli cell tumors, but these occur less commonly.

The normal ovulatory cycle consists of a follicular phase compressed into a 25-27-hr period. During this brief period, subtle and stringently regulated release of gonadotropins (follicle stimulating hormone (FSH) and luteinizing hormone (LH)) promote follicular development and maturation which culminate in ovulation[22]. During this period there is a decrease in FSH receptors in the ovarian granulosa cells with a concomitant increase of LH responsiveness of these cells. Many commercial strains of laying hens will ovulate almost daily through 1 or 2 years of egg production.

Fredrickson[15] classified ovarian tumors in the hen into 4 stages depending on their gross characteristics. In stage 1, the growths are nodular, very firm, white and closely resemble atretic follicles. Ovarian adenocarcinomas tend to be less symmetric than atretic follicles and may be buried within the ovarian stroma or growing on the surface of follicles (Figure 2). In stage 2, the growths increase in size and coalesce so that the ovary takes on the appearance of a cauliflower, and loses its maturing follicles. It is these ovaries that seed the abdominal cavity with tumor cells. When large numbers of individual foci grow on serosal surfaces of the oviduct, mesentery, intestines, and pancreas, the patient is at stage 3. In stage 4, growth of implanted cells appears to be rapid, with pronounced reaction of the muscularis of the oviduct and intestine. The sequelae are contraction of the mesentery, bowel wall thickening, and ascites. As much as 500mL of fluid may be present in the coelomic cavity. Cystic structures, filled with clear

to translucent, amber-colored fluid can project from the surface of the ovary. Fredrickson[15] reported that these cysts are not tumorigenic but are associated with the development of OAC.

More recently, Barua[23] applied the tumor staging system used in women in accordance with the International Federation of Gynecology and Obstetrics (FIGO, Rio de Janeiro, 1988) to ovarian tumors of the hen. In stage I, the tumors were confined to the ovary, firm, and resembled cauliflower-like nodules with no or minimal accompanying ascites. In stage II, tumors had metastasized to the oviduct with occasional seeding of the pelvic sidewall and moderate ascites, and in stage III, tumors had metastasized to both abdominal and peritoneal organs including the small and large intestine, mesentery, abdominal undersurface of the diaphragm, and surface of the liver and there was moderate to profuse ascites. Lastly, in stage IV, there was evidence of carcinomatosis and massive ascites, and tumors had metastasized to most of the pelvic, abdominal, and thoracic organs including liver, spleen, and lung.

Ovarian Adenocarcinoma: Pathology and Pathogenesis in Humans. The ovaries in women are paired, and the surface is generally smooth in early reproductive life, becoming more convoluted as the person ages[1]. In women, ovarian surface epithelium (OSE) covers the entire ovarian surface and varies morphologically from simple squamous to cuboidal to low pseudostratified columnar[24]. Embryologically derived from the mesodermal epithelium of the gonadal ridges, OSE cells are continuous with the flattened mesothelium of the peritoneum[25] and are separated from the underlying stromal compartment of the ovary by a basement membrane.

The process of ovulation is controlled by the hypothalamus through the release of gonadotropin releasing hormone (GnRH) from the hypothalamus, which stimulates the anterior lobe of the pituitary gland to secrete LH and FSH. In the follicular (pre-ovulatory) phase of the menstrual cycle, the ovarian follicle undergoes a series of transformations that is stimulated by the secretion of FSH. Ovulation is triggered by a spike in the amount of LH released from the pituitary gland. Ovulation occurs during the follicular phase of the menstrual cycle, and is the transitionary period from the follicular phase into the luteal phase. This process (menstrual cycle) is 28 days in women.

There are three major groups of ovarian tumors that occur in women. The most commonly occurring type is epithelial derived tumors (85-90% of cases)[26], with germ cell and stromal tumors being less common. Stromal tumors tend to occur in younger women. Invaginations of the epithelium result in crypts or gland-like structures that can become pinched off to form epithelial inclusion cysts within the underlying stromal compartment[27]. This process may occur following the postovulatory proliferation of OSE, during follicular attrition, and/or as a result of inflammation caused by carcinogens or chemical irritants like talcum powder[28]. The incidence of inclusion cysts increases with advancing age and is common in postmenopausal women. Although generally benign in nature, these epithelial rearrangements are widely thought to be the potential origin of many epithelial cancers. The more frequent appearance of epithelial invaginations and inclusion cysts in women with a hereditary risk of ovarian cancer has strengthened this hypothesis[29]. In addition, some microscopic borderline malignant and malignant tumors have been observed to arise directly within these sites of epithelial rearrangements, and are often associated with dysplasia in the same or contralateral ovary[30-31].

According to the "incessant ovulation hypothesis", continuous ovulation, with its repeated rounds of surface rupture and OSE cell mitosis to repair the wound, renders the cells susceptible to malignant transformation[12]. Godwin et al[32]and Roby et al[33] demonstrated evidence supporting this theory by illustrating the susceptibility of OSE cells to mutagenic events during mitosis in primary cultures of normal rat and mouse OSE cells which had been repeatedly subcultured to maintain continued proliferation. These cells acquired features associated with malignant transformation, including loss of substrate-independent growth, loss of contact inhibition, and the ability to form tumors in nude mice. Epidemiologic studies support the hypothesis by revealing that a decrease in the number of ovulations reduces the risk of ovarian cancer [18,34-35].

Kurman and Shih[36] proposed a new model for the pathogenesis of OAC based on clinical, pathological and molecular genetics by dividing ovarian tumors into two broad groups, designated Type I and Type II. Type I tumors are slow growing and generally confined to the ovary at diagnosis. They develop from well established precursor lesions that are termed “borderline” tumors. Type I tumors include serous carcinoma, mucinous, endometrioid, clear cell, and low-grade micropapillary carcinomas. They are genetically stable tumors and are characterized by mutations in a number of different genes including KRAS, BRAF, PTEN, and beta-catenin. In contrast, Type II tumors are rapidly growing, highly aggressive neoplasms for which well defined precursor lesions have not been described. The vast majority of what is considered “ovarian cancer” belongs to the Type II category. Tumors in this category include high-grade serous carcinoma, malignant mixed mesodermal tumors (carcinosarcomas), and undifferentiated carcinomas.

Recent development of molecular biomarkers has enhanced detection and prognosis of ovarian cancers in women. The following biomarkers are present in human OAC and also stained OAC in hens. These biomarkers include antibodies that detect cytokeratins (AE1/AE3, pan cytokeratin), growth factor receptors (EGFR, erbB-2) and oncofetal tumor markers (Lewis Y, CEA and Tag 72). More specifically, epidermal growth factors (EGFR, erbB-2) and p185 (a product of a proto-oncogene) stained tumor cells diffusely, while cytokeratins AE1/AE3 and pan cytokeratin and the onco-fetal tumor markers were focally positive in the tumor. Antibodies against proliferating cell nuclear antigen (PCNA- a proliferation marker), transforming growth factor alpha (TGF-α- an activating ligand present on the epidermal growth factor receptor), and p27 (a cell cycle inhibitor) have stained hen OAC and have been useful as surrogate endpoints in chemoprevention trials in women[17]. Biomarker antibodies for OAC in women that were not cross-reactive in the hen included CA 125, Ki-67, and Muc 1[17].

CA 125 (cancer antigen or carbohydrate 125), also known as MUC16, is a protein that is most commonly expressed by the female reproductive tract (ovaries, endometrium, fallopian tubes), lungs, breast and gastrointestinal tract. Elevated levels of CA125 in the blood of some patients have been associated with specific types of cancers, but it is best known as a biomarker for ovarian cancer. However, its elevation is not exclusive to tumors as it can also be elevated in benign conditions such as endometriosis[37] and pregnancy[38]. Up to 20% of OAC cases do not express CA 125[39]. Ki-67 is a marker strictly for cell proliferation, but it is also found in normal tissues. However, the fraction of Ki-67 positive tumor cells (known as the Ki-67 labelling index) is mostly associated with carcinomas of the prostate and breast. Lastly, Muc 1 is a mucin that penetrates

membranes of epithelial cells, and protects the body from infection by binding to pathogens. Over-expression, aberrant intracellular localization and changes in glycosylation of Muc1 have been associated with carcinomas especially of the colon[40].

Another protein of interest is ovalbumin, which is the major protein formed in normal oviductal tissue of hens. Giles et.al[19] found that hens diagnosed with adenocarcinoma of the ovaries expressed ovalbumin in the ovary. They reported the presence of ovalbumin in 100% of hen ovaries with OAC in the absence of any oviductal involvement. This finding suggests that ovarian tumors may de-differentiate during the disease process and acquire characteristics of Mullerian duct-derived epithelia, a phenomenon that has also been described in women[41]. Thus, in this respect, ovarian tumors in the hen, and epithelial derived OAC in women show similar behavior, further supporting the claim that the laying hen is a good model for OAC in women.

Epidemiology of Ovarian Adenocarcinoma. Several specific risk factors have been identified for OAC in women. Inherited gene mutations, such as mutations of BRCA1 and BRCA2, were originally identified in families with multiple cases of breast cancer. However, women with these mutations also have a significantly increased risk of developing OAC. Other risk factors include a family history of OAC not due to any known gene alterations, and a personal history of breast cancer. Age has also proven to be a significant risk factor, in that OAC develops most often in postmenopausal women, with the risk increasing with age into the late 70's. Women who have had at least one pregnancy or have used oral contraceptives have lower risks of developing OAC. Infertility increases the risk of OAC, whether due to the pathology that led to infertility or

due to the use of fertility drugs. Hormone replacement therapy (HRT) in postmenopausal women, especially with estrogen-only therapies[42], increases the risk of OAC. Being obese also increases both the risk of OAC and the aggressiveness of the tumors. The use of androgens, such as danazol[43] which is used to treat endometriosis, may be linked to an increased risk of OAC.

Limited work has been done, however to identify risk factors for OAC in hens. One OAC risk factor that hens have in common with women is age. Previous reports indicate that the prevalence of OAC in hens increases with bird age[14-17]. Chickens with higher plasma estradiol concentrations and larger ovaries were at significantly increased risk for OAC[44]. In addition to the known risk factors for OAC in hens, genetics, flock husbandry and management practices, and history of exposure to pathogens should all be explored as potential risk factors. If additional risk factors are identified for hens, related factors could be investigated for women. These may be important determinants of the appropriateness of the hen as a human OAC research model.

Oncogenic Viruses. The occurrence of a viral infection and the subsequent development of cancer is not new. There are several examples in medicine of viral infections that induce cancer. Examples include: Bovine and Feline Leukemia Viruses, Rous Sarcoma Virus, Marek's Disease Virus, and Avian Leukosis Virus (ALV). In people, Human Papilloma Virus, Hepatitis Virus, Epstein-Barr Virus, Human Immunodeficiency Virus-1, and Human T-cell Lymphotropic Virus are all associated with the development of cancer.

Recently, human endogenous retroviruses (HERVs) have been identified as being associated with OAC. Wang-Johanning and colleagues[6] reported that the expression of

HERV-K *env* mRNA was greater in ovarian epithelial tumors than in normal ovarian tissues. In addition, other classes of HERVs were also detected in the same ovarian cancer tissues.

Viruses, particularly retroviruses, can contribute to oncogenesis by transduction of oncogenes, Cis and Trans activation of host genes, direct stimulation of cell growth by viral envelope proteins or retrovirus-mediated immunosuppression[9]. The polymerase (pol) region of the retrovirus is responsible for encoding the viral enzymes required for replication, e.g. reverse transcriptase for transcription of viral RNA into DNA, RNAse for removal of the RNA strand from the RNA-DNA double strand, integrase for integration of the virus into the host cell genome, and protease for proteolytic cleavage of the primary translation products[9]. The specific pathogenesis of several viral induced cancers has been described in detail in the literature.

Bovine Leukemia Virus (BLV) is a C-type oncogenic retrovirus belonging to the deltaretrovirus genus. It affects cattle and sheep and leads to a neoplastic proliferation of polyclonal B lymphocytes in a disease known as bovine enzootic leukosis. It is mainly horizontally transmitted via mechanical blood transfer. It is thought to induce oncogenesis by way of the viral Tax protein, which functions as a transcription factor regulating the expressions of both viral and host genes.

Feline Leukemia Virus (FeLV) belongs to the gammaretrovirus genus and includes three genotypes: FeLV A, B, and C. It affects cats and is spread by both vertical and horizontal transmission. It causes aplastic anemia, immunodeficiency syndrome, T-cell lymphoma and myeloid leukemia. The determinants of oncogenicity have been mapped to the U3 region of the long terminal repeat (LTR)[9].

Rous Sarcoma Virus belongs to the alpharetrovirus genus and affects chickens. The Rous Sarcoma Virus (RSV) genome has terminal repeats enabling its integration into the host genome and also over-expression of its genes[69]. The *src* gene is oncogenic and it triggers uncontrolled growth in abnormal host cells. It is an acquired gene, found to be present throughout the animal kingdom with high levels of conservation among species[69]. The *src* gene is taken up by RSV and incorporated into its genome conferring it with the advantage of being able to stimulate uncontrolled mitosis of host cells, providing abundant cells for fresh infection. The *src* gene is not essential for RSV proliferation but it greatly increases virulence when present[69].

Viral hepatitis-induced hepatic cancer in humans is caused by chronic infection with hepatitis B and C, which are horizontally transmitted. Hepatitis B is caused by a hepdna virus (a DNA virus), and Hepatitis C is caused by a hepacivirus (an RNA virus)[70]. Both viruses induce cancer by causing mutations in the p53 gene[70].

Like BLV, human T-cell lymphotropic virus (HTLV-1, Adult T-cell Leukemia, tropical spastic paraparesis) belongs to the deltaretrovirus genus. It affects humans and is transmitted by breast feeding, sexual contact, and transfusion of contaminated blood[71]. There are three main subtypes: acute, which has a poor prognosis, and chronic and smoldering, both of which have guarded prognoses. Its oncogenicity is primarily attributed to the action of the viral Tax protein, which regulates viral gene transcription by interacting with enhancer elements of the U3 region of the viral 5' LTR[45]. Tax is also responsible for regulating other cellular genes, thus impairing cellular homeostasis [46-47].

It has been theorized that endogenous retroviruses, were once harmful, infectious retroviruses that became established in the genome. Endogenous retro viruses have a

similar structure to infectious retroviruses. The Murine Leukemia Virus (MLV) for example has the typical genome structure of simple retroviruses, of which there are 4 genes: gag - encodes the viral core structural proteins; pro - encodes the viral protease; pol - encodes the viral enzymes including reverse transcriptase; and env which encodes the glycoproteins of the viral envelope.

The retroviral genome is bounded at each end by long terminal repeats (LTRs) that regulate viral gene transcription. Each LTR contains unique 3' (U3), repeat (R) and unique 5' (U5) elements. 'Unique' refers to the sequences being only at one end (3' or 5') of viral genomic RNA, whereas U3 and U5 are present at both ends of proviral DNA. The repeat is a short sequence (15–250 nucleotides) repeated at the 3' and 5' ends of both viral genomic RNA and proviral DNA. The primer-binding site (PBS) is close to the 50 LTR and is used by a specific transcription RNA (tRNA) molecule to initiate reverse transcription. Proviruses were disabled by mutations inhibiting the expression of proviral genes, causing them to evolve into harmless "junk" DNA over thousands of years[48]. Infectious retroviruses stimulate the cells in which they are expressed and enable the virus to evade an immune response by producing several accessory proteins that aid viral replication. Endogenous retroviruses possess the potential to express similar proteins and thus can become pathogenic. They can induce pathology in three ways. They can alter the immune system by suppressing it or stimulating it, by expressing accessory proteins that can directly affect infected cells, or they can disrupt genes at the integration sites[48]. Moyes *et. al* [48] proposed that genetic polymorphisms may be the answer as to how genes can cause disease in certain populations. They theorize that HERVs have polymorphisms that could affect the function of an expressed product. Insertionally polymorphic HERVs

are proviruses that are present only in a proportion of the human population. They are more likely to be pathogenic because their recent insertion might disrupt host genes, as the enhancer elements of retroviral LTRs can influence expression of neighboring cellular genes; as recent integrations, polymorphic HERVs are more likely to have retained functional coding sequences with the capacity to modulate cellular proliferation or the immune response[48].

Denesvre[10] demonstrated successfully the high sequence homologies and similar functional properties between endogenous ALV-J (ev/J 4.1 Rb) and ALV-J (exogenous infectious virus). This indicated that that the recent emergence of exogenous infectious ALV-J viruses is most likely due to re-combinational insertion of the ev/J endogenous sequences. They were also able to demonstrate that both the endogenous ev/J 4.1 Rb and exogenous ALV-J exhibited complete and full reciprocal interference to superinfection, indicating that they shared the same receptor. This may also be a possibility for ALV-E or other endogenous viruses in the chicken genome. Huda[11] found 11 new families of endogenous retroviruses in the Gallus Gallus genome. These new families occupy about 2% of the chicken genome. These endogenous viruses can interact with any number of events to cause disease. ALV-E may be just one of them.

Although laying hens develop OAC with histologic features and epidemiological characteristics that are consistent with human OAC, an association with a viral risk factor similar to that found in humans has yet to be established in hens. Fredrickson[15] conducted a 3.5 year study that investigated the incidence of reproductive tract neoplasia in specific pathogen free (SPF) White Leghorn flocks and discovered that 32% (149 of 466) developed ovarian tumors, with 8% (39) being oviductal tumors and 5% (22) being

benign leiomyomas of the suspending ligament of the oviduct. Overall 45% of the birds had tumors of the reproductive tract. Occurrence was unusual in birds less than 2 years of age, and the dominant neoplasia type was malignant OAC (24%). The hens used in this study were classified as free of ALV based upon the results of COFAL tests. COFAL is a complement fixation test for detection of group specific ALV antigens[49]. However, interpretation of COFAL test results must consider that the COFAL test requires a large amount of antigen and may suffer from interference by other substances[50]. When compared to other methods of ALV diagnosis, COFAL proved to be relatively insensitive and its findings did not correlate well with the other tests[50]. These limitations in the validity of COFAL test results may be even more pronounced when attempting to detect endogenous ALV virus compared to the exogenous subtypes. Fredrickson's finding of a high prevalence of OAC in birds that were COFAL negative for ALV does not support the hypothesis that an endogenous ALV may be an important risk factor for OAC. However the validity of this conclusion is now questionable given the relative insensitivity of the test and its questionable ability to detect the endogenous virus. As previously described, ALV has been associated with several types of sarcoma in chickens.

Avian Leukosis Virus was discovered in 1908 in Copenhagen[9]. The ALV are alpharetroviruses that are both horizontally and vertically transmitted. The ALV env gene encodes a polyprotein that is processed into two glycoproteins: gp85env and gp37env. Variation in the nucleotide sequences of gp85env define the ALV subgroups A, B, C, D, and E [51-55]. These subgroups cause neoplastic diseases such as B-cell lymphoma/leukemia, occasionally erythroleukemia (erythroblastosis) or myeloid

leukemia (myeloblastosis or myelocytomatosis). Sporadically they may cause connective tissue tumors (fibrosarcoma, nephroblastoma, etc.)[7-8]. They act oncogenically through the mechanism of promoter/enhancer insertion or by transduction of an oncogene. Many currently known proto-oncogenes were first identified as parts of rapidly transforming ALVs (myc, myelocytoma; erb, erythroblastosis; myb, myeloblastosis; src,sarcoma, etc.)[56]. Subgroup J contains sequences homologous to both endogenous and exogenous viral elements[56-58]. ALV-J has been observed with increasing mortality in adult birds with myeloid leukosis and tumor infiltration and enlargement of the liver, spleen, kidneys and other organs[13].

Oncogenic and exogenous Avian Leukosis/sarcoma groups A,B, C, and D are both horizontally and vertically transmitted in chickens, whereas subgroup E viruses are described as non-oncogenic, endogenous viruses that are transmitted vertically in a non-infectious form in a Mendelian fashion along with host genes [59-60]. However, this characterization as non-oncogenic may need to be reconsidered in light of the recent discovery of multiple human endogenous retrovirus (HERV) envelope proteins in association with OAC in women[6]. The discovery of an analogous association between endogenous ALV-E and OAC in hens would further validate the chicken as a valid research model for human OAC.

The domestic hen is one of only a few species in which spontaneous OAC occurs at a prevalence similar to that seen in women. Histopathologically, the tumors in hens and humans are very similar and similar tissue markers are expressed. Recently, Wang-Johanning[6] and colleagues reported a possible association between human endogenous retrovirus K (HERV-K) and OAC in women. Expression of the HERV-K env mRNA

was greater in ovarian epithelial tumors than in normal ovarian tissues, and other classes of HERVs were also expressed in the same ovarian cancer tissues. An association was demonstrated, but no causal relationship has been established. Identification of a retroviral etiologic agent responsible for the development of OAC would represent a major paradigm shift. However, demonstration of a causal relationship between endogenous retroviruses and OAC will require experimental manipulation of a suitable animal model. It is plausible that endogenous retroviruses may be associated with OAC in domestic chickens, but no such association has been reported in the current literature.

Thus, the next logical step would be to identify a relationship between a viral risk factor, endogenous or otherwise, with OAC in hens. The current study examines the presence of ALV and expression of a partial gp85env for ALV-E as risk factors for OAC in hens.

Chapter 3. Avian Leukosis Virus and Ovarian Adenocarcinoma in Hens

Introduction

Laying hens spontaneously develop OAC with histologic features, biomarker staining and epidemiological characteristics similar to those seen in women. These similarities have resulted in the hen being proposed as a suitable animal model for human OAC. Recently, HERVs have been found to be associated with ovarian cancer in women[6]. However, no viral risk factor has yet been identified in association with OAC in laying hens. ALV-E is an endogenous retrovirus of chickens that maybe analogous to HERV's in humans. The identification of an association between OAC in hens and an endogenous retrovirus risk factor would further validate the use of the hen as a model for human OAC and facilitate research into the pathogenesis of OAC. The goal of this study is to assess the association between OAC in laying hens and ALV infection in general and ALV subgroup E in particular.

Materials and Methods

Study Population: The study population was composed of 177 hens of > 100 weeks of age obtained from the UIUC Poultry Research Laboratory. This flock of laying hens consists of White Leghorn laying hens being reared in standard commercial caged-layer conditions. There are three age strata of caged laying hens in the subset of the flock that is above 100 weeks of age: 104 weeks, 130 weeks and 165 weeks or older. A pilot study was conducted to determine the prevalence of OAC and ALV in the flock. Serology, bacteriology, necropsies, and histopathology were conducted on a sample of thirty 165

week old birds that had been selected to be culled from the flock. The data obtained from the pilot study were used to establish the final sample size for the overall project.

Preliminary analysis of the data from the pilot study indicated that a total of 160 birds would be required to detect an association between ALV seropositivity and OAC at 80% power with 95% confidence. Therefore, 147 hens from the UIUC Poultry Research Laboratory were obtained to supplement data from the 30 hens in the pilot study (total of 177 hens). To ensure a representative sample of each age stratum, hens were selected using a random sampling technique. Laying hens at the UIUC Poultry Research Laboratory are housed in sections of cages with each section housing hens of the same age stratum. A random number list was generated in Microsoft Excel® that corresponded to the cage numbers. Each cage contained 4-6 hens of the same age. One hen was removed per cage. A new random list was created for each sample collection session. Hens were transported to an on-farm necropsy room just prior to sampling. Blood was obtained via jugular or cardiac venipuncture. The blood was collected into anti-coagulant free glass tubes, using a 19- or 20-gauge 1-inch needle. Hens were then humanely euthanized in a carbon dioxide (CO_2) chamber. Birds undergoing cardiac venipuncture were placed in the CO_2 chamber for 1 min until unconscious before obtaining blood. Once euthanized, the hens were necropsied within 5 min. Birds were examined for gross lesions of the coelomic organs. The coelomic cavity was examined for excess fluid and the spleen was either placed in a cellophane bag on ice, or snap frozen with liquid nitrogen. Spleens were stored at -80^0 C, within 2 hrs of being harvested. The ovaries and reproductive tract were examined grossly for tumors and placed in 10% buffered formalin. Other abdominal organs (liver, intestinal tract and kidneys) were examined for

evidence of metastasis. All tissues that appeared to be associated with tumors were harvested and placed in 10% buffered formalin.

Variables recorded for each hen included: age stratum (104 weeks, 130 weeks or 165 weeks old); presence or absence of gross lesions; ALV ELISA test status; and presence or absence of OAC, based on histologic examination. Data were stored in a Microsoft Excel®. Fisher's Exact test, X^2 analyses and multivariate logistic regression were used to determine associations between age stratum, ALV seropositive status, ALV-Eenv mRNA expression and histologic confirmation of OAC. Odds ratios were used to describe the strength of these associations. The statistical significance level was set at a p value of ≤ 0.05.

Antigen Capture ELISA: Blood was allowed to clot, centrifuged at 8,000 x g for 15 min and the serum was pipetted into 5 ml polypropylene tubes (BD Falcon™[A]). Tubes were stored at -80^0 C. Serologic testing was conducted using a commercially-available antigen capture ELISA test to screen for ALV (IDEXX Flockchek LL Antigen test ®[B]) according to the manufacturer's directions. The test detects antigen p27 which is common to all subgroups of ALV (A, B, C, D, E and J) and can be conducted on cloacal swabs, egg albumen, and serum. Serum samples are much more likely to detect endogenous ALV-E, while cloacal swabs tested with the antigen capture ELISA are more likely to detect exogenous ALV subgroups (A,B, C, D, and J)[63]. The tests were conducted using positive and negative controls provided by the manufacturer (contained in the test kit), and ELISA

[A] BD Falcon™. New Jersey USA
[B] IDEXX FLOCKCHEK Avian Leukosis Antigen Test Kit manual. Idexx Laboratories Maine USA

plates were read with a spectrophotometer (FLUOStar Optima microplate reader[C]) at the absorbance value of 650 nm. The results were interpreted by the sample to positive (S/P) ratio, where an S/P ratio of ≤ 0.20 was considered negative. An S/P ratio of > 0.20 indicated presence of p27 antigen and was considered positive.

Histopathology: The formalin-fixed tissues were trimmed, processed and stained with hematoxylin and eosin and examined for histologic evidence of OAC. Histologic classification of OAC was determined by the presence of numerous clumps or nests of dark staining epithelial cells supported by an underlying stroma of spindyloid cells. The epithelial cells may arrange themselves to form acini or tubules with round eosinophilic globules (ovalbumin) being present. All tumors were examined histologically and classified according to their anatomical features as ovarian, oviductal or infundibular.

RNA Extraction: The spleens were numbered according to the date and bird in the order that they were collected. Whole frozen spleens were broken at -80^0 C into pieces weighing 40 – 400mg (whole spleen weighed ~1.5 g). One piece from each spleen was placed into a 15 ml polypropylene tube of which 1 ml of Trizol®[D] was added per 100 mg of spleen. The suspension was then homogenized using a mechanical homogenizer (ULTRA-TURRAX T25 IKA®[E]) until it was homogenous. Once the sample was homogenized, it was allowed to stand on the bench for 10 min at room temperature to allow Trizol® to digest the tissue at a maximum rate[4]. The homogenizer was washed

[C] FLUOStar Optima microplate reader. Georgia USA.
[D] TRIzol® manual. Invitrogen products California USA
[E] IKA®. Janke and Kunkel Staufen Germany

between samples with alcohol and water. After 10 min had elapsed, the sample was centrifuged at 4,500 x g for 5 min. After the centrifugation was complete, the sample was immediately placed on ice and 1.0 ml of the supernatant was transferred into a 1.5 ml polypropylene tube. Two hundred microliters of chloroform was then added to the tube. The suspension with the added chloroform was then vortexed for 6 seconds (secs) followed by centrifugation at 16,000 x g at 4^0 C for 20 min using an Eppendorf 5415 R centrifuge®[F].

Centrifugation caused the sample to separate into 3 layers. The top layer contained RNA (clear), the middle layer protein (white) and the bottom layer DNA (sanguineous). Four hundred microliters of the supernatant containing the RNA was removed and placed into a new 1.5 ml polypropylene tube. The remaining RNA supernatant along with some of the interface was removed and placed into a 0.5 ml polypropylene tube and centrifuged at 16,000 x g at 4^0 C for 10 min. Once centrifugation was complete, RNA (200µl) supernatant was again removed and added to the corresponding polypropylene tube to increase the quantity of RNA available.

Nucleic acids were then precipitated with two hundred and fifty microliters of isopropanol. The tubes were then inverted to mix the samples and the mixture was then stored overnight at -20^0 C. The Qiagen RNeasy RNA Clean up mini kit™[G] was used to complete the RNA extraction procedure.

The 1.5 ml polypropylene tubes containing the RNA precipitate and isopropanol, were placed on ice and allowed to thaw on ice for ~ 30 min. Once thawed to liquid phase the samples were centrifuged at 16,000 x g at $4\ ^0$C for 30 min. The tubes, which now

[F] Eppendorf 5415 R® centrifuge. Eppendorf® Hamburg Germany
[G] The Qiagen RNeasy RNA Clean up mini kit™. Qiagen Products California USA

contained pellets of RNA in a clear suspension then placed again on ice. Five hundred microliters of the supernatant was removed at a time with filter tipped pipettes. Five hundred microliters of 100% ethanol was then added to the pellet and the tube was centrifuged placed at 4^0 C at 16,000 x g for 10 min. The liquid fraction comprised of water and alcohol was removed via a pipette. The tubes were then left to dry open on its side on the bench for 10 min. One hundred microliters of diethylpyrocarbonate (DEPC) water was added to the dried RNA pellets. The mixture was then vortexed for 6 secs and placed in a water bath at 65^0 C for 5 min, vortexed again and re-placed in the water bath. This step was repeated for up to 20 min in the water bath to facilitate dissolving the RNA pellet.

Once dissolved, 350 µl of RLT Buffer™[H] was added. Once mixed, 250 µl of 100% ethanol were added and mixed. Up to 700 µl of liquid mixture were then added to a spin column. The spin column was then centrifuged at 16,000 x g for 15 sec in a microcentrifuge (210 A Denville Scientific Inc.™[I]) The fluid in the bottom tube was discarded. Up to 700 µl of the liquid mixture were added again to the spin column, and the centrifugation and discard steps were repeated until the entire same sample was processed.

Five hundred microliters of RPE Buffer™[8] (containing 100% ethanol) were added to the spin column, followed by centrifugation for 15 secs at 16,000 x g. The elute in the bottom tube was discarded. This step was repeated for a total of two washes. The spin column was then centrifuged at 16,000 x g for 2 min and the bottom collection tube was

[H] The Qiagen RNeasy RNA Clean up mini kit™. Qiagen Products California USA
[I] 210 A Denville Scientific Inc.™ New Jersey USA

removed and discarded altogether. The spin column was then placed into a new 1.5 ml polypropylene tube.

RNA was then eluted from the column by adding 150 µl of RNase free water[J] were added, drop-by-drop, to each spin column just above the filter. The mixture was left to stand for 10 min on the bench and then centrifuged at 16,000 x g for 1 min in the microcentrifuge. The filtered fluid containing the RNA was transferred to a 1.5 ml polypropylene tube and placed on ice.

After priming the filter pipette tip, 4 µl of RNA-enriched fluid were added to 156 µl of distilled water in a separate 1.5 ml polypropylene tube and mixed by pipetting. The filter from the spin column was retained in the event that the amount of RNA was insufficient. These RNA samples were diluted in distilled water were moved quickly to a spectrophotometer (Bio Rad Smart Spec™ 3000[K]). The spectrophotometer was blanked with 165 µl of distilled water. The water was then discarded and the sample placed in the cuvette and absorbance read at 260, 280 and 320 nm.

A sample with an A260 (260 nm) reading < 0.15 was considered to have an inadequate concentration of RNA. All samples with A260 > 0.7 were diluted with distilled water and re-measured. The ratio of A260 /A280 at neutral pH was used to indicate the quality of the RNA, with >1.5 indicating acceptable quality. The RNA samples were stored at -80^0 C. The concentration for RNA was calculated according to the manufacturer's directions[J.]

[J] The Qiagen RNeasy RNA Clean up mini kit™. Qiagen Products California USA
[K] Bio Rad Smart Spec™ 3000 California USA

Reverse Transcription: Superscript ® III Reverse Transcriptase Invitrogen™ [L] was used to conduct the reverse transcription. In a 1.5 ml polypropylene tube 1 µl of 10mM deoxyribonucleotide triphosphate mix (dNTP), 1 µl of random hexamers[12], and 2 µg of sample RNA were made up to a total volume of 13 µl with double distilled water. The mixture was then placed in a water bath at 65^0 C for 5 min, chilled on ice for 1 min, and centrifuged for 15 secs, before adding 4 µl of 5X first strand buffer[12], 1 µl of 0.1 M DTT[12], 1 µl of Rnase out, and 1 µl of SuperScript III[12] were added. The mixture was then vortexed for 6 secs to facilitate mixing, and centrifuged for 15 secs. The tube was then placed in a water bath at 55^0 C for 50 min, after which it was placed into another water bath at 70^0 C for 15 min. Reverse transcription was considered complete at this point, with the product being complementary DNA (cDNA). One hundred and eighty microliters of distilled water was then added to the tube to create a 1:10 dilution (20:180), mixed and the tube was mixed and stored at -80^0 C.

Polymerase Chain Reaction: See Appendix C for primers. All primers designed by the investigators of this study was conducted using Primer3 Output[11] software applied to the gp 85 env of the published complete sequence for ALV-E NSAC-1 in Genbank (Accession number FJ93550.1). Eukaryotic elongation factor 1 alpha (EFLα) is a highly conserved gene that is highly expressed and was used to check the quality of the cDNA . Sample #113 was chosen to be the positive control based on gross and histological evidence of OAC and ALV positive status on the ALV ELISA antigen test. Sample # 93 was chosen as our negative control because of lack of gross and histological evidence for

[L] Superscript ® III Reverse Transcriptase. Invitrogen™ California USA

OAC and seronegativity on the ALV ELISA antigen test. Initially, the published protocols for the primers PE1, PE2, PU1 and PU21 and ALV E and ALV All were adhered to and conducted on the positive control (sample #113), negative control (sample #93) and 10 samples. However, these reactions did not reveal consistent results. A gradient protocol was then initiated to test all the primers to determine the optimum annealing temperatures. The crude primers were diluted in Tris- acetate EDTA buffer (TAE: 40 mM Tris-acetate: 1mM EDTA) to a concentration of 0.1 nmol/µl for storage at -80^0 C, and a further 1:10 dilution was made for use as the working stock. For example, 40 nmol of crude primer was dissolved in 400 µl of TAE buffer for storage, and 20 µl of that stock was further diluted in 180 µl of distilled water to be used as the working stock. The working stock was then stored at -80^0 C.

The PCR assay included the following reagents in each reaction tube: 2.5 µl of 10X Blue Juice™ [M] (Gel Loading Buffer), 1.0 µl of 50mM $MgCl_2$, 0.5 µl of dNTP's, 1.0 µl (50pmol) of sense primer, 1.0 µl (50 pmol) of anitsense primer, 0.3 µlof Taq polymerase, 5 µl of template (cDNA from sample) and 13.7 µl of distilled water for a total volume of 25 µl.

A gradient PCR was conducted for all the primers using our positive control cDNA (sample # 113) as the template. First, samples were denatured at 95^0 C for 30 secs. Then, samples were cycled 40 times as follows. All samples were denatured at 95^0 C for 30 secs, then aliquots of template were annealed at one of five temperatures: 54.8^0 C, 57.7^0 C, 60.3^0 C, 62.1^0 C, and 64^0 C, followed by an extension cycle at 72^0 C for 20 secs.

[M] 10X Blue Juice™ . Invitrogen™ California USA

The final extension during the 40^{th} cycle was performed for an additional 10 min. Samples were then incubated at 4^0 C until gel electrophoresis was performed.

Results of the gradient PCR for primers: PE1, PE2, PU1, PU2 ALV E, ALV All, DIS1, DIS2, DISS1 or DISS2 yielded multiple bands of varying product sizes or no product evidenced by the absence of a band on the electrophoresis gels. However, for the ALV-E env and EF1α primers, it produced a bright single band of the appropriate size (215 bp for ALV-E env and 258bp for EF1α) across all temperatures, with the brightest band occurring at temperatures >62^0 C.

The following protocol was designed based on the optimum annealing temperature as indicated by the gradient, and the Primer 3 Output™ for the ALV-E env and EF1α primers. All samples were denatured at 95^0 C for 3 min. The samples were then cycled 4 times as follows. All samples were denatured at 95^0 C for 30 secs, annealed at 62^0 C for 30 secs, followed by an extension cycle of 72^0 C for 27 secs. The samples were then cycled 41 times as follows. All samples were denatured at 95 ^{0} C for 30 secs and then annealed at 62^0 C for 30 secs. The final extension was at 72^0 C for 10 min and then the samples were incubated at 4^0 C until gel electrophoresis was performed.

Once the DNA was amplified, electrophoresis was conducted on the product using 2% agarose gels. The gels were made by mixing 6g of agarose powder in 300 ml of Tris-EDTA buffer (TE buffer: 10 mM tris HCl :1 mM EDTA pH 8. Fifteen microliters of ethidium bromide were added to the gel in solution and left to set. Once the gel was set, it was immersed in TE buffer. The wells were checked for any cracks, breakage, collapse or leakage, visually and by adding 1 µl of 10X Blue Juice™ to each well. PCR tubes (0.2

ml) were pre-labeled to match the sample numbers that were placed in the PCR machine, and 1µl of 10X Blue Juice® was added to each tube. Ten microliters of DNA product from the PCR was added with a filter tip pipette tip to the corresponding 0.2 ml tube containing the 10X Blue Juice™ and mixed. The mixture was then loaded into the wells, one well per sample. One well on either end and in the middle of the gel was loaded with 5 µl of 100bp ladder (Track it™ [N]). The positive and negative controls were also loaded in two wells on the left. The ladders were loaded as such, to facilitate interpretation of the bands on the gels once the electrophoresis was complete. The gels were run at 120 mV, until the yellow dye of the ladder had run past the edge of the gel. Photographs of the gel were taken using Kodak MI™ system[O]. The digital images were printed and stored for analyses. A sample was considered positive if it produced a single bright band at the appropriate size according to the 100 bp ladder (Figures 5 and 6). The sample results were recorded for further analyses.

Results

In the pilot sample of 30 birds that were 165 weeks of age, 18 birds were identified that were OAC positive based on histologic examination of the ovaries. Birds with OAC were 2.6 times more likely to test positive for ALV using the IDEXX FlockChek antigen capture ELISA on serum samples collected prior to euthanasia, however in this small pilot study this association was not statistically significant (p=0.068). There were no other associations between OAC and the occurrence of any other gross or histopathological lesions. Post priori power analysis revealed that the

[N] Track it™ Invitrogen™ California USA
[O] Kodak Molecular Imaging Software. Care Stream Health. Connecticut USA

sample of 30 hens with 18 cases and 12 controls had a statistical power of .23, indicating that the probability of Type II error was 77%. Sample size calculations reveal that an additional 130 hens was required to achieve the target 80% statistical power." An additional 147 hens $\geq$ 100 weeks of age were selected from the UIUC Poultry Research Laboratory using a stratified random procedure.

The overall prevalence of OAC was 22.6% (40/177) in the birds sampled, with a prevalence of 3.22% (1/31) in the 104 week stratum, 10.81% (4/37) in the 130 week stratum and 32.11% (35/109) in the 165 week stratum (Figure 3). The overall prevalence of ALV was 26.4% (42/159), with a prevalence of 43.3% (13/30) among hens in the 130 week stratum and 29% (29/100) in the 165 week stratum. No ALV positive hens were identified from the 104 week stratum. The overall prevalence for ALV-E env mRNA expression was 86.4% (108/125), with a prevalence of 77.8% (21/27) in the 104 week stratum, 100% (30/30) in the 130 week stratum and 83.8% (57/68) in the 165 week stratum (Figure 4). Eighteen hemolyzed samples were removed from the analyses of the association between OAC and ALV. The presence of OAC was associated with the probability of being ALV positive ($p<0.0001$). OAC-positive birds were 5.2 times more likely to be ALV positive (95% confidence interval (C.I.) 2.3, 11.18) (Table 1). Twenty-two samples were removed from analysis of the association between OAC and ALV-E env mRNA expression due to inadequate RNA. Hens with OAC were 3.86 times more likely to express ALV-E env mRNA, but this was not significant ($p=0.303$) (Table 2). Hens expressing ALV-E env mRNA were 9.62 times more likely to be positive for ALV ($p=0.040$) (Table 3).

OAC was associated (X^2 = 15.2, p<0.0001) with age (Fig. 3). Age stratum was also associated (X^2 = 15.2 p=0.001) with ALV (Table 4). ALV positive hens were significantly more likely to be older. Among the age strata, an association (X^2= 6.81 p=0.033) was observed between age and ALV-E env mRNA expression (Fig. 4). Since age was associated with OAC, ALV and ALV-E env mRNA expression, it was a potential confounder. Stratified analysis was conducted to control for the potential confounding affect of age. Analyses conducted within each stratum for OAC and ALV showed that for associations between ALV seropositivity and OAC, the only significance was found in the 165 week old stratum (p<0.001) (Tables 5-7). Also analyses conducted within the age stratum for associations between OAC and ALV-E env mRNA expression, showed that there was a trend toward significance at the highest age stratum where hens that were ≥ 165 weeks old that had OAC were 8.86 times more likely to express ALV-E env mRNA, but this was not significant (p=0.056) (Tables 8-10).

The results of the multivariate logistic regression model are summarized in Table 11. Age stratum, ALV test status, and ALV-E env mRNA expression were introduced to the model. All possible interactions between the independent variables were evaluated but no significant interactions were identified. Age stratum and ALV test status were significantly associated with occurrence of OAC. Controlling for ALV test status and ALV-E env mRNA expression birds with OAC had a 3.6% increase in odds of being in a higher age stratum (95% C.I. 1.0076, 1.0563). Controlling for age stratum and Eenv mRNA expression, birds with OAC were 3.3 times more likely to be ALV positive (95% C.I. 1.1067, 10.1193). The odds ratio of the association between OAC and ALV-E env

mRNA expression was of a similar magnitude (odds ratio = 3.2) however this association was not statistically significant (95% C.I. 0.3837, 27.4777) (Table 11).

Discussion

The first objective of this study was to determine the prevalence of OAC, ALV, and ALV-E env mRNA expression in a stratified random sample of aged laying hens from the UIUC Poultry Research Laboratory. The overall prevalence of OAC was 22.6% in this sample of birds with a trend of increasing prevalence with increased age. This finding is consistent with the current literature on the epidemiology of OAC in both hens and women. Frederickson[15] reported that OAC was unusual in hens less than two years of age, with Rodriguez-Burford[17] reporting the prevalence to be 4% among two-yr-old hens. In Frederickson's study, 466 layers ranging from 2-7 years of age had an overall OAC prevalence of 19% and a trend of increasing prevalence with age: 12% at mean age 3.9 years, 32% at mean age 4.2 years, and 50% at mean age 6.1 years. Other studies have also identified this relationship.[16] In 4-yr-old birds, an OAC prevalence of 39% has been reported.[14] In women, there is an age-related increase in incidence of OAC, from 0.7 per 100,000 in women younger than 20-yrs to 6.6 at 20-49 yrs, 26.9 in women 50-64 years, 48.6 in women 65-74 years and as high as 55.6 in women older than 75 years.[64-66]

The prevalence of ALV in the flock was similar to the OAC prevalence (26.4%). The prevalence of ALV was not uniform across age strata. ALV-E is an endogenous retrovirus thought to be present within the genome of most domestic poultry. In this sample the prevalence of ALV-E env mRNA expression in splenic tissue was 86.4%. Both ALV seropositive test status and ALV-E env mRNA expression were not uniformly

manifest across age strata, unlike the pattern of increasing prevalence with increased age that was demonstrated with the prevalence of OAC. The 130 week age stratum had the highest prevalence of both ALV seropositive test status and ALV-E env mRNA expression (43.3% and 100% respectively). The prevalence of both of these was lower in the 104 (ALV prevalence = 0 and ALV-E env mRNA expression prevalence = 77.8%) and 165 week age strata (ALV prevalence = 29% and ALV-E env mRNA expression prevalence = 83.8%).

The second objective was to assess the association between the presence of OAC and ALV seropositive status. OAC-positive birds were 5.2 times more likely to be ALV positive (95% confidence interval (C.I.) 2.3, 11.18) ($p<0.0001$). This was the first time that an association between OAC and a viral risk factor has been reported. It is contradictory to earlier research by Fredrickson[15] who reported a prevalence of OAC of 24% in hens that had been classified as free of ALV based on the results of the COFAL test. The inconsistency between the results of the present study and that by Fredrickson and colleagues may be due to limited sensitivity of the COFAL test. Other investigators have found COFAL proved to be a relatively insensitive diagnostic test for ALV and that the results did not correlate well with the other tests.[50] These limitations may be even more pronounced when attempting to detect endogenous ALV virus compared with the exogenous subgroups. COFAL detects the group specific ALV antigens that require culture, purification, titration and dilution for analysis, and is not used clinically for screen purposes [49-50]. Whereas the ALV ELISA test kit which has a reported 99.2% sensitivity and 100% specificity and a detection limit of 2 ng, can be used clinically, with ease for screening for ALV .[61-63]

The third objective was to assess the association between presence of OAC and the expression of ALV-Eenv mRNA. Across all age strata there was no association between OAC and expression of ALV-E mRNA expression (Odds ratio = 3.8, p = 0.303). However, when assessing the association between OAC and expression of ALV-E mRNA expression in the 165 week age stratum alone, despite the much smaller sample size, the odds ratio increased to 8.86 and the p value declined to 0.056. While ALV-E env mRNA expression was common in both OAC positive and negative birds (Table 4), only 1 OAC positive bird failed to express ALV-E env mRNA. This suggests that expression of ALV-E mRNA may be necessary for OAC to occur but perhaps not sufficient to cause OAC. One possible explanation for this association may be that ALV-E expression must have to occur in the presence of an as yet unknown risk factor to cause disease. An alternative explanation may be that the site of ALV-E insertion into the genome may be an important risk factor for whether or not its presence induces OAC. Hens expressing ALV-E env mRNA were 9.62 times more likely to be positive for ALV (p=0.040). The significant association between ALV-E env mRNA expression and ALV test status and their similar patterns of age distribution within the sample are not surprising since the antigen capture ELISA was detecting any subgroup of ALV and the RT-PCR was detecting ALV-E.

This study has identified important risk factors associated with the presence of OAC in aged laying hens. However, there are limitations inherent within the study design. The cross-sectional nature of the study design precludes the establishment of a cause and effect relationship. Age was associated with the presence of OAC, with ALV test status and ALV-E env mRNA expression. Thus it is potentially an important

confounding variable. A prospective cohort study would be necessary to establish a temporal relationship between exposure to ALV (or ALV-E) and the subsequent development of OAC. However this may prove difficult if endogenous ALV-E were to be included because it is considered ubiquitous in the chicken genome.

The strength of the associations reported in this study may have been reduced due to potential misclassification bias. Ovarian tissues were evaluated in the same fashion across age strata. OAC was diagnosed in this study based on the aforementioned histological criteria. Multiple sections of the each ovary were examined histologically, however the entire ovary for each bird was not examined. As a result, small nests of neoplastic epithelial cells or tumors may have gone unrecognized causing OAC positive birds to have been misclassified as OAC negative. Ideally the entire ovary should be examined. The large disparity between OAC prevalence in 130 week old birds and that in 165 week old birds may be an indicator of misclassification error. This study reported the prevalence of OAC in 130 week old birds was one-third that found in 165 week old birds, from 10.81% in the 130 week old hens to 32.11% in the 165 week stratum. It is reasonable to presume that a fraction of the 130 week old birds that appeared to be OAC negative on both gross and histological examination were actually in an early stage of OAC.

Determination of the association with ALV-E expression was accomplished using a primer for ALV-E env mRNA designed by the investigators. A limited number of primers for the detection of ALV-E have been published. However, those evaluated in this study did not produce products or produced products that yielded multiple bands of various sizes. This may have been due to errors in sample handling by the investigators.

Spleens may not have been frozen fast enough or handled in a manner to preserve the RNA, therefore yielding insufficient or poor quality RNA that was unsuitable for conducting the RT-PCR with those primers. Investigation into the melting temperatures for the sense and anti-sense of the published primers, showed a disparity of up to 10^{o} C between a pair of complementary primers. This disparity can lead to a variety of nucleotide sequences being produced during the PCR reaction as the reaction temperatures increase and cool during the annealing stage. Some of the published primers had very large intended product sizes (>1.2 kb). Large products lend themselves to interference from insufficient quantity or poor quality RNA. This may result in vast variations in the intended product sequence as the primers are tested across different samples. This study contributes another primer for the detection of ALV-E. The Eenv primer designed here yielded a smaller product size with little disparity in annealing temperatures between the sense and antisense primers. Most importantly, the Eenv primer appeared to reliably produce appropriate products in the face of sub-optimum tissue handling for RNA extraction, while the other primers did not.

Chapter 4. Conclusions and Future Directions

There were three objectives of this study.

1. To determine the prevalence of OAC, ALV, and ALV-E mRNA expression in aged birds in the UIUC Poultry Research Laboratory.
2. To determine the association between ALV serologic test status and OAC in aged birds in the UIUC Poultry Research Laboratory.
3. To determine the association between expression of ALV-E env mRNA and OAC in aged birds in the UIUC Poultry Research Laboratory.

The study determined that the prevalence of OAC in the UIUC Poultry Research Laboratory in hens that were > 100 weeks of age was 22.6% and that hens with OAC were significantly more likely to be older. The prevalence of ALV was 26.4% and that of ALV-E env mRNA expression was 86.4%. ALV and ALV-E env mRNA expression were not uniformly distributed across the 3 age strata that were examined. Hens in the >165 age stratum were more likely to be positive for both ALV and ALV-E env mRNA expression. Hens with OAC were 5.2 times more likely to be ALV positive ($p<0.0001$). While not statistically significant 165 week old hens that were OAC positive were 8.9 times more likely to expression ALV-Eenv mRNA ($p=0.056$).

The identification of seropositive test status for ALV via antigen capture ELISA as a potential risk factor for OAC is an important new finding obtained from this study. This is the first time that a viral risk factor has been implicated as being associated with OAC in the hen. Further research is needed to investigate the association between ALV-E and OAC in hens. The findings from this study indicate

that at least one subtype of ALV may play a role in the pathogenesis of OAC in the hen. If ALV-E is implicated as the important subgroup in the pathogenesis, then an endogenous retroviral association may indeed occur in both hens and women.

The associations between OAC, ALV and ALV-Eenv mRNA expression become particularly intriguing given the recently reported association between human endogenous retroviruses (HERVs) and human cancers[48] including: breast cancer[67] colon cancer, germ cell tumors and prostate adenocarcinoma[68]. Further experimentation is warranted to explore possible insertion sites, functional properties, and receptor sites for ALV –E and to distinguish them from other ALV subgroups. Because Avian Leukosis Virus subgroup E is an endogenous retrovirus of chickens that is analogous to HERVs in humans, our findings along with the theories of insertional polymorphisms, re-combinational insertion, and the massively unexplored new families of endogenous retroviruses within the chicken genome demands further investigation into the ongoing quest for a model for OAC in humans.

BIBLIOGRAPHY

1. National Cancer Institute. 2009. http://www.cancer.gov/cancertopics/types/ovarian

2. National Cancer Institute, 2008. Dev Can: Probability of Developing Cancer or Dying of Cancer Software, Version 6.3.0 Statistical Research and Applications Branch, National Cancer Institute. http://srab.cancer.gov/devcan

3. American Cancer Society. 2009. http://www.cancer.org/docroot/PRO/content/PRO_1_1_Cancer_Statistics_2009_Presentation.asp

4. U.S. Cancer Statistics Working Group. *United States Cancer Statistics: 1999–2006 Incidence and Mortality Web-based Report*. Atlanta: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention and National Cancer Institute; 2010. Available at: www.cdc.gov/uscs.

5. Vanderhyden, BC, Shaw TJ, Ethier J. Animal models of ovarian cancer. Reprod Biol Endocrinol 2003,1: 67.

6. Wang-Johanning F, Liu J, Rycag K, Huang M, Tsai K, Rosen D, Chen D, Lu DW, Barnhart KF, Johanning GL. Expression of multiple human endogenous retrovirus surface envelope proteins in ovarian cancer. Int J Cancer 2006, 120: 81-90.

7. Payne LN. Biology of avian retroviruses. In: The Retroviridae Vol. 1, Edited by: Levy JA. Plenum Press: New York. 1992, 299-404.

8. Payne LN, Venugopal K. Neoplastic diseases: Marek's disease, avian leukosis and reticuloendotheliosis. Rev Sci Tech Off Int Epiz 2000, 19: 544–564.

9. Burnmeister T. 2001. Oncogenic retroviruses in animals and humans. Rev Med Virol 2001, 11: 369-380

10. Denesvre C, Soubieux D, Pin G, Hue D, Dambrine G. Interference between avian endogenous ev/J 4.1 and exogenous ALV-J retroviral envelopes. J Gen Virol 2003, 84: 3233-3238

11. Huda A, Polavarapu N, Jordan KI, Mc Donald JF. Endogenous retroviruses of the chicken genome. Biol Direct 2008, 3: 9.

12. Fathalla MF. Incessant ovulation – a factor in ovarian neoplasia? Lancet 1971, 2: 163.

13. Giles J, Olson LM, Johnson PA. Characterization of ovarian surface epithelial cells from the hen: a unique model for ovarian cancer. Experi Biol and Med 2006, 231:1718-1725.

14. Barnes MN, Berry WD, Straughn JM, Kirby TO, Leath CA, Huh WK, Grizzle WE, Patridge EE. A pilot study of ovarian cancer chemoprevention using medroxyprogesterone acetate in an avian model of spontaneous ovarian carcinogenesis. Gynecol Oncol 2002, 87: 57-63.

15. Fredrickson TN. Ovarian tumors of the hen. Environ Health Perspect 1987, 73: 35-51.

16. Papsolomontos P, Appleby E, Mayor O. Pathologic findings in condemned chickens: A survey of 1000 carcasses. The Vet Rec 1969, 85: 459–64.

17. Rodriguez-Burford, C., M. N. Barnes, W. Berry, E. E. Partridge, and W. E. Grizzle. Immunohistochemical expression of molecular markers in an avian model: a potential model for preclinical evaluation of agents for ovarian cancer chemoprevention. Gynecol. Oncol 2001, 81: 272–279.

18. Engstrom P, Meyskens F. Cancer Prevention. In: Principles and Practice of Gynecologic Oncology. Edited by: Hoskins W, Perez C. Young R. 1997, 197-210.

19. Giles JR, Shivaprasad HL, Johnson, PA. Ovarian tumor expression of an oviductal protein in the hen: a model for human serous ovarian adenocarcinoma. Gynecol. Oncol. 2004, 95: 530–533.

20. Langdon, S. P., and S. S. Lawrie. Establishment of ovarian cancer cell lines. In: Ovarian Cancer Methods and Protocols. Edited by Bartlett JMS. Humana Press, Totowa, NJ 2000, 155-159.

21. http://chickscope.beckman.uiuc.edu/explore/embryology/day05/ovary.html

22. Bahr JM, Johnson AL. Regulation of the follicular hierarchy and ovulation. The J of Experi Zool 1984, 232: 495-500.

23. Barua A, Bitterman P, Abramowicz JS, Dirks AL, Bahr JM, Hales DB, Bradaric MJ, Edassery SL, Rotmensch J, Luborsky JL. Histopathology of ovarian tumors in laying hens: a preclinical model of human ovarian cancer. Int J of Gynecol Cancer 2009, 19: 531-539

24. Papadaki L and Beilby JO: The fine structure of the surface epithelium of the human ovary. J Cell Sci 1971, 8: 445-465.

25. Moore KL. The pelvis and perineum. In: Clinically Oriented Anatomy Edited by Satterfield TS, Napora L, Lumpkin K. Baltimore, Williams & Williams; 1992:281-289.

26. Mayo Clinic. http://www.mayoclinic.com/health/ovarian-cancer

27. Nicosia SV: The aging ovary. Med Clin North Am 1987, 71:1-9.

28. Hamilton TC: Ovarian cancer, Part I: Biology. Curr Probl Cancer 1992, 16:1-57

29. Salazar H, Godwin AK, Daly MB, Laub PB, Hogan WM, Rosenblum N, Boente MP, Lynch HT, Hamilton TC. Microscopic benign and invasive malignant neoplasms and a cancer-prone phenotype in prophylactic oophorectomies. J Natl Cancer Inst 1996, 88: 1810-1820.

30. Deligdisch L and Gil J: Characterization of ovarian dysplasia by interactive morphometry. Cancer 1989, 63: 748-755.

31. Scully RE: Early de novo ovarian cancer and cancer developing in benign ovarian lesions. Int J Gynaecol Obstet 1995, 49 (Suppl): S9-15.

32. Godwin AK, Testa JR, Handel LM, Liu Z, Vanderveer LA, Tracey PA, Hamilton TC. Spontaneous transformation of rat ovarian surface epithelial cells: association with cytogenetic changes and implications of repeated ovulation in the etiology of ovarian cancer. J Natl Cancer Inst 1992, 84: 592-601.

33. Roby KF, Taylor CC, Sweetwood JP, Cheng Y, Pace JL, Tawfik O, Persons DL, Smith PG, Terranova PF. Development of a syngeneic mouse model for events related to ovarian cancer. Carcinogenesis 2000, 21: 585-591.

34. Whittemore AS, Harris R and Itnyre J. Characteristics relating to ovarian cancer risk: collaborative analysis of 12 US case-control studies IV: The pathogenesis of epithelial ovarian cancer: Collaborative Ovarian Cancer Group. Am J Epidemiol 1992, 136: 1212-1220.

35. La Vecchia C and Franceschi S: Oral contraceptives and ovarian cancer. Eur J Cancer Prev 1999, 8: 297-304.

36. Kurman RJ, Shih L. Pathogenesis of ovarian cancer. Lessons from morphology and molecular biology and their clinical implications. Int J Gynecol Pathol. 2008, 27: 151-160.

37. Bagan P, Berna P, Assouad J, Hupertan V, Le Pimpec Barthes F, Riquet M. Value of cancer antigen 125 for diagnosis of pleural endometriosis in females with recurrent pneumothorax. Eur. Respir. J. 2008, 31: 140–142.

38. Sarandakou A, Protonotariou E, Rizos D. Tumor markers in biological fluids associated with pregnancy. Crit Rev Clin Lab Sci 2007, 44: 151–78.

39. Morin PJ. Molecular markers in epithelial ovarian cancer. Curr Clin Oncol: Molecular Pathology of Gynecologic Cancer. Edited by Giordano A, A Bovicelli A, Kurman R, Humana press. 2007 pp 29-45

40. Niv Y. MUC1 and colorectal cancer pathophysiology considerations. World J. Gastroenterol. 2008, 14: 2139–41.

41. Auersperg N, Wong AST, Choi KC, Kang SK, Leung PCK. Ovarian surface epithelium: biology, endocrinology, and pathology. Endocr Rev 2001, 22: 255-288.

42. Zhou B, et al. Hormone replacement therapy and ovarian cancer risk: A meta-analysis. Gyn Oncol. 2008, 108: 641-651.

43. Olsen CM, Green AC, Nagle CM, Jordan SJ, Whiteman DC, Bain CJ, Webb PM. Epithelial ovarian cancer: testing the 'androgen hypothesis'. Endocr Relat Cancer 2008, 15: 1061-1068

44. Johnson PA, Giles JR. Use of genetic strains of chickens in studies of ovarian cancer. Poultry Sci 2006 85: 246-250.

45. Zhao LJ, Giam CZ. Interaction of the human T-cell lymphotropic virus type I (HTLV-I) transcriptional activator Tax with cellular factors that bind specifically to the 21-base-pair repeats in the HTLV-I enhancer. Proc Natl Acad Sci USA 1992; 88: 11445–11449.

46. Grassmann R, Fleckenstein B, Desrosiers RC. Viral transformation of human T lymphocytes. Adv Cancer Res 1994; 63: 211–244.

47. Rosenblatt JD, Miles S, Gasson JC, et al. Transactivation of cellular genes by human retroviruses. Curr Top Microbiol Immunol 1995, 193: 25–49.

48. Moyes D, Griffiths DJ, Venables PJ. Insertional polymorphisms: a new lease of life for endogenous retroviruses in human disease. Trends in genetics 2007, 23: 326-333

49. Sarma PS, Turner HC, Huenber RJ. An avian leukosis group-specific complement fixation rection. Application for the detection and assay of non-cytopathogenic leukosis viruses. Virol 1964, 23: 313-321.

50. Sandelin K, Estola T, Ristimaki S, Ruoslahti E, Vaheri A. Radioimmunoassay of the group-specific antigen in detection of avian leukosis virus. 1974. J. Gen. Virol 25: 415-420.

51. Coffin JM, Champion M, Chabot F. Nucleotide sequence relationships between the genomes of an endogenous and an exogenous avian tumor virus. J. Virol 1978, 28: 972–991.

52. Dorner AJ, Coffin JM. Determinants for receptor interaction and cell killing on the avian retrovirus glycoprotein gp85. Cell 1986, 45: 365–374.

53. Bova CA, Swanstrom R. Host range determinants of avian retrovirus envelope genes. In: Positive Strand RNA Viruses. Alan R. Liss, New York, 1987, 509–518.

54. Bova CA, Manfredi JP, Swanstrom R. en6 genes of avian retroviruses: nucleotide sequence and molecular recombinants define host range determinants. Virology 1986, 52: 343–354.

55. Bova CA, Olsen JC, Swanstrom R. The avian retrovirus en6 gene family: molecular analysis of host range and antigenic variants. J. Virol 1988. 62: 75–83.

56. Payne LN, Brown SR, Bumstead N, Howes K, Frazier JA, Thouless ME. A novel subgroup of exogenous avian leukosis virus in chickens. J. Gen. Virol 1991, 72: 801–807.

57. Bai J, Howes K, Payne LN, Skinner MA. Sequence of host-range determinants in the env gene of a full-length, infectious proviral clone of exogenous avian leukosis virus HPRS-103 confirms that it represents a new subgroup (designated J). J. Gen. Virol 1995a, 76: 181–187.

58. Bai J, Payne LN, Skinner MA. HPRS-103 (exogenous avian leukosis virus, subgroup J) has an en6 gene related to those of endogenous elements EAV-0 and E51 and an E element found previously only in sarcoma viruses. J. Virol 1995b, 69: 779–784.

59. Weiss RA. In: Nakahara, W., Hirayama, T., Nishioka, K., Sugano, H. (Eds.). Analytic and Experimental Epidemiology of Cancer. University of Tokyo Press, Tokyo. Proceedings of the 3rd International Symposium of the Princess Takamatsu Cancer Research Fund, 1973 pp. 201–233.

60. Calnek BW, Barnes HJ, Beard CW, Reid WM, Yoder HW. Diseases of Poultry, 10th edn. Iowa State University Press, Ames, IA.1991 pp 367-368

61. Pham TD, Spencer JL, Johnson ES. Detection of avian leukosis virus in albumen of chicken eggs using reverse transcription polymerase chain reaction. J Virol Methods. 1999, 78: 1-11.

62. Silva RF, Fadly AM, Taylor SP. Development of a polymerase chain reaction to differentiate avian leukosis virus (ALV) subgroups: Detection of an ALV contaminant in commercial Marek's disease vaccines. Avian Diseases 2007 51: 663-667.

63. Payne LN, Gillespie AM, Howes K. 1993. Unsuitability of chicken sera for detection of exogenous ALV by the group-specific antigen ELISA. Vet Rec 1993, 132: 555–557.

64. American Cancer Society. Cancer facts & figures 2008. Atlanta, Ga.:American Cancer Society; 2008

65. Centers for Disease Control and Prevention. United States cancer statistics: 1999-2005 cancer incidence and mortality data. U.S. Department of Health and Human Services. http://www.cdc.gov/uscs. Accessed February 26, 2009

66. Ries LA, Melbert D, Krapcho M, et al., eds. SEER cancer statistics review, 1975-2004. Bethesda, Md.: National Cancer Institute; 2007. http://seer.cancer.gov/csr/1975_2004. Accessed March 2, 2009

67. Turbeville MA, Rhodes JC, Hyams DM, Distler CM, Steele PE. Expression of a putative immunosuppressive protein in human tumors and tissues. Pathobiology 1996, 64: 233–238

68. Aguinaldo AM, Turbeville JM, Linford LS, Rivera MC, Garey JR, Raff RA, Lake JA. Evidence for a clade of nematodes, arthropods and other moulting animals. Nature 1997;387: 489–493

69. Payne LN, Fadly AM. Leukosis/Sarcoma Group. In: Diseases of Poultry. Edited by Calnek BW, Barnes HJ, Beard CW, McDonald LR, Saif YM. Iowa State University Press.1991 pp 415-421

70. Hsu IC, Tokiwa T, Bennett W, Metcalf RA, Welsh JA, Sun T, Harris CC. p53 gene mutation and integrated hepatitis B viral DNA sequences in human liver cancer cells. Carcinogenesis 1993 15: 987-992

71. Clark J, Saxinger C, Gibbs W, Lofters W, Lagranade L, Deceulaer K, Ensroth A, Robert-Guroff M, Gallo R, Blattner W. Seroepidemiologic studies of human T-cell leukemia/lymphoma virus type I in Jamaica". *Int J Cancer* 1985 **36**: 37–41

Appendix A. Figures

Figure 1. Diagram of the female reproductive tract of the hen[21]

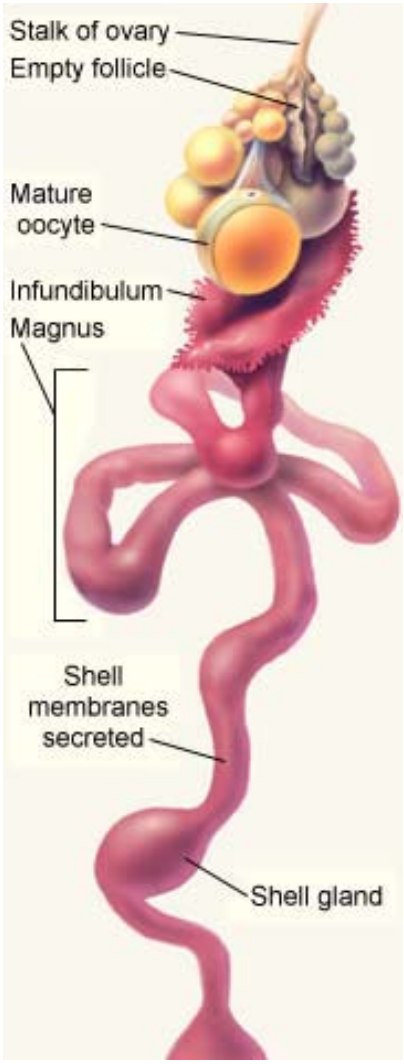


Figure 2. Gross appearance of ovarian adenocarcinoma in a 165 week old hen obtained from the University of Illinois Urbana-Champaign Poultry Research Laboratory laying flock.

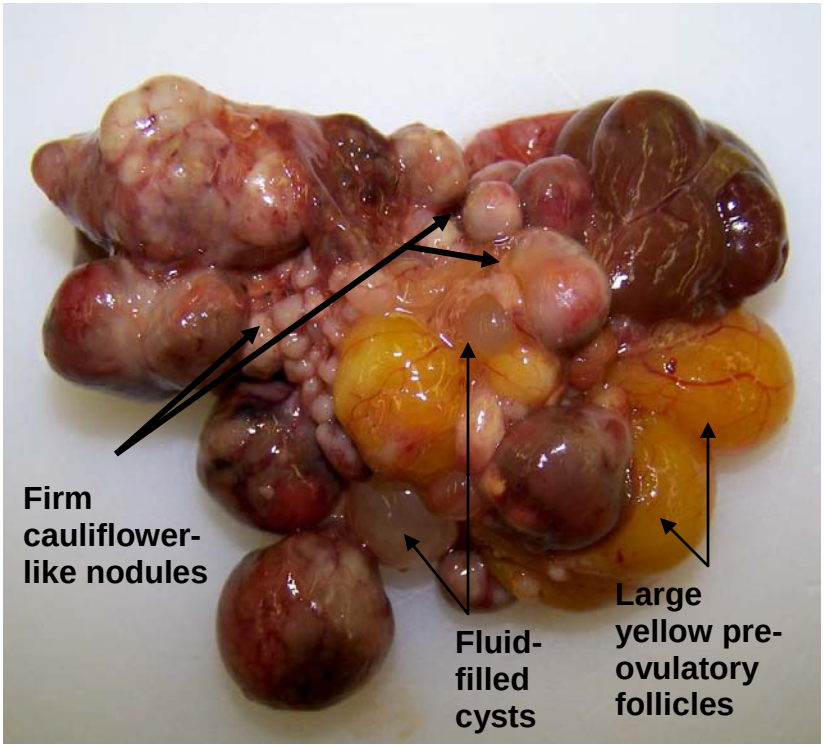


Figure 3. Prevalence of ovarian adenocarcinoma diagnosed by histologic examination in a stratified random sample of 177 White Leghorn hens obtained from the University of Illinois Poultry Research Laboratory laying flock.

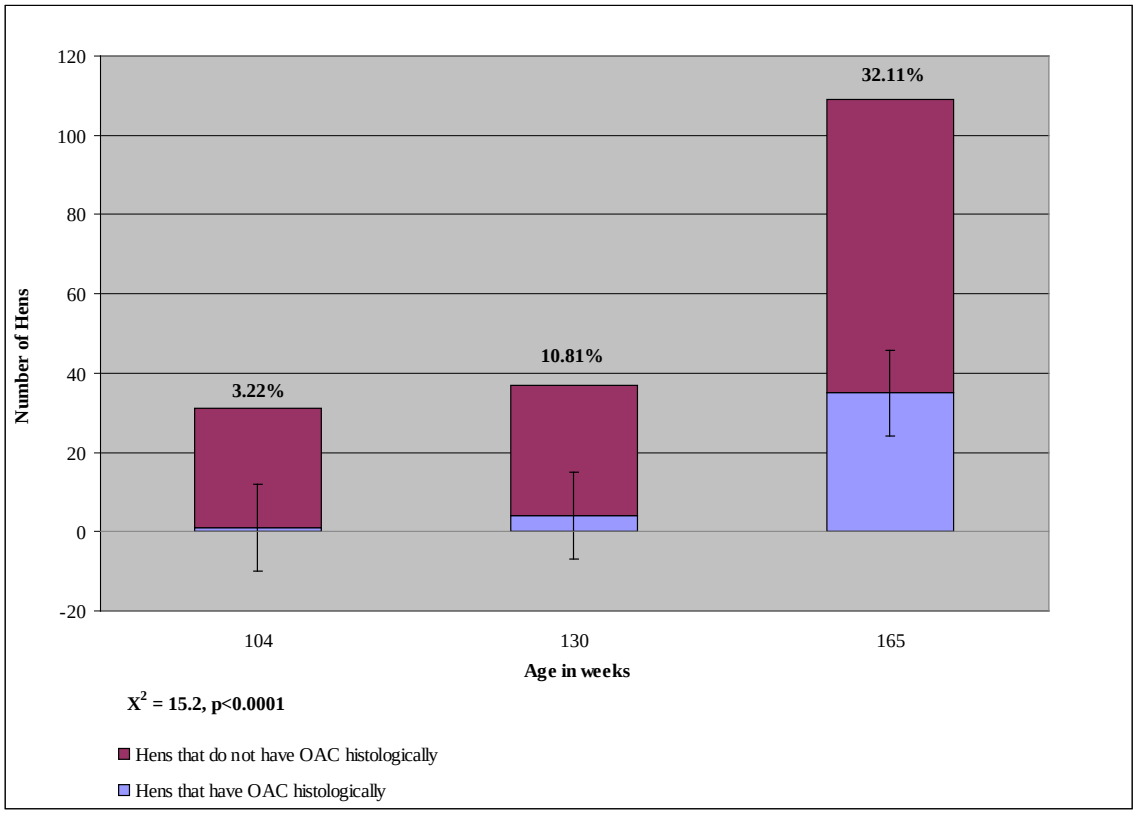


Figure 4. Prevalence of Avian Leukosis Virus subgroup E-env mRNA expression in a stratified random sample of 159 White Leghorn hens obtained from the University of Illinois Poultry Research Laboratory laying flock.

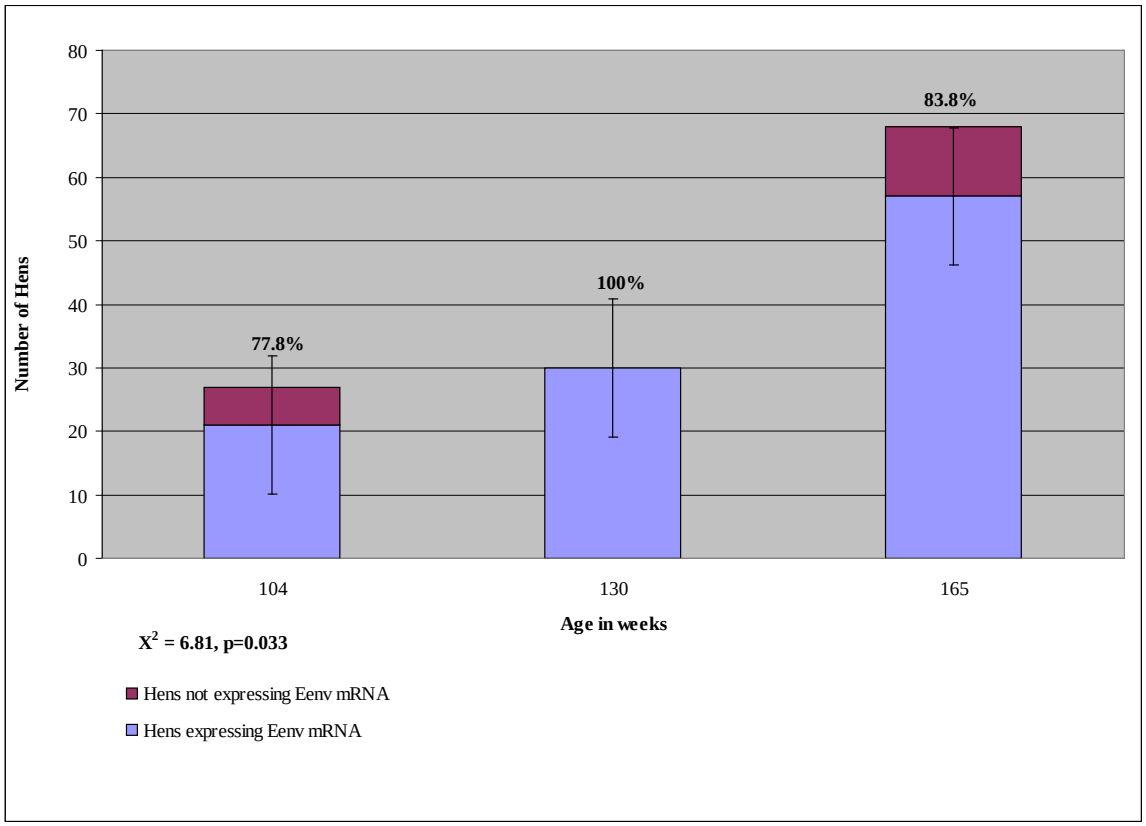


Figure 5. Detection of Eukaryotic Translation Factor 1 alpha (EF1α) using primers EF1α 1 and 2. Lane "a" is the positive control. Lane "b" is the 100 bp ladder. Lanes "c" through "t" are PCR products from spleen samples of White Leghorn hens obtained from the University of Illinois Poultry Research Laboratory laying flock. Lane "u" is the 100 bp ladder.

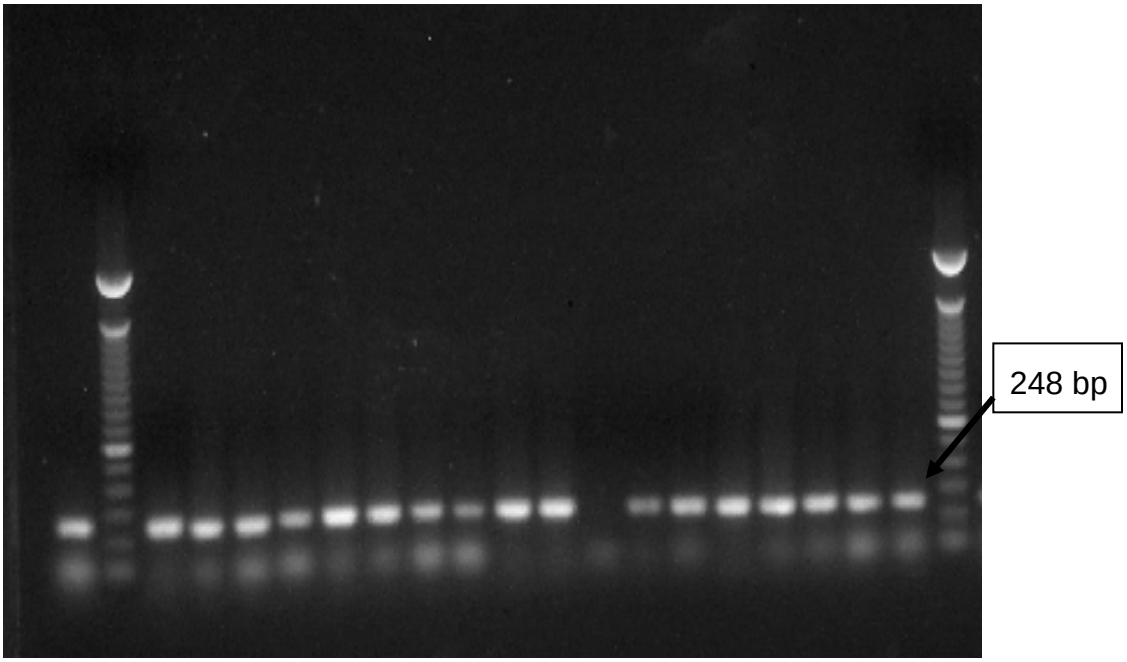
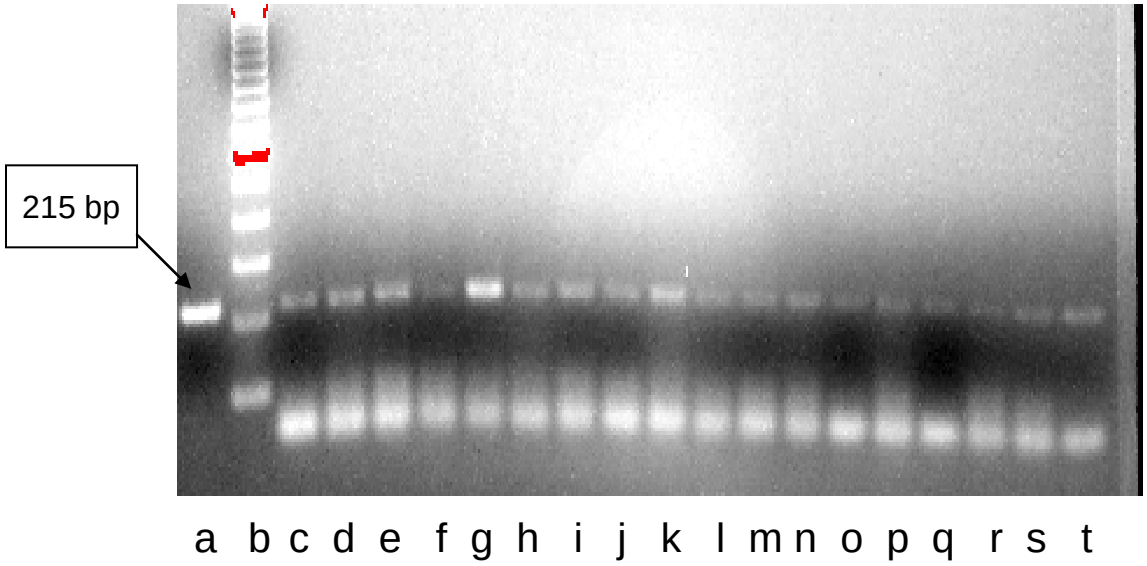


Figure 6. Detection of Avian Leukosis Virus subgroup E *env* mRNA using primers Eenv 1 and 2. Lane "a" is the positive control. Lane "b" is the 100 bp ladder. Lanes "c" through "t" are PCR products from spleen samples of White Leghorn hens obtained from the University of Illinois Poultry Research Laboratory laying flock.



Appendix B. Tables

Table 1. Association between presence of ovarian adenocarcinoma (OAC) and of Avian Leukosis Virus (ALV) serologic test status in 159 White Leghorn hens > 100 weeks of age obtained from the University of Illinois Poultry Research Laboratory laying flock.

	OAC Positive	OAC Negative	Odds Ratio
ALV positive	21	21	5.2
ALV negative	19	98	

$X^2 = 18.1$, $p<0.0001$

Table 2. Association between presence of ovarian adenocarcinoma (OAC) and of Avian Leukosis Virus-E env mRNA expression (ALV-E) in 125 White Leghorn hens > 100 weeks of age obtained from the University of Illinois Poultry Research Laboratory laying flock.

	OAC Positive	OAC Negative	Odds Ratio
ALV-E env positive	21	87	3.86
ALV-E env negative	1	16	

Fisher's Exact p=0.303

Table 3. Association between Avian Leukosis Virus-E env mRNA expression (ALV-E) and presence of Avian Leukosis Virus determined by antigen capture ELISA of serum samples from 125 White Leghorn hens > 100 weeks of age obtained from the University of Illinois Poultry Research Laboratory laying flock.

	Eenv +	Eenv -	Odds Ratio
ALV +	23	0	9.62
ALV -	85	17	

Fisher's Exact p=0.04

Table 4. Association between age stratum and presence of Avian Leukosis Virus determined by antigen capture ELISA of serum samples from 159 White Leghorn hens > 100 weeks of age obtained from the University of Illinois Poultry Research Laboratory laying flock.

Age Weeks	ALV Positive	ALV Negative
104	0	29
130	13	17
165	29	71

$X^2 = 15.2,\ p < 0.001$

Table 5. Association between presence of ovarian adenocarcinoma (OAC) and of Avian Leukosis Virus (ALV) serologic test status in 29 White Leghorn hens in the 104 week age stratum obtained from the University of Illinois Poultry Research Laboratory laying flock.

	OAC Positive	OAC Negative	Odds Ratio
ALV Positive	0	0	19.0
ALV Negative	1	28	

Fisher's Exact p=1.0

Table 6. Association between presence of ovarian adenocarcinoma (OAC) and of Avian Leukosis Virus (ALV) serologic test status in 30 White Leghorn hens in the 130 week age stratum obtained from the University of Illinois Poultry Research Laboratory laying flock.

	OAC Positive	OAC Negative	Odds Ratio
ALV Positive	3	1	4.8
ALV Negative	10	16	

Fisher's Exact p=0.29

Table 7. Association between presence of ovarian adenocarcinoma (OAC) and of Avian Leukosis Virus (ALV) serologic test status in 30 White Leghorn hens in the ≥165 week age stratum obtained from the University of Illinois Poultry Research Laboratory laying flock.

	OAC Positive	OAC Negative	Odds Ratio
ALV Positive	18	11	5.2
ALV Negative	17	54	

$X^2 = 13.16$ $p < 0.001$

Table 8. Association between presence of ovarian adenocarcinoma (OAC) and of Avian Leukosis Virus subgroup E env mRNA expression (ALV-E env) PCR status in 27 White Leghorn hens in the 104 week age stratum obtained from the University of Illinois Poultry Research Laboratory laying flock.

	OAC Positive	OAC Negative	Odds Ratio
ALV-E env positive	1	20	0.95
ALV-E env negative	0	6	

Fisher's Exact p=1.0

Table 9. Association between presence of ovarian adenocarcinoma (OAC) and of Avian Leukosis Virus subgroup E env mRNA expression (ALV-E env) PCR status in 30 White Leghorn hens in the 130 week age stratum obtained from the University of Illinois Poultry Research Laboratory laying flock.

	OAC Positive	OAC Negative	Odds Ratio
ALV-E env positive	4	26	0.17
ALV-E env negative	0	0	

Fisher's Exact p=1.0

Table 10. Association between presence of ovarian adenocarcinoma (OAC) and of Avian Leukosis Virus subgroup E env mRNA expression (ALV-E env) PCR status in 68 White Leghorn hens in the $\geq$ 165 week age stratum obtained from the University of Illinois Poultry Research Laboratory laying flock.

	OAC Positive	OAC Negative	Odds Ratio
ALV-E env positive	17	41	8.86
ALV-E env negative	0	10	

Fisher's Exact p=0.056

Table 11. Logistic regression model considering age Stratum, Avian leukosis virus (ALV), and Avian Leukosis Virus subgroup E env (ALV-E env) mRNA expression in 125 White Leghorn hens > 100 weeks of age obtained from the University of Illinois Poultry Research Laboratory laying flock.

Variable	df	Coefficient	Standard Error	Odds Ratio	95% CI
Intercept	1	-8.1406			
Age stratum	1	0.0349	0.0140	1.0355	1.0076, 1.0643
ALV ELISA	1	1.2079	0.5646	3.3465	1.1067, 10.1193
Eenv PCR	1	1.1778	1.0896	3.2472	0.3837, 27.4777

Model Statistics: df= 3; X^2= 14.5509; p=0.0022

Appendix C. Primers Used for ALV-E env mRNA detection

Target	Forward Primer (5'-3')	Reverse primer (5'-3')	Product (bp)	Subgroup detection
PU1/PU2[1]	CTRCARCTGYTAGGYTCCCAGT	GYCAYCACTGTCGCCTRTCCG	229	All
PE1/PE2[1]	GYCAYCACTGTCGCCTRTCCG	GCACATCTCCACAGGTGTAAAT	265	ALV-E
ALV all[2]	CGAGAGTGGCTCGCGAGATGG	ACACTACATTTCCCCCTCCCTAT	2400	All
ALV E[2]	CGAGAGTGGCTCGCGAGATGG	GGCCCCACCCGTAGACACCACTT	1250	ALV-E
DIS	GCGAGGAATGCAGGAAATTAC	GGCATATTGCTGTGTCATCG	410	ALV-E
DISS	CGCGTAACTGAGGGACTAGG	GGCATATTGCTGTGTCATCG	159	ALV-E
Eenv	TTTGGGGTCCTACAGCAAGAAT	GTGAGCCAGAAGCAAGAAGTCA	215	ALV-E
EF1α	CCCGAAGTTCCTGAAATCTG	CTGAGGTGGCAGCTGATGTA	248	

1. Pham TD, Spencer JL, Johnson ES. Detection of avian leukosis virus in albumen of chicken eggs using reverse transcriptase polymerase chain reaction. J Virol Methods 1999 78: 1-11
2. Silva RF, Fadly AM, Taylor SP. Development of a polymerase chain reaction to differentiate avian leukosis virus (ALV) subgroups: Detection of an ALV contaminant in commercial Mareck's disease vaccines. Avian Diseases 2007 51: 663-667

Appendix D. Product Sequence for ALV-E env mRNA primer

AGTCCAAAAGGTAGCAGCTGCGCAAGCCTTAAGAGAAATTGAGAGACTAGC
CTGTTGGTCCGTTAAACAGGCTAACTTGACAACATCACTCCTCGGGGACTTAT
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